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Cytotoxic and Antineoplastic Activity of Timosaponin A-III for Human Colon Cancer Cells

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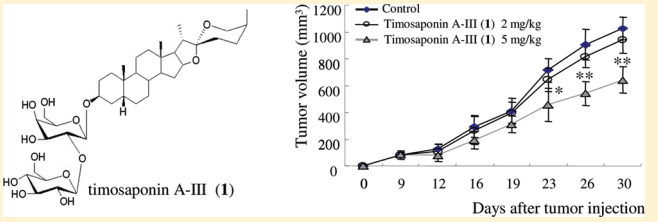
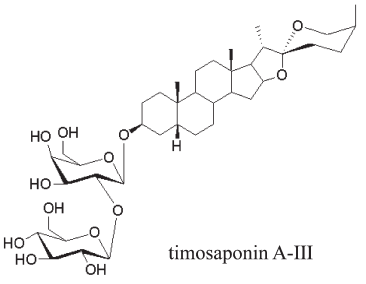
ABSTRACT: The potential antitumor activity of timosaponin A-III (**1**), a steroidal saponin from the rhizomes of *Anemarrhena asphodeloides*, was investigated in human colorectal cancer HCT-15 cells both in cell culture and in an in vivo murine xenograft model. Compound **1** inhibited the proliferation of cancer cells with cell-cycle arrest and induction of apoptosis. Cell-cycle arrest in the G0/G1 and G2/M phase by **1** was correlated with the down-regulation of cyclin A, cyclin B1, cyclin-dependent kinase 2 (CDK2), CDK4, proliferating cell nuclear antigen, and c-Myc. The increase of the sub-G1 peak by **1** was also closely related to the induction of apoptosis, which was evidenced by the induction of DNA fragmentation, activation of caspases, induction of cleaved poly-(ADP ribose) polymerase, and suppression of Bcl-xL and Bcl-2 expression. In an in vivo xenograft model, treatment with **1** (2 or 5 mg/kg body weight, three times/week, ip administration) for four weeks significantly suppressed tumor growth in athymic nude mice bearing HCT-15 cells, without any overt toxicity. Cell-cycle arrest and induction of apoptosis might be plausible mechanisms of actions for the observed antineoplastic activity of **1**.



Colorectal cancer is one of the most widely diagnosed cancer types worldwide, with approximately one million new cases per year.[1] Although the global incidence of colorectal cancer has gradually declined, the number of colorectal cancer patients has increased remarkably in Asian countries in recent years due to an enhanced westernized diet.[2,3] This epidemiological evidence suggests the necessity of continuous efforts to control colorectal cancer. In a similar manner to other solid tumors, the high mortality rate from colorectal cancers is also correlated with high metastasis. Efforts have been made to develop new approaches including chemotherapy and radiotherapy to conquer colorectal cancer, but these are limited by their side effects. Therefore, additional work is needed to decrease this cancer burden.

Anemarrhena asphodeloides Bunge (Liliaceae), a medicinal plant distributed widely in mainland China, Japan, and Korea, has been used traditionally as an agent for its antidepressant, antidiabetes, anti-inflammatory, antiplatelet aggregation, and antipyretic effects.[4,5] Phytochemical investigation of this species has revealed steroidal saponin components including timosaponin A-III (**1**), sarsasaponin, anemarsaponin, xanthones, and a lignan.[6−8] Recent studies have shown that saponins can inhibit cancer cell proliferation and thus might be potential candidates for development as chemotherapeutic agents. Polyphyllin D, a steroidal saponin from *Paris polyphylla*, exhibited growth inhibition in human breast cancer cells.[9] The saponins balanitin-6 and -7 from *Balanites aegyptiaca* exerted antiproliferative activity against human lung and glioblastoma cells.[10] Asparanin A from *Asparagus officinalis* induced cell-cycle arrest and apoptosis in human hepatocellular carcinoma cells.[11] In this same regard, the steroidal saponins of *A. asphodeloides* may be considered of interest. In particular, the water-soluble constituents of *A. asphodeloides* exhibited growth inhibitory effects and the induction of apoptosis against several gastric cancer cell lines.[12] Nyasol and timosaponin A-III (**1**), two major components of *A. asphodeloides*, also showed antiangiogenic and antiproliferative effects for cancer cell lines, and **1** was found to induce autophagy in HeLa cancer cells.[13] However, the anticancer potential of **1** remains to be further elucidated.



(**1**)

One of the characteristics of cancer cells is the uncontrolled proliferation through the deregulation of cell cycle and beyond apoptotic cell death. The cell cycle is finely regulated by consecutive activation and inactivation of cyclin-dependent

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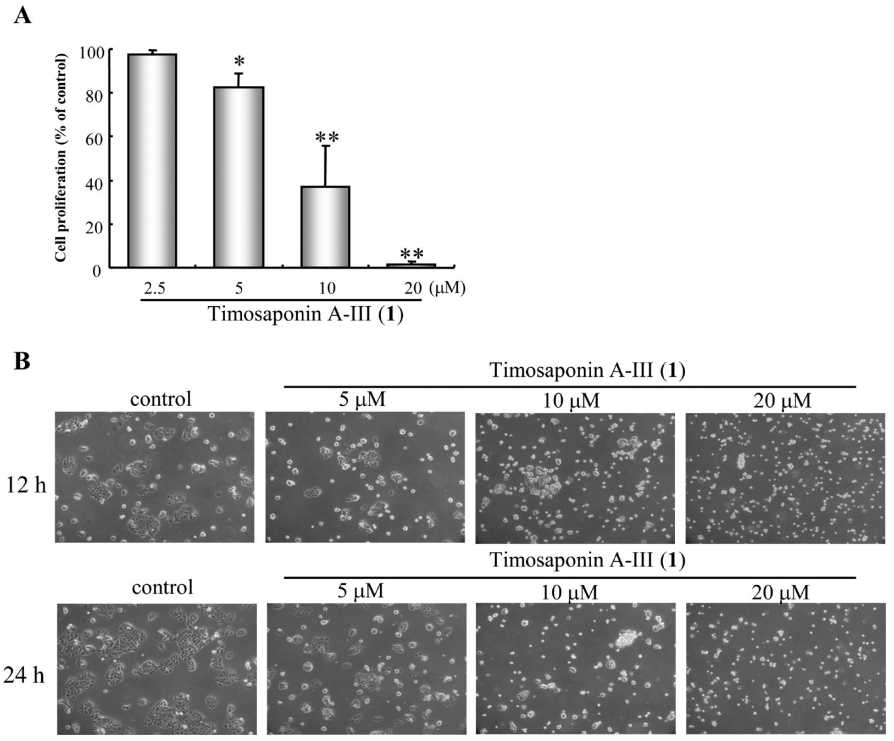

Figure 1. Growth inhibitory effects of timosaponin A-III (**1**) on HCT-15 human colon cancer cells (A). Human colon cancer cells (HCT-15) were plated at a density of 10 000 cells in 96-well plates in RPMI supplemented with 10% FBS and incubated with the test compound at the indicated concentrations for 3 days. The % values of cell survival were calculated from each mean absorbance of sample-treated cells/absorbance of control cells. Data are represented as the means ± SE (n = 3) ($^*p < 0.05$, $^{**}p < 0.01$ indicate statistically significant differences from the control group). (B) Morphological changes by **1** in HCT-15 cells. Cells were treated with indicated concentrations of **1** (0−20 μM) for 12 and 24 h. Morphology was observed under the phase-contrast microscope and photographed.

kinases (CDKs), resulting from expression of the various cyclins required at each cellular phase.[14,15] Specific CDK/cyclin complexes and CDK inhibitors control the progression of the cell cycle at each stage, so the control of the cell cycle might be a target in inhibiting cell proliferation.[16] The other point of controlling the proliferation of cancer cells is to evoke the apoptotic process, or programmed cell death.[17,18] Therefore, the induction of cell-cycle arrest and apoptosis of cancer cells might be plausible strategies to suppress the uncontrolled proliferation of cancer cells using phytochemicals. In the present study, we herein report the biological activity of **1** and its mechanism of action against HCT-15 human colon cancer cells in both cell culture and in vivo in a nude mouse xenograft model.

RESULTS AND DISCUSSION

To evaluate the effects of timosaponin A-III (**1**) on the growth of human colon cancer cells, the growth inhibitory potential was determined by a colorimetric sulforhodamine B (SRB) protein dye staining method. Compound **1** exhibited potent growth inhibition of several human colorectal cancer cells with IC_{50} values below 15 μM in all cell lines tested (HCT-15, 6.1 μM; HCT-116, 5.5 μM; HT-29, 10.3 μM; SW-480, 13.1 μM; SW-620, 11.1 μM). In the case of normal lung epithelial (MRS-5) and fibroblast (Hs68) cells, the IC_{50} values of **1** were over 50 μM. This result suggests that the growth inhibitory effect of **1** is more potent against various colon cancer cells than normal cells. According to the potent inhibitory effect of **1** against HCT-15 cells, further studies were performed using HCT-15 cells to elucidate its mechanism of action. Compound **1** exhibited a growth inhibitory effect against HCT-15 cells in a concentration-dependent manner with an IC_{50} value of 6.1 μM (Figure 1A). The concentrations of **1** up to 10 μM exhibited mainly cell-cycle arrest. In contrast, over 20 μM of **1** caused a cytotoxic effect with the observation of floating dead cells. As shown in Figure 1B, cells treated with **1** exhibited morphological changes with distinct rounded shapes and detachment in a time- and dose-dependent manner when compared to vehicle-treated control cells.

Cell proliferation and growth are generally controlled by the progression of three well-defined phases (G0/G1, S, and G2/M) of the cell cycle. To investigate whether **1** affects cell-cycle progression, HCT-15 cells were treated with **1** (up to 20 μM) for 12 or 24 h, and the distribution of cells in various compartments of the cell cycle was analyzed by flow cytometry. Cell-cycle arrest in the G0/G1 and G2/M phase by **1** appeared at both 12 and 24 h, but the degree was slightly different according to the incubation time and concentration. As shown in Figure 2, when treated with 10 μM **1** for 24 h, the G0/G1 phase was effectively increased from 37.0% to 47.9% and the G2/M phase was also increased from 22.4% to 30.4%, whereas the S phase was decreased significantly from 34.1% to 15.3%. In addition, when treated with **1** at 20 μM, the sub-G1 phase was observed at both 12 and 24 h, indicating the induction of apoptotic cell death. These data show that **1** induces double blockade of the cell cycle at the G0/G1 and G2/M phases at a 10 μM concentration and induction of cell death at 20 μM, a relatively high concentration.

To confirm whether the elevation of the sub-G1 fraction by **1** was associated with apoptosis, a DNA fragmentation assay was

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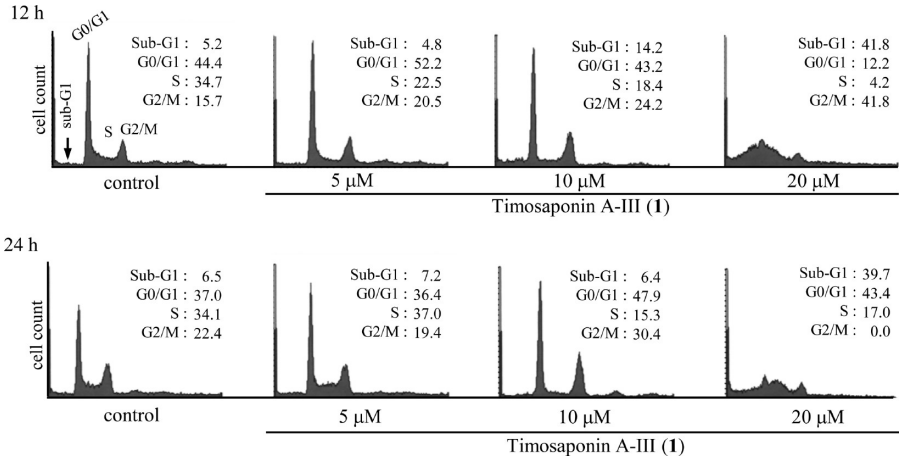

Figure 2. Effect of timosaponin A-III (**1**) on cell-cycle distribution in cultured HCT-15 cells. Flow cytometric analysis of the DNA content treated with **1** (0−20 μM) for 24 h.

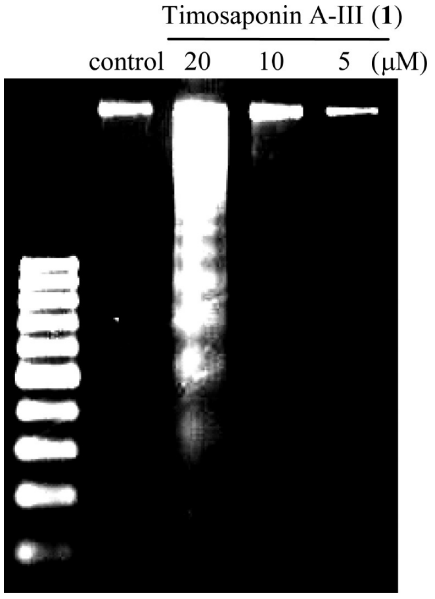

Figure 3. Effect of timosaponin A-III (**1**) on DNA fragmentation in cultured HCT-15 cells. Cells were incubated for 12 h in the presence of **1** (0−20 μM). DNA was extracted and separated using agarose gel electrophoresis, as described in the Experimental Section.

performed. DNA was extracted from HCT-15 cells 24 h after treatment with various concentrations of **1** (0−20 μM) and electrophoresed in agarose gel and visualized under a UV transmission image analyzer. As shown in Figure 3, DNA fragmentation characteristic of apoptosis was clearly detected after exposure to 20 μM of **1**. The data were coincident with the results of the accumulation of sub-G1 peaks in the cell-cycle distribution.

Cell-cycle progression is regulated by cyclin/cyclin-dependent kinase (CDK) complexes.[19] Although CDK expression is relatively constant during all of the cell cycle, the expression of cyclins changes according to the particular phase. For example, cyclin D is increased during the mid/late G1 phase, and a subsequent increase of cyclin E is needed for entry to the S phase. Up-regulation of cyclins A and B leads to S and to G2/M phase entry. Cyclins/CDKs are also regulated by several factors, such as kinase (WEE1, PLK) and phosphatase (CDC25C).[20−23] To investigate further whether the cell-cycle arrest and induction of apoptosis mediated by **1** are related to the expression of cell-cycle regulator proteins, Western blotting analysis was performed. As indicated in Figure 4A, **1** induced the arrest of cell cycle progression at the G0/G1 phase, which was correlated with the decrease of c-Myc and CDK4 expression, and suppression of Rb phosphorylation. In addition, the down-regulation of cyclins A and B1 by treatment of **1** was related to G2/M phase arrest.

Compound **1** down-regulated the expression of cyclin A and CDK4 proteins that are correlated with the G1 to S transition, but, unexpectedly, cyclin D1 and cyclin E expression was increased but the PCNA levels were not altered. Yoo et al.[24] demonstrated that paclitaxel induces G1 phase arrest by suppressing the expression of CDK4 without any changes in cyclin D1 levels. Likewise, although the cyclin D1 level increased until a 10 μM concentration of **1** was reached, the down-regulation of CDK4 might result in the decrease of the cyclin D1-CDK4 complex. Subsequently, the formation of the PCNA/cyclin D1/CDK4 complex might be reduced, and eventually the G0/G1 phase arrest induced. In addition, the level of c-Myc, an oncogene that causes the expression of cyclins and differentiation, was suppressed by **1**, and this was also correlated with the suppressive expression of cyclin A, a target gene of c-Myc. This result is quite similar to the finding that natural cardenolides down-regulate c-Myc expression as a potential target for their cytototic activity in prostate cancer cells.[25] Moreover, the levels of cyclin B1 and phosphorylation of cdc2 (Tyr-15), which are involved in the progression of the M phase, were suppressed by **1**. The suppression of cyclin B1 expression might affect the formation of the cyclin B/cdc2 complex, and this event may evoke also the arrest of the G2/M phase of the cell cycle.

Induction of apoptosis by **1** seems to be associated with the activation of the cascade of caspases. In general, apoptosis, programmed cell death, occurs via either the extrinsic (death receptor) or the intrinsic (mitochondrial) pathway with the activation of caspases. Moreover, the induction of proapoptotic proteins or the suppression of antiapoptotic proteins is a crucial event of apoptosis through mitochondria. Therefore, the change of caspases and Bcl-2 family expression by **1** was confirmed by Western blotting. Compound **1** suppressed the expression of procaspases-8, -9, and -3 and simultaneously increased the corresponding active cleavage forms of caspases in a dose- and

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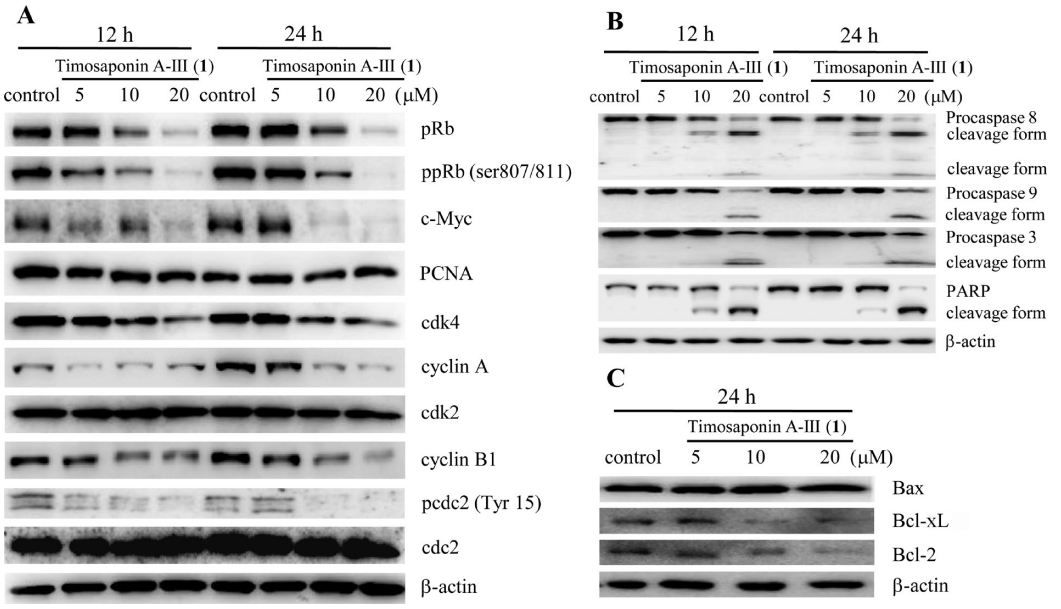

Figure 4. Changes in the expression of biomarkers of cell-cycle regulation (A) and apoptosis (B). HCT-15 cells were treated with **1**, and protein expression was measured by Western blot analysis.

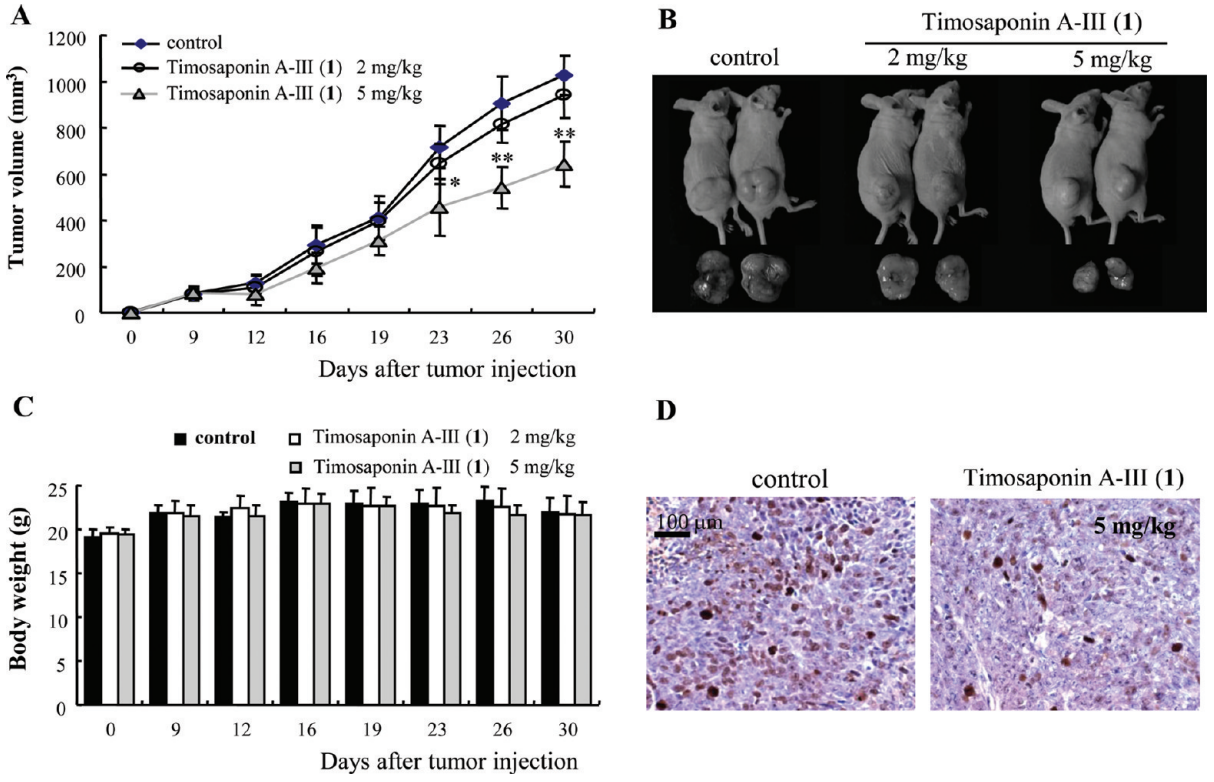

Figure 5. Antineoplastic activity of **1** in a nude mouse model. (A and B) HCT-15 cells were implanted into BALB/c-nu (*nu/nu*) mice, and **1** (2 or 5 mg/kg) was administered ip three times a week for four weeks. Tumor growth was monitored and the tumor volume measured at the indicated time ($^{*}p < 0.05$, $^{**}p < 0.01$ indicates statistically significant differences from the control group). (C) Body weight changes were also monitored during the testing period. (D) Immunohistochemical analysis of Ki-67 cell proliferation biomarker from the tumor tissues. Bar: 100 μm.

time-dependent manner. Subsequently, the induction of PARP cleavage, a target protein of caspase-3,[26] was also manifested with 20 μM **1**, indicating that this compound evokes apoptotic cell death (Figure 4B). In addition, the expression of Bcl-2 and Bcl-xL, antiapoptotic proteins, was suppressed by **1** at 24 h, whereas the level of Bax was not altered (Figure 4C). These events may increase the membrane permeability of the mitochondria and thus induce the release of pro-apoptotic proteins including cytochrome *c*, suggesting that **1** might induce apoptosis via mitochondrial-association events.

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The in vivo antineoplatic activity of **1** was assessed in nude mouse xenograft-bearing HCT-15 human colon cancer cells. Compared with tumor growth in control mice, the growth of tumors was significantly inhibited after treatment of 5 mg/kg **1** ($p < 0.05$; Figure 5A and B). The inhibition rates of tumor size compared with control volume were 8.3% and 37.3% at 2 and 5 mg/kg **1**, respectively. No overt toxicity or body weight change was apparent in the **1**-treated group compared to the controls (Figure 5C). Immunohistochemical analysis of tumors with Ki-67 antibody showed that **1** inhibited the expression of the proliferation biomarker Ki-67 in xenograft tumor cells (Figure 5D).

In summary, this study demonstrates the cytotoxic and antineoplatic effects of timosaponin A-III (**1**) against HCT-15 human colon cancer cells and its mechanism of action of cell-cycle arrest and induction of apoptosis. These findings are supportive of timosaponin A-III (**1**) as a promising candidate natural product anticancer agent.

EXPERIMENTAL SECTION

General Experimental Procedures. Trichloroacetic acid (TCA), sulforhodamine B, propidium iodide, trypsin inhibitor, RNase A, and anti-β-actin primary antibody were purchased from Sigma (St. Louis, MO). Roswell Park Memorial Institute medium 1640 (RPMI 1640), fetal bovine serum (FBS), nonessential amino acid solution (10 mM, 100X), trypsin-EDTA solution (1X), and antibiotic-antimycotic solution (PSF) were from Gibco-BRL (Grand Island, NY). Rabbit anti-CDK2, anti-CDK4, anti-cyclin A, cyclin B1, mouse anti-CDK1 (cdc2), anti-c-myc, horseradish peroxidase (HRP)-conjugated anti-mouse IgG, and horseradish peroxidase (HRP)-conjugated anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-caspase-3, anti-caspase-9, anti-phospho-cdc2 (tyr15), and mouse anti-caspase-8 primary antibody were obtained from Cell Signaling (Danver, MA). Mouse monoclonal anti-PARP, anti-cyclin D1, and anti-cyclin E were from BD Biosciences (San Diego, CA). Mouse monoclonal anti-Ki-67 was purchased from DakoCytation (Copenhagen, Denmark).

Plant Material. The rhizomes (ca. three years cultivated) of *A. asphodeloides* were purchased in September 2008 from Omni Herb.com Oriental Herb Store in Seoul, South Korea. The plant material was identified by Professor Je-Hyun Lee (College of Oriental Medicine, Dongguk University). A voucher specimen (No. EA270) was deposited at the Natural Product Chemistry Laboratory, College of Pharmacy, Ewha Womans University.

Extraction and Isoration. The rhizomes of *A. asphodeloides* (20 kg) were extracted with MeOH under reflux for 4 h. The MeOH solutions were concentrated in vacuo to yield a dried MeOH extract (4 kg). The MeOH extract (4 kg) was suspended in distilled water and fractionated with *n*-hexane, EtOAc, and *n*-BuOH, successively. The *n*-BuOH extract (150 g) was subjected to a silica gel (3000 g) flash column chromatography using $CHCl_3$−MeOH (99:1 → 1:1) as a solvent system, to afford 20 fractions (B1−B20). Fraction B15 (8 g) was separated on a silica gel (180 g) column eluting with $CHCl_3$−MeOH (50:1 to 1:1) to furnish 15 subfractions (B15.1 to B15.15). Subfraction B15.4 (3.5 g) was subjected to passage over a silica gel (90 g) column, eluting with $CHCl_3$−MeOH−H_2O (10:1:0.1 → 9:2:0.1 → 8:2:0.2 → 7:2:0.5), to give timosaponin A-III (**1**) (0.20 g, 0.13% w/w). This compound was identified as **1** by comparison of its physical and spectroscopic data with published values.[27]

HPLC Analysis. High-performance liquid chromatography (J'sphere ODS-H80, 150 × 4.6 mm i.d. 4 μm) of timosaponin A-III (**1**) (t_R 12.56 min) was performed with acetonitrile−H_2O (0.05% TFA) gradient (0 → 20 min (20:80), 20 → 21 min (20:80 → 100:0), 21 → 30 min (100:0), total 30 min, flow rate: 1 mL/min, detector: ELSD). The purity determined for **1** was greater than 97%.

Cell Culture. Human colon cancer (HCT-15, HCT 116, HT-29, SW480, and SW620) cells were obtained from the Korean Cell Line Bank (Seoul, Korea). Human lung MRC-5 and Hs68 fibroblast cells were obtained from ATCC (Manassas, VA). Cells were grown in medium (RPMI for HCT-15, HCT 116, and HT-29 cells; DMEM for MRC-5 and Hs68 cells) supplemented with 10% heat-inactivated fetal bovine serum (FBS), and antibiotics−antimycotics (PSF; 100 units/mL penicillin G sodium, 100 μg/mL streptomycin, and 250 ng/mL amphotericin B). SW480 and SW620 cells were grown in RPMI 1640 medium containing 25 mM HEPES, 10% FBS, and PSF. Cells were maintained at 37 °C in a humidified atmosphere with 5% CO_2.

Evaluation of Growth Inhibitory Potential. Cells (5×10^4 cells/mL) were treated with various concentrations of **1** for 3 days. After treatment, cells were fixed with 10% TCA solution, and cell viability was determined with a sulforhodamine B (SRB) protein staining method.[28] The results are expressed as percentages, relative to solvent-treated control incubations, and the IC_{50} values were calculated using nonlinear regression analysis (percent survival versus concentration).

DNA Fragmentation Assay. HCT-15 cells were plated in a 100 mm culture dish at a density of 1×10^6 cells/dish. Twenty-four hours later, fresh medium containing test sample was added to the cultured dishes. After treatment for 24 h, the cells were washed with PBS and lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 1.0% NP-40. After centrifugation, 1% SDS and RNase A (5 μg/μL) were added to the supernatants and then incubated at 56 °C for 2 h. Subsequently, proteinase K (2.5 μg/μL) was added and then incubated at 37 °C for 2 h. DNA was precipitated with a 0.5 volume of 10 M ammonium acetate and 2.5 volumes of cold ethanol at −20 °C overnight. Precipitated DNA was dissolved in 50 μL of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA. DNA samples (4 μg) were resolved by electrophoresis in 2% agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, OR), and visualized.[29]

Analysis of Cell-Cycle Dynamics by Flow Cytometry. Cell-cycle analysis by flow cytometry was performed as described previously.[29] Briefly, HCT-15 cells were plated at a density of 1×10^6 cells per 100 mm culture dish and incubated for 24 h. Fresh medium containing test samples was added to the culture dishes. After 12 or 24 h, the cells were harvested (trypsinization and centrifugation), fixed with 80% ethanol, and incubated with a staining solution containing 0.2% NP-40, RNase A (50 μg/mL), and propidium iodide (50 μg/mL) in phosphate−citrate buffer (pH 7.2). Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer (BD Bioscienes, San Jose, CA). At least 20 000 cells were used for each analysis, and results are displayed as histograms. The distribution in each phase of cell cycle was determined using the ModFit LT 2.0 program.

Evaluation on the Expression of Biomarkers of Cell-Cycle Regulators. Cells were exposed with various concentrations of **1**. After incubation, cells were lysed and protein concentrations were determined by the BCA method.[30] Each protein (30−50 μg) was subjected to 10% SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were treated for 1 h with blocking buffer [5% nonfat dry milk in phosphate-buffered saline−0.1% Tween 20 (PBST)]. Membranes were then incubated with indicated antibodies overnight at 4 °C and washed three times for 5 min with PBST. After washing, membranes were incubated with HRP-conjugated anti-mouse IgG diluted 1:1500 in PBST for 3 h at room temperature, washed three times for 5 min with PBST, and detected using ECL reagent (Lab Frontier, Seoul, Korea). Blots were imaged by LAS3000 (Fuji Film Corp., Tokyo, Japan)

In Vivo Tumor Xenograft Study. Male nude mice (5 weeks old, BALB/c-nu (*nu/nu*)) were purchased from the Central Laboratory Animal Inc. (Seoul, Korea). All animal experiments and care were conducted in a manner conforming to the Guidelines of the Animal Care and Use Committee of Ewha Womans University approved by the

Korean Association of Laboratory Animal Care (permission number: EWHA2008-2-05).

HCT-15 cells were injected subcutaneously into the flanks of the mice (2×10^6 cells in 200 μL of medium), and tumors were allowed to grow. When the tumor volume was reached (ca. 80 mm^3), the treatment was initiated. The mice were randomized into vehicle control and treatment groups of five animals per each. Compound **1** (2 or 5 mg/kg body weight) dissolved in a volume of 200 μL of solution (ethanol−cremophor−H_2O, 0.5:0.5:99) was administered intraperitoneally three times a week. The control group was treated with an equal volume of vehicle. Tumor volume was monitored for 30 days three times per week using calipers, and tumor volume was estimated according to the following formula: tumor volume (mm^3) = $3.14 \times L \times W \times H/6$, where L is the length, W is the width, and H is the height.

The immunohistochemical analysis of tumor tissues was performed for the detection of cell proliferation with the biomarker Ki-67 antibody.[31] Sections from xenograft tumor tissues were incubated at 4 °C overnight with the antibody for Ki-67 (1:250). The primary antibody was localized by incubation with a biotinylated anti-mouse secondary antibody for 4 h, followed by streptavidin-conjugated peroxidase, and then visualized with 3,3-diaminobenzidine chromogen solution. Sections were counterstained with Mayer's hematoxylin and mounted. Brown-red labeled nuclei within each section were observed under the inverted phase-contrast microscope and photographed.

Statistical Analysis. Data are presented as means ± SE for the indicated number of independently performed experiments. Statistical significance ($p < 0.05$) was assessed by one-way analysis of variance (ANOVA) coupled with the Dunnett's t-test.

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