The Mechanism of NPC-14686-Induced $[\text{Ca}^{2+}]_i$ Rises and Non-$\text{Ca}^{2+}$-Triggered Cell Death in MG63 Human Osteosarcoma Cells

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Abstract

NPC-14686 has been shown to have anti-inflammatory effect in previous studies, but the mechanisms are unclear. The effect of NPC-14686 on cytosolic $\text{Ca}^{2+}$ concentrations ($[\text{Ca}^{2+}]_i$) and viability in MG63 human osteosarcoma cells was explored. The $\text{Ca}^{2+}$-sensitive fluorescent dye fura-2 was applied to measure $[\text{Ca}^{2+}]_i$. NPC-14686 at concentrations of 100-500 µM induced a $[\text{Ca}^{2+}]_i$ rise in a concentration-dependent manner. The response was reduced by 80% by removing $\text{Ca}^{2+}$. NPC-14686 induced Mn$^{2+}$ influx leading to quenching of fura-2 fluorescence. NPC-14686-evoked $\text{Ca}^{2+}$ entry was suppressed by nifedipine, econazole, SK&F96365, and protein kinase C inhibitor. Inhibition of phospholipase C with U73122 abolished NPC-14686-induced $[\text{Ca}^{2+}]_i$ rise. At 20-50 µM, NPC-14686 decreased cell viability, which was not reversed by chelating cytosolic $\text{Ca}^{2+}$ with 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid/acetoxy methyl (BAPTA/AM). Annexin V/propidium iodide staining data suggest that NPC-14686 (30-50 µM) induced apoptosis in a concentration-dependent manner. NPC-14686 also increased levels of reactive oxygen species. Together, in human osteosarcoma cells, NPC-14686 induced a $[\text{Ca}^{2+}]_i$ rise by inducing phospholipase C-dependent $\text{Ca}^{2+}$ release from the endoplasmic reticulum and $\text{Ca}^{2+}$ entry via protein kinase C-sensitive store-operated $\text{Ca}^{2+}$ channels. NPC-14686 induced cell death that might involve apoptosis via mitochondrial pathways.

Key Words: apoptosis, $\text{Ca}^{2+}$, human osteosarcoma cells, NPC-14686

Introduction

Burch et al. (2) reported in vivo evidence that N-(fluorenly-9-methoxycarbonyl) amino acids blocked antigen arthritis in rabbits and reversed oxazolone edema. NPC-14686 (Fmoc-L-homophenylalanine) is one of these compounds and has been shown to prevent gastric mucosal lesions in rats (20). Previous
studies showed that NPC-14686 induced intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) rises and caused cell death in different cancer cells such as human prostate cancer cells (11) and human hepatoma cells (13). Collectively, NPC-14686 caused cytotoxicity in different cancer cells, but the underlying mechanisms are not clear.

Ca\textsuperscript{2+} ions play a crucial role in different biological responses. A rise in [Ca\textsuperscript{2+}], can initiate many pathophysiological cellular processes such as secretion, protein expression, proliferation, contraction, etc. However, an unregulated [Ca\textsuperscript{2+}] rise may cause cell death via apoptosis (1, 8). Previous studies showed that NPC-14686 increased intracellular free Ca\textsuperscript{2+} levels in human hepatoma cells (13) and canine renal tubular cells (14), and caused cell death in human prostate cancer cells (11). Although NPC-14686 induced [Ca\textsuperscript{2+}], rises and caused cytotoxicity in different cancer cells, whether NPC-14686 could increase [Ca\textsuperscript{2+}], and evoke death in osteosarcoma cells is unknown. MG63 human osteosarcoma cell is a good model for research on osteoblasts. Several chemicals have been shown to induce [Ca\textsuperscript{2+}] rises and alter cell viability in this cell, such as 3,3’-diindolylmethane (18) and thymol (4). Furthermore, NPC-14686 may have potential anti-inflammatory or cytotoxic effect on MG63 cells. Therefore, this study explored the effect of NPC-14686 on [Ca\textsuperscript{2+}], and viability in MG63 cells.

Fura-2 was used as a fluorescent Ca\textsuperscript{2+}-sensitive dye to measure [Ca\textsuperscript{2+}], changes in the present study. NPC-14686-induced both Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} release in MG63 cells were explored. The [Ca\textsuperscript{2+}], rise was characterized, the concentration-response plots were established, and the pathways underlying NPC-14686-evoked Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+} release were explored. The effect of NPC-14686 on cell viability was also investigated.

**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). All other reagents were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise indicated.

**Cell Culture**

MG63 human osteosarcoma cells obtained from Bioresource Collection and Research Center (Taiwan, ROC) were cultured in modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

**Solutions Used in [Ca\textsuperscript{2+}], Measurements**

Ca\textsuperscript{2+}-containing medium (pH 7.4) had 140 mM NaCl, 5 mM KCl, 1 mM MgCl\textsubscript{2}, 2 mM CaCl\textsubscript{2}, 10 mM Heps, and 5 mM glucose. Ca\textsuperscript{2+}-free medium contained similar chemicals as Ca\textsuperscript{2+}-containing medium except that CaCl\textsubscript{2} was replaced with 0.3 mM EGTA and 2 mM MgCl\textsubscript{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 or basal [Ca\textsuperscript{2+}],

**[Ca\textsuperscript{2+}], Measurements**

Confluent cells grown on 6 cm dishes were trypsinized and made into a suspension in culture medium at a density of 10\textsuperscript{6} cells/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\textsuperscript{2+}-containing medium twice and was made into a suspension in Ca\textsuperscript{2+}-containing medium at a density of 10\textsuperscript{7} cells/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\textsuperscript{2+}-containing or Ca\textsuperscript{2+}-free medium, by recording excitation signals at 340 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\textsuperscript{2+}], after completion of the experiments, the detergent Triton X-100 (0.1%) and CaCl\textsubscript{2} (5 mM) were added to the cuvette to obtain the maximal fura-2 fluorescence. Then the Ca\textsuperscript{2+} chelator EGTA (10 mM) was added to chelate Ca\textsuperscript{2+} in the cuvette to obtain the minimal fura-2 fluorescence. Control experiments showed that cells bathed in a cuvette with 100 µM NPC-14686 had a viability of 95% after 20 min of fluorescence measurements. [Ca\textsuperscript{2+}], was calculated as previously described (5-7, 9, 10, 17). Mn\textsuperscript{2+} quenching of fura-2 fluorescence was performed in Ca\textsuperscript{2+}-containing medium containing 50 µM MnCl\textsubscript{2}. MnCl\textsubscript{2} was added to cell suspension in the cuvette 30 sec before the fluorescence recording was started. Data were recorded at excitation signal at 360 nm (Ca\textsuperscript{2+}-insensitive) and emission signal at 510 nm at 1-sec intervals as described...
Cell Viability Assays

The measurement of cell viability was based on the ability of cells to cleave tetrazolium salts by dehydrogenases. Augmentation in the amount of developed color directly correlated with the number of live cells. Assays were performed according to manufacturer’s instructions specifically designed for this assay (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-lodophenyl]-2(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 ($A_{570}$) 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 ®Flour 488 Annexin V/Propidium Iodide (PI) Staining for Apoptosis

Annexin V/PI staining assay was employed to further detect cells in early apoptotic and late apoptotic/necrotic stages. Cells were exposed to NPC-14686 at concentrations of 0, 30, 40 µM or 50 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 HEPES, 140 mM of NaCl, 2.5 mM of CaCl$_2$ (pH 7.4). Alexa Fluor 488 annexin V/PI staining solution (Probes Invitrogen, Eugene, OR, 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 nm and 575 nm band pass filters, respectively. A total of 50,000 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/necrosis 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.

Detection of Intracellular ROS by Flow Cytometry

Cells were plated in triplicate at a density of 2 × 10$^4$ cells/well in 6-well plates (Falcon, BD Biosciences, Franklin Lakes, NJ, USA). After overnight incubation, cells were treated with 0, 30, 40 or 50 µM NPC-14686 for 24 h. Cells were harvested, washed twice with cold PBS, and then dichlorofluorescein diacetate (DCFH-DA) and dihydroethidine (DHE) were added at a final concentration of 50 µg/ml in Ca$^{2+}$-containing medium. Cells were incubated for 30 min at 37°C. After cells were washed twice with cold PBS, 1 ml cold PBS was added. These two fluorescent probes were commonly used for detection of intracellular oxidants. During an intracellular oxidative burst, ROS species are usually generated, leading to the conversion of the non-fluorescent probes into fluorescent molecules. The oxidation product of DCFH is dichlorofluorescein (DCF), with the green emission at 529 nm, while that of DHE is ethidium, emitting red fluorescence at 590 nm with a FACS Calibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). Data were later analyzed using the flow cytometry analysis software WinMDI 2.8 (by Joe Trotter, freely distributed software) by gating 10$^2$-10$^4$ areas of the X and Y coordinates.

Statistics

Data are reported as mean ± SEM of three 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$^{2+}$],

The effect of NPC-14686 on basal [Ca$^{2+}$]$_i$ was examined. Fig. 1A shows that the basal [Ca$^{2+}$]$_i$ level was 49 ± 2 nM. At concentrations between 100 and 500 µM, NPC-14686 induced a [Ca$^{2+}$]$_i$ rise in a concentration-dependent manner in Ca$^{2+}$-containing medium. At a concentration of 500 µM, NPC-14686 evoked a [Ca$^{2+}$]$_i$ rise that attained to a net increase of 130 ± 2 nM followed by a slow decay. The [Ca$^{2+}$]$_i$ rise saturated at 500 µM NPC-14686 because at a concentration of 750 µM, NPC-14686
Evoked a similar response as that induced by 500 µM.

Fig. 1B shows that in the absence of extracellular Ca²⁺, 500 µM NPC-14686 induced a [Ca²⁺]ᵢ rise of 68 ± 2 nM; and at a concentration of 300 µM, NPC-14686 induced a [Ca²⁺]ᵢ rise of 36 ± 2 nM. Fig. 1C shows the concentration-response plots of NPC-14686-induced responses. The EC₅₀ value was 212 ± 2 µM or 290 ± 4 µM in Ca²⁺-containing medium or Ca²⁺-free medium, respectively, by fitting to a Hill equation.

NPC-14686-Induced Mn²⁺ Influx

Experiments were performed to confirm that NPC-14686-evoked [Ca²⁺]ᵢ rise involved Ca²⁺ influx. Mn²⁺ enters cells through similar mechanisms as Ca²⁺ but quenches fura-2 fluorescence at all excitation wavelengths. Therefore, quenching of fura-2 fluorescence excited at the Ca²⁺-insensitive excitation wavelength of 360 nm by Mn²⁺ implicates Ca²⁺ influx. Fig. 2 shows that 500 µM NPC-14686 evoked an instant decrease in the 360 nm excitation signal by 81 ± 2 (n = 3) arbitrary units. This suggests that Ca²⁺ influx participates in NPC-14686-evoked [Ca²⁺]ᵢ rise.

Pathways of NPC-14686-Induced Ca²⁺ Entry

Experiments were conducted to explore the Ca²⁺ entry pathway of the NPC-14686-induced [Ca²⁺]ᵢ rise.
Three Ca²⁺ entry inhibitors: nifedipine (1 µM), econazole (0.5 µM) and SK&F96365 (5 µM); phorbol 12-myristate 13-acetate (PMA; 1 nM; a protein kinase C activator); and GF109203X (2 µM; a protein kinase C inhibitor) were applied 1 min before NPC-14686. The concentrations of these compounds are widely used in many studies. Except PMA, the other compounds all significantly inhibited NPC-14686-induced [Ca²⁺]_<i> rise (Fig. 3).

**The Role of Phospholipase C (PLC) in NPC-14686-Induced [Ca²⁺]_<i> Rise**

Because PLC-dependent production of inositol 1,4,5-trisphosphate is a key process for releasing Ca²⁺ from the endoplasmic reticulum, the role of PLC in NPC-14686-induced [Ca²⁺]_<i> rise was examined. U73122, a PLC inhibitor (23), was used to see whether the activation of this enzyme was required for NPC-14686-induced Ca²⁺ release. Fig. 4A shows that ATP (10 µM) induced a [Ca²⁺]_<i> rise of 49 ± 2 nM. First, it was needed to assure that U73122 indeed inhibited PLC in our system. ATP is a PLC-dependent agonist of [Ca²⁺]_<i> rise in most cell types. Fig. 4B shows that incubation with 2 µM U73122 did not change basal [Ca²⁺]_<i> but abolished ATP-induced [Ca²⁺]_<i> rise. This suggests that U73122 effectively suppressed PLC activity. Fig. 4B also shows that incubation with 2 µM U73122 did not alter basal [Ca²⁺]_<i>, but abolished NPC-14686-induced [Ca²⁺]_<i> rise. U73343 (2 µM), a U73122 analogue, failed to have an inhibition (not shown).

**Effect of NPC-14686 on Cell Viability**

Given that acute incubation with NPC-14686 induced a substantial and lasting [Ca²⁺]_<i> rise, and that
unregulated \([\text{Ca}^{2+}]\)_i rise often alters cell viability, experiments were performed to examine the effect of NPC-14686 on viability of MG63 cells. Cells were treated with 0-50 \(\mu\text{M}\) NPC-14686 for 24 h, and the tetrazolium assay was performed. In the presence of 30-50 \(\mu\text{M}\) NPC-14686, cell viability decreased in a concentration-dependent manner (Fig. 5). At concentrations \(\geq 100 \mu\text{M}\), NPC-14686 killed all cells (not shown).

**Relationship between NPC-14686-Induced \([\text{Ca}^{2+}]\)_i Rise and Cell Death**

The next issue was whether the NPC-14686-induced cell death was caused by a preceding \([\text{Ca}^{2+}]\)_i rise. The intracellular \(\text{Ca}^{2+}\) chelator BAPTA/AM (24) was used to prevent a \([\text{Ca}^{2+}]\)_i rise during NPC-14686 treatment. Fig. 5A shows that 5 \(\mu\text{M}\) BAPTA/AM loading did not alter the control value of cell viability. BAPTA/AM loading did not reverse NPC-14686-induced cell death. Furthermore, NPC-14686 (50 \(\mu\text{M}\)) did not induce a \([\text{Ca}^{2+}]\)_i rise in BAPTA/AM-treated cells (Fig. 5B). Therefore, NPC-14686-induced cell death is not caused by a preceding rise in \([\text{Ca}^{2+}]\)_i.

**The Role of Apoptosis in NPC-14686-Induced Cell Death**

Annexin V/PI staining was applied to detect apoptotic cells after NPC-14686 treatment. Fig. 6, A and B show that treatment with 30, 40 or 50 \(\mu\text{M}\) NPC-14686 induced apoptosis in a concentration-dependent manner.

**The Role of ROS in NPC-14686-Induced Cell Death**

ROS are involved in the internal pathway of apoptosis. To investigate whether NPC-14686 induced oxidative stress in MG63 cells, the levels of intracellular ROS including hydrogen peroxide (H\(_2\)O\(_2\)) and superoxide anion (O\(_2^{-}\)) in NPC-14686-treated cells were measured by flow cytometry using DCFH-DA and DHE fluorescent dyes, respectively. It was found that 40-50 \(\mu\text{M}\) NPC-14686 treatment increased the intracellular levels of H\(_2\)O\(_2\) (Fig. 7, A and B) and superoxide anion (Fig. 7, C and D) in a concentration-dependent manner.

**Discussion**

Our study is the first to show that NPC-14686 induced a \([\text{Ca}^{2+}]\)_i rise in MG63 osteosarcoma cells. The data show that NPC-14686 induced a concentration-dependent \([\text{Ca}^{2+}]\)_i rise. NPC-14686 increased \([\text{Ca}^{2+}]\)_i, by depleting intracellular \(\text{Ca}^{2+}\) stores and causing...
Fig. 6. NPC-14686-induced apoptosis/necrosis as measured by Annexin V/PI staining. (A) Cells were treated with 0, 30, 40, or 50 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. Data are mean ± SEM of three separate experiments. *P < 0.05 compared to control.

Fig. 7. (A, B) Effect of NPC-14686 on the hydrogen peroxide level. 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescence was measured after treatment with 0, 30, 40 or 50 µM NPC-14686 in serum-free culture media for 24 h. The fluorescence was quantified using the BD Cell Quest software. Data were mean ± SEM of four separate experiments. *P < 0.05 compared to control. (C, D) Effect of NPC-14686 on the superoxide anion level. Dihydroethidine (DHE) fluorescence in cells was measured after treatment with 0, 30, 40 or 50 µM NPC-14686 in serum-free culture media for 24 h. The fluorescence was quantified using the BD Cell Quest software. The data are represented as DCFH-DA (or DHE) fluorescence percentage that refers to cells positive to DCFH-DA (or DHE). Data were mean ± SEM of four separate experiments. *P < 0.05 compared to control.
Ca²⁺ entry from extracellular milieu because removing extracellular Ca²⁺ reduced a part of NPC-14686-induced [Ca²⁺]i rise. Removal of extracellular Ca²⁺ reduced the NPC-14686-induced [Ca²⁺]i rise throughout the measurement period, suggesting that Ca²⁺ entry occurred during the whole stimulation interval (200 sec).

The mechanism of NPC-14686-induced Ca²⁺ influx was explored. The results suggest that NPC-14686 might cause Ca²⁺ entry via stimulating store-operated Ca²⁺ entry which is induced by depletion of intracellular Ca²⁺ stores, based on the inhibition of NPC-14686-induced [Ca²⁺]i rise by nifedipine, econazole and SK&F96365. These three compounds have often been applied as blockers of store-operated Ca²⁺ entry in different cell types (12, 15, 21, 22). Furthermore, the effect of regulation of protein kinase C activity on NPC-14686-induced [Ca²⁺]i rise was explored. Inhibition of protein kinase C inhibited NPC-14686-induced [Ca²⁺]i rise. Protein kinase C is known to associate with Ca²⁺ signaling in many cases (3, 16). It seems that PLC-dependent pathways played a significant role in NPC-14686-induced Ca²⁺ release, since the response was abolished by U73122. Thus it appears that NPC-14686-induced Ca²⁺ release was caused by an IP₃-dependent Ca²⁺ release from the endoplasmic reticulum.

This study also shows that NPC-14686 was cytotoxic to MG63 cells. Ca²⁺ overloading is known to initiate processes leading to alteration in cell viability (1). However, cell viability could be altered in a Ca²⁺-independent manner (4-7). Our data also show that NPC-14686-induced cell death is not caused by a preceding rise in [Ca²⁺]. Furthermore, Annexin/PI staining data suggest that NPC-14686-induced cell death involved apoptosis. The present findings suggest that NPC-14686-induced cell apoptosis might not be mediated through Ca²⁺ signaling pathway in MG63 cells. Note that NPC-14686 started to increase [Ca²⁺]i only at a concentration of 100 µM; whereas at a concentration of 50 µM, NPC-14686 had caused death of 70% of cells. These data are not contradictory because [Ca²⁺]i measurements were completed within 250 sec after addition of NPC-14686; whereas viability was assayed after overnight treatment with NPC-14686.

It is known that apoptosis consists of external and internal pathways. Thus, the role of mitochondria in NPC-14686-induced apoptosis was explored by measuring ROS levels. Our data suggest that NPC-14686 at concentrations that induced [Ca²⁺]i rise also induced ROS production. Thus it is likely that ROS production is involved in NPC-14686-induced apoptosis. This is the first evidence showing that NPC-14686 caused apoptosis and elevated ROS levels in any cell type.

The mechanisms underlying NPC-14686-induced [Ca²⁺]i rise appear to be different among cell types. In prostate cancer cells and renal tubular cells, NPC-14686-induced Ca²⁺ release was not via PLC activation, whereas in osteosarcoma cells PLC was responsible for NPC-14686-induced Ca²⁺ release. Furthermore, the EC₅₀ values were different among cell types. A previous study explored the plasma concentration of NPC-14686 after oral administration. The doses administered were 20 mg/day in healthy rats. No BioResponse (BR) NPC-14686-related adverse effects were reported at doses up to 20 mg. A single 20 mg dose of BR-NPC-14686 resulted in a mean Cmax of ~ 30 µM after 3 h (20). However, in inflammatory rats, the plasma concentration of NPC-14686 after oral administration might be 2-fold higher than in healthy rats. Although there was no evidence showing the effect of NPC-14686 on human plasma level, our study may have clinical relevance in vivo exposures.

Together, our data show that NPC-14686 induced Ca²⁺ release from endoplasmic reticulum in a PLC-dependent manner and also caused Ca²⁺ influx via protein kinase C-dependent store-operated Ca²⁺ entry pathway. NPC-14686 also evoked cell death via Ca²⁺-independent apoptotic pathways that involved ROS. A rise in [Ca²⁺], can lead to numerous Ca²⁺-associated cellular responses, thus the effect of NPC-14686 on Ca²⁺ movement and apoptosis should be considered in other types of in vitro research.

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References


