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6-Shogaol, an Active Constituent of Dietary Ginger, Induces Autophagy by Inhibiting the AKT/mTOR Pathway in Human Non-Small Cell Lung Cancer A549 Cells

Jen-Yu Hung,[†,‡,§] Ya-Ling Hsu,[†,§] Chien-Te Li,[∥,§] Ying-Chin Ko,[⊥,#,▽] Wen-Chiu Ni,[†] Ming-Shyan Huang,*[,‡,#] and Po-Lin Kuo*[,#,○,z]

[†]Graduate Institute of Medicine, College of Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, [‡]Division of Pulmonary and Critical Care Medicine, Kaohsiung Medical University Hospital, No. 100, Tz-You 1st Road, Kaohsiung, Taiwan, [∥]Division of Chest Medicine, Changhua Christian Hospital, No. 135, Nanxiao Street, Changhua 500, Taiwan, [⊥]Department of Public Health, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, [#]Center of Excellence for Environmental Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, [▽]Division of Environmental Health and Occupational Medicine, National Health Research Institutes, No. 100, Shih-Chuan 1st Road, Kaohsiung, Taiwan, [○]Institute of Clinical Medicine, College of Medicine, Kaohsiung Medical University, No. 100, Shih-Chuan 1st Road, Kaohsiung 807, Taiwan, and [¶]Department of Medical Research, Kaohsiung Medical University Hospital, No. 100, Tz-You 1st Road, Kaohsiung 807, Taiwan.
[§]These authors contributed equally to this work

This study is the first study to investigate the anticancer effect of 6-shogaol in human non-small cell lung cancer A549 cells. 6-Shogaol inhibited cell proliferation by inducing autophagic cell death, but not, predominantly, apoptosis. Pretreatment of cells with 3-methyladenine (3-MA), an autophagy inhibitor, suppressed 6-shogaol mediated antiproliferation activity, suggesting that induction of autophagy by 6-shogaol is conducive to cell death. We also found that 6-shogaol inhibited survival signaling through the AKT/mTOR signaling pathway by blocking the activation of AKT and downstream targets, including the mammalian target of rapamycin (mTOR), forkhead transcription factors (FKHR) and glycogen synthase kinase-3β (GSK-3β). Phosphorylation of both of mTOR's downstream targets, p70 ribosomal protein S6 kinase (p70S6 kinase) and 4E-BP1, was also diminished. Overexpression of AKT by AKT cDNA transfection decreased 6-shogaol mediated autophagic cell death, supporting inhibition of AKT beneficial to autophagy. Moreover, reduction of AKT expression by siRNA potentiated 6-shogaol's effect, also supporting inhibition of AKT beneficial to autophagy. Taken together, these findings suggest that 6-shogaol may be a promising chemopreventive agent against human non-small cell lung cancer.

KEYWORDS: 6-Shogaol; AKT; mTOR; autophagy; human non-small cell lung cancer

INTRODUCTION

Autophagic cell death is an important physiological process occurring in all eukaryotic cells (*1*, *2*). Autophagic cell death is characterized by massive degradation of cellular contents, including portions of the cytoplasm and intracellular organelles, by means of complicated intracellular membrane/vesicle reorganization and lysosomal hydrolases (*1*−*3*). Autophagic cell death is involved in development and stress responses, and has been observed in several human diseases such as neurodegenerative disease, muscular disorders and pathogen resistance (*2*, *3*). Furthermore, like apoptosis, autophagic cell death is found to be suppressed in malignant tumors and involved in tumorigenesis. A number of studies have reported that autophagy is activated in response to various anticancer therapies (*4*, *5*). Several molecular and cell signaling pathways have been implicated in regulating autophagy, such as BECN1, DAPK (death-associated protein kinase), DRP1 (death-associated related protein kinase 1), MAPK (mitogen-activated kinase) and PI3K-AKT-mTOR (phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin) pathways (*1*, *2*, *6*). However, details of the autophagic cell signaling pathway's mechanisms are poorly understood.

PI3K/AKT signaling has been found to be involved in the survival and proliferation of a variety of tumor cells, including lung cancers (*7*, *8*). Hyperactivation of PI3K/AKT has resulted in altering the response of tumor cells to chemotherapy and irradiation (*9*, *10*). PI3K is a heterodimer of the regulatory p85 and catalytic p110 subunits. A variety of growth factors can activate PI3K through activation of their cognate receptors, leading to the activation of AKT signaling. AKT mediates a variety of

*To whom correspondence should be addressed. M.-S.H.: phone, +886-7-320-8158; fax, +886-7-316-1210; e-mail, shyan310@gmail.com. P.-L.K.: phone, +886-7-312-1101 ext 5528; fax, +886-7-321-0701; e-mail, kuopolin@seed.net.tw.

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biological functions, including glucose uptake, protein synthesis, and inhibition of cell death (*8*). Furthermore, AKT can mediate cell survival and growth by regulating both post-translational mechanisms and gene transcription. AKT activity is regulated by phosphorylation on two regulatory residues, threonine 308 (Thr 308) in the activation loop of the catalytic domain, and serine 473 (Ser 473) in the regulatory domain (*8*). Once activated, AKT promotes cell survival by inhibiting types I (apoptosis) and II (autophagy) cell death by phosphorylation of several signaling proteins, including cAMP-response-element-binding protein, mTOR proteins, forkhead transcription factors (FKHR) and glycogen synthase kinase-3β (GSK-3β) (*1*, *2*, *8*, *11*). mTOR is a serine-threonine kinase that regulates the function of transcriptional regulators p70 ribosomal protein S6 kinase (p70S6 kinase) and 4E-BP1 (*8*, *12*). Many studies have shown that inhibition of AKT and its downtarget mTOR signaling contributes to the initiation of autophagy (*11*). Therefore, mTOR signaling has emerged as an important and attractive therapeutic target for cancer therapy.

Ginger (*Zingiber officinale*) is a well-known plant used in cooking worldwide, and has long been reputed to have medicinal properties. It is an herbaceous, rhizomatous perennial plant widely distributed throughout tropical and the subtropical regions (*13*) and is cultivated on a large scale in Nigeria, India, Bangladesh, Sri Lanka, Taiwan and other East Asian countries (*13*). Zerumbone, an active ingredient in ginger, has anti-growth and anti-inflammatory effects on several human cancer cell lines (*14*, *15*). The pungent vallinoids of ginger, 6-gingerol and 6-paradol, exhibit antiproliferation activity in colon, pancreatic, prostate, gastric and leukemia cancer cells (*15*−*18*). 6-Shogaol has also been shown to exert anti-inflammatory and anti-*Helicobacter pylori* effects (*19*, *20*), and has also been found to induce apoptosis in human colorectal carcinoma cells (*21*). In this study, we examined the cell growth inhibition activity of 6-shogaol on the human non-small cell lung cancer cell line, A549. To establish 6-shogaol's anticancer mechanism, we assayed levels of autophagy-related molecules which are strongly associated with the cell death signal transduction pathway and affect the chemosensitivity of tumor cells to anticancer agents.

MATERIALS AND METHODS

Test Compound and Cell Culture. 6-Shogaol was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), dissolved in dimethyl sulfoxide (DMSO) and stored at −20 °C. The purity was >98%, as assessed by HPLC. Control cultures received the carrier solvent (0.1% DMSO).

A549 (American Type Culture Collection [ATCC] CCL185) was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (GIBCO, Gaithersburg, MD), 10 U/mL of penicillin, 10 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (Life Technologies, Inc., Grand Island, NY). IMR-90 (ATCC CCL-186) fibroblast cells were cultured in minimum essential medium (Eagle) with Earle's balanced salt solutions (BSS), 2 mM L-glutamine, 1.5 mg/mL sodium bicarbonate, 0.1 mM nonessential amino acids, 1.0 mM sodium pyruvate (GIBCO, Gaithersburg, MD), 10 U/mL of penicillin, 10 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, and 10% FCS. Both cell lines were cultured in monolayer cultures at 37 °C and 5% CO_2.

Cell Proliferation and Clonogenic Assay. Inhibition of cell proliferation by 6-shogaol was measured by XTT (sodium 3′-[1-(phenylamino-carbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate) assay. Cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h incubation, the cells were treated with vehicle alone (0.1% DMSO) and 6-shogaol (10, 20, 40, and 80 μM) for 48 h. Fifty microliters of XTT test solution, prepared by mixing 5 mL of XTT-labeling reagent with 100 μL of electron coupling reagent, was then added to each well. Absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm. Data were calculated as the percentage of inhibition using the following formula: inhibition % = [100 − (ODt/ODs) × 100] %. ODt and ODs indicated the optical density of the test substances and the solvent control, respectively.

To determine long-term effects, cells were treated with 6-shogaol at various concentrations for 12 h. After being rinsed with fresh medium, cells were allowed to form colonies for 14 days and then stained with crystal violet (0.4 g/L; Sigma, St. Louis, MO). Clonogenic assay was used to elucidate possible differences in long-term effects of 6-shogaol on human lung cancer cells.

Apoptosis Assay. Cells (1×10^6) were treated with vehicle alone (0.1% DMSO) and 6-shogaol (50 and 100 μM) for 24 and 48 h and then collected by centrifugation. Apoptotic cells were quantitatively assessed by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick endlabeling (TUNEL) method, which examines DNA-strand breaks during apoptosis using the BD ApoAlert DNA Fragmentation Assay Kit. Briefly, cells were incubated with 0, 50, and 100 μM 6-shogaol for the indicated times. The cells were trypsinized, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After being washed, the cells were incubated with the reaction mixture for 60 min at 37 °C. The stained cells were then analyzed with an EPICS flow cytometer. The total cell population was gated, and apoptosis was measured by calculating the percentage of TUNEL-positive cells.

Detection and Quantification of Acidic Vesicular Organelles with Acridine Orange Staining. Autophagy is the process of sequestering cytoplasmic proteins into lytic components and is characterized by the formation and promotion of acidic vesicular organelles (AVO). To assess the occurrence of acidic vesicular organelles, we treated tumor cells with 6-shogaol for the indicated times and then stained them with acridine orange. Briefly, cells were incubated with acridine orange (1 μg/mL) for 15 min and then examined under fluorescence microscopy (*5*, *22*).

Detection of Autophagic Vacuoles with Monodansylcadaverine. Autophagic vacuoles were also detected with monodansylcadaverine (MDC) by incubating cells with MDC (50 μM) in PBS at 37 °C for 10 min. After incubation, cells were washed four times with PBS and immediately analyzed by fluorescence microscopy using an inverted microscope (Nikon Eclipse TE 300, Germany) equipped with a filter system (excitation wavelength 380 nm, emission filter 525 nm) (*23*). To determine quantification of MDC incorporation, at least 100 cells from each treatment group were examined under fluorescence microscopy, and the percentage of MDC incorporation in cells was calculated (*5*).

EGFP-LC3 Plasmid Transfection. To study the formation of autophagic vacuoles, the localization of LC3, a specific marker of autophagosomes, was monitored. LC3 was expressed as an amino-terminal fusion with green fluorescent protein (EGFP) using Lipofectamine (Invitrogen) for A549 cells. The plasmid encoding EGFP-LC3 was provided by Professor Tamotsu Yoshimori (Department of Cellular Regulation, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan).

Electron Microscopy. Cells were directly fixed with 1% glutaraldehyde and postfixed with 2% osmium tetroxide. The cell pellets or sections were embedded in Epon resin. Representative areas were chosen for ultrathin sectioning and viewed with a JEM 1010 transmission electron microscope (JEOL, Peabody, MA).

Immunoblot and AKT Activity Assays. Cells were treated with 100 μM 6-shogaol for the indicated times. For immunoblot, the cells were lysed on ice for 40 min in a solution containing 50 mM Tris, 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 2 mM Na_3VO_4, 2 mM EGTA, 12 mM β-glycerolphosphate, 10 mM NaF, 16 μg/mL benzamidine hydrochloride, 10 μg/mL phenanthroline, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, and 1 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO). The cell lysate was centrifuged at 14000*g* for 15 min, and the supernatant fraction was collected for immunoblot. Equivalent amounts of protein were resolved by SDS−PAGE (10−12%) and transferred to PVDF membranes. After blocking for 1 h in 5% nonfat dry milk in Tris-buffered saline, the membrane was incubated with the desired primary antibody for 1−16 h. The membrane was then treated with appropriate peroxidase-conjugated secondary antibody, and the immunoreactive proteins were detected using an enhanced chemiluminescence kit (Amersham, USA) according to the manufacturer's instructions.

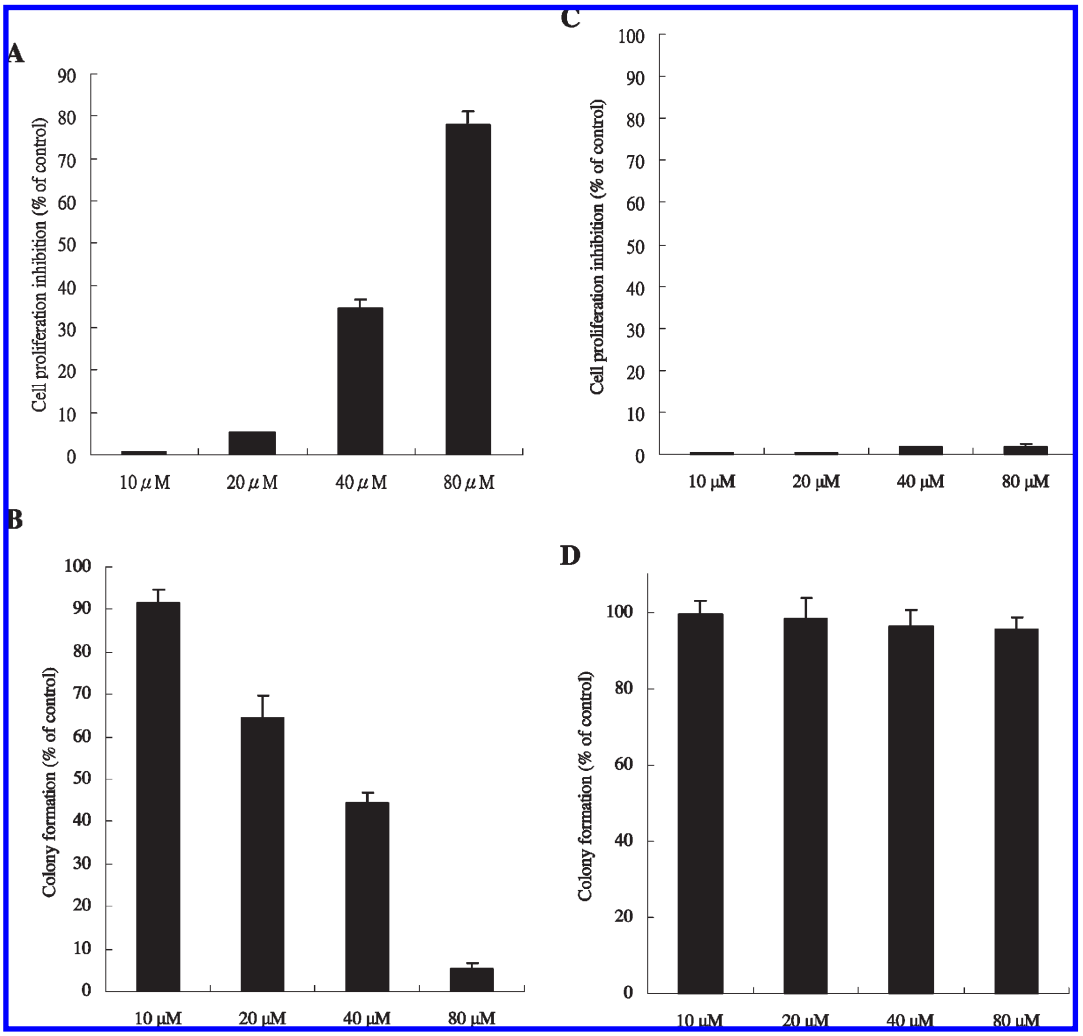

Figure 1. The effects of 6-shogaol on cell proliferation inhibition and colony formation in A549 cells. (**A**) Cell proliferation inhibition effect of 6-shogaol in A459 cells. (**B**) Influence of A549 cells on the number of colony-forming cells, as evaluated by clonogenic assay. (**C**) The effect of 6-shogaol on the cell proliferation of IMR-90 cells. (**D**) The effect of 6-shogaol on the colony formation of IMR-90 cells. Cell growth inhibition activity of 6-shogaol was assessed by XTT. For colony-forming assay, the clonogenic assay was performed as described in Materials and Methods. Results are expressed as the percentage of cell proliferation relative to the proliferation of the control. The data shown are the mean from three independent experiments. Each value is the mean ± SD of three determinations.

AKT, phospho-AKT, mTOR, phospho-mTOR, p70S6K and phospho-p706SK antibodies, and AKT activity assay kit were obtained from Cell Signaling Technology (Beverly, MA). AKT activities were determined using an AKT activity assay kit according to the manufacturer's instructions.

Stable Transfection of Activated AKT-tag cDNA. Transfection of A549 cells was carried out using Lipofectamine 2000 reagent (Life Technologies). A549 cells were exposed to the mixture of Lipofectamine 2000 reagent and AKT cDNA plasmid or an empty vector for 6 h. After transfection, cells resistant to neomycin were selected by incubating with medium containing 1 mg/mL G418 (Geneticin) (Life Technologies). Individual A549 clones were then isolated and tested for constitutive c-Myc expression. AKT-positive A549 cells were selected and maintained in the presence of G418 (400 μg/mL), as were control-cDNA cells (*23*).

siRNA Knockdown of AKT Expression. Lung cancer cell monolayers were transfected with AKT1 siRNA duplexes or nonspecific control siRNA duplexes (Upstate Biotechnology Inc., NY) using Lipofectamine 2000 (Invitrogen). Immunoblot analyses showed that AKT levels remained low but detectable, and expression of β-actin was unaffected by siRNA treatment.

Statistical Analysis. Data were expressed as means ± SD. Statistical comparisons of the results were made using analysis of variance (ANOVA). Significant differences ($p < 0.05$) between the means of control and 6-shogaol treated cells were analyzed by Dunnett's test.

RESULTS

6-Shogaol Inhibits Cell Proliferation and Clonogenic Survival in A549 Cells. To investigate 6-shogaol's potential cell growth inhibition in lung cancer, we first examined the effect of 6-shogaol on cell proliferation and clonogenic survival in A549 cells. As shown in **Figure 1A**, 6-shogaol inhibited cell growth in A549 cells in a concentration-dependent manner. The IC_{50} value of 6-shogaol was 55.4 μM.

Additional experiments were performed to determine the antitumor activities of A549 inhibition when analyzed by *in vitro* clonogenic assays. *In vitro* clonogenic assays showed a close correlation with *in vivo* assays of tumorigenicity in nude mice (*24*). **Figure 1B** shows the effects of 6-shogaol on the relative clonogenicity of the control and the 6-shogaol-treated A549 cells. Clonogenicity of A549 cells was reduced in a concentration-dependent manner after exposure to 6-shogaol.

To further examine the selection of 6-shogaol mediated inhibition of cell proliferation, we also evaluated the effect of 6-shogaol on the normal lung cell line, IMR-90. The results showed that treatment of IMR-90 cells with 6-shogaol not only failed to affect cell proliferation (**Figure 1C**) but also failed to affect colony

formation of IMR-90 cells (**Figure 1D**). These results demonstrate that 6-shogaol possesses selectivity between normal and cancer cells.

6-Shogaol Does Not Predominantly Induce Apoptosis in A549 Cells. A previously published study showed that 6-shogaol induces apoptosis in colorectal carcinoma cells (*21*). Consequently, we investigated whether 6-shogaol could also induce apoptosis in lung cancer cells. The TUNEL results showed that 6-shogaol at concentrations of 50 μM and 100 μM induces only a small amount of apoptotic cell death in A549 cells after 24 and 48 h treatment (**Figure 2A**).

To verify the possiblility of apoptosis in 6-shogaol treated lung cancer cells, we used pan-caspase inhibitor to block caspase activity in A549 cells, and determined whether cell proliferation inhibition changed after 6-shogaol treatment. Blocking caspase activation resulted in a slight decrease in 6-shogaol mediated proliferation inhibition in A549 cells, suggesting that 6-shogaol may only induce a small number of lung cancer cells to undergo apoptosis (**Figure 2B**).

6-Shogaol Induces Autophagy in A549 Cells. Growing evidence indicates that nonapoptotic programmed cell death is principally attributed to autophagy (type II programmed cell death) (*2*). However, the majority of 6-shogaol treated A549 cells do not primarily display features typical of apoptosis. We therefore assessed whether 6-shogaol induces autophagy in lung cancer. As shown in **Figure 3A**, 6-shogaol treatment resulted in the appearance of AVO when cells were stained with acridine orange after 24 h treatment. In addition, 3-methyladenine (3-MA), an autophagy inhibitor, decreased the accumulation of red fluorescence in both control and 6-shogaol treated cells. Because MDC accumulates in mature autophagic vacuoles such as autophagolysosomes, but not in the early endosome compartment, MDC staining can detect autophagic vacuoles. As shown in **Figure 3B**, treatment of cells with 6-shogaol increased accumulation of MDC in comparison with the control. These results corroborate the observation that 6-shogaol treatment induces autophagic cell death in A549 cells. In addition, 3-MA decreased the accumulation of blue fluorescence in both control and 6-shogaol treated cells. To determine the accumulation of the autophagic vacuoles, we performed quantification of MDC incorporation. At least 100 cells from each treatment group were examined under a fluorescence microscopy, and the percentage of MDC incorporation in the cells was calculated. As shown in **Figure 3C**, we demonstrate that 6-shogaol induces autophagy of A549 cells in a dose-dependent manner. In addition, 3-MA decreased the strength of blue fluorescence from 80.5% to 13.4% in 100 μM 6-shogaol treated A549 cells at 24 h.

To examine alternative mechanisms of cell death, we studied the effect of 6-shogaol on the localization of LC3, an autophagy marker. During autophagy, cytosolic LC3-I is cleaved to form the membrane-associated LC3-II, which is involved in the formation of autophagosomes. In A549 cells transfected with a plasmid encoding EGFP-LC3, 6-shogaol redistributed EGFP-LC3 into vesicular structures, visualized by fluorescence microscopy, which is indicative of autophagic cell death (**Figure 3D**).

We also tested whether autophagy occurs in 6-shogaol treated cells by using transmission electron microscopy (TEM). The TEM results showed that, in most cells, the nuclei maintained their integrity and displayed dispersed chromatin, which is not consistent with apoptosis. Normal A549 cells had numerous membrane-bound vesicles, often containing organelles and other cellular fragments (**Figure 3E**). In contrast, exposure of cells to 50 and 100 μM 6-shogaol resulted in the appearance of autophagocytic vacuoles after 24 h treatment, and these autophagocytic vacuoles contained extensively degraded organelles (**Figure 3E**).

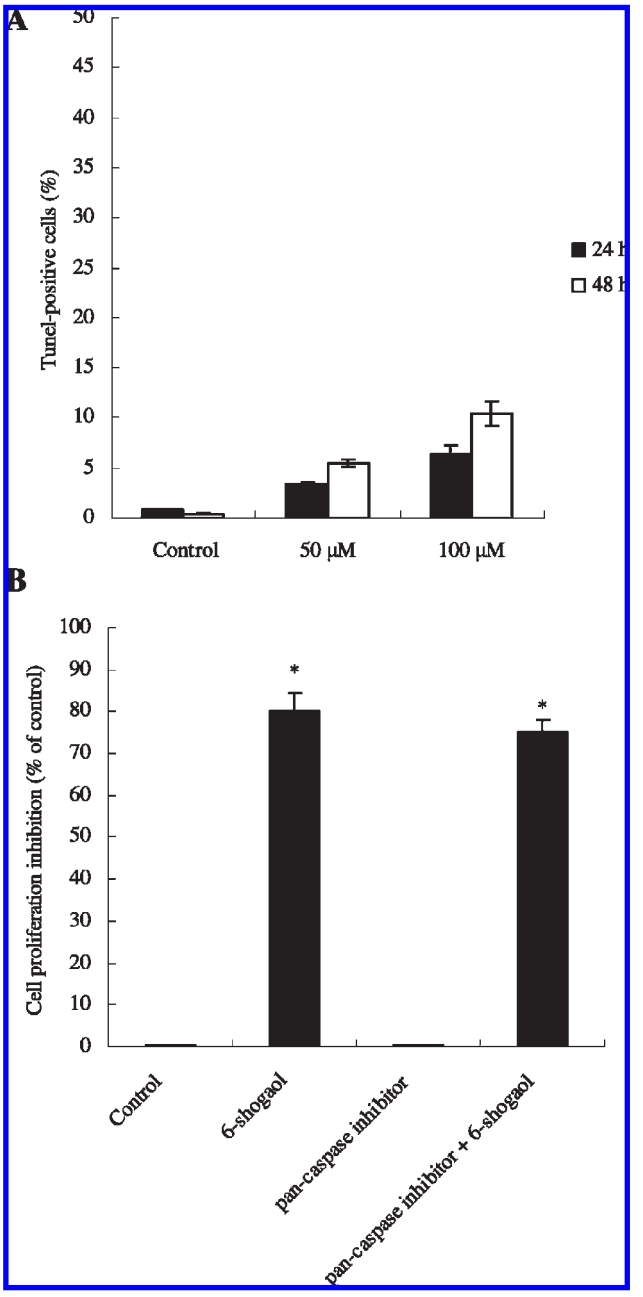

Figure 2. 6-Shogaol induces a small amount of apoptosis in A549 cells. (**A**) The effect of 6-shogaol on apoptosis in A549 cells. (**B**) The effect of pan-caspase inhibitor on 6-shogaol mediated proliferation inhibition. For (**A**), cells were treated with vehicle alone (0.1% DMSO), and 50 and 100 μM 6-shogaol for 24 and 48 h. The TUNEL-positive cells were then examined by flow cytometry. For blocking experiments, cells were pre-incubated with pan-caspase inhibitor (20 μM) for 1 h before the addition of 100 μM 6-shogaol for an additional 48 h. The proliferation of A549 cells was assessed by XTT. The data shown are the mean from three independent experiments. Each value is the mean $\pm$ SD of three determinations. The asterisk indicates a significant difference between control and 6-shogaol-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

The Role of Autophagy in 6-Shogaol Mediated Cell Death. To confirm the involvement of autophagy in 6-shogaol mediated cell death, we assessed the effect of 3-MA on 6-shogaol mediated cytotoxicity. As shown in **Figure 4**, 3-MA decreased autophagy induced by 100 μM 6-shogaol in A549 cells.

6-Shogaol Inhibits the Expression and Activity of AKT/mTOR Pathway. We investigated whether AKT/mTOR, which is

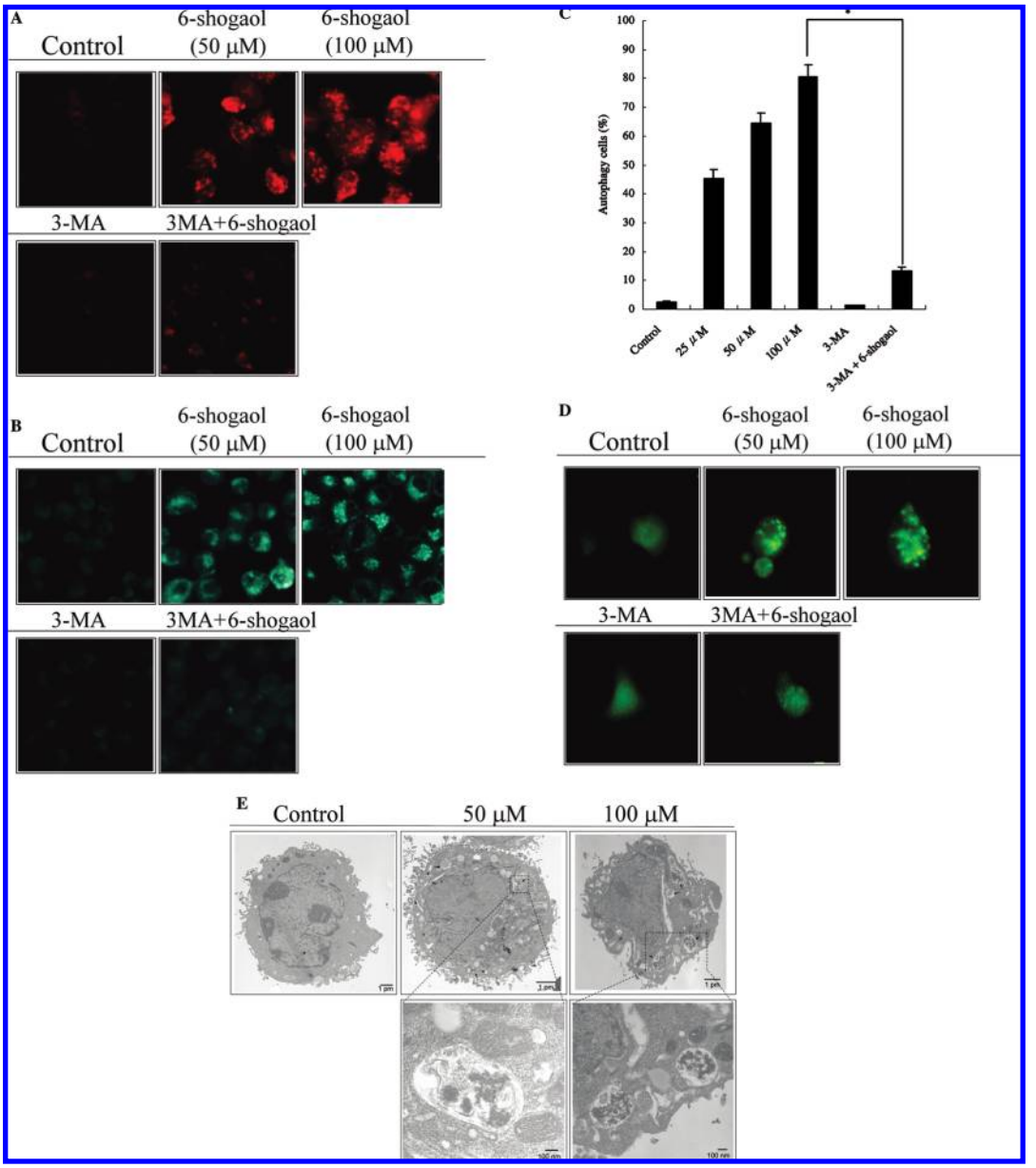

Figure 3. 6-Shogaol induces autophagy in A549 cells. 6-Shogaol treated cells were stained with acridine orange (**A**) and MDC (**B**). (**C**) The quantification of MDC staining using a fluorescence microscopy. (**D**) The aggregation of LC3 in 6-shogaol treated cells. (**E**) The autophagocytic vacuoles were examined by transmission electron microscopy. For (**A**) and (**B**), cells were treated with 6-shogaol for 24 h. After staining, cells were examined clearly visible by a fluorescence microscopy. For (**C**), the quantification of MDC incorporation, at least 100 cells from each treatment group were examined under a fluorescence microscopy, and the percentage of MDC incorporation in cells was calculated. For (**D**), EGFP-LC3 transfected cells were treated with 6-shogaol for 24 h, then cells were examined clearly visible by means of fluorescence microscopy. For blocking experiments, cells were preincubated with 3-MA (5 mM) for 1 h before the addition of 100 μM 6-shogaol for an additional 24 h. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between control and 6-shogaol-treated cells, * $p < 0.05$.

important in regulating cell proliferation and autophagy, is involved in 6-shogaol mediated cell death in A549 cells. 6-Shogaol caused a significant time-dependent decrease in the phosphorylation (Ser 473 and Thr 308) of AKT protein in A549 cells, but did not cause any change in the protein levels of total AKT. However, exposure of A549 cells to 6-shogaol diminished levels of the phosphorylated (activated) form of mTOR (Ser 2448 and Ser 2481), a downstream target of AKT which may inhibit cell growth and induce autophagy. Similar responses were observed for the phosphorylated forms of two other AKT downstream targets, GSK-3β (Ser 9) and FKHR (Ser 256). To determine the change of mTOR in 6-shogaol treated A549 cells, we also examined the phosphorylation of two downstream effectors of mTOR signaling, p70S6K and 4E-BP1. The results showed that phosphorylation levels of both p70S6K (phospho-p70S6K) and 4E-BP-1 (phospho-4E-BP1) decreased, revealing a potent inhibitory effect of 6-shogaol on AKT/mTOR signaling (**Figure 5A**).

6-Shogaol mediated inhibition of AKT was further confirmed by determining phosphorylation of one of its substrates, GSK-3β. As shown in **Figure 5B**, when compared with the control, the Ser 21/9 phosphorylation of GSK-3β decreased after A549 cells were exposed for 45 min to 6-shogaol. Phosphorylation of GSK-3β decreased relative to the control at all 4 time points (**Figure 5B**). These results suggest the inhibitory potential of 6-shogaol against AKT/mTOR pathway, which is highly activated during the development and progression of lung cancer.

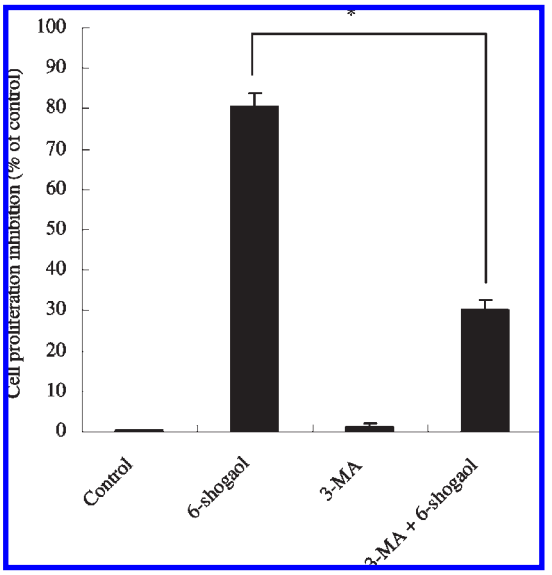

Figure 4. The role of autophagy in 6-shogaol mediated cell proliferation. Cells were preincubated with 3-MA (5 mM) for 1 h before the addition of 100 μM 6-shogaol for an additional 48 h. The data shown are the means from three independent experiments. Each value is the mean ± SD of three determinations. The asterisk indicates a significant difference between control and 6-shogaol-treated cells, as analyzed by Dunnett's test ($p < 0.05$).

The Role of AKT Pathway in 6-Shogaol Mediated Autophagy. To confirm the central role of the AKT signaling pathway as a target of 6-shogaol induced autophagy, we transfected A549 cells with a constitutively active form of AKT cDNA (**Figure 6A**). AKT-overexpressing cells were treated with 6-shogaol, and the induction of autophagy was assayed. Transfected cells expressing active AKT cDNA were considerably more resistant to 6-shogaol induced autophagy than cells transfected with control cDNA. That is, 6-shogaol was notably unable to cause autophagy in cells transfected with active AKT cDNA, whereas 6-shogaol maintained its autophagy effect on A549 cells transfected with control cDNA (**Figure 6B**,**C**). Therefore, it can be firmly concluded that 6-shogaol induces autophagic cell death in A549 cells by suppression of the AKT pathway.

Reduction of AKT Expression by siRNA Potentiated 6-Shogaol's Effect. To confirm the central role of the AKT signaling pathway as a target of 6-shogaol-induced autophagy, we transfected A549 cells with a pool of siRNA targeting AKT. As shown in **Figure 7A**, AKT siRNA reduced AKT phosphorylation approximately 70% in comparison with control siRNA. Reduction of AKT phosphorylation by transfection of cells with AKT siRNA had no significant effect on A549 autophagy, suggesting that the remaining AKT activity is high enough to maintain survival of the cells. However, treatment with 6-shogaol significantly increased the number of autophagy cells in AKT siRNA-transfected cells to more than in the control siRNA-transfected cells (**Figure 7B**,**C**). This may be because 6-shogaol's treatment has lowered the level of AKT activity to below the threshold for maintaining normal cell survival under such conditions. In short, this siRNA result further confirms that the AKT signaling pathway is indeed the target of 6-shogaol treatment.

DISCUSSION

Because lung cancer is a leading cause of death worldwide and the long-term survival rate for lung cancer patients is one of the lowest for any cancer, new therapies are urgently needed (*25*). Our study found that 6-shogaol effectively inhibits tumor cell

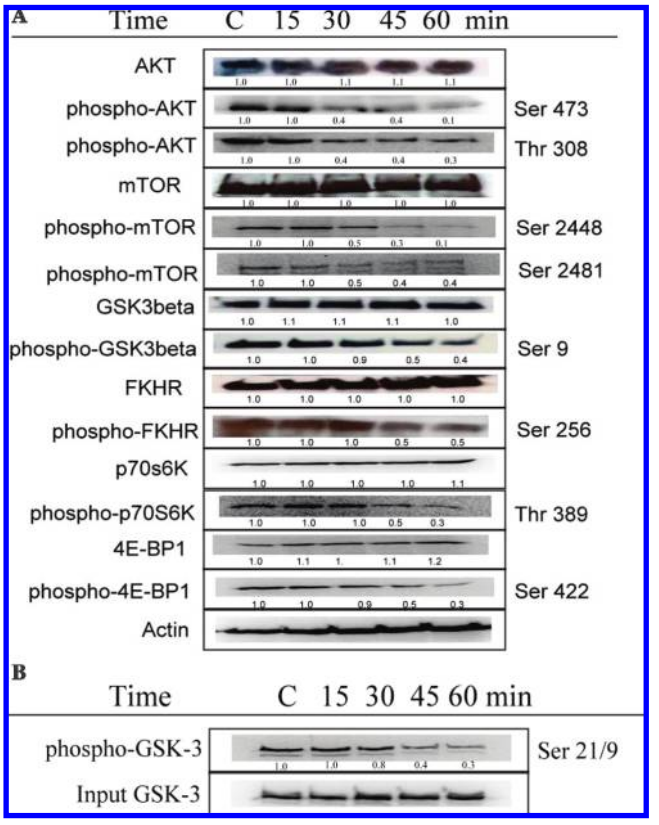

Figure 5. The effect of 6-shogaol on AKT/mTOR pathway. (**A**) The effect of 6-shogaol on AKT, GSK-3β, FKHR, mTOR, p70S6K, and 4E-BP1 levels and their phosphorylated forms. (**B**) The effect of 6-shogaol on AKT activity. For (**A**), the cells were treated with 100 μM 6-shogaol at different times. Cell lysates were prepared, and immunoblot was performed using antibodies against various AKT/mTOR and phospho-AKT/mTOR signaling proteins. For (**B**), the AKT kinase activity was determined using an AKT assay activity kit from Cell Signaling Technology (Beverly, MA) according to the manufacturer's instructions. Results shown are representative of three independent experiments.

proliferation, concomitant with induction of autophagic cell death.

In this study, we show that 6-shogaol induces autophagic cell death, but not primarily apoptosis, in A549 cells. Some types of cancer cells exhibit autophagic changes after treatments with various chemotherapeutic agents (*4*−*6*, *26*). Autophagy begins with the sequestering of cytosolic components, often including intracellular organelles within double-membrane structures. The vacuoles (also called autophagosomes) undergo acidification after maturation. Finally, autophagosomes fuse with lysosomes and their material is digested by lysosomal hydrolases (*1*, *2*). Our results show that the AVO formations in A549 cells are characterized by acridine orange and MDC stain after exposure to 6-shogaol. Moreover, 6-shogaol mediated autophagy is blocked by 3-MA, an autophagy inhibitor. In contrast, the typical characteristic of apoptosis, DNA fragmentation determined by TUNEL, is either slight or not observable in 6-shogaol treated A549 cells. It is still unclear whether autophagy suppresses tumorigenesis or provides cancer cells with a protective response under unfavorable conditions, although several studies have reported that autophagy is triggered in cancers in response to various anticancer agents, including As_2O_3, tamoxifen and temozolomide (*4*, *5*, *27*, *28*). However, determining whether or not the initiation of different types of cell death is influenced by different stimuli, cell types and cell content requires further investigation.

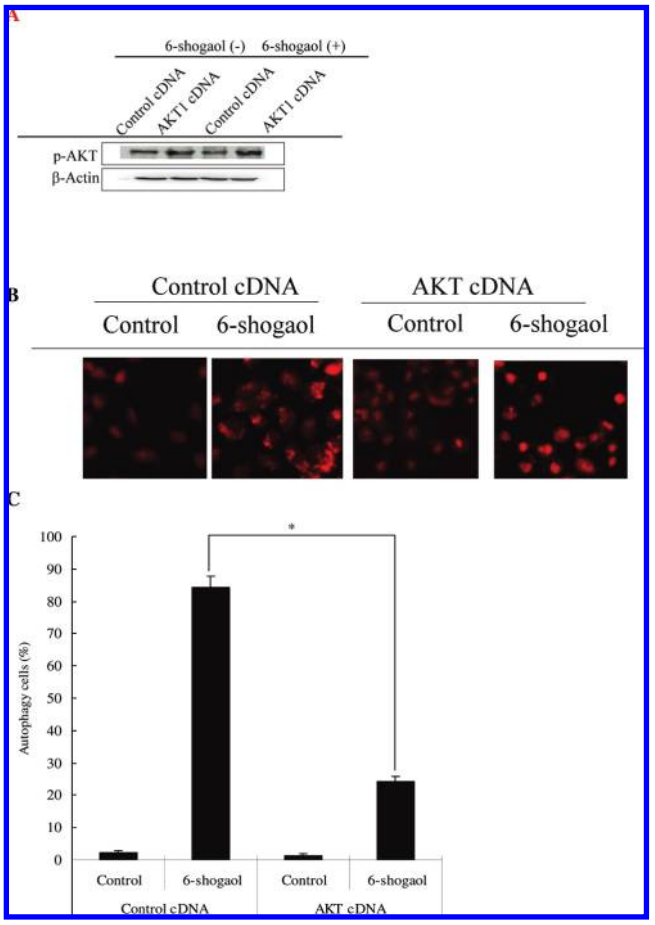

Figure 6. The role of AKT on 6-shogaol mediated autophagy. (**A**) Upregulation of AKT phosphorylation by active AKT cDNA transfection. (**B**) The induction of autophagy of 6-shogaol in AKT cDNA transfected cells. (**C**) The quantification of MDC staining using a fluorescence microscopy. A549 cells were transfected with control-cDNA or active AKT cDNA and then treated with 6-shogaol (100 μM) for the indicated times (30 min for phospho-AKT assay and 24 h for autophagy assay). The degree of autophagic cell death was assessed by acridine orange and MDC stain. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between control and 6-shogaol treated cells, * $p < 0.05$.

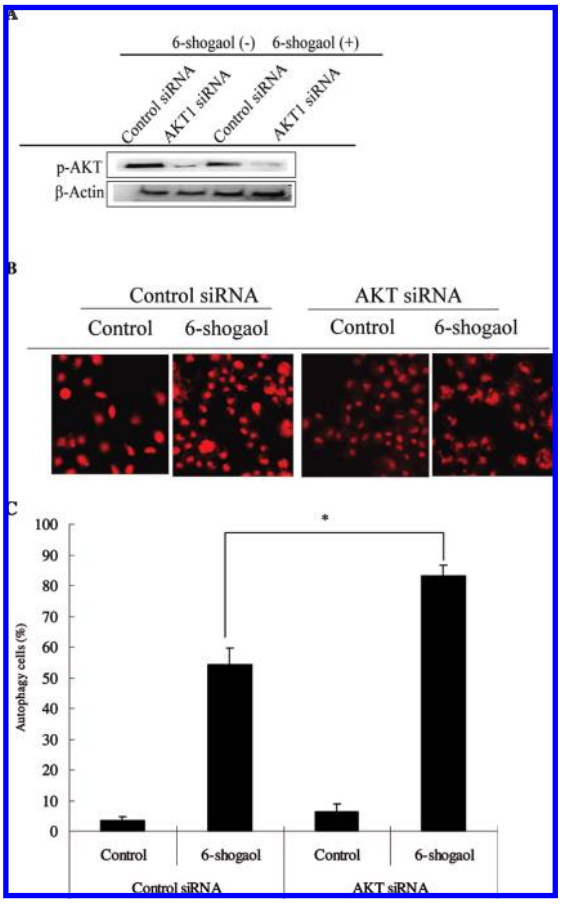

Figure 7. Reduction of AKT expression by siRNA potentiated 6-shogaol's effect. (**A**) Genetic suppression of AKT phosphorylation by AKT siRNA transfection. (**B**) 6-Shogaol induced autophagy in control and AKT siRNA transfected cells. (**C**) The quantification of MDC staining using a fluorescence microscopy. A549 cells were transfected with control oligonucleotide or AKT siRNA, then treated with 6-shogoal (100 μM) for the indicated times (30 min for phospho-AKT assay and 24 h for autophagy assay). The degree of autophagic cell death was assessed by acridine orange and MDC stain. Results shown are representative of three independent experiments. The asterisk indicates a significant difference between control and 6-shogaol treated cells, * $p < 0.05$.

AKT signaling is the major pathway in lung cancer cells and plays a variety of physiologic roles, including cell growth, cell cycle regulation, migration, and survival (*8*, *9*, *29*). Activated AKT in turn signals to a variety of key downstream molecules, including mTOR, GSK-3β and FKHR, the consequence of which is to inhibit cell death and promote cell survival (*30*). Recent studies have indicated that inhibition of the AKT/mTOR pathway has consistently been associated with triggering autophagy in cancer cells (*11*, *12*). Our results show that 6-shogaol treatment decreases AKT activation and activity. The inhibitory effects of 6-shogaol on AKT signaling are correlated with the loss of phosphorylation of AKT's downstream targets, GSK-3β and FKHR. In addition, exposure to 6-shogaol also inactivated mTOR and reduced phosphorylation of its downstream targets p70S6K and 4E-BP1. Moreover, enforced expression of AKT by active AKT cDNA transfection significantly diminished 6-shogaol mediated autophagic cell death. In contrast, selective knockdown AKT expression by AKT siRNA-based inhibition increased 6-shogaol induced autophagy. Together, these findings indicate that 6-shogaol induces autophagic cell death through AKT/mTOR inhibition in human non-small cell lung cancer A549 cells.

In conclusion, this study demonstrates (a) the potential cell growth inhibition of an active constituent of dietary ginger, 6-shogaol, in human non-small cell lung cancer A549 cells; (b) that 6-shogaol inhibits cell proliferation by inducing cells to autophagic cell death, but not, predominantly, apoptosis; (c) that 6-shogaol inhibits survival signaling through the AKT/mTOR pathway by blocking the activation of AKT and downstream targets; and finally (d) that overexpression of AKT by AKT cDNA transfection decreased 6-shogaol-mediated autophagic cell death, supporting inhibition of AKT beneficial to autophagy. These data provide a basic mechanism for the chemopreventive properties of 6-shogaol in human non-small cell lung cancer cells. Future *in vivo* studies using human patients would help ascertain whether this cell growth inhibition effect of 6-shogaol would contribute its overall chemotherapy effects in the fight against lung cancer, and may possibly have future therapeutic applications.

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