Effect of [6]-Shogaol on Cytosolic Ca^{2+} Levels and Proliferation in Human Oral Cancer Cells (OC2)

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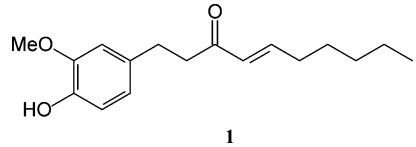
The effect of [6]-shogaol (**1**) on cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_i$) and viability has not been explored previously in oral epithelial cells. The present study has examined whether **1** alters $[Ca^{2+}]_i$ and viability in OC2 human oral cancer cells. Compound **1** at concentrations ≥ 5 μM increased $[Ca^{2+}]_i$ in a concentration-dependent manner with a 50% effective concentration (EC_{50}) value of 65 μM. The Ca^{2+} signal was reduced substantially by removing extracellular Ca^{2+}. In a Ca^{2+}-free medium, the **1**-induced $[Ca^{2+}]_i$ elevation was mostly attenuated by depleting stored Ca^{2+} with thapsigargin (an endoplasmic reticulum Ca^{2+} pump inhibitor). The $[Ca^{2+}]_i$ signal was inhibited by La^{3+} but not by L-type Ca^{2+} channel blockers. The elevation of $[Ca^{2+}]_i$ caused by **1** in a Ca^{2+}-containing medium was not affected by modulation of protein kinase C activity, but was inhibited by 82% with the phospholipase A2 inhibitor aristolochic acid I (20 μM). U73122, a selective inhibitor of phospholipase C, abolished **1**-induced $[Ca^{2+}]_i$ release. At concentrations of 5−100 μM, **1** killed cells in a concentration-dependent manner. These findings suggest that [6]-shogaol induces a significant rise in $[Ca^{2+}]_i$ in oral cancer OC2 cells by causing stored Ca^{2+} release from the thapsigargin-sensitive endoplasmic reticulum pool in an inositol 1,4,5-trisphosphate-dependent manner and by inducing Ca^{2+} influx via a phospholipase A2- and La^{3+}-sensitive pathway.

Recent epidemiological studies have shown that the populations of Southeast Asian countries have much lower risks of colon, gastrointestinal, prostate, breast, and other cancers than their European and American counterparts.[1] It is believed that constituents of the Asian diet such as garlic, ginger, and chillies may play important roles in cancer prevention. Ginger (*Zingiber officinale* Roscoe, Zingiberaceae) has been used widely as a spicy condiment, and as a medicinal herb in traditional medicine.[2] The underground stems or rhizomes of this plant have been utilized in oriental medicine for the treatment of the common cold, disorders of the gastrointestinal tract, neuralgia, rheumatism, colic, and motion discomfort.[3,4] The nonvolatile pungent ingredients from ginger include gingerol, shogaol, and zingerone.[4] Indeed, many phenolic substances present in fruits and vegetables, and in medicinal plants, have potential cancer chemopreventive activities, as supported by both in vitro and in vivo experiments.[4−7] These agents are known to have the ability to suppress the transformative, hyperproliferative, and inflammatory processes of carcinogenesis.

The pungent phenolic constituents derived from ginger possess many interesting pharmacological and physiological activities. Of these substances, 6-shogaol [(*E*)-1-(4-hydroxy-3-methoxyphenyl)-dec-4-en-3-one] (**1**), a major biologically active compound of ginger, has potential antiemetic, anthelmintic, antitussive, and anti-inflammatory activities.[8−10] Evidence reveals that **1** causes apoptotic cell death via an oxidative stress-mediated caspase-dependent mechanism in human hepatoma and colorectal carcinoma cells.[11,12] Moreover, **1** was reported previously to have inhibitory effects on inflammatory nitric oxide synthase, COX-2 gene expression, and NF-kappa B transcription.[13] This compound also induces growth arrest in the mitotic stage and damages microtubules in gastric cancer cells.[14] However, the molecular mechanism underlying the Ca^{2+} signal is still not fully resolved.



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The aim of the present study was to elucidate the effect of [6]-shogaol (**1**) on $[Ca^{2+}]_i$ and cytotoxicity in OC2 human oral cancer cells. OC2 human oral cancer cells have been applied in the in vitro study of oral cells.[15] For example, as using this cell line, orthodontic bonding adhesive was found to be cytotoxic;[16] acetylsalicylic acid-induced apoptosis occurred via activation of p53 signaling;[17] root canal sealers induced cytotoxicity and necrosis;[18] the potential carcinogen, safrole, caused independent $[Ca^{2+}]_i$ increases and cell proliferation;[19] tamoxifen-induced $[Ca^{2+}]_i$ was found to rise, and Ca^{2+}-independent cell death occurred;[20] and the Chinese herb *Antrodia camphorata* induced ERK and JNK MAPK phosphorylation and apoptosis.[21]

Using fura-2 as a fluorescent Ca^{2+} indicator, it is shown herein that [6]-shogaol (**1**) induced a concentration-dependent $[Ca^{2+}]_i$ elevation in both the presence and absence of extracellular Ca^{2+} in human oral cancer OC2 cells. The time-course and the concentration−response relationship, the Ca^{2+} sources of the Ca^{2+} signal, and the role of phospholipases A2/C in the signal were evaluated. Furthermore, the cytotoxic effect of **1** on cell viability was also investigated.

Results and Discussion

[6]-Shogaol (**1**), at concentrations up to 100 μM, increased $[Ca^{2+}]_i$ in a concentration-dependent manner in the presence of extracellular Ca^{2+}. Figure 1A shows a typical recording of the $[Ca^{2+}]_i$ elevation induced by **1**. At a concentration of 0.1 μM, **1** had no effect on $[Ca^{2+}]_i$ (i.e., equivalent to baseline, 0 μM). At a concentration of 100 μM, the $[Ca^{2+}]_i$ rise had a net value of 295 $\pm$ 2 nM at 80 s followed by a gradual decay. Figure 1C (filled circles) shows the concentration−response curve of the **1**-induced response.

Experiments were performed to evaluate the relative contribution of extracellular Ca^{2+} entry and Ca^{2+} release from stores in the [6]-shogaol (**1**) response. Figure 1B shows that removal of extracellular Ca^{2+} largely suppressed the **1**-induced $[Ca^{2+}]_i$ elevation. The concentration−response relationship of **1**-induced $[Ca^{2+}]_i$ elevation in the presence and absence of extracellular Ca^{2+} is shown in Figure 1C. Ca^{2+} removal inhibited the $[Ca^{2+}]_i$ elevation caused by 100 μM **1** by 68% as the maximum value ($n = 5$; $p < 0.05$).

The role of the endoplasmic reticulum Ca^{2+} stores in the observed [6]-shogaol (**1**)-induced $[Ca^{2+}]_i$ elevation was examined because

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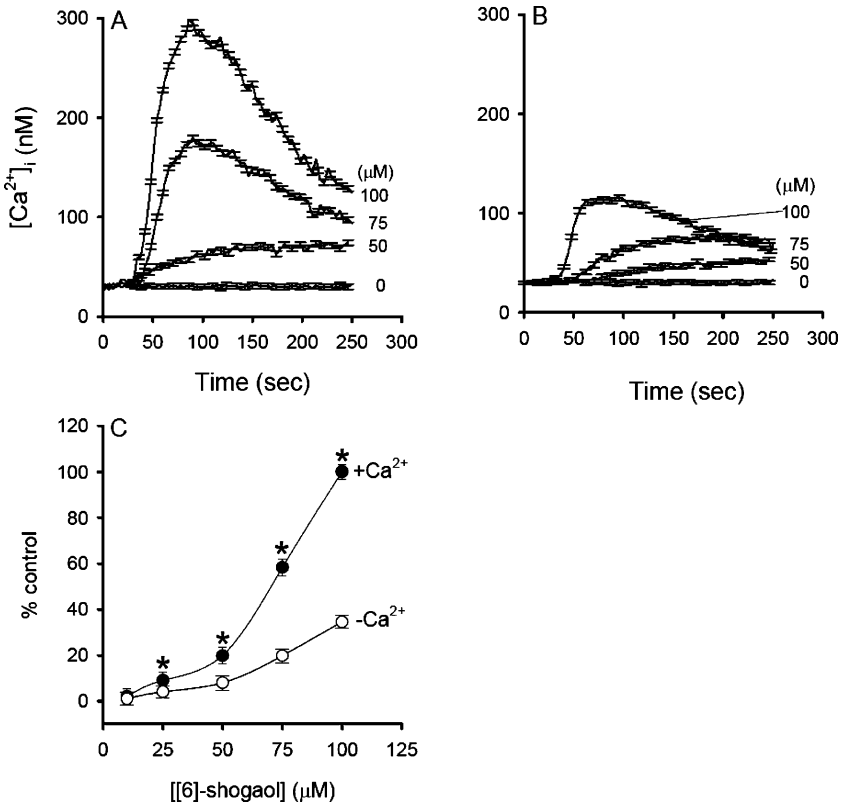

Figure 1. Effects of [6]-shogaol (**1**) on $[Ca^{2+}]_i$ in human oral cancer cells. (A) Concentration-dependent effects of **1**, with the concentration of the reagent indicated. Experiments were performed in Ca^{2+}-containing medium. Compound **1** was added at 30 s and was present throughout the measurements for 250 s. (B) Effect of extracellular Ca^{2+} removal on **1**-induced $[Ca^{2+}]_i$ elevation. The concentration of **1** is indicated. (C) Concentration−response plots of **1**-induced $[Ca^{2+}]_i$ rises in Ca^{2+}-containing medium (filled circles) and Ca^{2+}-free medium (open circles). The data are presented as the percentage of control, which is the net $[Ca^{2+}]_i$ rise induced by 100 μM **1** in Ca^{2+}-containing medium. Data are means ± SEM of five experiments (*$p < 0.05$ compared to open circles).

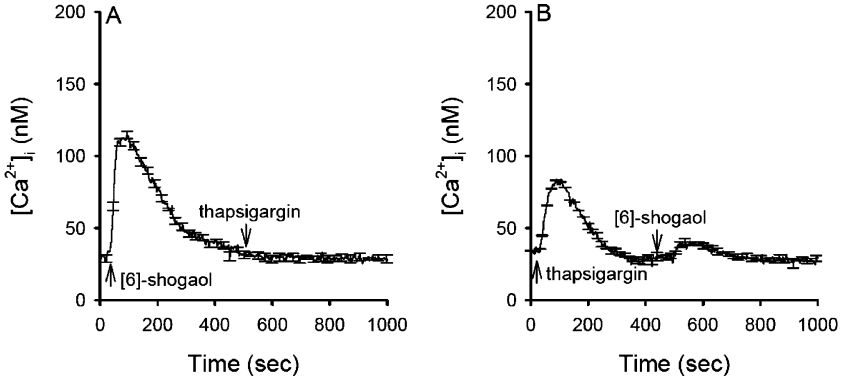

Figure 2. Effect of thapsigargin on [6]-shogaol (**1**)-induced $[Ca^{2+}]_i$ elevation. (A) 1 μM thapsigargin and **1** (100 μM) were added at 30, 250, and 280 s, respectively. (B) ATP (10 μM) was added at 30 s. All experiments were performed in Ca^{2+}-free medium. Data are means ± SEM of five experiments.

previous studies have shown that these stores play a key role in Ca^{2+} release in OC2 cells.[19−21] Figure 2A shows that in a Ca^{2+}-free medium, the addition of 1 μM thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+} pump[22,23] after a 100 μM **1**-induced Ca^{2+} signal, did not induce a $[Ca^{2+}]_i$ rise. Conversely, Figure 2B shows that addition of thapsigargin induced a $[Ca^{2+}]_i$ rise of 84 ± 2 nM. After depletion of Ca^{2+} stores in the endoplasmic reticulum for 470 s, addition of 100 μM **1** barely caused any $[Ca^{2+}]_i$ release.

Experiments were performed to distinguish the pathway underlying [6]-shogaol (**1**)-induced Ca^{2+} influx. Figure 3 shows that pretreatment with the Ca^{2+} entry blocker La^{3+} (10 μM) decreased the **1**-induced $[Ca^{2+}]_i$ (100 μM) elevation to 29% ($n = 5$; $p < 0.05$). At a concentration of 1 μM, diltiazem, nicardipine, nifedipine, and verapamil had no effect ($n = 5$, Figure 3).

To determine if the [6]-shogaol (**1**)-induced Ca^{2+} signal was modulated by protein kinases and phospholipases, H-89 (10 μM), an inhibitor of protein kinase A; aristolochic acid I (20 μM), an inhibitor of phospholipase A2; phorbol 12-myristate 13-acetate (PMA; 1 nM), a protein kinase C activator; and GF109203X (2 μM), a protein kinase C inhibitor, were applied to cells for 1 min before addition of 100 μM **1**. Figure 4A shows that pretreatment of cells with 20 μM aristolochic acid I decreased the **1** (100 μM)-induced $[Ca^{2+}]_i$ elevation to 19% ($n = 5$; $p < 0.05$). However, H-89, PMA, and GF109203X failed to alter the **1**-induced $[Ca^{2+}]_i$ elevation in Ca^{2+}-containing medium (Figure 4B).

Previous studies have shown that in OC2 cells, stored Ca^{2+} can be released by pathways dependent on or independent of phospholipase C activity.[19−21] Thus, an effort was made to explore whether

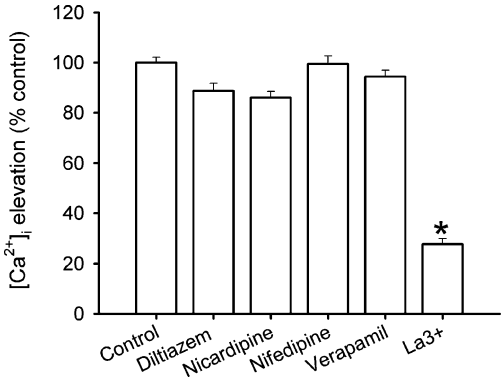

Figure 3. Effect of Ca^{2+} blockers on [6]-shogaol (**1**)-induced $[Ca^{2+}]_i$ elevation. All experiments were performed in Ca^{2+}-containing medium. The concentration was 10 μM for La^{3+} and 1 μM for other channel blockers. The blocker was added 1 min prior to 100 μM **1**. The data are presented as the percentage of the control, which is the net area under the curve (30−250 s) of the $[Ca^{2+}]_i$ rise induced by 100 μM **1**. Data are means ± SEM of five experiments (*$p <$ 0.05 compared to control).

phospholipase C plays a role in shogaol (**1**)-induced Ca^{2+} release. Figure 5A shows that the IP_3-dependent Ca^{2+} mobilizer ATP (10 μM)[24] increased in $[Ca^{2+}]_i$ Ca^{2+}-free medium, suggestive of the occurrence of IP_3-coupled Ca^{2+}-releasing pathways. The effect of inhibiting phospholipase C on the response by **1** was investigated. Figure 5B shows that incubation with the phospholipase C inhibitor U73122 (2 μM) for 190 s did not elevate basal $[Ca^{2+}]_i$, but rather abolished the 10 μM ATP-induced $[Ca^{2+}]_i$ increases ($n = 5$, $p <$ 0.05). Conversely, U73343 (10 μM), an inactive U73122 analogue,[24] did not affect ATP-induced $[Ca^{2+}]_i$ elevation (data not shown). This suggests that U73122 effectively inhibited phospholipase C-coupled IP_3 formation. After U73122 and ATP pretreatment, 100 μM [6]-shogaol failed to increase $[Ca^{2+}]_i$ ($n = 5$). These data suggest that [6]-shogaol (**1**) increases $[Ca^{2+}]_i$ by activation of phospholipase C.

Given that acute incubation with [6]-shogaol (**1**)-induced substantial $[Ca^{2+}]_i$ increases, and that unregulated $[Ca^{2+}]_i$ elevations are often associated with cytotoxicity,[25,26] experiments were performed to examine the effect of [6]-shogaol (**1**) on the viability of OC2 cells. Oral cancer cells were treated with up to 100 μM **1** for 24 h, and the crystal violet method[27] was utilized. Figure 6 shows that 5−100 μM **1** decreased viability in a concentration-dependent manner ($n = 5$; $p < 0.05$).

This study is the first to investigate the effects of [6]-shogaol (**1**) on $[Ca^{2+}]_i$ in OC2 human oral cancer cells. The data obtained suggest that **1** evokes a concentration-dependent $[Ca^{2+}]_i$ elevation in Ca^{2+}-containing medium. The Ca^{2+} signal was contributed to by both Ca^{2+} influx and release from the intracellular Ca^{2+} stores, because removing extracellular Ca^{2+} reduced the $[Ca^{2+}]_i$ signal by 68%. The **1**-induced Ca^{2+} influx seems to be mediated by nonselective Ca^{2+} channels because it was sensitive to La^{3+} but not to L-type Ca^{2+} channels. At 10 μM, La^{3+} blocks most Ca^{2+} channels;[28] and at 1 μM, nifedipine and other structurally similar chemicals effectively block L-type Ca^{2+} channels.[29]

[6]-Shogaol (**1**) and thapsigargin appear to share a common endoplasmic reticulum Ca^{2+} store, because the response to **1** was mostly inhibited by depletion of the endoplasmic reticulum Ca^{2+} store with thapsigargin, and conversely, thapsigargin failed to release additional Ca^{2+} after treatment with **1**. Similar observations were also examined as a result of manipulation by [6]-gingerol, [10]-gingerol, and diallyl sulfide.[30−32] The endoplasmic reticulum is one of the major intracellular Ca^{2+} stores and the organelle where proteins and lipids are synthesized and modified.[33,34] Ca^{2+} dyshomeostasis of the endoplasmic reticulum, protein misfolding, or oxidative stress can lead to endoplasmic reticulum stress-induced cell death.[34,35]

The present data also show that aristolochic acid I, a phospholipase A2 inhibitor,[36] inhibited significantly 100 μM [6]-shogaol (**1**)-induced $[Ca^{2+}]_i$ elevation. Evidence shows that phospholipase A2 activity is associated with Ca^{2+} fluxes. It is reported that activation of this enzyme is required prior to the influx of extracellular Ca^{2+} into the CYP2E1-expressing HepG2 cells.[37] Ca^{2+} overload was also observed by snake phospholipase A2 neurotoxins in nerve terminals of cultured neurons.[38] Most importantly, recent evidence shows that this enzyme controls endothelial store-operated Ca^{2+} entry and vascular tone in intact aorta,[39] and enhances store-operated Ca^{2+} entry in dystrophic skeletal muscle fibers.[40] Additionally, phospholipase A2 mediates store-operated Ca^{2+} entry in rat cerebellar granule cells.[41] Thus, these reports are consistent with the present data that phospholipase A2 activity was required for the [6]-shogaol-induced Ca^{2+} signal in OC2 cells.

Recent evidence shows that phospholipase C (PLC) mediates several biological activities including the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2),[42] the mobilization of Ca^{2+}, and the formation of diacylglycerol, without affecting cAMP levels or the activities of protein kinase C (PKC) in several cell types.[43−45] It is reported that C1P (C_2-ceramide 1-phosphate) enhances calcium entry but is attenuated by the PLC inhibitor U73122 in rat pituitary GH_4C_1 cells.[45] Moreover, PLC-dependent intracellular Ca^{2+} release is required to mediate capsaicin-caused apoptosis in HepG2 cells.[46] This is consistent with our observation that PLC may mediate [6]-shogaol (**1**)-induced intracellular Ca^{2+} mobilization. However, the exact mechanism by which **1** activates PLC is still unclear. This issue remains to be determined in future studies.

[6]-Shogaol (**1**) has been shown to be cytotoxic to several cell lines.[11−13] Data from the present investigation show that in the presence of 10−100 μM (**1**), the viability of OC2 cells decreased in a concentration-dependent manner. Ca^{2+} overloading is known to initiate processes leading to cell death.[47] Additionally, cell death is induced in a Ca^{2+}-dependent[26] or -independent[30] manner, depending on the stimulating agent and cell type. [6]-Shogaol (**1**) is the dehydrated form of [6]-gingerol.[4] In a previous report, [6]-gingerol was found to induce Ca^{2+} mobilization in Madin-Darby canine kidney cells.[32] The $[Ca^{2+}]_i$ elevation induced by [6]-gingerol comprised an immediate rise and a sustained phase in 250 s. In comparison with [6]-gingerol, the $[Ca^{2+}]_i$ elevation induced by **1** reached a maximum value at a time point of 80 s followed by a gradual decline within the 250 s interval. The [6]-gingerol-induced Ca^{2+} influx was blocked by the L-type channel blocker (nicardipine), but not La^{3+}. However, the [6]-gingerol-evoked Ca^{2+} signaling was insensitive to the phospholipase C blocker, U73122. Additionally, [6]-shogaol (**1**) ($IC_{50} = 15.6$ μM) is more cytotoxic than [6]-gingerol ($IC_{50} = 74.2$ μM) for oral cancer OC2 cells (unpublished data). However, both compounds mobilize the thapsigargin-sensitive internal Ca^{2+} pool. These results suggested that even a subtle change in the phenylalkanoid structure of [6]-shogaol (**1**) could have a significant impact on the resultant signaling pathway and antiproliferative activity.

Collectively, the present study shows that in oral cancer OC2 cells, [6]-shogaol (**1**) caused $[Ca^{2+}]_i$ elevations in a concentration-dependent manner by evoking phospholipase C-dependent Ca^{2+} release from the endoplasmic reticulum and also by causing Ca^{2+} influx via phospholipase A2- and La^{3+}-sensitive Ca^{2+} channels. These effects may play a crucial role in the overall physiological action of [6]-shogaol (**1**).

Experimental Section

General Experimental Procedures. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were obtained in MeCN using a JASCO V-530 spectrophotometer. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^1H (400 MHz, using $CDCl_3$ as solvent for measurement), ^{13}C (100 MHz), DEPT,

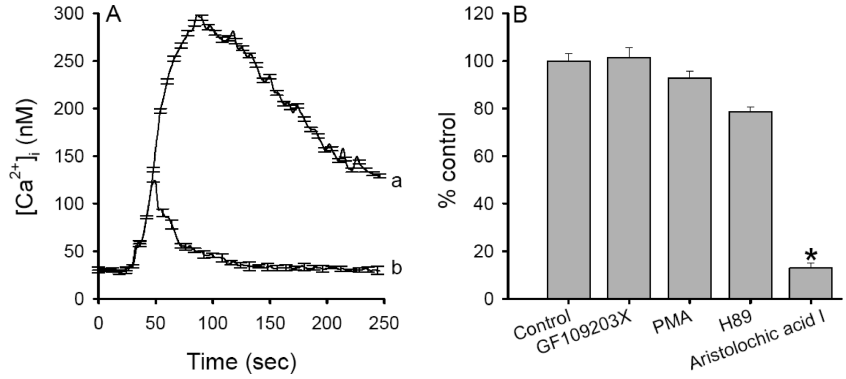

Figure 4. Effect of phospholipase A2 inhibitor and protein kinase C modulators on [6]-shogaol (**1**)-induced $[Ca^{2+}]_i$ elevation. (A) Trace a: **1** (100 μM) was added at 30 s. Trace b: aristolochic acid I (20 μM) was added to cells 1 min before [6]-shogaol. (B) These chemicals were added 1 min prior to **1** (100 μM) treatment in Ca^{2+}-containing medium. The data are presented as percentage of control, which was the $[Ca^{2+}]_i$ elevation induced by 100 μM **1**. Data are means ± SEM of 3−5 replicates.

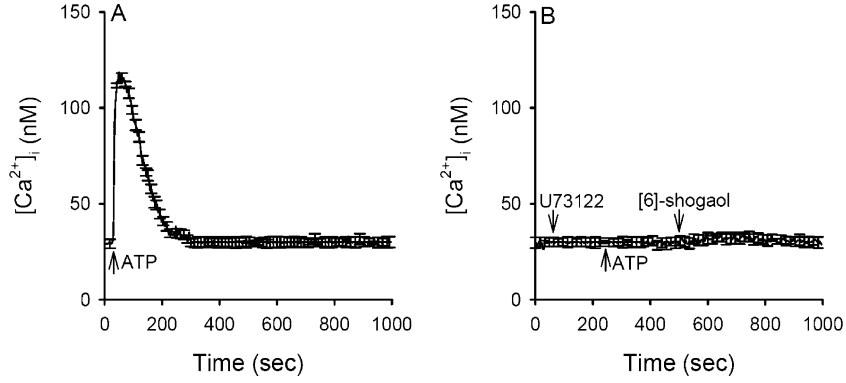

Figure 5. Effect of U73122 on [6]-shogaol (**1**)-induced $[Ca^{2+}]_i$ elevation. (A) ATP (10 μM) was added at 30 s. (B) U73122 (2 μM), ATP (10 μM), and **1** (100 μM) were added at 30, 250, and 280 s, respectively. All experiments were performed in Ca^{2+}-free medium. Data are means ± SEM of five experiments.

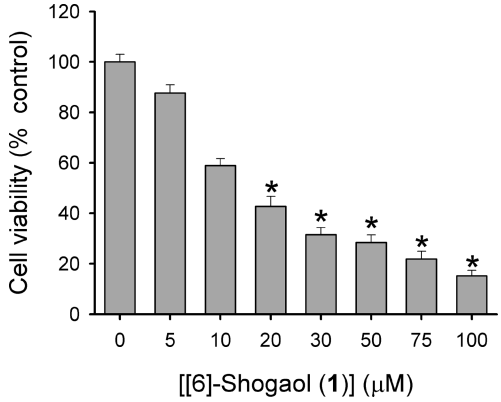

Figure 6. Crystal violet assay of the effect of [6]-shogaol (**1**) on viability of OC2 cells. Cells were treated with 0−100 μM of **1** for 24 h, and the crystal violet elution method was performed. Data are expressed as percentage of control, which is the increase in cell number in **1**-free groups. *$p < 0.05$ compared to control.

HETCOR, COSY, NOESY, and HMBC NMR spectra were obtained on a Unity Plus Varian NMR spectrometer. LRFABMS and LREIMS were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC-MS spectrometer with a direct inlet system. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC, and precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC. Spots were detected by spraying with 50% H_2SO_4 and then heating on a hot plate.

Reagents for cell culture were obtained from Gibco (Gaithersburg, MD). Fura-2/AM was from Molecular Probes (Eugene, OR). U73122 (1-[6-[[(17β)-3- methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione) and U73343 (1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-2,5-pyrrolidinedione) were from Biomol (Plymouth Meeting, PA). Aristolochic acid I and other reagents were obtained from Sigma (St. Louis, MO), and all solvents were of HPLC grade.

Plant Material. The rhizomes of *Zingiber officinale* (ginger) were purchased from a local market in Kaohsiung, Taiwan, in July 2005, and were identified by Dr. Yen-Ray Hsui of the Division of Silviculture, Taiwan Forestry Research Institute, Taipei, Taiwan. A voucher specimen (Hsui-*Zo*-1) was deposited at the Department of Medical Technology, School of Medical and Health Sciences, Fooyin University, Kaohsiung County, Taiwan.

Extraction and Isolation. The rhizomes (4.1 kg) of *Z. officinale* were chipped and air-dried and extracted repeatedly with $CHCl_3$ at room temperature. The combined $CHCl_3$ extracts were then evaporated and further separated into 14 fractions by column chromatography on silica gel with a gradient of *n*-hexane/$CHCl_3$. Fr. 11, eluted with $CHCl_3$−MeOH (40:1), was subjected to silica gel column chromatography [*n*-hexane−acetone (50:1)] and yielded pure [6]-shogaol (**1**, 70 mg), which was identified by spectroscopic data analysis and comparison with literature values.[48] The purity of **1** was >95% as determined by HPLC.

Cell Culture and Test Compound Treatment. OC2 cells were obtained from the American Type Culture Collection. Cells were cultured in RPMI medium. The medium was supplemented with 10% heat-inactivated fetal calf serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were kept at 37 °C in 5% CO_2-containing humidified air.

Measurement of $[Ca^{2+}]_i$. Trypsinized cells (10^6/mL) were allowed to recover in the culture medium for 1 h before being loaded with 2 μM fura-2/AM for 30 min at 25 °C in the same medium. The cells

were washed once with serum-free DMEM medium and resuspended in Ca^{2+}-containing medium (pH 7.4) containing (mM): NaCl, 140; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 2; HEPES, 5; D-glucose, 5. 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 (Kyoto, Japan) by recording the excitation signals at 340 and 380 nm and the emission signal at 510 nm at 1 s intervals. Maximum and minimum fluorescence values were obtained by adding 0.1% Triton X-100 and 10 mM EGTA sequentially at the end of each experiment. $[Ca^{2+}]_i$ was calculated as described previously assuming a K_d of 155 nM.[49] In experiments performed in the absence of extracellular Ca^{2+}, cells were bathed in Ca^{2+}-free medium in which $CaCl_2$ (2 mM) was substituted with 0.1 mM EGTA.

Cell Proliferation Assay. Cytotoxicity tests on OC2 cells were carried out using a crystal violet elution method, as described previously.[27] In brief, the human oral cancer cells were plated at $1 \times l0^4$ in 24-well microtiter plates (Corning Costar Italia, Milan, Italy) and grown at 37 °C overnight in a 5% CO_2 incubator. Twenty-four hours after plating, at 70% confluence and before the cultures became multilayered, the growth medium was removed and replaced with appropriate concentrations of [6]-shogaol (**1**); then cell numbers were counted using the crystal violet elution method. The drugs were dissolved as a stock solution in DMSO and diluted for use [final DMSO concentration less than 0.5% (v/v)]; DMSO at the same concentration was used as the control.

Statistics. Data are presented as means ± standard deviation (SD) and were analyzed using one-way ANOVA with Scheffe's test. A p value of less than 0.05 was considered as statistically significant.

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