Growth Inhibition and Induction of G1 Phase Cell Cycle Arrest in Human Lung Cancer Cells by a Phenylbutenoid Dimer Isolated from *Zingiber cassumunar*

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In our previous study, a novel phenylbutenoid dimer (±)-*trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (PSC), isolated from *Zingiber cassumunar* Roxb. (Zingiberaceae), inhibited proliferation of various human cancer cells with the IC_{50} values ranging 10 to 30 μM. Prompted by these anti-proliferative effects, we performed additional studies in A549 human lung cancer cells in order to investigate the mechanism of action. PSC arrested cell cycle progression at the G0/G1 phase in a concentration- and time-dependent manner. PSC dose-dependently induced cyclin-dependent kinase (CDK) inhibitor p21 expression, whereas the expression of cyclin D1, cyclin A, CDK4, CDK2, and proliferating cell nuclear antigen (PCNA) were decreased by treatment with PSC. These results suggest that one of the anti-proliferative mechanisms of PSC is to suppress cell cycle progression by increasing p21 expression and down-regulating cyclins and CDKs. This study characterizes additional biological activity of this novel phenylbutenoid dimer and expands its therapeutic potential for cancer as a chemotherapeutic agent derived from natural products.

Key words phenylbutenoid dimer; *Zingiber cassumunar*; A549 cell; G1 phase cell cycle arrest

Cancer is the leading cause of death in Korea and in the rest of the world. Despite therapeutic advances, cancer mortality has increased.[1)] Numerous studies are in progress to develop more potent and selective anticancer agents either by synthesis or from natural products.

In the course of searching for active ingredients with anticancer potential from natural products, we found that the chloroform extract and isolates from *Zingiber cassumunar* inhibited cell proliferation in various human cancer cell lines.[2,3)] *Zingiber cassumunar* Roxb., an indigenous Indonesian plant, belongs to the Zingiberaceae family. Zingiberaceae species have been used as spices and traditional medicines in the treatment of stomach problems, epilepsy, sore throat, cough, wounds, liver complaints, rheumatism, muscular pains, malignancies, and other disorders.[4,5)] Previous studies isolated phenylbutenoids, cassumumins, and cassumunarins from the extracts of rhizomes of *Zingiber cassumunar*.[2,6,7)] These constituents exhibited anti-inflammatory, anti-oxidant, and anti-tumor promoter activities.[5,6,8,9)] Recently, we reported for the first time the isolation of a phenylbutenoid dimer, (±)-*trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohexene (PSC), from *Zingiber cassumunar*. This dimer was found to inhibit cell proliferation in various human cancer cells.[3)] Consequently, in this study we further investigated the mechanism of action of PSC using A549 human lung cancer cells.

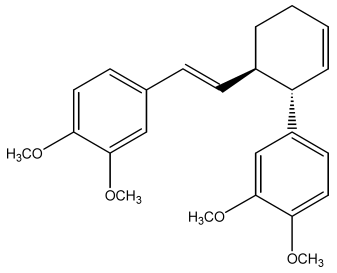

Fig. 1. Chemical Structure of a Phenylbutenoid Dimer, (±)-*trans*-3-(3,4-Dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohexene (PSC)

MATERIALS AND METHODS

Chemicals Trichloroacetic acid (TCA), sulforhodamine B, propidium iodide, RNase A, and mouse monoclonal anti-β-actin antibody were purchased from Sigma (St. Louis, MO, U.S.A.). Minimal essential medium with Earle's salt (MEME), fetal bovine serum (FBS), trypsin-EDTA solution, and antibiotic–antimycotic solution were from GIBCO-BRL (Grand Island, NY, U.S.A.). Mouse monoclonal anti-p21, anti-p53, anti-PCNA antibody, rabbit polyclonal anti-CDK2, anti-CDK4, anti-cyclin A, anti-cyclin D1 antibody, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.).

Isolation of Test Compound The test compound, a phenylbutenoid dimer, (±)-*trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohex-1-ene (PSC), was isolated from the chloroform extract of *Zingiber cassumunar* by bioassay-guided fractionation as described previously (Fig. 1).[3)]

Cell Culture Human lung carcinoma A549 cells, obtained from the American Type Culture Collection (ATCC), were cultured in MEME supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Evaluation of Growth Inhibitory Potential A549 cells (5×10^4 cells/ml) were treated with various concentrations of

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PSC for 3 d. After treatment, cells were fixed with 10% TCA solution, and cell viability was determined by the sulforhodamine B (SRB) protein staining method.[10] The result was expressed as a percentage relative to solvent-treated control incubations, and the IC_{50} values were calculated using nonlinear regression analysis (percent survival *versus* concentration).

Cell Cycle Analysis A549 cells were plated at a density of 2×10^5 cells per 60 mm culture dish and incubated for 24 h. Fresh media containing various concentrations of PSC were added to culture dishes. Cells were harvested, fixed with 100% methanol overnight, and incubated with a staining solution containing 0.2% NP-40, RNase A (30 μg/ml), and propidium iodide (50 μg/ml) in phosphate-buffered saline (PBS). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 20000 cells were used for each analysis, and results were displayed as histograms.[11]

Western Blot Analysis A549 cells were plated in 60 mm culture dishes at a density of 2.5×10^5 cells/dish. Cells were exposed to various concentrations of PSC for 24 and 36 h. Total cell lysates were obtained by boiling with 2X sample loading buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2 mM sodium orthovanadate, and 2% β-mercaptoethanol). The protein concentration of each lysate was determined by the BCA method. Each protein (20 μg) was subjected to 12% SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were incubated with blocking buffer (5% non-fat dry milk in PBS–0.1% Tween 20 (PBST)) for 1 h. Membranes were then incubated with indicated antibodies overnight at 4 °C, then washed for 1 h with PBST. Membranes were incubated with the corresponding secondary antibodies for 1 h at room temperature. After washing for 1 h with PBST, proteins were detected using ECL detection reagent. Quantification of each blot was performed using the Multi Gauge V3.0 program (Fuji Photo Film Co., Japan). Relative density (RD) was determined by normalization of the density of each blot with that of corresponding β-actin.

Statistics Data were presented as the means±S.D. for the indicated number of independently performed experiments. Figure data are shown as one representative of at least three independent experiments.

RESULTS

Inhibitory Effect of PSC on the Proliferation of A549 Human Lung Cancer Cells In order to investigate whether the PSC inhibits the proliferation of A549 human lung cancer cells, cells were treated with various concentrations of PSC for 24, 48, and 72 h. The proliferation of A549 cells was inhibited in a concentration- and time-dependent manner by treatment with PSC (IC_{50}=15.6 μM at 72 h) (Fig. 2). The density of cells was also decreased in a concentration-dependent manner by PSC (data not shown). The IC_{50} value (72 h) of a known anticancer agent ellipticine was 0.8 μM in the same experiment.

Effect of PSC on the Cell Cycle Progression To examine the effect of PSC on the cell cycle progression, cell cycle analysis was performed in A549 human lung cancer cells. Cells were treated with various concentrations of PSC (6.5, 13, 26 μM) for 24 or 36 h. At 24 h, cells treated with lower concentrations (6.5, 13 μM) of PSC did not show distinct changes in cell cycle distribution. However, at a higher concentration (26 μM), cells moderately accumulated in the G0/G1 phase of the cell cycle (57.18% for control; 64.29% for 26 μM PSC), indicating concentration-dependent G0/G1 phase cell cycle arrest. At 36 h, cell cycle arrest in G0/G1 phase was more pronounced by treatment with 13 and 26 μM PSC (57.87% for control; 63.91% for 13 μM PSC; 66.99% for 26 μM PSC), whereas the distribution of cell cycle was not affected by PSC at the lowest concentration (6.5 μM). The sub-G1 peak, an indicator of apoptotic cell death, was not observed in all of tested cells. These results suggest that the anti-proliferative effect of PSC is not related to the induction of cell death but rather to the induction of cell cycle arrest at the G0/G1 phase in a concentration- and time-dependent manner (Fig. 3).

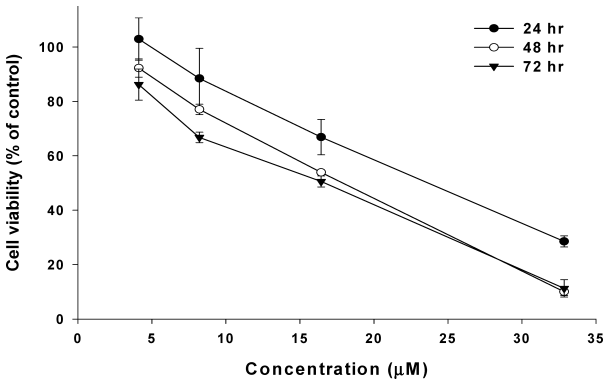

Fig. 2. Inhibition of Cell Proliferation by PSC in A549 Human Lung Caner Cells

Cells were treated with various concentrations of PSC for the indicated times (24, 48 or 72 h). Regulation of cell proliferation was determined by SRB assay. Data are represented as the means±S.D. (n=3).

Effect of PSC on the Expression of Proteins Regulating Cell Cycle Progression Cells were treated with various concentrations (6.5, 13, 26 μM) of PSC for 24 and 36 h. To examine the effects of PSC on the expression of proteins related to the regulation of cell cycle progression, total cell lysates were analyzed by Western blot analysis. As shown in Fig. 4, the CDK inhibitor p21 was induced by treatment with PSC for 36 h, whereas the expression level of p53 was not distinctly changed. The expression level of PCNA was decreased in a concentration-dependent manner at 24 and 36 h. Down-regulation of CDK2, CDK4 and cyclin D1, which promote cell cycle progression through the G1 phase into S phase, was also observed in a concentration-dependent manner at 24 h. In addition, a concentration-dependent decrease of cyclin A, which binds to CDK2 and promotes progression through the S phase of cell cycle, was observed by treatment with PSC for 24 and 36 h.

DISCUSSION

Zingiber cassumunar has been used in traditional medicine to treat stomach problems, inflammation, fever, immune diseases, and respiratory problems.[4,5] Some compounds isolated from *Z. cassumunar* have been shown to have anti-inflammatory and anti-oxidant activities. However, their anticancer activity has not been investigated yet. We recently re-

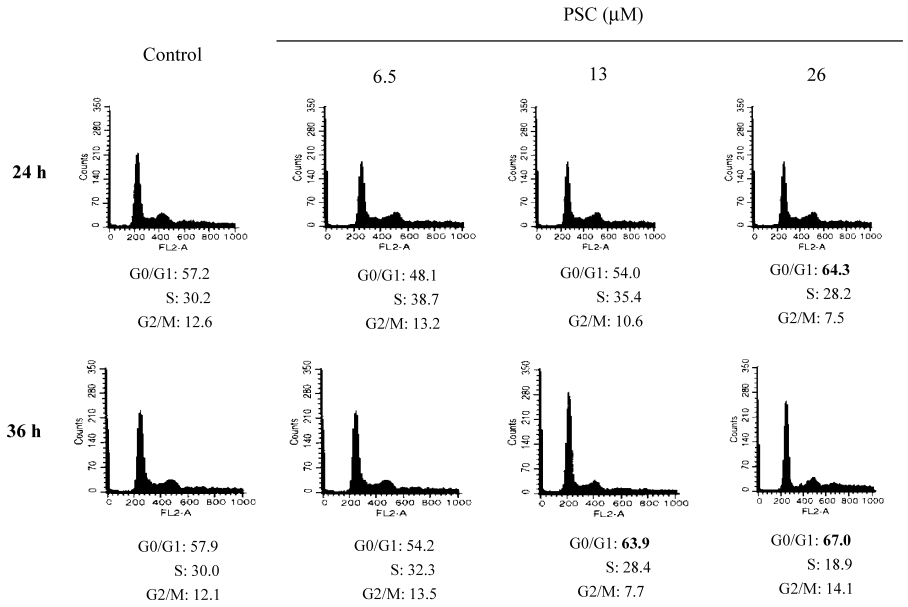

Fig. 3. Induction of Cell Cycle Arrest in G0/G1 Phase by PSC

Cells were treated with the indicated concentrations of PSC for 24 and 36 h. The distribution of the cell cycle was analyzed by FACS calibur, and the percentage of distribution in distinct phases of cell cycle was determined using ModFIT software. Data are shown as one representative of at least three independent experiments.

ported the growth-inhibitory potential of the chloroform extract of *Z. cassumunar* and several compounds isolated from *Z. cassumunar* in cultured human cancer cells.[2,3] In particular, the phenylbutenoid dimer, (±)-*trans*-3-(3,4-dimethoxyphenyl)-4-[(*E*)-3,4-dimethoxystyryl]cyclohexene (PSC), exhibited the most potent inhibitory effect against the proliferation of A549 human lung cancer cells (IC_{50}=15.6 μM at 72 h). Since lung cancer is the leading cause of cancer death in Korea as well as in the world, we investigated the anti-proliferative mechanism of PSC in A549 human lung cancer cells. PSC inhibited cell proliferation in a concentration- and time-dependent manner, which is consistent with our previous study (Fig. 2).[3] Based on these results, we further investigated the mechanism of action of PSC in relation to the regulation of cell cycle progression.

It is known that cell proliferation is mediated by cell cycle progression. One of characteristics of cancerous cells that distinguish them from normal cells is their uncontrolled growth by mutation or deregulation of cell cycle checkpoints. Thus, the inhibition of cell cycle progression *via* activation of cell cycle control might be an appropiate target for the regulation of cancer.[12,13] Cell cycle control is a highly regulated process that involves a complex cascade of cellular events including activation of cyclins and CDKs.[14] CDK4/cyclin D complex promotes the progression through G1 phase into S phase.[13—15] CDK2 is associated with entry of S phase by binding cyclin E and makes a complex with cyclin A through S phase.[16] The activity of CDK/cyclin complexes is further regulated by PCNA that binds to cyclin D, cyclin E, and cyclin A.[17] In addition, the activity of CDK/cyclin complexes is negatively regulated by binding to cyclin-dependent kinase inhibitors (CKIs) which are largely classified into the INK4 family (p16, p15, p18, p19) and the Cip/Kip family (p21, p27, p57).[18] Among them, p21 is known to induce cell cycle arrest in G0/G1 phase *via* inhibition of activity of the CDK4/cyclin D complex.[19] In this study, cell cycle analysis showed that PSC induced arrest of cell cycle progression in

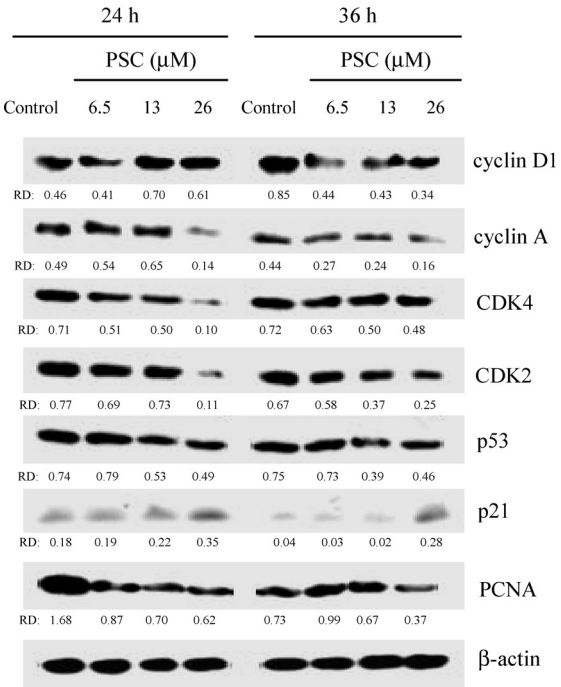

Fig. 4. Effect of PSC on the Expression of Cell Cycle-Related Proteins in A549 Cells

Cells were treated with various concentrations of PSC for 24 and 36 h. The level of protein expression was analyzed by Western blot analysis as described in Materials and Methods. Relative density (RD) indicates the band density of corresponding protein expression normalized with the band density of β-actin. Data are shown as one representative of at least three independent experiments.

G0/G1 phase in a time-dependent manner (Fig. 3). The sub-G1 peak, a marker of cell death, was not detected in PSC-treated cells, suggesting that the mechanism of PSC's inhibition of growth was not related to the induction of apoptosis. Indeed, PSC down-regulated the expression of PCNA, CDK4, cyclin D1, CDK2, and cyclin A, and up-regulated the expression of p21 with up to 36 h exposure (Fig. 4). The level of p53 expression was not affected by treatment with PSC,

indicating that up-regulation of p21 expression was p53-independent as has been reported in several studies.[13,20] Therefore, PSC-mediated G0/G1 phase cell cycle arrest is related to the inhibition of the formation of CDK/cyclin complexes by down-regulation of their expression, and the suppression of CDK activity by induction of p21 expression.

In summary, these results suggest that one of the anti-proliferative mechanisms of PSC in A549 human lung cancer cells is to induce G0/G1 phase cell cycle arrest. This study presents an additional biological activity of PSC and points to the therapeutic potential of this novel phenylbutenoid dimer in the development of cancer chemotherapeutic agents derived from natural products.

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