

## Full Paper

**Xanthorrhizol, a Natural Sesquiterpenoid, Induces Apoptosis and Growth Arrest in HCT116 Human Colon Cancer Cells**You-Jin Kang<sup>1</sup>, Kwang-Kyun Park<sup>2</sup>, Won-Yoon Chung<sup>2</sup>, Jae-Kwan Hwang<sup>3</sup>, and Sang Kook Lee<sup>1,\*</sup><sup>1</sup>College of Pharmacy, Ewha Womans University, Seoul 120-750, Korea<sup>2</sup>College of Dentistry, <sup>3</sup>Department of Biotechnology, Yonsei University, Seoul 120-749, Korea

Received May 8, 2009; Accepted September 14, 2009

**Abstract.** Xanthorrhizol is a sesquiterpenoid from the rhizome of *Curcuma xanthorrhiza*. In our previous studies, xanthorrhizol suppressed cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression, inhibited cancer cell growth, and exerted an anti-metastatic effect in an animal model. However, the exact mechanisms for its inhibitory effects against cancer cell growth have not yet been fully elucidated. In the present study, we investigated the growth inhibitory effect of xanthorrhizol on cancer cells. Xanthorrhizol dose-dependently exerted antiproliferative effects against HCT116 human colon cancer cells. Xanthorrhizol also arrested cell cycle progression in the G0/G1 and G2/M phase and induced the increase of sub-G1 peaks. Cell cycle arrest was highly correlated with the downregulation of cyclin A, cyclin B1, and cyclin D1; cyclin-dependent kinase 1 (CDK1), CDK2, and CDK4; proliferating cell nuclear antigen; and inductions of p21 and p27, cyclin-dependent kinase inhibitors. The apoptosis by xanthorrhizol was markedly evidenced by induction of DNA fragmentation, release of cytochrome c, activation of caspases, and cleavage of poly-(ADP-ribose) polymerase. In addition, xanthorrhizol increased the expression and promoter activity of pro-apoptotic non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1). These findings provide one plausible mechanism for the growth inhibitory activity of xanthorrhizol against cancer cells.

**Keywords:** apoptosis, cell cycle arrest, HCT116 human colon cancer cell, non-steroidal anti-inflammatory drug-activated gene-1 (NAG-1) expression, xanthorrhizol

**Introduction**

Natural products have played an important role in drug discovery. In particular, cancer chemotherapeutic agents are mainly derived from natural products or natural product-oriented synthetic derivatives (1–5). In our program to discover cancer chemotherapeutic or chemopreventive agents from plant extracts, we recently found that xanthorrhizol is relevant to this capacity. Xanthorrhizol, a sesquiterpenoid isolated from the rhizome of *Curcuma xanthorrhiza* (Zingiberaceae), exhibits various biological activities including antibacterial effect, antifungal effect, preventive effects against cisplatin-induced hepatotoxicity and nephrotox-

icity, and neuroprotective activity (6–8). In our previous studies on carcinogenesis, xanthorrhizol suppressed cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression in lipopolysaccharide (LPS)-induced RAW 264.7 cells and exhibited anti-metastasis activity in a mouse spontaneous lung metastasis model (9, 10). Recently, we also reported cancer chemopreventive activity mediated by xanthorrhizol and a plausible mechanism of action in a mouse two-stage carcinogenesis model (11). However, the underlying mechanisms of action through which xanthorrhizol inhibits cancer cell growth are yet not fully clarified.

Non-steroidal anti-inflammatory drug (NSAID)-activated gene-1 (NAG-1) has recently been identified as a pro-apoptotic and anti-tumorigenic protein induced by NSAIDs using the polymerase chain reaction (PCR)-based subtractive hybridization method (12). NAG-1 is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ )

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Published online in J-STAGE

doi: 10.1254/jphs.09141FP

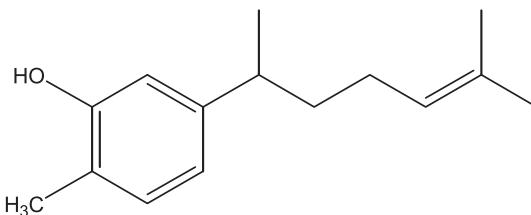
superfamily and identical to previously identified macrophage inhibitory cytokine-1, placental TGF- $\beta$ , prostate-derived factor, growth differentiating factor-15, and placental bone morphogenetic protein (12–14). Especially, NAG-1 is highly expressed in mature intestinal epithelial cells, but is significantly suppressed in human colorectal cancer and neoplastic tumors (15, 16). In addition, expression of NAG-1 gene in several cancer cells including prostate, breast, lung, and colon induced apoptosis and growth inhibition of cancer cells; and NAG-1 expression also resulted in the suppression of tumor growth (15) and induction of apoptosis in a xenograft and/or a chemical-induced tumor model *in vivo*. These findings strongly suggest that NAG-1 is functioning in the growth suppressive anti-cancer effects in cancer cells, particularly in the colon. Several compounds including resveratrol, indole-3-carbinol, epicatechin gallate, genistein, diallyldisulfide, peroxisome proliferators-activated receptor  $\gamma$  (PPAR- $\gamma$ ) ligand, conjugated linoleic acid, and NSAIDs such as indomethacin showed anti-tumor activity against colon cancer through the upregulation of NAG-1 (12, 13, 17, 18). Recent studies also demonstrated that NAG-1 expression is regulated by several signaling pathways such as p53, phosphatidylinositol 3-kinase (PI3K) / AKT / glycogen synthase kinase-3 $\beta$  (GSK3 $\beta$ ), protein kinase C (PKC), and activating transcription factor 3 (ATF3) (15, 17).

In the present study, based on the cancer chemopreventive potential of xanthorrhizol, we investigated the growth inhibitory effect of xanthorrhizol in colon cancer cells and analyzed the mechanisms of action by elucidating cell cycle distribution and regulation of NAG-1 expression.

## Materials and Methods

### Chemicals and reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ribonuclease A (RNase A), propidium iodide, bisbenzimid H 33258 (Hoechst 33258), and mouse monoclonal anti- $\beta$ -actin primary antibody were purchased from Sigma (St. Louis, MO, USA). Rosewell Park Memorial Institute (RPMI) medium 1640, fetal bovine serum, non-essential amino acid solution (10 mM, 100 $\times$ ), trypsin-EDTA solution (1 $\times$ ) and antibiotic-antimycotics solution (PSF) were from Invitrogen Co. (Grand Island, NY, USA). Mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) and anti-cyclin-dependent kinase 1 (CDK1); rabbit polyclonal anti-p21, anti-p27, anti-CDK2, anti-CDK4, anti-cyclin A, and anti-cyclin B1 primary antibody; and horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit IgG were from Santa Cruz Biotechnology (Santa



**Fig. 1.** Chemical structure of xanthorrhizol.

Cruz, CA, USA). Mouse monoclonal anti-PARP and anti-cytochrome c antibody were from BD Biosciences (San Diego, CA, USA). Mouse anti-caspase-8 and rabbit anti-caspase-3, and anti-caspase-9 antibody were from Cell Signaling Technology (Beverly, MA, USA). Rabbit anti-NAG-1 antibody was from Upstate (Charlottesville, VA, USA). pNAG-1-Luc was a gift from Dr. S.J. Baek (University of Tennessee, Knoxville, TN, USA). The dual luciferase assay system was purchased from Promega (Madison, WI, USA).

Xanthorrhizol (Fig. 1) isolated from the rhizome of *Curcuma xanthorrhiza* (7), which was provided by J.-K. Hwang, a co-author.

### Cell lines and cell culture

Human colon carcinoma HCT 116 cells, obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea), were cultured in RPMI supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100  $\mu$ g/ml streptomycin, and 250 ng/ml amphotericin B. Cells were maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

### Cell growth inhibitory assay

HCT 116 cells (2  $\times$  10<sup>4</sup> cells/well in 24-well plates) were incubated for 48 h and then re-fed with fresh medium containing various concentrations of test compound for an additional 3 days. After treatment, MTT (5 mg/ml in phosphate-buffered saline) was added to media (final concentration of 500  $\mu$ g/ml) and further incubated for 4 h. Media were discarded, and 200  $\mu$ l of DMSO was added to each well to dissolve formazan crystals. The absorbance was measured at 570 nm. The result was expressed as a percentage relative to solvent-treated control incubations, and the IC<sub>50</sub> values were calculated using non-linear regression analysis (percent survival vs. concentration).

### DNA fragmentation assay

HCT 116 cells were plated in 100-mm culture dish at a density of 6.5  $\times$  10<sup>5</sup> cells/dish. Forty-eight hours later, the cells were treated with various concentrations of test agent for either 24 or 48 h. After treatment, the cells

were collected by centrifugation, washed with PBS, and lysed in buffer containing 50 mM Tris-HCl, pH 7.5, 20 mM EDTA, and 1% NP-40. After centrifugation, 1% SDS and RNase A (0.5 µg/µl) were added to the supernatants and then incubated at 56°C for 1 h. Subsequently, proteinase K (5 µg/µl) was added and then incubated at 37°C for 2 h. The samples were mixed with 0.5 volume of 10 M ammonium acetate and 2.5 volumes of cold ethanol at -70°C for 1 h. Precipitated DNA was dissolved in 30 µl of 10 mM Tris buffer (pH 8.0) containing 1 mM EDTA. DNA samples (10 µg) were resolved by electrophoresis in 2% agarose gel, stained with SYBR Gold (Molecular Probes, Eugene, Oregon, USA), and visualized under Alpha Imager™ (Alpha Innotech Co., San Leandro, CA, USA).

#### *Double staining*

HCT 116 cells were treated with xanthorrhizol for 24 or 48 h. After incubation, cells were collected and washed twice with PBS. Cells were stained with annexin V-FITC and propidium iodide (PI) solution by use of an annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells were diluted with 1× binding buffer at the density of  $1 \times 10^6$  cells/ml. A 100-µl aliquot of cell suspension was transferred into a 15 ml round-bottom polystyrene tube, and 5 µl of annexin V-FITC solution and 5 µl of PI solution was added to the cell suspension and further incubated for 20 min at room temperature in the dark. Stained cells were diluted with 1× binding buffer and immediately analyzed by flow cytometer.

#### *Cell cycle analysis by flow cytometry*

Cell cycle analysis by flow cytometry was performed as previously described (9). Briefly, HCT 116 cells were plated at a density of  $6.5 \times 10^5$  cells per 100-mm culture dish and incubated for 48 h. Fresh media containing test samples were added to culture dishes. After additional incubation for either 24 or 48 h, cells were collected by centrifugation and resuspended with PBS. Cells were centrifuged at  $2,000 \times g$  for 5 min, washed again with PBS, and then fixed with 80% ethanol. Fixed cells were washed with phosphate-citrate buffer (pH 7.2) and incubated with a staining solution containing 0.2% NP-40, RNase A (30 µg/ml), and propidium iodide (50 µg/ml) in phosphate-citrate buffer (pH 7.2) for 30 min. Cellular DNA content was analyzed by flow cytometry using a Becton Dickinson laser-based flow cytometer. At least 20,000 cells were used for each analysis, and results were displayed as histograms. Cell cycle distribution was analyzed using ModFit LT 2.0 program (BD Biosciences, San Jose, CA, USA).

#### *Western blot analysis*

Cells were exposed to the test agent at various concentrations or for various time intervals. After incubation, cells were lysed and protein concentrations were determined by the BCA method. Each protein sample (30–50 µg) was subjected to 8%–15% SDS-PAGE. Proteins were transferred onto PVDF membranes by electroblotting, and membranes were incubated for 1 h with blocking buffer [5% skimmed milk in phosphate-buffered saline–0.1% Tween 20 (PBST)]. Membranes were then incubated with the indicated antibodies overnight at 4°C (mouse anti-β-actin, 1:1500; mouse anti-cytochrome c, 1:500; mouse anti-PCNA, 1:1000; rabbit anti-cyclin A, 1:1000; rabbit anti-cyclin B1, 1:1000; mouse anti-cyclin D1, 1:1000; mouse anti-CDK1, 1:1000; rabbit anti-CDK2, 1:1000; rabbit anti-CDK4, 1:1000; rabbit anti-p21, 1:750; rabbit anti-p27, 1:750; rabbit anti-phospho-mTOR (ser 2448), 1:1000; rabbit anti-mTOR, 1:1000; rabbit anti-phospho-Akt (ser 473), 1:1000; rabbit anti-Akt, 1:1000; rabbit anti-GSK3β (ser 9), 1:1000; mouse anti-GSK3β, 1:1000; mouse anti-PARP, 1:1000; mouse anti-caspase-8, 1:1000; rabbit anti-caspase-9, 1:1000; rabbit anti-caspase-3, 1:500; rabbit anti-NAG-1, 1:1000) and then washed three times (each for 5 min) with PBST. After washing, membranes were incubated with the corresponding HRP-conjugated IgG (diluted 1:2000 in PBS) for 1 h at room temperature, washed three times (each for 5 min) with PBST, and the luminescence detected by LAS-3000 (Fuji Film Corp., Tokyo) using ECL reagent (Amersham Corp., Arlington Heights, IL, USA). The expression of β-actin was used as an internal standard.

#### *Cell-based reporter gene assay*

Transient transfections were carried out using Lipofectamine 2000 (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions. HCT116 cells were plated at a density of  $1 \times 10^5$  cells per well in a 24-well plate and incubated for 18 h. Plasmid mixtures (0.5 µg NAG-1 promoter linked to luciferase and 0.05 µg pRL-SV40 vector) were transfected for 5 h. After removal of plasmid-containing media, the cells were further cultured in a fresh medium for 19 h. Subsequently, the cells were treated with various concentrations of test compound for 24 h. The cells were harvested in 1× passive lysis buffer, and luciferase activity was normalized to the *Renilla* luciferase activity using a dual luciferase assay kit (Promega).

#### *Statistical analyses*

Data were presented as means ± S.E.M. for the indicated number of independently performed experiments. Statistical significance ( $P < 0.05$ ) was assessed by one-

way analysis of variance (ANOVA) coupled with Dunnett's *t*-tests.

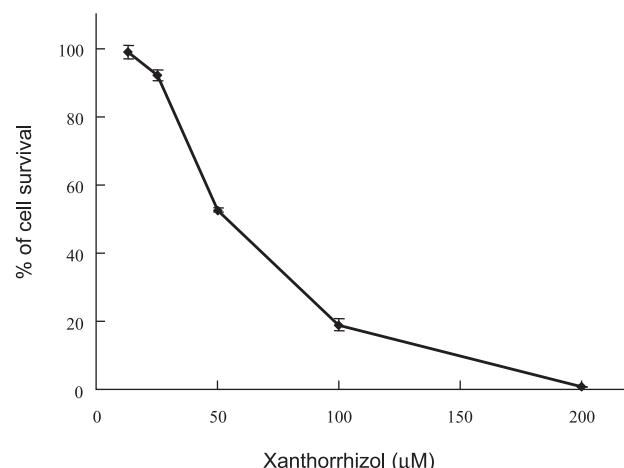
## Results

### Growth inhibitory effects of xanthorrhizol on HCT116 human colon cancer cells

In order to investigate the effects of xanthorrhizol on the growth of human colon cancer cells, HCT116 cells were treated with various concentrations of xanthorrhizol for 3 days, and the viable cells were measured by the MTT method. As shown in Fig. 2, xanthorrhizol inhibited the growth of HCT116 cells in a dose-dependent manner ( $IC_{50} = 54.8 \mu\text{M}$ ). Xanthorrhizol concentrations up to  $50 \mu\text{M}$  caused mainly cell cycle arrest. In contrast, over  $50 \mu\text{M}$  of the compound caused a cytotoxic effect, with floating dead cells being observed.

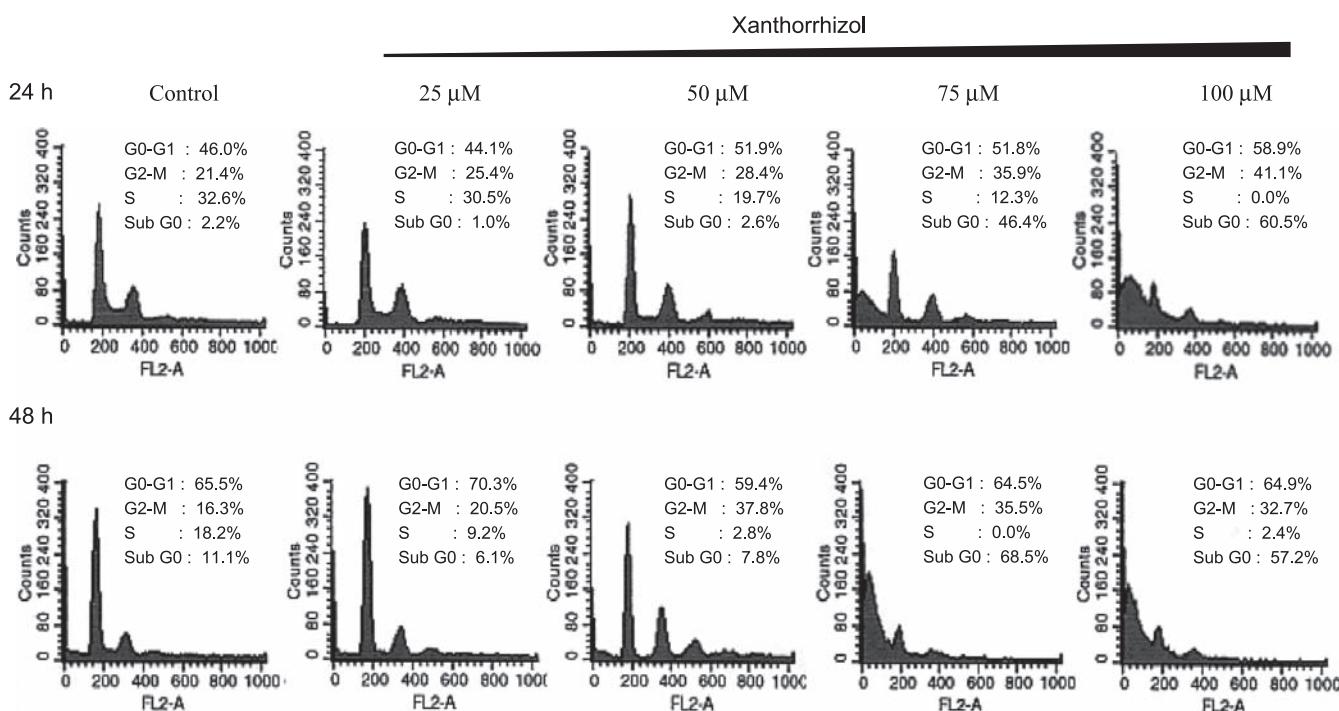
### Effect of xanthorrhizol on the cell cycle distribution of HCT116 cells

HCT116 cells were treated with various concentrations ( $0 - 100 \mu\text{M}$ ) of xanthorrhizol for 24 or 48 h, and the distribution of cells in various compartments of the cell cycle was analyzed by flow cytometry. When treated with the compound under the  $IC_{50}$  value (25 or  $50 \mu\text{M}$ ) or the earlier incubation time points (24 h), cells were generally accumulated in either the G1 or G2/M



**Fig. 2.** Growth inhibitory effects of xanthorrhizol in HCT116 human colon cancer cells. HCT116 cells were treated with various concentrations of xanthorrhizol ( $0 - 200 \mu\text{M}$ ) for 72 h. Growth inhibitory effect was determined by the MTT assay. The values for % of cell survival were calculated by comparison with the vehicle-treated control cells. Data are each expressed as the mean  $\pm$  S.D. of 4 replicates.

phase. However, treatment with the higher concentrations (75 or  $100 \mu\text{M}$ ) or longer exposure (48 h) to the compound dramatically increased cells in the sub-G1 phase, indicative of apoptotic peaks, during the incubation time (Fig. 3 and Table 1).



**Fig. 3.** Effects of xanthorrhizol on the cell cycle progression in HCT116 cells. HCT116 cells were treated with vehicle or various concentrations of xanthorrhizol (25, 50, 75, or  $100 \mu\text{M}$ ) for 24 or 48 h. The cell cycle distribution was analyzed by FACScalibur and depicted with the histogram. The data are representative of three independent experiments.

**Table 1.** Cell cycle analysis of xanthorrhizol treated HCT-116 cells

Time	Treatment ( $\mu\text{M}$ )	Cell cycle			
		Sub-G0/G1 (%)	G0/G1 (%)	S (%)	G2/M (%)
24 h	0 (Control)	2.2	46	32.6	21.4
	25	1	44.1	30.5	25.4
	50	2.6	51.9	19.7	28.4
	75	46.4	51.8	12.3	35.9
	100	60.5	58.9	0	41.1
48 h	0 (Control)	11.1	65.5	18.2	16.3
	25	6.1	70.3	9.2	20.5
	50	7.8	59.4	2.8	37.8
	75	68.5	64.5	0.0	35.5
	100	57.2	64.9	2.4	32.7

#### Effects of xanthorrhizol on the expression of cell cycle regulator proteins

Based on the analysis of the FACS data, cell cycle arrest seems to be evoked by treatment with xanthorrhizol at the lower concentrations (under  $\text{IC}_{50}$  value) or for a short time. To examine whether the cell cycle arrest is associated with the expression of cell cycle regulatory proteins, cells were treated with various concentrations of the compound for 24 or 48 h, and then Western blot analysis was performed. As shown in Fig. 4 the expression of cyclin D1 and CDK4, which is associated with the transition of G1 to S phase, was suppressed in a time- and dose-dependent manner. The expression level of cyclin A and CDK2, which is connected with the

transition of S or M phase, was also downregulated with the treatment of xanthorrhizol. Cyclin B1 and CDK1 are associated with the transition of G2 to M phase, and the level of cyclin B1 and CDK1 was also suppressed by xanthorrhizol at concentrations less than the  $\text{IC}_{50}$ . However, the CDK inhibitors p21 and p27, which are related with the retardation of cell cycle progression in the G1 or G2/M phase, were induced, and these events might regulate the cell cycle arrest by the compound. In addition, PCNA, a biomarker for the cell proliferation, was also suppressed by xanthorrhizol, indicating the growth inhibition of cell proliferation by the compound.

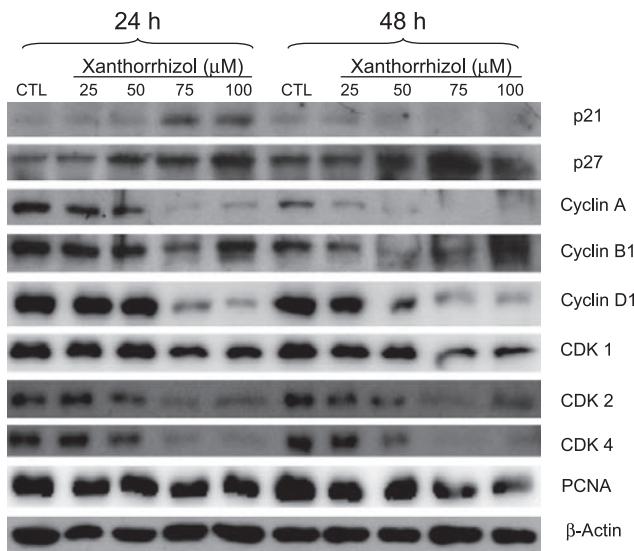
#### Apoptosis induction by xanthorrhizol in HCT116 cells

To further determine whether the cytotoxic effect of xanthorrhizol was associated with apoptosis, DNA was extracted from HCT116 cells at 24 or 48 h after exposure to the compound and subjected to agarose-gel electrophoresis. DNA fragmentation characteristic of apoptosis was clearly detected by exposure to xanthorrhizol in a dose- and time-dependent manner as illustrated in Fig. 5A.

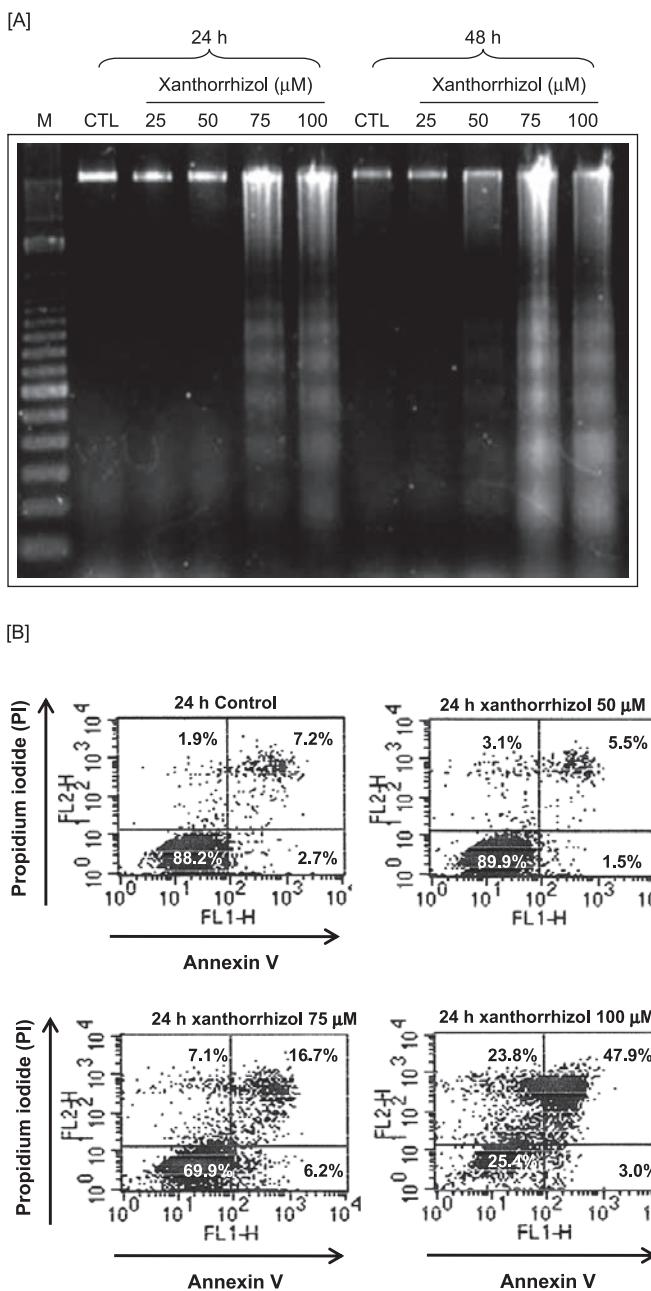
In addition, to examine the induction of apoptosis by xanthorrhizol, phosphatidylserine (PS) exposure was also determined. As shown in Fig. 5B, compared to vehicle-treated control cells, xanthorrhizol (75 and 100  $\mu\text{M}$ ) significantly increased the cells stained with both annexin V-FITC and propidium iodide, suggesting apoptotic cell death.

#### Effects of xanthorrhizol on the expression of apoptosis related proteins

To examine whether the expressions of apoptosis-related proteins are associated with the sub-G1 peaks of FACS analysis and DNA fragmentation by treatment



**Fig. 4.** Effect of xanthorrhizol on the expression of cell cycle regulatory proteins in HCT116 cells. HCT116 cells were treated with various concentrations of xanthorrhizol for 24 or 48 h. Cell lysates were subjected to Western blotting.



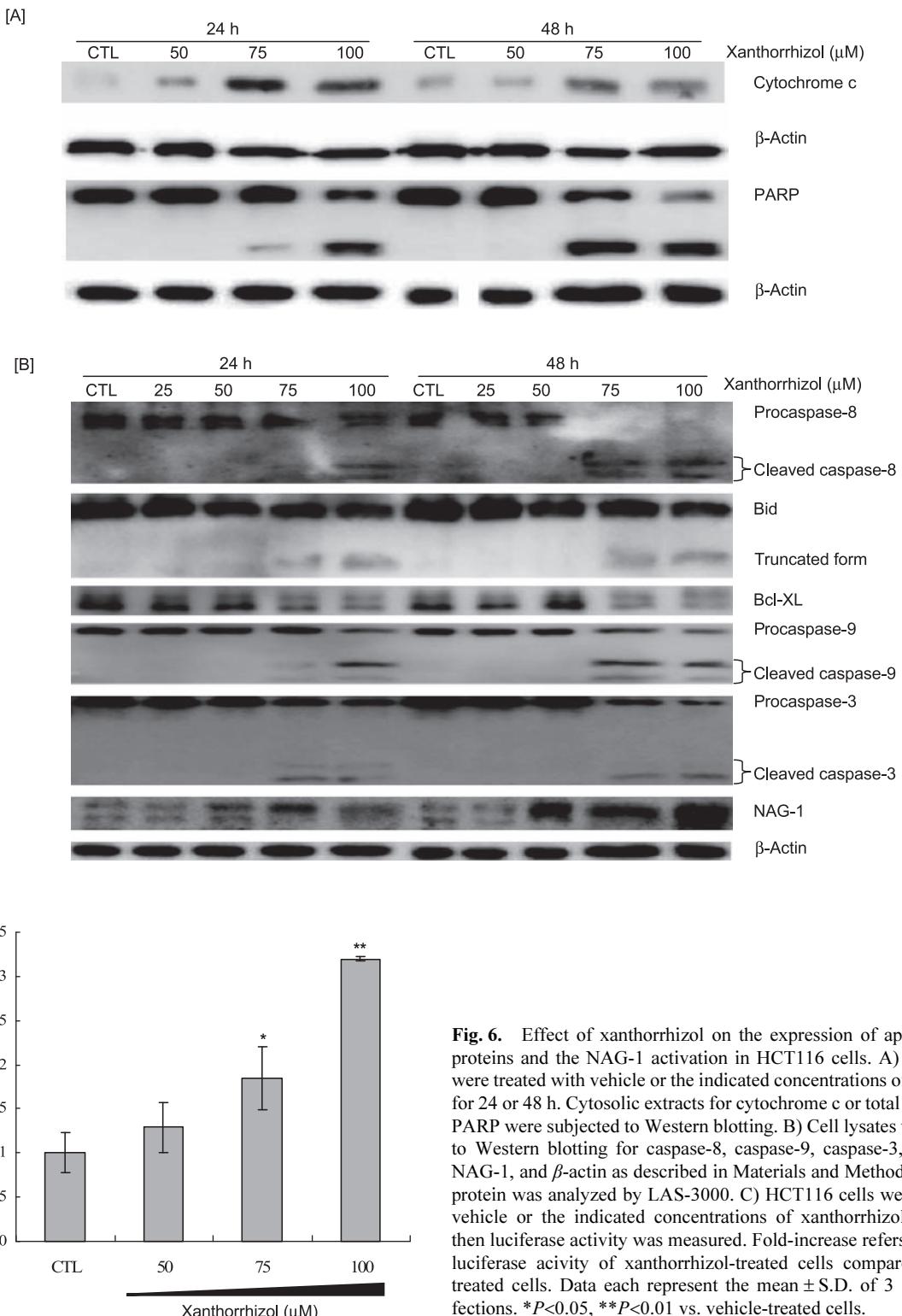
**Fig. 5.** Induction of apoptosis by treatment with xanthorrhizol in HCT116 cells. HCT116 cells were treated with vehicle or the indicated concentration of xanthorrhizol for 24 or 48 h. A) Xanthorrhizol induced DNA fragmentation in HCT116 cells. Extracted DNA was separated by agarose gel electrophoresis and visualized under the UV transilluminator. B) HCT116 cells stained by both annexin V-FITC and propidium iodide (PI) were increased by xanthorrhizol. All of the adherent and floating cells were collected, and then the cells were further incubated with annexin V-FITC and PI for 20 min and immediately subjected to cytometric analysis.

of xanthorrhizol, the cells were treated with various concentrations of test compound ( $0 - 100 \mu\text{M}$ ) for 24 or 48 h. As shown in Fig. 6A, the cytosolic release of cytochrome c mediated by the increase of membrane permeability was detected, indicating the apoptosis evoked by xanthorrhizol might be related to the mitochondria-associated events. These results were also associated with the downregulation of anti-apoptotic protein Bcl-XL. Xanthorrhizol also promoted the cleavage of caspase-8 and truncation of Bid (tBid). To further characterize the apoptotic phenomena, the cascade proteins along with the activation of procaspase-9 and procaspase-3 were determined. As illustrated in Fig. 6B,

procaspase-9 and -3 expressions were clearly decreased and the active forms caspase-9 and -3 were also increased in a dose- and time-dependent manner. Subsequently, the PARP cleavage, which is a target of caspase-3, was also induced and thus involved in the induction of apoptosis. In addition, pro-apoptotic protein NAG-1 was also induced in a dose-dependent manner.

#### *Effects of xanthorrhizol on the promoter activity of NAG-1*

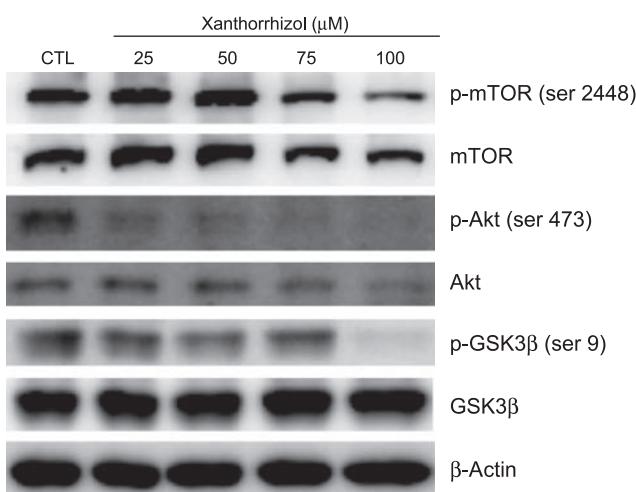
To further obtain evidence for whether the NAG-1 protein expression is related to the induction of promoter activity of NAG-1, the cells were transiently co-



**Fig. 6.** Effect of xanthorrhizol on the expression of apoptosis-related proteins and the NAG-1 activation in HCT116 cells. A) HCT116 cells were treated with vehicle or the indicated concentrations of xanthorrhizol for 24 or 48 h. Cytosolic extracts for cytochrome c or total cell lysates for PARP were subjected to Western blotting. B) Cell lysates were subjected to Western blotting for caspase-8, caspase-9, caspase-3, Bid, Bcl-XL, NAG-1, and  $\beta$ -actin as described in Materials and Methods. The level of protein was analyzed by LAS-3000. C) HCT116 cells were treated with vehicle or the indicated concentrations of xanthorrhizol for 24 h and then luciferase activity was measured. Fold-increase refers to the ratio of luciferase activity of xanthorrhizol-treated cells compared to vehicle-treated cells. Data each represent the mean  $\pm$  S.D. of 3 separate transfections. \*P<0.05, \*\*P<0.01 vs. vehicle-treated cells.

transfected with pNAG-1 and pRL-SV40 and then luciferase activity was measured by dual luciferase activity assay. As shown in Fig. 6C, xanthorrhizol enhanced the NAG-1 promoter activity in a dose-dependent manner. To further confirm xanthorrhizol's effect

on the expression of NAG-1 gene, the plausible regulatory signaling pathway was examined. As shown in Fig. 7, xanthorrhizol suppressed the activation of the AKT pathway and thus affected to the subsequent downstream signaling molecules including GSK3 $\beta$  and mTOR



**Fig. 7.** Effect of xanthorrhizol on the expression of AKT/GSK3 $\beta$ /mTOR signaling in HCT116 cells. HCT116 cells were treated with vehicle or the indicated concentrations of xanthorrhizol for 24 h. Cell lysates were subjected to Western blotting for AKT, GSK3 $\beta$ , mTOR, and  $\beta$ -actin as described in Materials and Methods. The level of protein was analyzed by LAS-3000.

activation. Therefore, these signaling molecules might be in part related to the expression of the NAG-1 gene.

## Discussion

Cancer is the leading cause of death worldwide. Especially, colorectal cancer is one of the most abundant causes of cancer mortality in the Western countries. One plausible strategy for controlling colorectal cancers is cancer chemoprevention by intake of dietary factors. A variety of phytochemicals derived from natural products has been reported to modulate the growth of colorectal cancer cells and thus are considered to be cancer chemopreventive agents (19, 20). Xanthorrhizol is a naturally occurring sesquiterpenoid that has been demonstrated to have a variety of biological activities such as antibacterial and antifungal activities, neuroprotective activity, and protective effects against chemotherapeutic drug-induced hepatotoxicity and nephrotoxicity (6–8). In our previous studies, we showed that xanthorrhizol may be a possible cancer chemopreventive agent that suppresses COX-2 and iNOS expressions and also exhibited an anti-metastasis effect and anti-tumor promoter activity in a mouse two-stage skin carcinogenesis model. In the anti-metastasis activity, xanthorrhizol suppressed expressions of MMP-9 and COX-2, which are proteins highly associated with the metastatic process. Based on the anti-tumor and anti-metastatic effects of xanthorrhizol, we attempted to elucidate the underlying mechanisms of action of xanthorrhizol in the regulation of the growth of human colon cancer cells.

Xanthorrhizol inhibits the proliferation of cultured human colorectal cancer HCT 116 cells and the growth inhibition was associated with the cell cycle arrest and induction of apoptosis. In particular, the lower concentrations of xanthorrhizol (up to 50  $\mu$ M) were found to be effective in the inhibition of cell proliferation with cytostatic activity, but the higher concentration ( $>50 \mu$ M) had cytotoxic effects (Fig. 2). The mechanism of the growth inhibitory effect of xanthorrhizol in cancer cells appeared to be related to the induction of cell cycle arrest either in the G1 or G2/M phase, depending on the test concentration and incubation time (Fig. 3). At 24 h, G1 phase cell cycle arrest was found at the test concentration of 50  $\mu$ M (control: G1 45%, treatment: G1 52%), but at 48-h incubation, G1 cell cycle was found at 25  $\mu$ M and G2/M phase cell cycle was more manifested at the test concentration of 50  $\mu$ M (control: G2/M 16%, treatment: G2/M 38%). Cell cycle checkpoint protein expression showed that the induction of cell cycle arrest was highly correlated with the downregulation of cyclin A/B1/D1, CDK 1/2/4, and PCNA and also induction of p21 and p27 (Fig. 4). In addition, the cytotoxic effect and apoptotic cell death by xanthorrhizol was pronounced with treatment by concentrations over the IC<sub>50</sub> value (75 or 100  $\mu$ M) and the induction of apoptosis was dependent on the concentration and duration of incubation with the test compound. At 48 h, the treatment of 100  $\mu$ M of xanthorrhizol evoked the apoptotic peaks with over 80% in the sub-G1 phase of the cell cycle distribution. The apoptosis was also confirmed by the observation of DNA fragmentation (Fig. 5A) and PS exposure (Fig. 5B). One of the well-known phenomena of apoptosis is an increase in mitochondrial membrane permeability, thus leading to the cytosolic release of cytochrome c. The treatment of xanthorrhizol dose-dependently induced release of cytochrome c as shown in Fig. 6A. The release of cytochrome c is also evoked by the activation of cascade proteins including caspases. Xanthorrhizol-mediated apoptosis exhibits consequential activation of caspase-8, -9, and caspase-3; truncation of bid; and inhibition of bcl-XL (Fig. 6B), and thus induces the cleavage of PARP, a target protein of caspases.

Recent studies reported that NAG-1 is considered a pro-apoptotic protein, which is induced in either a p53-dependent or p53-independent manner (17, 21). The induction of NAG-1 protein was clearly demonstrated with the apoptosis-evoking concentrations of xanthorrhizol and the longer exposure of the compound. NAG-1 promoter activity studied by transient transfection of pNAG-1-Luc revealed that xanthorrhizol activates NAG-1 promoter activity (Fig. 6C) and thus induces apoptosis in the human colon cancer cells. Although

the mechanisms of expression of NAG-1 gene are not clear, recently several signaling pathways including PI3K/AKT/GSK3 $\beta$  were suggested (15). The present study showed that xanthorrhizol also suppressed the activation of AKT/GSK3 $\beta$ /mTOR signaling, suggesting one plausible mechanism of action in the regulation of the NAG-1 gene (Fig. 7). Further studies are still needed to clarify the exact regulatory mechanism of NAG-1 expression.

In summary, this study suggests that the growth inhibitory effects of xanthorrhizol against human colon cancer cells are related to the cell cycle arrest and induction of apoptosis. With the potential cancer chemopreventive potential of xanthorrhizol in vitro and in vivo, this study further suggests one plausible mechanism of action in the growth inhibitory effect of xanthorrhizol in human colon cancer cells.

### Acknowledgments

This work was supported by a grant No. R15-2006-020 from the National Core Research Center (NCRC) program of the Ministry of Education, Science & Technology (MEST) and the Korea Science and Engineering Foundation (KOSEF) through the Center for Cell Signaling & Drug Discovery Research at Ewha Womans University.

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