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Effect of NPC-14686 (Fmoc-l-Homophenylalanine) on Ca^{2+} Homeostasis and Viability in OC2 Human Oral Cancer Cells

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Abstract

The effect of the anti-inflammatory compound NPC-14686 on intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and viability in OC2 human oral cancer cells was investigated. The Ca^{2+}-sensitive fluorescent probe fura-2 was used to examine $[Ca^{2+}]_i$. NPC-14686 induced $[Ca^{2+}]_i$ rises in a concentration-dependent fashion. The effect was reduced approximately by 10% by removing extracellular Ca^{2+}. NPC-14686-elicited Ca^{2+} signal was decreased by nifedipine, econazole, SKF96365, and GF109203X. In Ca^{2+}-free medium, incubation with the endoplasmic reticulum Ca^{2+} pump inhibitor thapsigargin or 2,5-di-tert-butylhydroquinone (BHQ) abolished NPC-14686-induced $[Ca^{2+}]_i$ rises. Conversely, pretreatment with NPC-14686 abolished thapsigargin or BHQ-induced $[Ca^{2+}]_i$ rises. Inhibition of phospholipase C with U73122 abolished NPC-14686-induced $[Ca^{2+}]_i$ rises. At 20-100 μM, NPC-14686 inhibited cell viability, which was not reversed by chelating cytosolic Ca^{2+} with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid-acetoxymethyl ester (BAPTA/AM). NPC-14686 between 20 μM and 40 μM also induced apoptosis. Collectively, in OC2 cells, NPC-14686 induced $[Ca^{2+}]_i$ rises by evoking phospholipase C-dependent Ca^{2+} release from the endoplasmic reticulum and Ca^{2+} entry *via* protein kinase C-regulated store-operated Ca^{2+} channels. NPC-14686 also caused Ca^{2+}-independent apoptosis.

Key Words: apoptosis, Ca^{2+}, endoplasmic reticulum, human oral cancer cells, NPC-14686, phospholipase C

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Introduction

Numerous studies have shown that non-steroidal anti-inflammatory drugs (NSAIDs) use several mechanisms to exert anti-tumor effects on different cancer models (14, 21). NSAIDs exert their antineoplastic activity by specifically inhibiting prostaglandin synthesis which catalyzes the rate-liming step, *i.e.* the synthesis of prostaglandins from arachidonic acid substrates (25). Furthermore, aspirin, a type of NSAIDs, causes cytotoxicity and induces apoptosis in colon cancer cells resulting in the suppression of malignant transformation and tumor growth (26). NPC-14686 (Fmoc-L-homophenylalanine) (Fig. 1) belongs to NSAIDs, which has been shown to induce various physiological effects. The inflammation-induced cytotoxicity caused by T-lymphocyte activation and leukocyte infiltration was inhibited by NPC-14686 (2). NPC-14686 induced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) rises and caused cell death in different cancer cells including human prostate cancer cells (12) and hepatoma cells (13). Collectively, NPC-14686 caused cytotoxicity in different cancer cells, but the underlying mechanisms are not clear.

Ca^{2+} ions play an important role in the regulation of many cellular responses in almost all cells (1), such as protein activation, secretion, contraction, fertilization, proliferation, *etc.* (6). However, a unregulated $[Ca^{2+}]_i$ rise may cause cell death such as apoptosis and necrosis, *etc.* (6). Apoptosis is a physiological cell death process that is involved in the selective elimination of mutated, infected or simply dispensable cells and cells that are present in inappropriate position, but failure of apoptosis can result in cancer (9). It has been shown that many anti-tumor drugs exert their effects by altering Ca^{2+} homeostasis and inducing apoptosis (8). The endoplasmic reticulum contributes to apoptosis through a change in its Ca^{2+} homeostasis that results in overloading the mitochondria with Ca^{2+} (8). However, Ca^{2+}-independent apoptosis could also be found in different cell types such as human ovarian carcinoma cells (20) and prostate cancer cells (19); therefore the role of a $[Ca^{2+}]_i$ rise in apoptosis needs to be established for each stimulant and cell type.

In the present study, the effect of NPC-14686 on $[Ca^{2+}]_i$ rises and apoptosis was explored in OC2 human oral cancer cells. The pharmacological effect of NPC-14686 on oral cancer cells has not been explored previously. The OC2 cell is a useful model for oral cell research. It has been shown that in this cell, $[Ca^{2+}]_i$ can increase in response to the stimulation of various agents such as methoxychlor (27), thimerosal (16) and fendiline (11), *via* causing Ca^{2+} entry and Ca^{2+} release. Therefore, it was of interest to study the effect of NPC-14686 on OC2 cells.

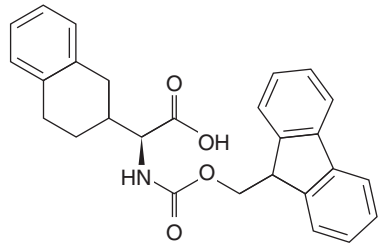

Fig. 1. The chemical structure of NPC-14686.

The Ca^{2+}-sensitive fluorescent dye fura-2 was used to measure $[Ca^{2+}]_i$ changes. The effect of NPC-14686 on $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium and Ca^{2+}-free medium, the concentration-response relationships, the pathways underlying Ca^{2+} entry and Ca^{2+} release, the internal Ca^{2+} stores, and the role of phospholipase C were explored. The effect of NPC-14686 on viability and apoptosis were also assessed.

Materials and Methods

Chemicals

The reagents for cell culture were from Gibco® (Gaithersburg, MD, USA). Fura-2/AM and BAPTA/AM were from Molecular Probes® (Eugene, OR, USA). NPC-14686 and all other reagents were from Sigma-Aldrich® (St. Louis, MO, USA) unless otherwise indicated.

Cell Culture

OC2 human oral cancer cells obtained from Bioresource Collection and Research Center (Taiwan) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Solutions Used in $[Ca^{2+}]_i$ Measurements

Ca^{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 5 mM glucose. Ca^{2+}-free medium contained similar chemicals as Ca^{2+}-containing medium except that $CaCl_2$ was replaced with 0.3 mM ethylene glycol tetraacetic acid (EGTA) and 2 mM $MgCl_2$. NPC-14686 was dissolved in dimethyl sulfoxide as a 1 M stock solution. The other chemicals were dissolved in water, ethanol or dimethyl sulfoxide. The concentration of organic solvents in the experimental solutions did not exceed 0.1%, and did not affect viability, apoptosis or basal $[Ca^{2+}]_i$.

$[Ca^{2+}]_i$ Measurements

$[Ca^{2+}]_i$ was measured as previously described

(4). Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10^6/ml. Cell viability was determined by trypan blue exclusion (adding 0.2% trypan blue to 0.1 ml cell suspension). The viability was greater than 95% after the treatment. Cells were subsequently loaded with 2 μM fura-2/AM for 30 min at 25°C in the same medium. After loading, cells were washed with Ca^{2+}-containing medium twice and was made into a suspension in Ca^{2+}-containing medium at a density of 10^7/ml. Fura-2 fluorescence measurements were performed in a water-jacketed cuvette (25°C) with continuous stirring; the cuvette contained 1 ml of medium and 0.5 million cells. Fluorescence was monitored with a Shimadzu RF-5301PC spectrofluorophotometer immediately after 0.1 ml cell suspension was added to 0.9 ml Ca^{2+}-containing or Ca^{2+}-free medium, by recording excitation signals at 340 nm and 380 nm and emission signal at 510 nm at 1-sec intervals. During the recording, reagents were added to the cuvette by pausing the recording for 2 sec to open and close the cuvette-containing chamber. For calibration of $[Ca^{2+}]_i$, after completion of the experiments, the detergent Triton X-100 (0.1%) and $CaCl_2$ (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca^{2+} chelator EGTA (10 mM) was added to chelate Ca^{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette had a viability of 95% after 20 min of fluorescence measurements. $[Ca^{2+}]_i$ was calculated as previously described (4, 10).

Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Increase in color intensity directly correlated with the number of live cells. Assays were conducted according to manufacturer's instructions (Roche Molecular Biochemical, Indianapolis, IN, USA). Cells were seeded in 96-well plates at a density of 10,000 cells/well in culture medium for 24 h in the presence of NPC-14686. The cell viability detecting reagent 4-[3-[4-Iodophenyl]-2-4(4-nitrophenyl)-2H-5-tetrazolio-1,3-benzene disulfonate] (WST-1; 10 μl pure solution) was added to samples after NPC-14686 treatment, and cells were incubated for 30 min in a humidified atmosphere. In experiments using BAPTA/AM to chelate cytosolic Ca^{2+}, cells were treated with 5 μM BAPTA/AM for 1 h prior to incubation with NPC-14686. The cells were washed once with Ca^{2+}-containing medium and incubated with/without NPC-14686 for 24 h. The absorbance of samples (A_{450}) was determined using an enzyme-linked immunosorbent assay (ELISA) reader. Absolute optical density was normalized to the absorbance of unstimulated cells in each plate and expressed as a percentage of the control value.

Alexa® Fluor 488 Annexin V/Propidium Iodide (PI) Staining for Apoptosis

Alexa Fluor® 488 Annexin V/Dead Cell Apoptosis Kit was from Molecular Probes® (Eugene, OR, USA). Annexin V/PI staining assay was employed to further detect cells in early apoptotic and late apoptotic stages. Cells were exposed to NPC-14686 at concentrations of 0, 20, or 40 μM for 24 h. Cells were harvested after incubation and washed in cold phosphate-buffered saline (PBS). Cells were resuspended in 400 μl reaction solution with 10 mM of HEPES, 140 mM of NaC1, 2.5 mM of $CaC1_2$ (pH 7.4). Alexa Fluor® 488 annexin V/PI staining solution (Probes Invitrogen, Eugene, Oregon, USA) was added in the dark. After incubation for 15 min, the cells were collected and analyzed in a FACScan flow cytometry analyzer. Excitation wavelength was at 488 nm and the emitted green fluorescence of Annexin V (FL1) and red fluorescence of PI (FL2) were collected using 530 and 575 nm band pass filters, respectively. A total of 20,000 cells were analyzed per sample. Light scatter was measured on a linear scale of 1024 channels and fluorescence intensity was on a logarithmic scale. The amount of early apoptosis and late apoptosis were determined, respectively, as the percentage of Annexin V^+/PI^- or Annexin V^+/PI^+ cells. Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software). X and Y coordinates refer to the intensity of fluorescence of Annexin and PI, respectively.

Statistics

Data are reported as mean ± SEM of three separate experiments. Data were analyzed by one-way analysis of variances (ANOVA) using the Statistical Analysis System (SAS®, SAS Institute Inc., Cary, NC, USA). Multiple comparisons between group means were performed by *post-hoc* analysis using the Tukey's HSD (honestly significantly difference) procedure. A *P*-value less than 0.05 was considered significant.

Results

Effect of NPC-14686 on $[Ca^{2+}]_i$

The effect of NPC-14686 (Fig. 1) on basal $[Ca^{2+}]_i$ was explored. Fig. 2A shows that the basal $[Ca^{2+}]_i$ level was 51 ± 2 nM. At concentrations between 100 and 200 μM, NPC-14686 evoked $[Ca^{2+}]_i$ rises in a

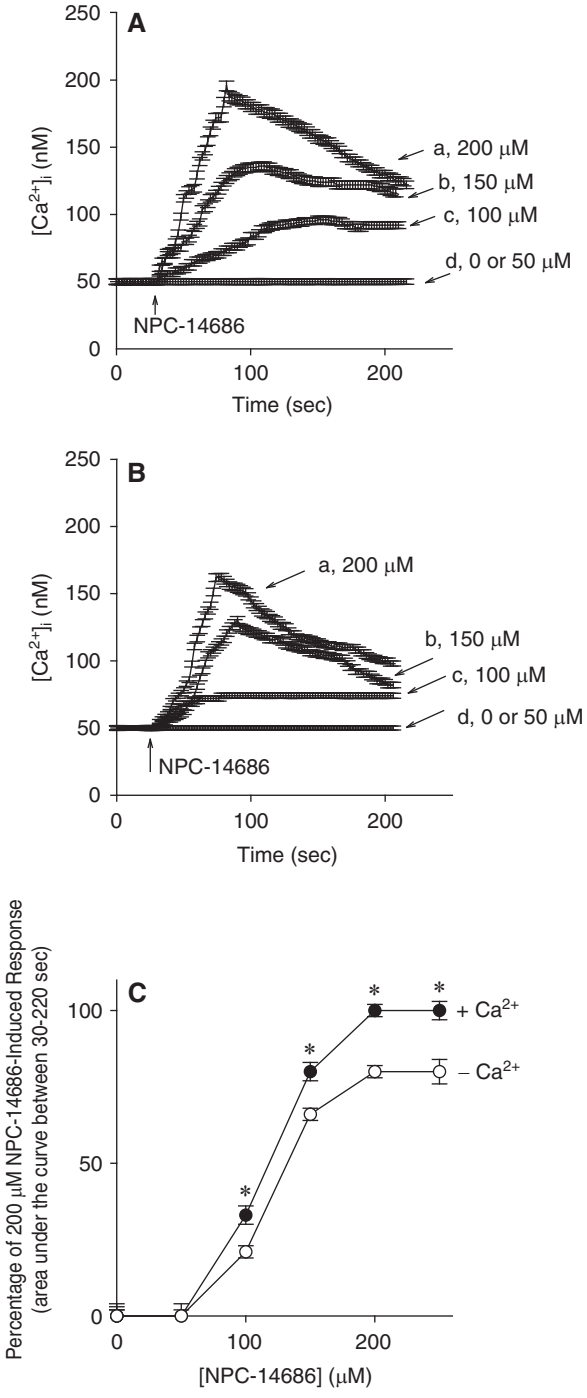

Fig. 2. Effect of NPC-14686 on $[Ca^{2+}]_i$ in fura-2-loaded OC2 cells. (A) NPC-14686 was added at 25 sec. The concentration of NPC-14686 was indicated. The experiments were performed in Ca^{2+}-containing medium. (B) Effect of NPC-14686 on $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}. NPC-14686 was added at 25 sec in Ca^{2+}-free medium. (C) Concentration-response plots of NPC-14686-induced $[Ca^{2+}]_i$ rises in the presence or absence of extracellular Ca^{2+}. Y axis is the percentage of the net (baseline subtracted) area under the curve (25-210 sec) of the $[Ca^{2+}]_i$ rises induced by 200 μM NPC-14686 in Ca^{2+}-containing medium. Data are mean ± SEM of three separate experiments. $*P < 0.05$ compared to open circles.

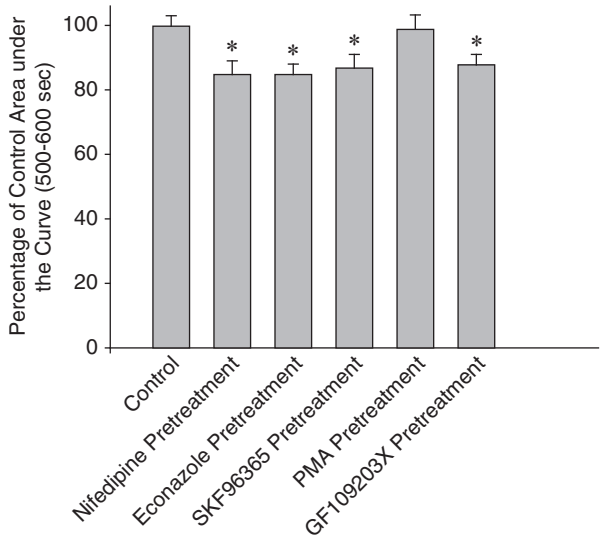

Fig. 3. Effect of Ca^{2+} channel modulators on NPC-14686-induced Ca^{2+} influx. The concentration was 1 μM for nifedipine, 0.5 μM for econazole, 5 μM for SKF96365, 10 nM for phorbol 12-myristate 13-acetate (PMA), and 2 μM for GF109203X. NPC-14686 (200 μM) was added at 25 sec to induce a $[Ca^{2+}]_i$ rise in Ca^{2+}-free medium. At the time point of 500 sec, 3 mM Ca^{2+} was added to the suspension. This immediately induced a $[Ca^{2+}]_i$ rise which was taken as control. Each of the inhibitors was added 30 sec before 3 mM Ca^{2+}. Data are expressed as the percentage of control (1st column) that is the area under the curve (500-600 sec) of 3 mM Ca^{2+}-induced $[Ca^{2+}]_i$ rises, and are mean ± SEM of three separate experiments. $*P < 0.05$ compared to the 1st column.

concentration-dependent manner in Ca^{2+}-containing medium. At 200 μM, NPC-14686 induced a $[Ca^{2+}]_i$ rise that attained to a net increase of 147 ± 2 nM (n = 3) followed by a slow decay. The Ca^{2+} response saturated at 200 μM NPC-14686 because 250 μM NPC-14686 did not induce a greater response (data not shown). Fig. 2B shows that in the absence of extracellular Ca^{2+}, 200 μM NPC-14686 induced a $[Ca^{2+}]_i$ rise of 112 ± 2 nM (n = 3). Fig. 2C shows the concentration-response plot of NPC-14686-induced response. The EC_{50} value was 112 ± 2 μM or 113 ± 1 μM in the presence or absence of extracellular Ca^{2+}, respectively, by fitting to a Hill equation (n = 3).

NPC-14686 Induced Ca^{2+} Entry via *Store-Operated Ca^{2+} Channels*

Because Fig. 2 shows that NPC-14686-induced Ca^{2+} response saturated at 200 μM, in the following experiments 200 μM NPC-14686 was used to deplete internal Ca^{2+} stores. Three Ca^{2+} entry inhibitors: nifedipine (1 μM), econazole (0.5 μM) and SKF96365 (5 μM) (7, 12, 13, 22, 30); phorbol 12-myristate 13 acetate (PMA; 1 nM; a protein kinase C activator); and

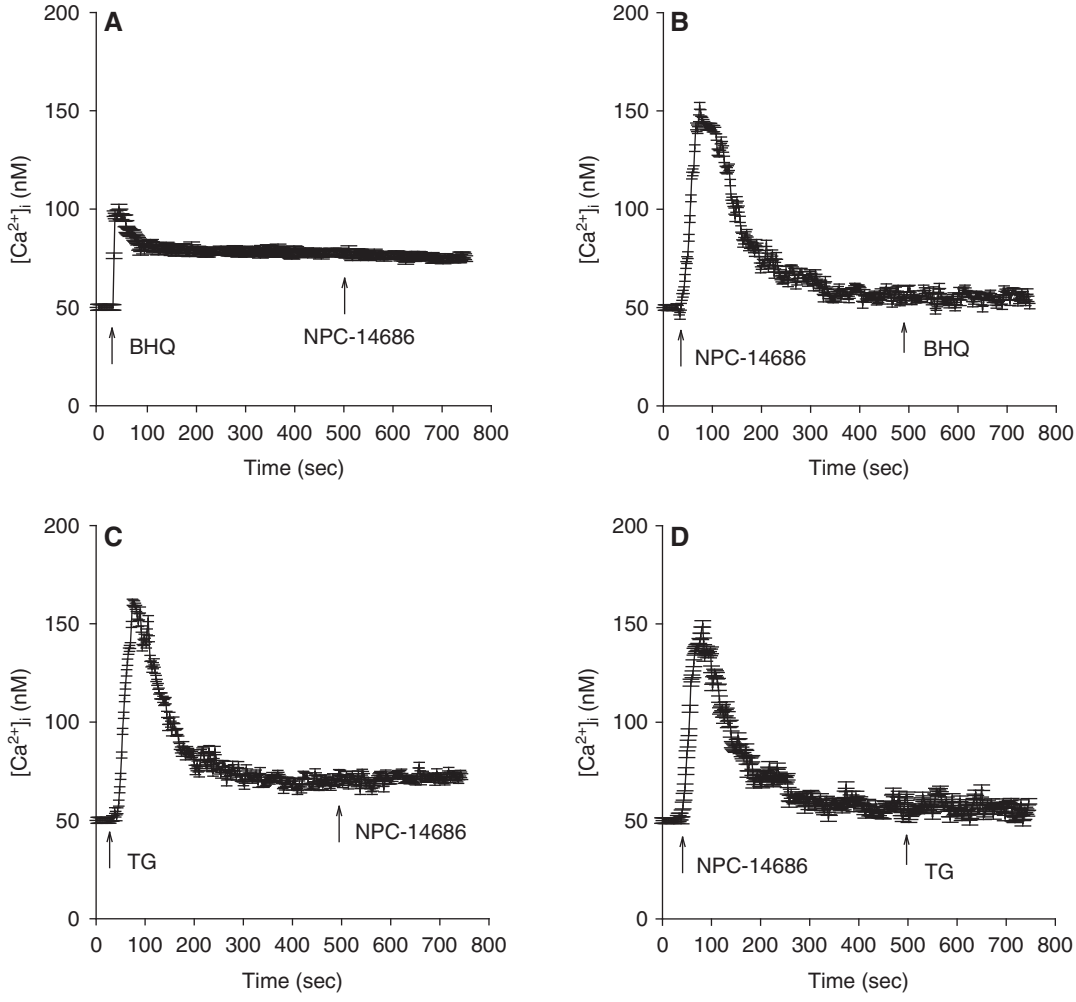

Fig. 4. Intracellular Ca^{2+} stores of NPC-14686-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. NPC-14686 (200 μM), BHQ (50 μM) and thapsigargin (1 μM) were added at time points indicated (A, B, C, D). Data are mean ± SEM of three separate experiments.

GF109203X (2 μM; a protein kinase C inhibitor) (12, 13) were used in this study. Cells were first incubated in Ca^{2+}-free medium, then NPC-14686 was added at 25 sec to induce a $[Ca^{2+}]_i$ rise. At the time point of 500 sec, 3 mM Ca^{2+} was added back to the suspension. This immediately induced a $[Ca^{2+}]_i$ rise which was taken as control. In other experiments, each of the inhibitors was added 30 sec before 3 mM Ca^{2+}. Nifedipine, econazole, SKF96365, and GF109203X all significantly inhibited Ca^{2+}-induced $[Ca^{2+}]_i$ rises by 10-15% (n = 3) (Fig. 3). PMA had no effect on Ca^{2+}-induced $[Ca^{2+}]_i$ rises (n = 3).

NPC-14686 Induced Ca^{2+} Release from the Endoplasmic Reticulum

The endoplasmic reticulum has been shown to be a major Ca^{2+} store in most cell types including OC2 cells (11, 16, 27). Thus the role of endoplasmic reticulum in NPC-14686-induced Ca^{2+} release in OC2 cells was explored. To exclude the contribution of Ca^{2+} entry, the experiments were performed in Ca^{2+}-free medium. Fig. 4A shows that addition of 50 μM 2,5-di-tert-butylhydroquinone (BHQ), an endoplasmic reticulum Ca^{2+} pump inhibitor (4, 29), induced a $[Ca^{2+}]_i$ rise of 51 ± 2 nM (n = 3). Subsequently added NPC-14686 at 500 sec failed to cause any $[Ca^{2+}]_i$ rises. Conversely, Fig. 3B shows that after incubation with 200 μM NPC-14686 for 470 sec, addition of BHQ also failed to induce a $[Ca^{2+}]_i$ rise (n = 3). Thapsigargin, another endoplasmic reticulum Ca^{2+} pump inhibitor (TG, 1 μM) (4, 23) was used to confirm the BHQ data. Fig. 4C shows that thapsigargin induced a $[Ca^{2+}]_i$ rise of 110 ± 2 nM (n = 3). Subsequently added NPC-14686 at 500 sec failed to induce a $[Ca^{2+}]_i$ rise. Fig. 4B shows that thapsigargin failed to induce a $[Ca^{2+}]_i$ rise after NPC-14686 incubation for 470 sec (n = 3).

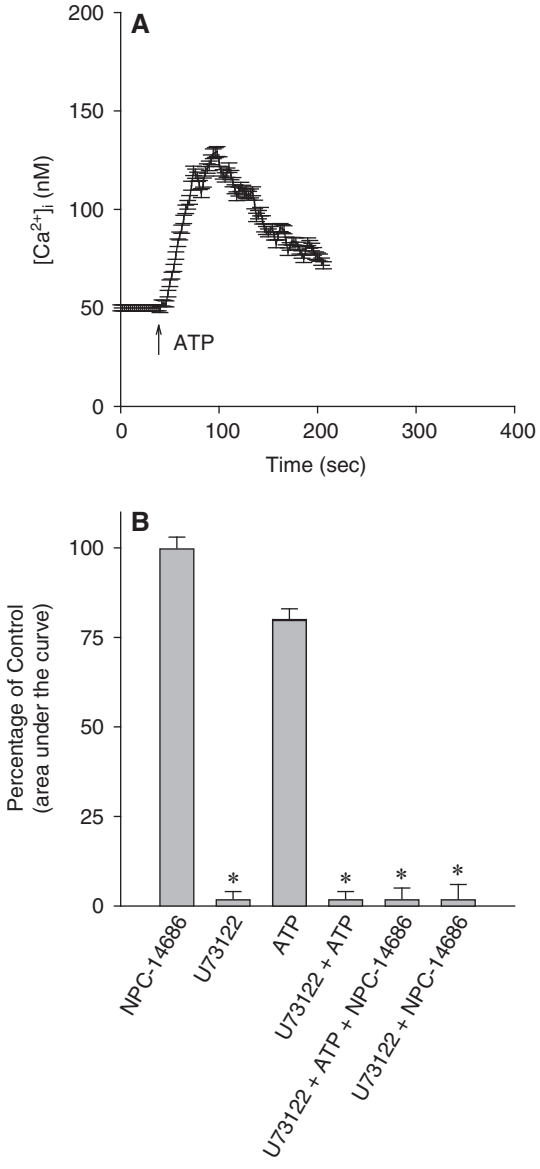

Fig. 5. Effect of U73122 on NPC-14686-induced Ca^{2+} release. Experiments were performed in Ca^{2+}-free medium. (A) ATP (10 μM) was added as indicated. (B) U73122 (2 μM), ATP (10 μM), and NPC-14686 (200 μM) were added as indicated. Data are mean ± SEM of three separate experiments. *$P < 0.05$ compared to first bar (control). Control is the area under the curve of 200 μM NPC-14686-induced $[Ca^{2+}]_i$ rises (25-250 sec).

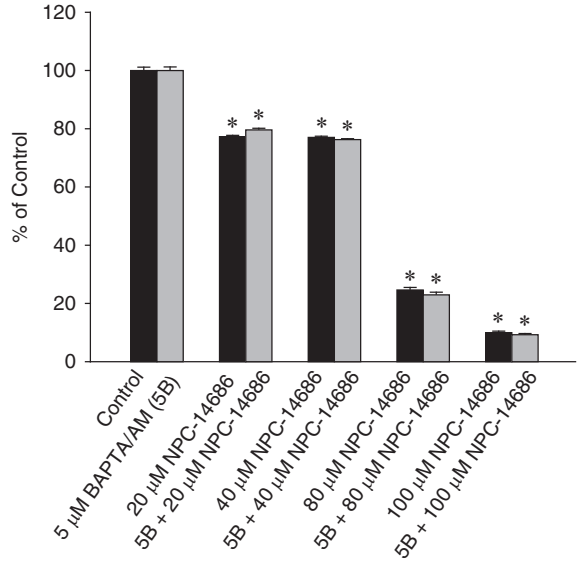

Fig. 6. Effect of NPC-14686 on viability of OC2 cells. Cells were treated with 0-100 μM NPC-14686 for 24 h, and the cell viability assay was performed. Data are mean ± SEM of three separate experiments. Each treatment had six replicates (wells). Data are expressed as percentage of control that is the increase in cell numbers in NPC-14686-free groups. Control had 10,125 ± 789 cells/well before experiments, and had 13,987 ± 788 cells/well after incubation for 24 h. *$P < 0.05$ compared to control. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with NPC-14686. Cell viability assay was subsequently performed.

NPC-14686 Induced $[Ca^{2+}]_i$ Rises via *a Phospholipase C-Dependent Pathway*

Phospholipase C-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing Ca^{2+} from the endoplasmic reticulum. Because NPC-14686 released Ca^{2+} from the endoplasmic reticulum, the role of phospholipase C in this event was examined. U73122 (24), a phospholipase C inhibitor, was used to explore if the activation of this enzyme was necessary for NPC-14686-induced Ca^{2+} release. Fig. 5A shows that ATP (10 μM) induced a $[Ca^{2+}]_i$ rise of 91 ± 2 nM (n = 3). ATP is a phospholipase C-dependent agonist of $[Ca^{2+}]_i$ rises in most cell types including oral cancer cells (3, 18). Fig. 5B shows that incubation with 2 μM U73122 did not change basal $[Ca^{2+}]_i$ but abolished ATP-induced $[Ca^{2+}]_i$ rises (n = 3). This suggests that U73122 effectively suppressed phospholipase C activity. Fig. 5B also shows that incubation with 2 μM U73122 did not alter basal $[Ca^{2+}]_i$ but abolished NPC-14686-induced $[Ca^{2+}]_i$ rises (n = 3). U73343 (2 μM), a U73122 analogue, failed to have an inhibition (not shown).

NPC-14686 Caused Cytotoxicity in OC2 Cells

Given that acute incubation with NPC-14686 induced a substantial and lasting $[Ca^{2+}]_i$ rise, and that unregulated $[Ca^{2+}]_i$ rises often alter cell viability (6), experiments were performed to examine the effect of NPC-14686 on cell viability. Cells were treated with 0-100 μM NPC-14686 for 24 h, and the tetrazolium assay was performed. In the presence of 20-100 μM NPC-14686, cell viability decreased by 20-90% (n = 3) (Fig. 6).

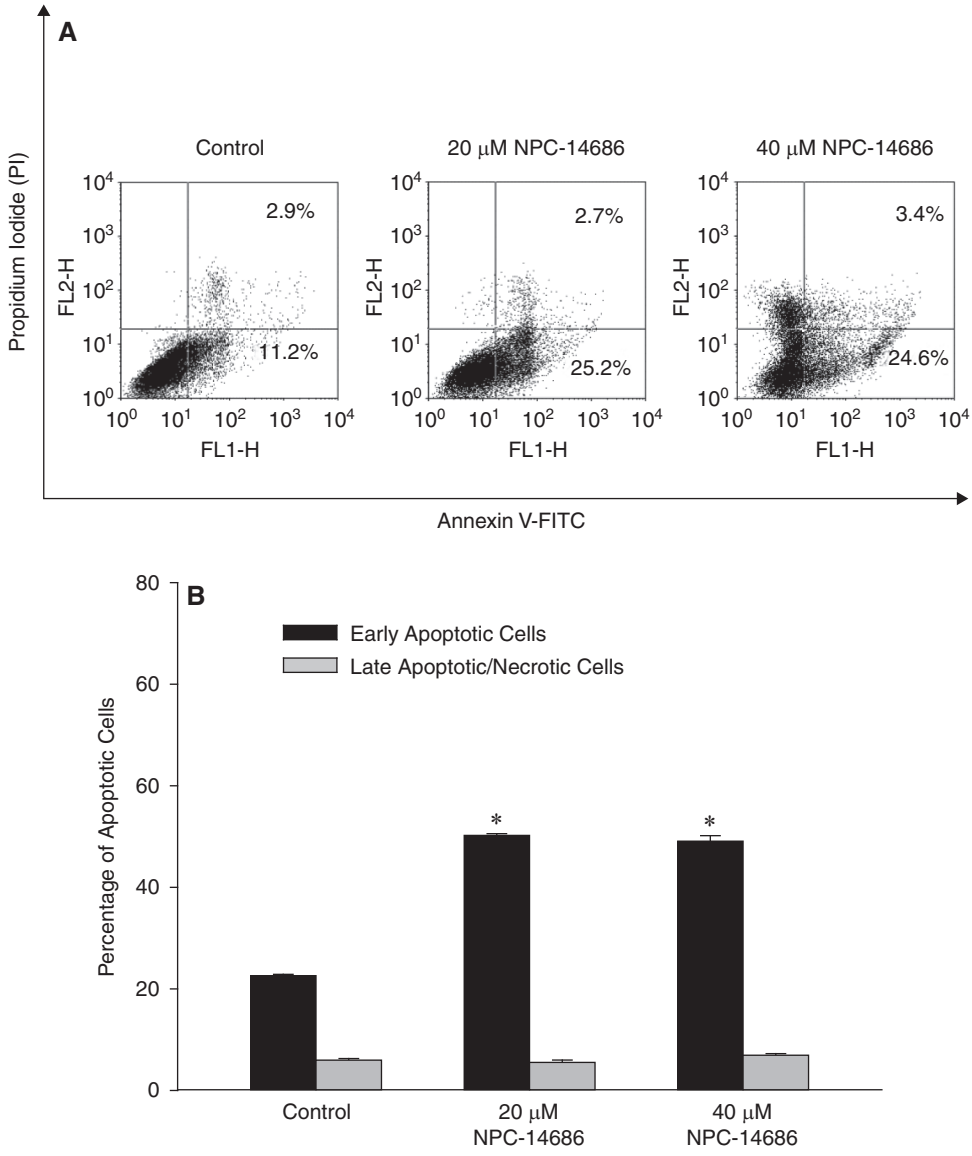

Fig. 7. NPC-14686-induced apoptosis/necrosis as measured by Annexin V/PI staining. (A) Cells were treated with 0-40 μM NPC-14686, respectively, for 24 h. Cells were then processed for Annexin V/PI staining and analyzed by flow cytometry. (B) The percentage of early apoptotic cells and late apoptotic/necrotic cells. In each group, the Ca^{2+} chelator BAPTA/AM (5 μM) was added to cells followed by treatment with NPC-14686. *$P < 0.05$ compared to control. Data are mean ± SEM of three separate experiments.

NPC-14686 Caused Ca^{2+}-Independent Cell Death in OC2 Cells

The next question was whether the NPC-14686-induced cell death was caused by a preceding $[Ca^{2+}]_i$ rise. The intracellular Ca^{2+} chelator BAPTA/AM (5 μM) (4, 28) was used to prevent a $[Ca^{2+}]_i$ rise during NPC-14686 treatment. Fig. 6 also shows that 5 μM BAPTA/AM loading did not alter the control value of cell viability. 5 μM BAPTA/AM loading for 25 h abolished 200 μM NPC-14686-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium (data not shown). This suggests that BAPTA loading still effectively chelated cytosolic Ca^{2+} after such a long time. In the presence of 20-100 μM NPC-14686, BAPTA loading for 25 h did not reverse NPC-14686-induced cell death (n = 3).

NPC-14686 Induced Apoptosis in OC2 Cells

Because the cytotoxic response was most significant between 20 and 40 μM NPC-14686, these concentrations were chosen for apoptotic experiments. Annexin V/PI staining was applied to detect apoptotic cells after NPC-14686 treatment. Fig. 7 shows that treatment with 20 and 40 μM NPC-14686 significantly induced apoptosis (n = 3).

Discussion

Our study shows that the anti-inflammatory drug NPC-14686 induced $[Ca^{2+}]_i$ rises in human oral cancer cells. NPC-14686 increased $[Ca^{2+}]_i$ mainly by depleting intracellular Ca^{2+} stores. A minor contribution was from Ca^{2+} entry because removing extracellular Ca^{2+} reduced only 10% of NPC-14686-induced $[Ca^{2+}]_i$ rises.

The mechanism of NPC-14686-induced Ca^{2+} entry was explored. Because NPC-14686-induced $[Ca^{2+}]_i$ rises were inhibited by nifedipine, econazole and SKF96365, it suggests that NPC-14686 might cause Ca^{2+} entry *via* store-operated Ca^{2+} entry pathway. Consistently, the $[Ca^{2+}]_i$ rise inhibited by these compounds (10%) corresponded to NPC-14686-induced Ca^{2+} influx. These three compounds have often been applied as blockers of store-operated Ca^{2+} entry in different cell types (7, 22, 30). The activity of many protein kinases is directly or indirectly coupled to Ca^{2+} (5, 17). Our data show that NPC-14686-induced $[Ca^{2+}]_i$ rises were decreased when protein kinase C activity was inhibited. In contrast, activation of protein kinase C did not enhance the $[Ca^{2+}]_i$ rise. This might be because the 200 μM NPC-14686-induced $[Ca^{2+}]_i$ rises had reached its maximum level. In previous studies, the protein kinase C inhibitor GF109203X increased the store-operated Ca^{2+} entry induced by phosphorylation of Orai1 in HEK 293 human embryonic kidney cells (15). However, in our studies, NPC-14686-induced $[Ca^{2+}]_i$ rises were inhibited by GF109203X in OC2 human oral cancer cells. Therefore, the effect of protein kinase C on store-operated Ca^{2+} channels varied between HEK cells and OC2 cells.

In terms of the Ca^{2+} stores involved in NPC-14686-induced Ca^{2+} release, the thapsigargin/BHQ-sensitive endoplasmic reticulum stores appear to be the dominant one. The data further show that the Ca^{2+} release was solely *via* a phospholipase C-dependent mechanism, since the release was abolished when phospholipase C activity was inhibited by U73122.

In previous studies, NPC-14686 at concentrations between 10 and 200 μM induced concentration-dependent $[Ca^{2+}]_i$ rises in human HA22T hepatoma cancer cells (13) and PC3 prostate cancer cells (12). In contrast, NPC-14686 between 100-200 μM induced concentration-dependent $[Ca^{2+}]_i$ rises in OC2 human oral cancer cells. In terms of phospholipase C involved in NPC-14686-induced Ca^{2+} release, U73122 did not inhibit NPC-14686-induced Ca^{2+} release in HA22T cells (13) and PC3 cells (12), but abolished it in OC2 cells. Phospholipase C triggers hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP_3 and diacylglycerol (DAG). The increased DAG concentration leads to the activation of protein kinase C while IP_3 binds to the IP_3 receptor (IP_3R), an intracellular Ca^{2+}-release channel located in the endoplasmic reticulum, thereby inducing Ca^{2+} release from internal stores. Since U73122 inhibited phospholipase C in MG63 and OC2 cells, it suggests that NPC-14686-induced Ca^{2+} release is *via* a phospholipase C-dependent pathway in these two cells, but not in HA22T and PC3 cells. Therefore, the effect of NPC-14686 on phospholipase C pathway appears to vary among different cell types.

Previous studies showed that store-operated Ca^{2+} influx inhibitors inhibited 200 μM NPC-14686-induced $[Ca^{2+}]_i$ rises by 25 ± 5% in HA22T cells (13). In OC2 cells, these inhibitors only inhibited 200 μM NPC-14686-induced $[Ca^{2+}]_i$ rises by 10 ± 3%. Thus the mechanisms underlying NPC-14686-induced $[Ca^{2+}]_i$ rises in OC2 cells and other cells are different.

Cell death could be Ca^{2+}-dependent or independent, depending on cell type and the stimulant (8, 19, 20). Our data show that NPC-14686-induced cell death was Ca^{2+}-independent. NPC-14686-induced cell death appeared to involve apoptosis. In this study, treatment with 20 μM and 40 μM NPC-14686 induced apoptosis. Therefore it appears that the concentration range needed for NPC-14686 to induce apoptosis was similar in these two cell types.

In our studies, $[Ca^{2+}]_i$ measurements and viability were two totally different assays. $[Ca^{2+}]_i$ measurements were conducted online and terminated within 4-15 min. After 20 min incubation with NPC-14686, cell viability was still >95%. In contrast, in viability assays, cells were treated with NPC-14686 overnight in order to obtain measurable changes in viability. This is why 100 μM NPC-14686 decreased cell viability by approximately 85% whereas 200 μM NPC-14686 did not alter viability in $[Ca^{2+}]_i$ measurements.

NPC-14686 has not been tested in patients, thus the achievable plasma level is unknown. Collectively, the results show that NPC-14686 induced Ca^{2+} release from endoplasmic reticulum in a phospholipase C-dependent manner and also caused Ca^{2+} influx *via* protein kinase C-sensitive store-operated Ca^{2+} entry in OC2 human oral cancer cells. NPC-14686 also induced apoptosis in a Ca^{2+}-independent manner. Given the importance of Ca^{2+} handling and apoptosis in cell function, caution should be exercised in using NPC-14686 in other *in vitro* or *in vivo* studies.

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References

1. Bootman, M.D., Berridge, M.J. and Roderick, H.L. Calcium sig-

nalling: more messengers, more channels, more complexity. *Curr. Biol.* 12: R563-R565, 2003.
2. Burch, R.M., Weitzberg, M., Blok, N., Muhlhauser, R., Martin, D., Farmer, S.G., Bator, J.M., Connor, J.R., Green, M. and Ko, C. N-(Fluorenyl-9-methoxycarbonyl) amino acids, a class of anti-inflammatory agents with a different mechanism of action. *Proc. Natl. Acad. Sci. USA* 88: 2612-2617, 1991.
3. Burnstock, G. and Di Virgilio, F. Purinergic signalling and cancer. *Purinergic Signal*. 9: 491-540, 2013.
4. Chang, K.H., Tan, H.P., Kuo, C.C., Kuo, D.H., Shieh, P., Chen, F.A. and Jan, C.R. Effect of nortriptyline on Ca^{2+} handling in SIRC rabbit corneal epithelial cells. *Chinese J. Physiol.* 53: 178-184, 2010.
5. Chu, Y., Fioravante, D., Thanawala, M., Leitges, M. and Regehr, W.G. Calcium-dependent isoforms of protein kinase C mediate glycine-induced synaptic enhancement at the calyx of held. *J. Neurosci.* 32: 13796-13804, 2012.
6. Clapham, D.E. Calcium signaling. *Cell* 131: 1047-1058, 2007.
7. Geng, S., Gao, Y.D., Yang, J., Zou, J.J. and Guo, W. Potential role of store-operated Ca^{2+} entry in Th_2 response induced by histamine in human monocyte-derived dendritic cells. *Int. Immunopharmacol.* 12: 358-367, 2012.
8. Giorgi, C., Romagnoli, A., Pinton, P. and Rizzuto, R. Ca^{2+} signaling, mitochondria and cell death. *Curr. Mol. Med.* 8: 119-130, 2008.
9. Glinsky, G.V. Apoptosis in metastatic cancer cells. *Crit. Rev. Oncol. Hematol.* 25: 175-186, 1997.
10. Grynkiewicz, G., Poenie, M. and Tsien, R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J. Biol. Chem.* 260: 3440-3450, 1985.
11. Huang, C.C., Huang, C.J., Cheng, J.S., Liu, S.I., Chen, I.S., Tsai, J.Y., Chou, C.T., Tseng, P.L. and Jan, C.R. Fendiline-evoked $[Ca^{2+}]_i$ rises and non-Ca^{2+}-triggered cell death in human oral cancer cells. *Hum. Exp. Toxicol.* 28: 41-48, 2009.
12. Huang, J.K., Liu, C.S., Chen, W.C., Liu, S.I., Hsu, S.S., Chang, H.T., Chou, C.T., Hsieh, C.H., Chang, C.H. and Jan, C.R. NPC-14686 (Fmoc-L-homophenylalanine)-induced Ca^{2+} increases and death in human prostate cancer cells. *Life Sci*. 78: 964-969, 2006.
13. Jan, C.R., Kuo, S.Y., Cheng, J.S., Lo, Y.K., Liu, C.P. and Chen, W.C. Effect of NPC-14686 (Fmoc-L-homophenylalanine) on intracellular Ca^{2+} levels in human hepatoma cells. *Life Sci.* 72: 2571-2580, 2003.
14. Janne, P.A. and Mayer, R.J. Chemoprevention of colorectal cancer. *N. Engl. J. Med.* 342: 1960-1968, 2000.
15. Kawasaki, T., Ueyama, T., Lange, I., Feske, S. and Saito, N. Protein kinase C-induced phosphorylation of Orai1 regulates the intracellular Ca^{2+} level *via* the store-operated Ca^{2+} channel. *J. Biol. Chem*. 285: 25720-25730, 2010.
16. Kuo, L.N., Huang, C.J., Fang, Y.C., Huang, C.C., Wang, J.L., Lin, K.L., Chu, S.T., Chang, H.T., Chien, J.M., Su, H.H., Chi, C.C., Chen, W.C., Tsai, J.Y., Liao, W.C., Tseng, L.L. and Jan, C.R. Effect of thimerosal on Ca^{2+} movement and viability in human oral cancer cells. *Hum. Exp. Toxicol.* 28: 301-308, 2009.
17. Mai, Y., Higashi, T., Terada, K., Hatate, C., Nepal, P., Horiguchi, M., Harada, T., Miwa, S. and Horinouchi, T. Nicotine- and tar-free cigarette smoke extract induces cell injury *via* intracellular Ca^{2+}-dependent subtype-specific protein kinase C activation. *J. Pharmacol. Sci.* 120: 310-314, 2012.
18. Nagamine, K., Ozaki, N., Shinoda, M., Asai, H., Nishiguchi, H., Mitsudo, K., Tohnai, I., Ueda, M. and Sugiura, Y. Mechanical allodynia and thermal hyperalgesia induced by experimental squamous cell carcinoma of the lower gingiva in rats. *J. Pain* 7: 659-670, 2006.
19. Nicotera, T.M., Schuster, D.P., Bourhim, M., Chadha, K., Klaich, G. and Corral, D.A. Regulation of PSA secretion and survival signaling by calcium-independent phopholipase $A_2\beta$ in prostate cancer cells. *Prostate* 69: 1270-1280, 2009.
20. Song, Y., Wilkins, P., Hu, W., Murthy, K.S., Chen, J., Lee, Z., Oyesanya, R., Wu, J., Barbour, S.E. and Fang, X. Inhibition of calcium-independent phospholipase A_2 suppresses proliferation and tumorigenicity of ovarian carcinoma cells. *Biochem. J.* 406: 427-436, 2007.
21. Taketo, M. Cyclooxygenase-2 inhibitors in tumorigenesis (part II). *J. Natl. Cancer Inst.* 90: 1609-1620, 1998.
22. Thakur, P., Dadsetan, S. and Fomina, A.F. Bidirectional coupling between ryanodine receptors and Ca^{2+} release-activated Ca^{2+} (CRAC) channel machinery sustains store-operated Ca^{2+} entry in human T lymphocytes. *J. Biol. Chem.* 287: 37233-37244, 2012.
23. Thastrup, O., Cullen, P.J., Drøbak, B.K., Hanley, M.R. and Dawson, A.P. Thapsigargin, a tumor promoter, discharges intracellular Ca^{2+} stores by specific inhibition of the endoplasmic reticulum Ca^{2+}-ATPase. *Proc. Natl. Acad. Sci. USA* 87: 2466-2470, 1990.
24. Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J.E. and Fisher, S.K. The aminosteroid U-73122 inhibits muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. A role for Gp in receptor compartmentation. *J. Biol. Chem.* 266: 23856-23862, 1991.
25. Thun, M.J., Henley, S.J. and Patrono, C. Nonsteroidal anti-inflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. *J. Natl. Cancer Inst.* 94: 252-266, 2002.
26. Thun, M.J., Namboodiri, M.M. and Heath, C.W. Jr. Aspirin use and reduced risk of fatal colon cancer. *N. Engl. J. Med.* 325: 1593-1596, 1991.
27. Tseng, L.L., Shu, S.S., Kuo, C.C., Chou, C.T., Hsieh, Y.D., Chu, S.T., Chi, C.C., Liang, W.Z., Ho, C.M. and Jan, C.R. Effect of methoxychlor on Ca^{2+} handling and viability in OC2 human oral cancer cells. *Basic Clin. Pharmacol. Toxicol.* 108: 341-348, 2011.
28. Tsien, R.Y. New calcium indicators and buffers with high selectivity against magnesium and protons: design, synthesis, and properties of prototype structures. *Biochemistry* 19: 2396-2404, 1980.
29. Wassenberg, J.J., Clark, K.D. and Nelson, D.L. Effect of SERCA pump inhibitors on chemoresponses in *Paramecium*. *J. Eukaryotic Microbiol.* 44: 574-581, 1997.
30. Zou, W., Meng, X., Cai, C., Zou, M., Tang, S., Chu, X., Wang, X. and Zou, F. Store-operated Ca^{2+} entry (SOCE) plays a role in the polarization of neutrophil-like HL-60 cells by regulating the activation of Akt, Src, and Rho family GTPases. *Cell. Physiol. Biochem.* 30: 221-237, 2012.