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Letter

Cytotoxic actions of N-(2,4,6-trichlorophenyl)maleimide (IT-354), an antifouling agent, in rat thymic lymphocytes

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ABSTRACT — Of antifoulants that are substitutes for organotin compounds such as tributyltin and triphenyltin, N-(2,4,6-trichlorophenyl)maleimide (IT-354) is listed as a much less toxic agent, although the available information concerning IT-354 toxicity is the results of acute toxicity tests in freshwater fish. In this study, the effects of IT-354 on rat thymic lymphocytes were examined using flow-cytometric techniques with appropriate fluorescent probes in order to estimate the effects of IT-354 on mammalian cells. Treatment of cells with 1-10 μM IT-354 for 1 hr did not increase the population of dead cells (cell lethality). However, 10 μM IT-354 significantly increased the population of living, annexin V-positive cells. Annexin V-positive, living cells are expected to be undergoing apoptosis. IT-354 at 3-10 μM significantly elevated intracellular Ca^{2+} and Zn^{2+} levels mainly by increasing Ca^{2+} influx and intracellular Zn^{2+} release. Furthermore, IT-354 significantly depolarized membranes and decreased cellular non-protein thiol content. Assessments using selected antifouling agents showed that the cellular actions of IT-354 are most likely similar to those of other commonly used antifouling agents. Therefore, the toxic potency of IT-354 on wild mammals is speculated to be similar to those of the other tested antifoulants.

Key words: IT-354, Intracellular Zn^{2+}, Intracellular Ca^{2+}, Lymphocytes

INTRODUCTION

N-(2,4,6-trichlorophenyl)maleimide (IT-354) is an antifouling agent used as a substitute for highly-toxic organotin compounds. Because IT-354 is used in Japan, but not worldwide (Finnie and Williams, 2009), its toxicity has not been evaluated as comprehensively as that of some other antifouling agents. Consequently, little information is available in the literature regarding the toxicity of IT-354. According to reports published by the Ocean Policy Research Foundation of Tokyo, Japan (2009, 2010), IT-354 is considered to have no effect on the environment because its predicted environmental concentration (PEC)/predicted no-effect concentration (PNEC) ratio is less than 1. Among currently used antifoulants, IT-354 is listed as a much less toxic agent. However, the reliability of the PNEC as a predictor of toxicity may be low because the only available toxicity data for IT-354 was collected *via* acute toxicity tests in freshwater fish (Ocean Policy Research Foundation, 2010). In addition, we were unable to locate information concerning the cytotoxicity of IT-354. Therefore, estimation of the actions of IT-354 on mammalian cells is necessary to allow accurate prediction of the influence of IT-354 on wild mammals. In this study, the effects of IT-354 on rat thymic lymphocytes were examined using flow-cytometric techniques with appropriate fluorescent probes.

MATERIALS AND METHODS

Animals and cell preparation

This study was approved by the Committee for Animal Experiments at Tokushima University (No. 14124), Tokushima, Japan. The cell suspension was prepared as previously reported (Chikahisa *et al*., 1996; Matsui *et al*., 2010). In brief, thymus glands dissected from ether-anesthetized rats were sliced under cold conditions (2-4°C). The slices were triturated in Tyrode's solution to dissociate the thymocytes. The cell suspension was incubated at 36-37°C for 1 hr before the experiment. Importantly, the zinc concentration in the solution that was obtained after removing the cells from the cell suspension by filtration was 216.9 ± 14.4 nM (Sakanashi *et al*., 2009). The cell suspension contained trace zinc derived

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from the cell preparation.

Chemicals

IT-354 was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). Annexin V-FITC, propidium iodide, FluoZin-3-tetra(acetoxymethyl)ester (FluoZin-3-AM), bis-(1,3-dibutylbarbituric acid) trimethine oxonol (Oxonol), and 5-chloromethylfluorescein diacetate (5-CMF-DA) were obtained from Molecular Probes Inc., Invitrogen (Eugene, OR, USA). Fluo-3-tetra(acetoxymethyl)ester (Fluo-3-AM) and 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). N-ethylmaleimide (NEM) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) unless mentioned.

Fluorescence measurements of cellular parameters

The methods for measuring cellular and membrane parameters using a flow cytometer (CytoACE-150; JASCO, Tokyo, Japan) and fluorescent probes were similar to those previously described (Chikahisa *et al*., 1996). Various concentrations of IT-354 (1-10 mM in 2 μL DMSO) were added to cell suspensions (2 mL per test tube), which were incubated at 36-37°C.

To assess cell lethality (dead cell population) using propidium iodide, the dye was added to the cell suspension at a final concentration of 5 μM. Exposure of phosphatidylserine on the outer surface of the cell membrane, a marker of the early stage of apoptosis, was detected using 10 μL/mL annexin V-FITC (Koopman *et al*., 1994). Changes in intracellular Ca^{2+} and Zn^{2+} levels were estimated using 500 nM Fluo-3-AM (Kao *et al*., 1989) and 500 nM FluoZin-3-AM (Gee *et al*., 2002), respectively. 5-CMF-DA at a concentration of 1 μM was used to monitor changes in cellular non-protein thiol content, presumably glutathione (Chikahisa *et al*., 1996). The excitation wavelength for the fluorescent probes was 488 nM. Fluorescence by FITC, Fluo-3, FluoZin-3, Oxonol, and 5-CMF was detected at 530 ± 20 nm, whereas propidium fluorescence was detected at 600 ± 20 nm. FluoZin-3, Fluo-3, Oxonol, and 5-CMF fluorescence were monitored in living cells that did not exhibit propidium fluorescence.

Statistical analysis and figure presentation

Statistical analyses were performed by ANOVA with post-hoc Tukey's multivariate analysis. P-values less than 0.05 were considered significant. In the results, values (including columns and bars in figures) were expressed as the mean and standard deviation of four samples.

RESULTS

Changes in cell lethality induced by IT-354

Treatment of cells with 10 μM IT-354 for 1 hr significantly increased the population of living, annexin V-FITC-positive cells (Area A of Fig. 1A) without increasing the population of dead cells (Areas P and PA of Fig. 1A). The annexin V-positive, living cells are expected to have exposed phosphatidylserine on their outer membranes, a marker of the early stage of apoptosis (Koopman *et al*., 1994). As shown in Fig. 1B, 10 μM IT-354 almost completely abolished the population of intact living cells without significantly increasing the population of dead cells at 1 hr after the start of treatment. Prolonged treatment with 10 μM IT-354, but not 1-3 μM IT-354, for 3 hr significantly increased the population of dead cells (Fig. 1C).

Augmentation of Fluo-3 and FluoZin-3 fluorescence by IT-354

A23187, a calcium ionophore, increased the population of annexin V-positive cells in a manner similar to IT-354 (Sakanashi *et al*., 2008), suggesting that IT-354 might increase the intracellular Ca^{2+} concentration of treated cells. To test this possibility, the effect of 1-10 μM IT-354 on Fluo-3 fluorescence was examined 1 hr after the start of drug application. As shown in Fig. 2A, treatment with 10 μM IT-354 shifted the histogram of Fluo-3 fluorescence in the direction of higher intensity, indicating an increased intracellular Ca^{2+} concentration. The dose-response relationship for augmentation of Fluo-3 fluorescence by IT-354 is shown in Fig. 2B. Tri-n-butyltin, the most toxic antifoulant, increased intracellular concentrations of Ca^{2+} and Zn^{2+} (Chikahisa and Oyama, 1992; Oyama *et al*., 2009). The effect of IT-354 on FluoZin-3 fluorescence, an indicator of intracellular Zn^{2+}, was also examined. Treatment with 10 μM IT-354 for 1 hr also shifted the histogram of FluoZin-3 fluorescence (Fig. 2A), indicating an increased intracellular Zn^{2+} concentration. Dose-dependent augmentation of FluoZin-3 fluorescence by 1-10 μM IT-354 is shown in Fig. 2B.

Contribution of Zn^{2+} to the augmentation of Fluo-3 and FluoZin-3 fluorescence by IT-354

The elevation of intracellular Zn^{2+} abundance by IT-354 may have increased the intensity of Fluo-3 fluorescence because Zn^{2+} was also bound to Fluo-3, resulting in augmentation of Fluo-3 fluorescence (Kao *et al*., 1989). To test this possibility, changes in the intensity of Fluo-3 flu-

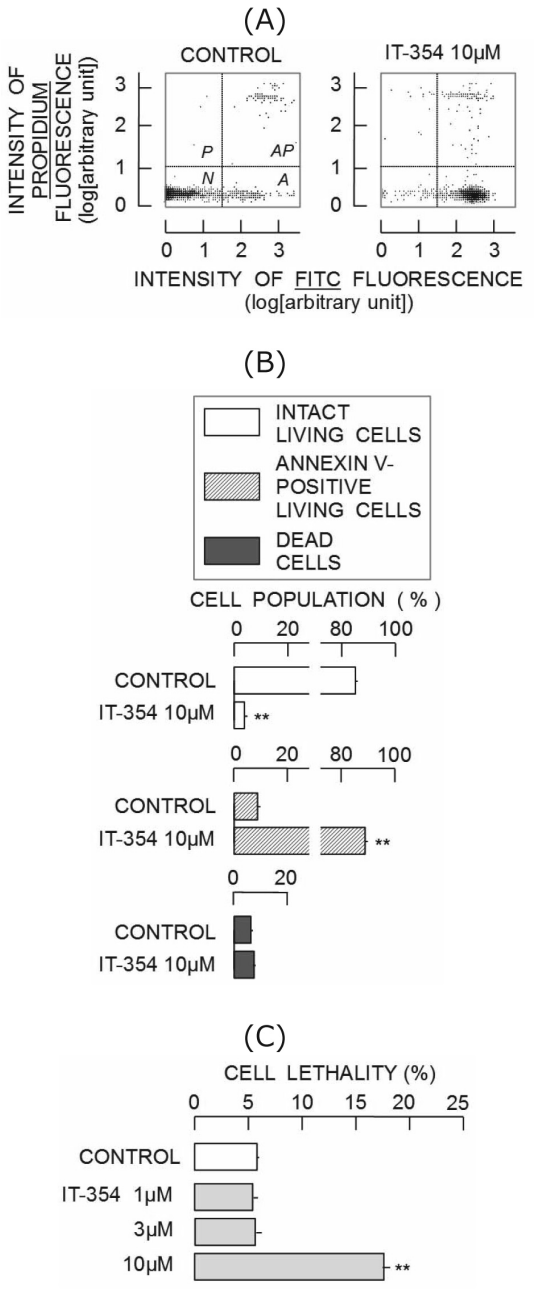

Fig. 1. Change in cell lethality induced by IT-354. (A) Change in cell population consisting of intact living cells (N), annexin-V-positive living cells (A), and dead cells (P and PA) by 10 μM IT-354. The effect of IT-354 was observed 1 hr after the start of drug application. Each cytogram was constructed with 2000 cells. (B) Percentage change in the cell population induced by 10 μM IT-354. (C) Change in cell lethality induced by 3 hr incubation with 10 μM IT-354. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with IT-354.

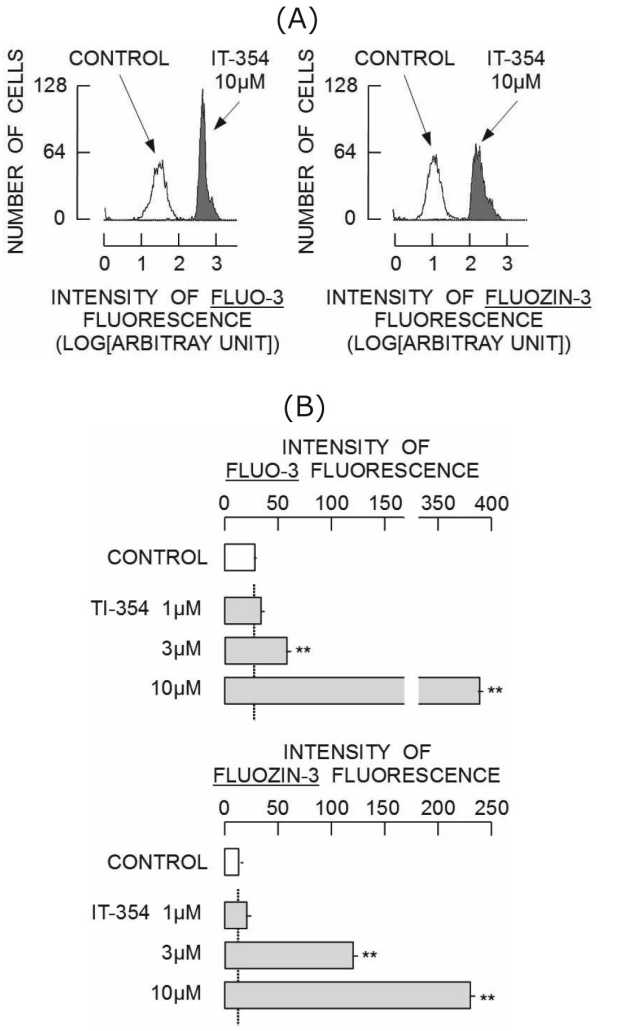

Fig. 2. Changes in Fluo-3 and FluoZin-3 fluorescence by induced IT-354. (A) Changes in the histograms of Fluo-3 (Left panel) and FluoZin-3 (Right panel) fluorescence induced by 10 μM IT-354. The effect was observed 1 hr after the start of drug application. (B) Concentration-dependent changes in Fluo-3 (upper panel) and FluoZn-3 fluorescence induced by IT-354. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with IT-354.

orescence caused by simultaneous application of IT-354 with a Zn^{2+}-chelator (DTPA or TPEN) were examined. As shown in Fig. 3A, 10 μM IT-354 increased the intensity of Fluo-3 fluorescence in the presence of 10 μM DTPA or 10 μM TPEN. Furthermore, the removal of Zn^{2+} by a Zn^{2+} chelator slightly potentiated the action of IT-354. It is likely that augmentation of Fluo-3 fluorescence by

10 μM IT-354 is dependent on elevation of intracellular Ca^{2+} levels. In the case of FluoZin-3 fluorescence (Fig. 3B), removal of external Zn^{2+} by DTPA did not affect the augmentation of FluoZin-3 fluorescence by IT-354, whereas TPEN, a chelator of intracellular Zn^{2+}, almost completely attenuated FluoZin-3 fluorescence. These results suggest that augmentation of FluoZin-3 fluorescence by IT-354 is largely dependent on the increase in intracellular Zn^{2+} concentration caused by intracellular Zn^{2+} release.

Contribution of Ca^{2+} to the augmentation of Fluo-3 and FluoZin-3 fluorescence by IT-354

To reveal the source of Ca^{2+} in the augmentation of Fluo-3 fluorescence by IT-354, the action of IT-354 was examined under external Ca^{2+}-free conditions. Removal of external Ca^{2+} decreased the intensity of Fluo-3 fluorescence in the control group. Augmentation of Fluo-3 fluorescence by IT-354 was greatly attenuated under external Ca^{2+}-free conditions (Fig. 3C), suggesting that augmentation of Fluo-3 fluorescence by IT-354 was caused mainly by the influx of external Ca^{2+}. However, a small augmentation of Fluo-3 fluorescence by IT-354 was observed even under external Ca^{2+}-free conditions, suggesting the involvement of intracellular Ca^{2+} release. Removal of external Ca^{2+} slightly increased the intensity of FluoZin-3 fluorescence in the absence and presence of IT-354 (Fig. 3D). Thus, it is unlikely that Ca^{2+} contributes to the augmentation of FluoZin-3 fluorescence by IT-354.

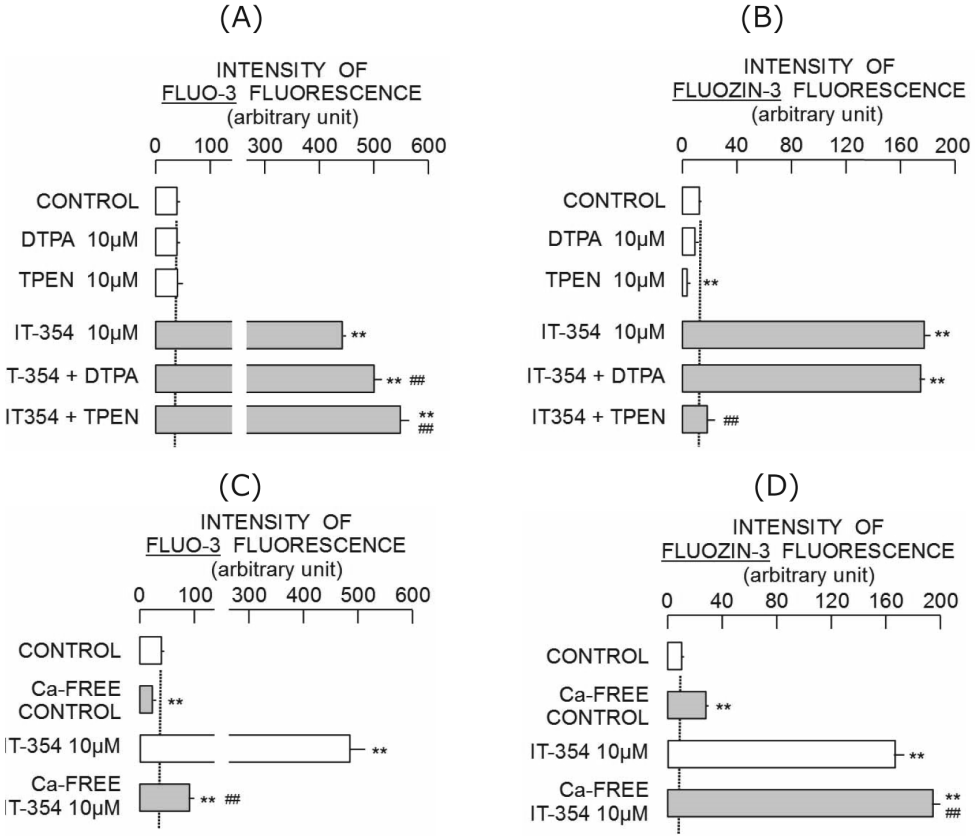

Fig. 3. Effects of Zn^{2+} and Ca^{2+} on changes in Fluo-3 and FluoZin-3 fluorescence induced by 10 μM IT-354. (A) Change in Fluo-3 fluorescence induced by IT-354 in the presence of DTPA or TPEN. (B) Change in FluoZin-3 fluorescence induced by IT-354 in the presence of DTPA or TPEN. (C) Change in Fluo-3 fluorescence induced by IT-354 under external Ca^{2+}-free conditions. (D) Change in FluoZin-3 fluorescence induced by IT-354 under external Ca^{2+}-free conditions. Effects were observed 1 hr after the start of drug application. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the test groups. Number signs (##) indicate significant differences ($P < 0.01$) between the groups of cells treated with IT-354 under different conditions.

Changes in Oxonol and 5-CMF fluorescence induced by IT-354

The results described above indicate that IT-354 greatly changes the ionic balance inside treated cells. Therefore, to assess the effect of IT-354 on membrane potential, the effect of 3-10 μM IT-354 on Oxonol fluorescence was examined. As shown in Fig. 4A, treatment with IT-354 for 1 hr significantly increased the intensity of Oxonol fluorescence, an indicator of membrane potential, suggesting that IT-354 induced membrane depolarization.

Excessive elevations of intracellular Ca^{2+} and/or Zn^{2+} levels induce oxidative stress in some cells, presumably reducing cellular glutathione abundance. However, increases in the intracellular Zn^{2+} concentration elevate the cellular content of glutathione (Kinazaki *et al*., 2011). Therefore, the effect of IT-354 on 5-CMF fluorescence was examined. The intensity of 5-CMF fluorescence in rat thymocytes and cellular glutathione content were very strongly correlated (correlation coefficient = 0.965) (Chikahisa *et al*., 1996). Treatment with 3 μM IT-354 attenuated 5-CMF fluorescence in some cells and augmented it in others. The standard deviation of the mean intensity of 5-CMF fluorescence was large in the group treated with 3 μM IT-354 (Fig. 4B). The intensity of 5-CMF fluorescence was significantly attenuated by 10 μM IT-354. These results suggest that the potency of IT-354 as a reducer of cellular glutathione content was greater than that of NEM, an agent used for chemical depletion of non-protein thiols (Fig. 4B).

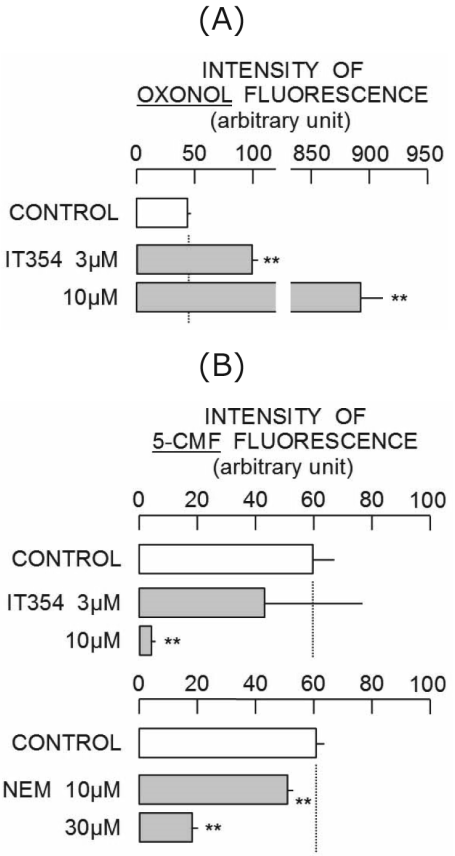

Fig. 4. Changes in Oxonol (A) and 5-CMF (B) fluorescence induced by IT-354. Effects were observed 1 hr after the start of drug application. NEM was used as a reference to reduce the cellular content of non-protein thiols. Asterisks (**) indicate a significant difference ($P < 0.01$) between the control group (CONTROL) and the group of cells treated with IT-354.

DISCUSSION

The results of the present study show that IT-354 elevates intracellular Ca^{2+} and Zn^{2+} levels mainly by increasing Ca^{2+} influx and intracellular Zn^{2+} release. The augmentation of Fluo-3 fluorescence by IT-354 was significantly reduced under external Ca^{2+}-free conditions (Fig. 3C). However, a small augmentation of Fluo-3 fluorescence was produced by IT-354 even under external Ca^{2+}-free conditions (Fig. 3C), indicating that IT-354 induced intracellular Ca^{2+} release. IT-354 similarly increased the intensity of FluoZin-3 fluorescence in the presence of DPTA, a chelator of external Zn^{2+} (Fig. 3B). Because both intracellular Ca^{2+} and Zn^{2+} are involved in the cellular functions of lymphocytes (Lewis, 2001; Hirano *et al*., 2008), these results suggest that excessive increases in intracellular Ca^{2+} and Zn^{2+} concentrations by IT-354 might disturb the normal cellular functions of lymphocytes, possibly resulting in immunotoxic action.

The lowest observed adverse effect level (LOAEL) of IT-354, dichlofluanid, tolylfluanid, Ziram, Zineb, and 4.5-dichloro-2-octyl-4-isothiazolin-3-one (DCOIT) is 49,000 μg/L, 2.7 μg/L, 16.0 μg/L, 1.8 μg/L, 32.0 μg/L, and 0.63 μg/L, respectively (Ocean Policy Research Foundation, 2009). Because the LOAEL of IT-354 is 10^2-10^5 times higher than those of other antifoulants, IT-354 seems to be much less toxic (Ocean Policy Research Foundation, 2010). However, IT-354 at a concentration of 10 μM (equivalent to 2,765 μg/L) increased the population of cells with membranes with exposed phosphatidylserine (Fig. 1A and 1B) and induced cell death (Fig. 1C) in rat thymocytes *in vitro*. Furthermore, 10 μM IT-354 induced significant membrane depolarization (Fig. 4A) and depletion of non-protein thiols (Fig. 4B).

Dichlofluanid and tolylfluanid at concentrations of 1-10 μM increase the intracellular Ca^{2+} concentration

of treated cells (Fukunaga *et al*., 2015). The potency of IT-354 as an elevator of intracellular Ca^{2+} abundance seems to be similar to the potency of phenylsulfamides dichlofluanid and tolylfluanid (Fukunaga *et al*., 2015). The potency of IT-354 as an elevator of intracellular Zn^{2+} concentration is similar to that of Zineb (Kanemoto-Kataoka *et al*., 2015). The concentrations of IT-354 that elevate intracellular Zn^{2+} levels are 10-30 times higher than those of DCOIT (Saitoh *et al*., 2015) and Ziram (Kanemoto-Kataoka *et al*., 2015). Therefore, the concentrations of IT-354 that alter Zn^{2+} and Ca^{2+} homeostasis in rat thymocytes under *in vitro* conditions are similar to those of phenylsulfamides and Zineb, but higher than those of DCOIT and Ziram. Thus, IT-354 at concentrations much lower than its LOAEL may exert toxic effects on mammalian species. The reported LOAEL of IT-354 may be specific for *Oryzias latipes*, a euryhaline fish (Ocean Policy Research Foundation, 2009).

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Conflict of interest---- The authors declare that there is no conflict of interest.

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