Biochemical Pharmacology 80 (2010) 1356–1364

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Inhibition of cell growth and potentiation of tumor necrosis factor-α (TNF-α)-induced apoptosis by a phenanthroindolizidine alkaloid antofine in human colon cancer cells

Hye-Young Min [a], Hwa-Jin Chung [a,b], Eun-Hye Kim [a], Sanghee Kim [b], Eun-Jung Park [a], Sang Kook Lee [a,b,*]

[a] College of Pharmacy and Division of Life & Pharmaceutical Sciences, Ewha Womans University, Seoul 120-750, Republic of Korea
[b] College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

ARTICLE INFO

Article history:
Received 15 May 2010
Accepted 21 July 2010

Keywords:
Antofine
Phenanthroindolizidine alkaloid
Anti-proliferation
Antitumor

ABSTRACT

Based on the potential of natural products as a source for the development of cancer chemotherapeutic agents, this study was performed to investigate the anti-proliferative and antitumor effects of antofine, a phenanthroindolizidine alkaloid derived from *Cynanchum paniculatum*. Antofine showed potent anti-proliferative effects in several human cancer cells with IC_{50} values in the nanomolar range. Treatment with antofine for 24 h did not result in the induction of apoptotic cell death but moderately induced cell cycle arrest at G0/G1 phase and inhibited the expression of cyclin D1, cyclin E, and CDK4. In addition, antofine inhibited the transcriptional activity of β-catenin/Tcf in human colon HCT116 cells, and the expression level of β-catenin and cyclin D1 was also down-regulated by antofine in human colon SW480 cells. Moreover, antofine potentiated tumor necrosis factor-α (TNF-α)-induced apoptosis, which was demonstrated by the increase of Annexin V-positive cell population and of the cleavage of poly (ADP-ribose) polymerase (PARP) and caspase-8. Antofine also effectively suppressed tumor growth in the HCT116 implanted xenograft nude mouse model. Taken together, these findings suggest that antofine might be a potential candidate for the development of cancer chemotherapeutic agents derived from natural products.

1. Introduction

Phenanthroindolizidine alkaloids are bioactive constituents of *Cynanchum*, *Pergularia*, *Tylophora* and other genera that belong to the Asclepiadaceae family [1]. Phenanthroindolizidine alkaloids, such as tylophorine, tylocrebrine, pergularinine and tylophorinidine, exhibit profound cytotoxic activities in cancer cells [2–4]. Their reported mechanisms include the suppression of DNA and protein synthesis, inhibition of dihydrofolate reductase activity and induction of apoptosis [5–7]. Other detailed mechanisms, including the inhibition of nuclear factor-kappaB (NF-κB) activation and the interaction of a phenanthroindolizidine alkaloid with bulged DNA through the intercalating mode, were also proposed in recent reports [4,8,9]. Antofine, a phenanthroindolizidine alkaloid, is known to be distributed in the Asclepiadaceae plants, such as *Vincetoxicum nigrum*, *Cynanchum vincetoxicum*, and *C. paniculatum* [3,10,11]. In previous studies, antofine exhibited antifungal, antibacterial and cytotoxic activities [3,9–11]. Antofine also suppressed the proliferation of human hepatocellular carcinoma cells by inhibiting activation of NF-κB and synthesis of DNA and proteins [9]. However, its anticancer potentials and mechanisms of action remain to be elucidated.

In the course of evaluating the anti-proliferative effects against human cancer cells with the methanol extracts derived from traditional medicinal plants, we found and further identified antofine as the active compound of the methanol extract obtained from the root of *C. paniculatum* [11,12]. Our previous study suggested that antofine inhibited cell proliferation of human colon and lung cancer cells [11]. We also recently investigated the structure-activity relationship of the anti-proliferative effects of antofine with several synthetic antofine analogues [13]. On the basis of earlier findings, in this study, the anti-proliferative effects and mechanisms of action of antofine were examined both *in vitro* and *in vivo*. The present results suggest the potential of antofine as a lead candidate of anticancer agents derived from natural products.

2. Materials and methods

2.1. Reagents

RPMI 1640 medium, Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), antibiotics–antimycotics

* Corresponding author at: College of Pharmacy, Seoul National University, San 56-1, Sillim-dong, Gwanak-gu, Seoul 151-742, Republic of Korea.
Tel.: +82 2 880 2475, fax: +82 2 762 8322.
E-mail address: sklee61@snu.ac.kr (S.K. Lee).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.07.026

solution and trypsin-EDTA were purchased from Invitrogen (Carlsbad, CA, USA). Sulforhodamine B (SRB), trichloroacetic acid, TRI reagent, propidium iodide (PI), ribonuclease A (RNase A), HEPES, mouse monoclonal anti-β-actin antibody and other reagents unless otherwise indicated were purchased from Sigma–Aldrich (St. Louis, MO, USA). Complete™ protease inhibitor cocktail was purchased from Roche Applied Science (Indianapolis, IN, USA). Annexin V-FITC apoptosis detection kit, BrdU flow kit, mouse monoclonal anti-cyclin D1, anti-cyclin E, anti-GSK-3β, anti-PARP, anti-cleaved PARP (Asp214) and anti-β-catenin antibodies were purchased from BD Biosciences (San Jose, CA, USA). Mouse monoclonal anti-PCNA antibody, rabbit polyclonal anti-cyclin B1 (H-433), anti-cyclin A (H-432), anti-CDK4 (H-22), anti-CDK2 (M2), anti-Bcl-2, and anti-Bcl-X_L antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-phospho-GSK-3α/β (Ser 21/9) antibody and mouse monoclonal anti-caspase-8 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Dual-luciferase reporter gene assay system and pRL-SV40 reporter plasmid were purchased from Promega (Madison, WI, USA).

2.2. Test material

(−)-Antofine was synthesized as described in recently published reports [14,15]. The structure is designated in Fig. 1. The compound was dissolved in 100% dimethyl sulfoxide (DMSO), and final concentration of vehicle (DMSO) was adjusted to less than 0.1%.

2.3. Cell culture

Human lung cancer (A549 and NCI-H358), colon cancer (HCT 116, HT-29, HCT-15 and SW480), stomach cancer (SNU-638), breast cancer (MDA-MB-231 and T47D), prostate cancer (PC-3 and LNCaP), fibrosarcoma (HT-1080), and leukemia (HL-60, K562) cells were provided from Korean Cell Line Bank (KCLB). Cells were grown in medium (DMEM for MDA-MB-231 and HT-1080 cells, RPMI 1640 medium for A549, NCI-H358, HCT 116, HT-29, HCT-15, SNU-638, T47D, PC-3, K562 and HL-60 cells) supplemented with 10% fetal bovine serum (FBS) and antibiotics–antimycotics (PSF; 100 units/ml penicillin G sodium, 100 μg/ml streptomycin, and 250 ng/ml amphothericin B). SW480 cells were grown in RPMI 1640 medium containing 25 mM HEPES, 10% FBS, and PSF. LNCaP cells were maintained in RPMI 1640 medium without phenol red supplemented with 10% FBS and PSF.

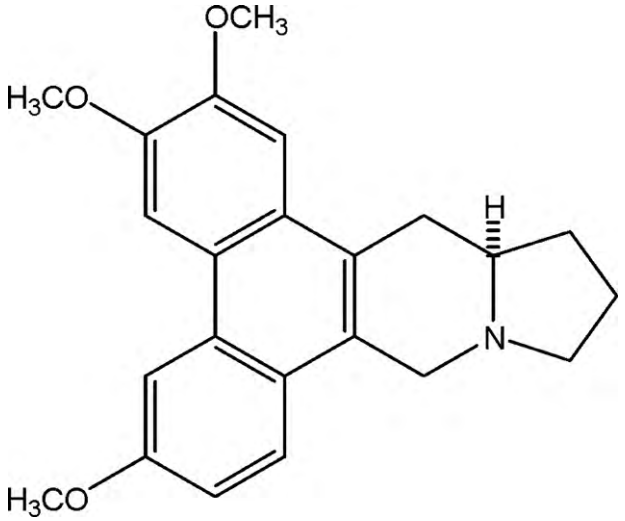

Fig. 1. The chemical structure of (−)-antofine.

Paclitaxel-resistant A549 cells (A549-PA) and 5-fluorouracil-resistant HCT 116 cells (HCT 116-FU) were generated by continuous exposures to gradually increasing concentrations of anticancer agents for more than 6 months. Cells were maintained in RPMI 1640 medium containing 100 nM paclitaxel and 100 μM 5-fluorouracil, respectively.

All cells were incubated at 37 °C, 5% CO_2 in a humidified atmosphere, and subcultured once or twice a week. Cells passed more than 3 times were used for experiments.

2.4. Sulforhodamine B (SRB) assay

To evaluate the growth-inhibitory potential of test compounds against various human cancer cells, SRB assay was performed. Cells were seeded in 96 well plates at a density of 5×10^3 cells/well and incubated for 24 h. Various concentrations of antofine were diluted in medium and added to cells for an additional 48 h. Cells were fixed with 10% trichloroacetic acid solution for 30 min at 4 °C, washed 5 times with tap water, and dried in the air. Cells were stained with 0.4% SRB in 1% acetic acid solution for 30 min at room temperature. After washing unbound dye and drying, stained cells were dissolved in 10 mM Tris (pH 10.0), and absorbance was measured at 515 nm. Cell viability was calculated by comparison with absorbance of vehicle-treated control group. IC_{50} value, the concentration for 50% cell survival, was determined by non-linear regression analysis using Tablecurve software.

2.5. Cell cycle analysis

HCT 116 cells (3.6×10^5 cells/dish in 60 mm dishes) were incubated with test samples for 24 and 48 h. All adherent or floating cells were collected and washed twice with PBS. Cells were fixed with 100% methanol overnight. Fixed cells were washed with PBS, and then stained with 50 μg/ml propidium iodide (PI) solution containing 50 μg/ml RNase A for 30 min at room temperature. Fluorescence intensity was analyzed by FACSCalibur® flow cytometer (BD Biosciences, San Jose, CA, USA). The percentages of the distributions in distinct phases of cell cycle were determined using ModFIT LT V2.0 software.

2.6. Double staining

HCT 116 cells were treated with test compounds for 24 h. In case of TNF-α stimulation, cells were treated with antofine 30 min prior to TNF-α treatment and then stimulated with TNF-α (final 10 ng/ml) for an additional 24 h. After incubation, cells were collected and washed twice with PBS. Cells were stained with annexin V-FITC and propidium iodide (PI) solution using an annexin V-FITC apoptosis detection kit (BD Biosciences) according to the manufacturer's recommendation. Briefly, cells were diluted with 1× binding buffer at a density of 1×10^6 cells/ml. Cell suspension (100 μl) was transferred into 15 ml round-bottomed polystyrene tubes, and annexin V-FITC solution (5 μl) and PI solution (5 μl) were added to cell suspensions 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 cytometry.

2.7. BrdU staining

5-Bromo-2′-deoxyuridine (BrdU) staining and detection were performed according to the manufacturer's instruction in the BrdU flow kit (BD Biosciences). HCT 116 cells (1.5×10^5 cells/well) were seeded into 6 well plates and incubated for 24 h. After washing with PBS, cells were further incubated with various concentrations of test samples for 24 h. Prior to harvesting, cells were exposed to

10 μM BrdU solution for 1 h. Cells were washed with PBS and trypsinized. Collected cells were briefly washed with PBS, and then fixed with Cytofix/cytoperm buffer for 15 min at room temperature. After washing with Perm/wash buffer, cells were fixed again with Cytoperm plus buffer for 10 min on ice and washed. Cells were fixed with Cytofix/cytoperm buffer for 5 min at room temperature. After washing, cells were incubated with 300 μg/ml DNase solution for 1 h at 37 °C. Cells were washed, and further incubated with anti-BrdU-FITC antibody (diluted in 1:50) and 7-amino-actinomycin D (7-AAD) solution for 20 min at room temperature. Stained cells were immediately analyzed by flow cytometry.

2.8. Western blot analysis

Cultured HCT 116 or SW480 cells were seeded into 60 mm dishes at a density of 3×10^5 cells/dish and incubated for 24 h. Cells were treated with various concentrations of antofine for 24 h. To evaluate the effect of antofine on TNF-α-induced apoptotic cell death, cells were pretreated with antofine for 30 min, and TNF-α was added to the cells for an additional 24 h. After harvesting, cells were washed twice with PBS, suspended with boiling 2× sample loading buffer (250 mM Tris–HCl (pH 6.8), 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol, 50 mM sodium fluoride, and 5 mM sodium orthovanadate) and further incubated for 5–20 min at 100 °C for complete lysis. After cooling at room temperature, samples were stored at −20 °C until the experiment. Protein concentration of cell lysates was determined by BCA method.

Equal amounts (25–50 μg) of protein samples were subjected to 8–15% SDS-PAGE. Separated proteins were electrically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA). Membranes were blocked with blocking buffer (5% non-fat dry milk in PBS containing 0.1% Tween-20 (PBST)) for 1 h at room temperature. After washing 3 times with PBST, membranes were incubated with primary antibodies diluted in 3% non-fat dry milk in PBS (1:1000–1:2000) overnight at 4 °C. Membranes were washed 3 times with PBST, and incubated with corresponding secondary antibodies diluted in 3% non-fat dry milk in PBS (1:2000–1:5000) for 1–2 h at room temperature. Membranes were washed 3 times with PBST, and then exposed to enhanced chemiluminescence (ECL) detection kit (LabFrontier, Suwon, Korea). Blots were detected by LAS 3000 (Fuji Film Corp., Japan).

2.9. Reporter gene assay

HCT 116 cells were co-transfected with 100 ng of TOPflash (β-catenin/Tcf reporter plasmids, Millipore, Billerica, MA, USA) and 5 ng of pRL-SV40 reporter plasmids for 24 h. After transfection, cells were incubated with various concentrations of antofine for 24 h. Luciferase activity was measured by dual-luciferase reporter gene assay system. Transfection efficiency was normalized by the values of *Renilla* luciferase activity.

2.10. In vivo tumor xenograft model

Six week-old female athymic mice (BALB/c nu/nu) were purchased from Orient Co., Ltd. (Seoul, Korea). HCT 116 cell suspension (2×10^6 cells in 0.1 ml of RPMI) was injected subcutaneously into the right flank of each mouse on day 0. Mice were treated when their tumor volume reached 95–100 mm^3. Animals were randomly divided into 3 groups (5 animals per group). Antofine (2 or 10 mg/kg/day) was dissolved in 1% cremophore in PBS and solvent alone served as control. All study medications were given by intra-peritoneal injections 3 times per week starting on day 10 after implanting the cancer cells. Tumors were excised from animals on 35 days after inoculation. To quantify tumor growth, 3 perpendicular diameters of the tumors were measured with caliper every 3–5 days, and the body weight of each mouse was monitored for toxicity. The tumor volume was calculated using the following formula: tumor volume (mm^3) = (width) × (length) × (height) × $\pi/6$.

2.11. 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 two independent experiments. Non-linear regression analysis for calculation of IC_{50} values was performed by Tablecurve program (ver. 1.0, AISN software). Statistical significance was analyzed by Student's *t*-test or one-way ANOVA (SigmaStat 3.1, Systat software Inc.). The difference was considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Inhibitory effects of antofine on the proliferation of human cancer cells

Phenanthroindolizidine alkaloids have exhibited notable antiproliferative activities against various human cancer cells [2,3,11]. Along this line, the effects of antofine on the growth of human cancer cells were primarily evaluated. As shown in Table 1, antofine markedly inhibited the growth of several human cancer cells with IC_{50} values below 30 nM in all cell lines tested. In addition to these data, the IC_{50} values of antofine in HCT-15, SW480, LNCaP, and PC-3 cells were 23.7, 4.7, 7.2, and 11.1 nM,

Table 1
Inhibitory effects of antofine on the proliferation of human cancer cells. Cells were treated with various concentrations of antofine for 48 h. Cell viability was determined by SRB assay. The IC_{50} values were calculated by non-linear regression analysis.

Cell line	Classification	IC_{50} (nM)			
		Antofine	Paclitaxel	Vinblastine	Camptothecin
HCT 116	Colon cancer cells	6.3	10.6	1.4	54.0
HT-29	Colon cancer cells	10.8	6.9	0.9	166.7
A549	Lung cancer cells	9.6	1.4	17.0	168.4
NCI-H358	Lung cancer cells	7.0	9.9	1.2	41.1
MDA-MB-231	Breast cancer cells	12.2	>100	>1000	>500
T47D	Breast cancer cells	19.7	17.5	>100	61.3
SNU-638	Stomach cancer cells	8.9	5.7	2.2	18.7
HT-1080	Fibrosarcoma	7.1	9.9	0.8	148
HL-60	Leukemia	10.7	10.0	1.0	15.1
A549-PA	Drug-resistant lung cancer cells	8.9	321.2	NT[a]	NT
HCT 116-FU	Drug-resistant lung cancer cells	8.3[b]	NT	NT	NT

[a] NT: not tested
[b] The IC_{50} values of 5-FU in HCT 116 and in HCT 116-FU cells were 19.4 μM and over 200 μM (% of survival at 200 μM 5-FU was 78.3%), respectively.

respectively. Antofine showed greater inhibitory activities in HCT 116 and HCT-15 cells among the colon cancer cells. Antofine was more effective on estrogen receptor (ER)-negative MDA-MB-231 cells than ER-positive T47D cells, whereas androgen-responsive LNCaP cells were more susceptible to antofine than androgen-insensitive PC3 cells. In case of MRC-5 human lung normal epithelial cells, antofine also inhibited the growth of MRC-5 cells, but the cell viability up to 1000 nM antofine was over 50% (data not shown). This result suggests that the growth-inhibitory effect of antofine was more potent against cancer cells than normal cells. In addition, it is well known that multidrug resistance is an obstacle to successful anticancer therapy [16]. Thus, we developed drug-resistant cancer cells (A549-PA and HCT 116-FU cells) and evaluated the effects of antofine on these resistant cells. Comparing the effects in drug-resistant cells to that of parental cells, the growth-inhibitory effect of paclitaxel or 5-fluororuacil was significantly attenuated in drug-resistant cells. Antofine, however, showed similar growth-inhibitory effects in both parental and drug-resistant cells (Table 1).

3.2. Time- and dose-dependent inhibition of cell growth by antofine without inducing cell death

Previous studies suggested that phenanthroindolizidine alkaloids inhibited cell proliferation through the inhibition of DNA and protein syntheses, suppression of dihydrofolate reductase activity, induction of apoptosis, and inhibition of transcriptional activation of NF-κB and AP-1 [5–7,9]. According to the potent inhibitory effect of antofine against the proliferation of HCT 116 human colon cancer cells, further studies were primarily performed using HCT 116 cells to investigate its anti-proliferative mechanisms of action.

Treatment with antofine exhibited remarkable suppression of cell proliferation in a time- and concentration-dependent manner (Fig. 2(A)). This effect appeared even at 24 h treatment, and the effect at 48 h incubation was very similar to that of 72 h incubation. In addition, the inhibitory effect was more pronounced at concentrations above 10 nM. The cell viability at 100 nM was only 15%. Comparing the absorbance of each group to that of 0 h control, the ratio (Abs. of each test group versus Abs. of 0 h control) was greater than 1 even at high concentrations (50 or 100 nM), indicating that antofine might suppress cell growth in a cytostatic manner (data not shown).

In general, most anticancer agents exhibit their effects via regulating cell cycle progression and subsequently inducing cell death, primarily apoptosis. To further investigate whether the potent anti-proliferative effect of antofine was mediated by induction of cell death, HCT 116 cells were treated with 100 nM antofine for 24 h, and morphological changes were observed using an inverted phase-contrast microscope. And then the cells were collected, stained with annexin V-FITC and propidium iodide (PI), and immediately analyzed by flow cytometry. As shown in Fig. 2(B), although the number of cells was markedly decreased by antofine treatment, most of the cells were attached and floating cells were barely detected. This was further confirmed by double staining of cells with annexin V-FITC and PI. It is known that the proportion of cells stained with PI is considered as the population of dead cells (late apoptotic or necrotic cells) and that an increase of annexin V-FITC-stained cells is an indicator of apoptosis induction [17]. Along with the morphological change, cells treated with antofine showed no obvious increase of annexin V and/or PI-stained cell populations, indicating that antofine did not induce cell death (Fig. 2(C)). These results suggest that induction of cell death might not be involved in the antofine-mediated growth inhibition in HCT 116 cells.

3.3. Effect of antofine on the regulation of cell cycle progression

It is known that cell proliferation is generally regulated by controlling cell cycle progression through promotion or inhibition of activities of cyclins/cdks complexes and/or their associated proteins [18]. To determine whether antofine affects cell cycle progression, cells were incubated with various concentrations of antofine for 24 and 48 h, and the effect of antofine on cell cycle distribution was analyzed by flow cytometry. As shown in Fig. 3(A) and Table 2, antofine neither increased the distribution of cells in the sub-G1 phase nor induced notable changes in cell cycle progression in all of cells tested. In HCT 116 cells, antofine induced cell cycle arrest in G0/G1 phase, and the distribution in the S phase

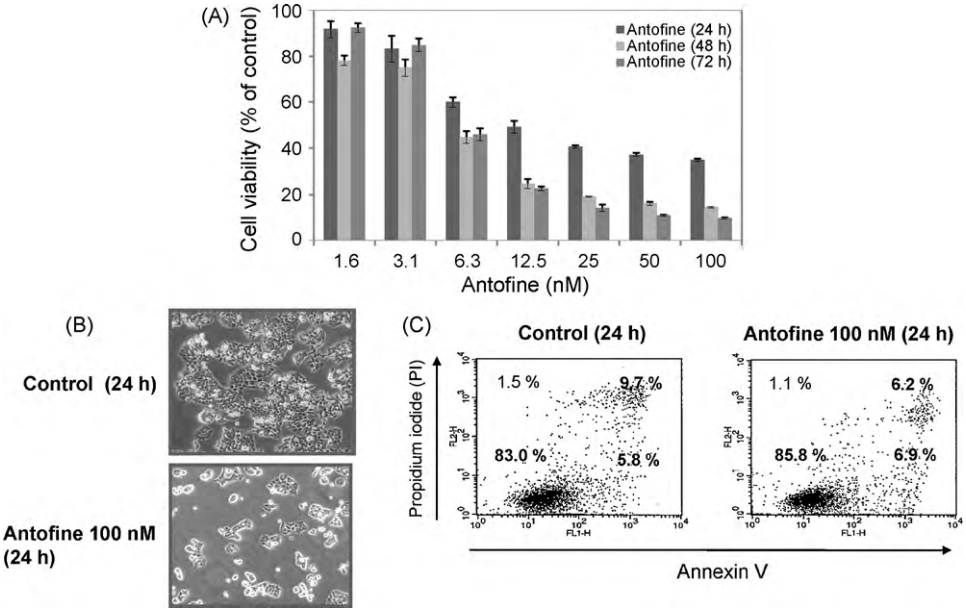

Fig. 2. Inhibitory effect of antofine on cell proliferation without inducing cell death. (A) Time- and concentration-dependent inhibition of cell viability by antofine in HCT 116 cells. Cells were treated with various concentrations of antofine for 24, 48 and 72 h. Cell viability at each concentration and time point was evaluated by SRB assay. (B) Cells were treated with antofine for 24 h, and morphological changes were observed using an inverted phase-contrast microscope and photographed. (C) Cells were treated with antofine for 24 h. Collected cells were incubated with propidium iodide and annexin V-FITC for 20 min at room temperature, then immediately analyzed by flow cytometry.

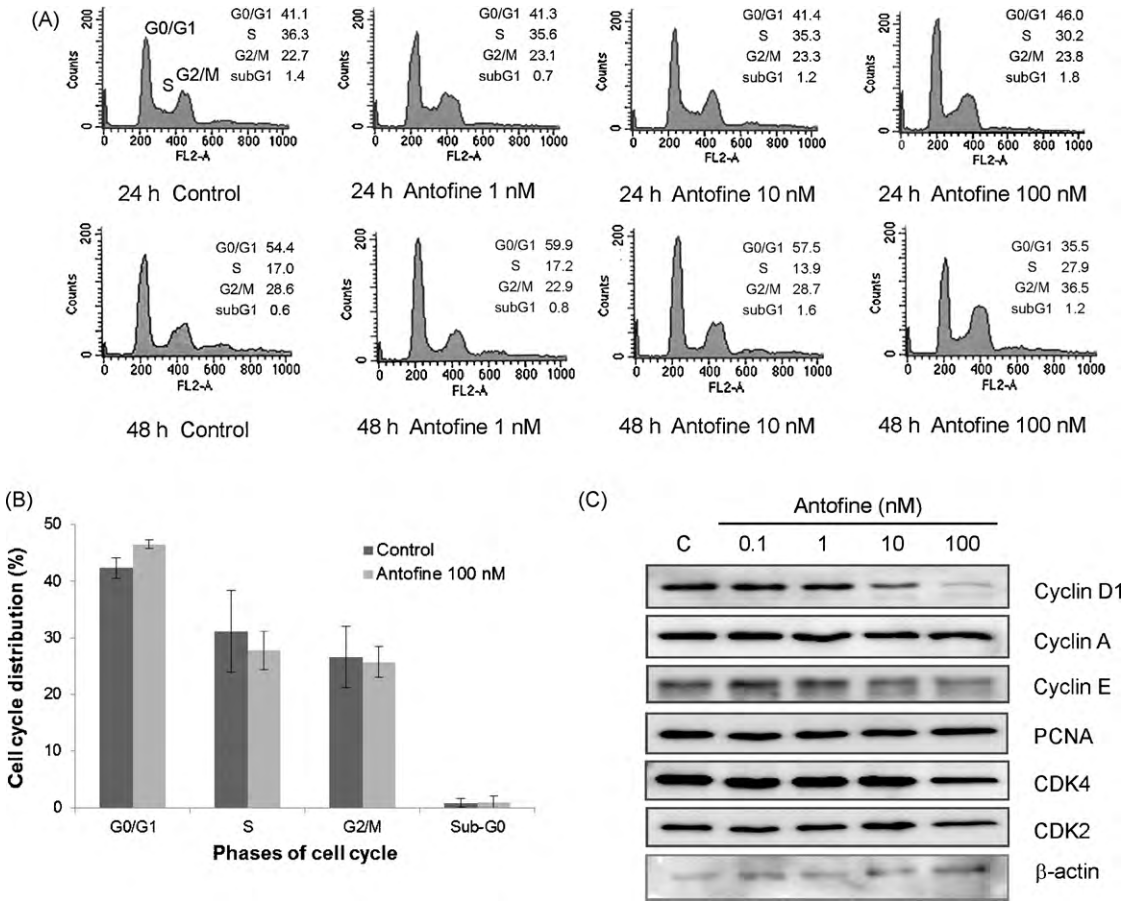

Fig. 3. Effect of antofine on cell cycle progression in HCT 116 cells. (A) HCT 116 cells were incubated with antofine for 24 and 48 h. After incubation, cells were fixed with 100% methanol and stained with PBS containing propidium iodide (50 μg/ml) and RNase A (50 μg/ml) for 30 min at room temperature. Cellular DNA contents were analyzed by flow cytometry. (B) Changes of cell cycle distribution by antofine in HCT 116 cells at 24 h treatment. The results from the analysis of cell cycle distribution were expressed as a graph. (C) HCT 116 cells were incubated with various concentrations of antofine for 24 h. After incubation, cells were lysed with 2X SDS sample buffer. Expression of cell cycle regulatory proteins was analyzed by Western blot analysis.

was decreased compared to control cells at the treatment for 24 h. However, the accumulation of cells in G0/G1 phase was not statistically significant ($P = 0.09$, two-tailed t-test) (Fig. 3(B)). At 48 h, low doses of antofine (1 and 10 nM) also induced an accumulation of cells in G0/G1 phase, while 100 nM antofine induced moderate cell cycle arrest in G2/M phase. Time- or dose-dependent modulation of cell cycle progression was not markedly observed in antofine-treated cells. Additional studies showed that

Table 2
Changes of cell cycle progression by antofine in several human cancer cells. HCT 116, A549, and HT-29 cells were incubated with increasing concentrations of antofine for 24 or 48 h. The distribution of cells at each phase of cell cycle was analyzed by flow cytometry as described in Section 2.

Cell line	Time (h)	Sample	Phase of cell cycle (%)			
			G0/G1	S	G2/M	sub-G1
HCT 116 cells	24	Antofine (nM)	0	41.1	36.3	22.7	1.4
			1	41.3	35.6	23.1	0.7
			10	41.4	35.3	23.3	1.2
			100	46.0	30.2	23.8	1.8
	48	Antofine (nM)	0	54.4	17.0	28.6	0.6
			1	59.9	17.2	22.9	0.8
			10	57.5	13.9	28.7	1.6
			100	35.5	27.9	36.5	1.2
A549 cells	24	Antofine (nM)	0	54.6	30.5	14.9	0.2
			12.5	59.2	25.6	15.2	0.2
			25	64.7	17.8	17.5	0.2
			50	60.6	18.8	20.6	0.2
			100	56	26.6	17.4	0.1
HT-29 cells	12	Antofine (nM)	0	59.1	22.7	18.3	0.1
			5	54.3	20.3	25.4	0.2
			50	63.3	12.9	23.8	0.2
	24	Antofine (nM)	0	68.0	14.5	17.5	1.0
			5	34.7	32.0	33.3	0.4
			50	42.3	34.4	23.3	0.2

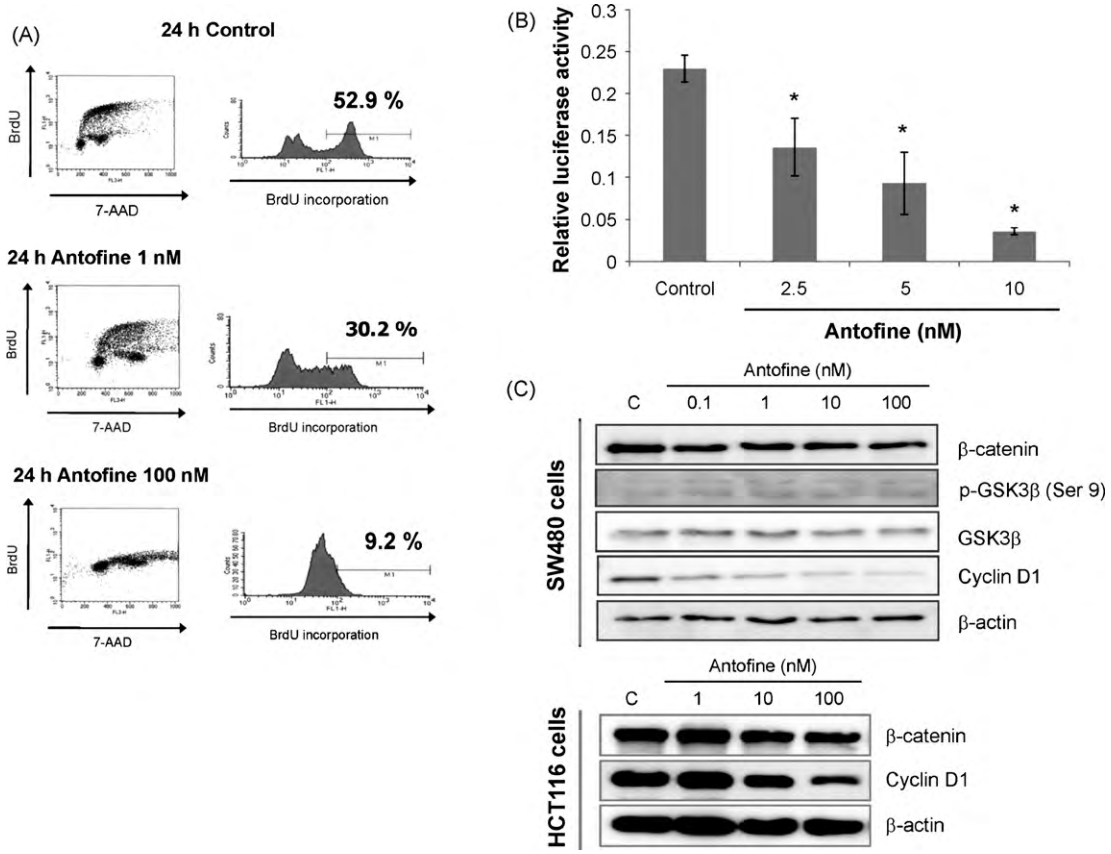

Fig. 4. Inhibition of DNA synthesis and modulation of Wnt signaling by antofine in HCT 116 cells. (A) HCT 116 cells were treated with antofine for 24 h and further incubated with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for an additional 1 h. Incorporation of BrdU was determined by fluorescence labeling with anti-BrdU-FITC antibody and 7-aminoactinomycin D (7-AAD) and flow cytometry. (B) HCT 116 cells were transfected with 100 ng of TOPflash (β-catenin/Tcf reporter plasmids) and 5 ng of pRL-SV40 reporter plasmids for 24 h. After transfection, cells were incubated with antofine for 24 h. Luciferase activity was measured by dual-luciferase reporter gene assay system. Transfection efficiency was normalized by values of *Renilla* luciferase activity. (*$P < 0.05$ compared with control) (C) HCT 116 and SW480 cells were incubated with various concentrations of antofine for 24 h. After incubation, cells were lysed with 2× SDS sample buffer. Protein expression was analyzed by Western blot analysis.

treatment with antofine for 24 h induced G0/G1 phase cell cycle arrest at the concentrations up to 50 nM and moderate G2/M phase arrest at 100 nM in A549 cells (Table 2). In case of HT-29 cells, antofine exhibited the accumulation of cells in G0/G1 phase at 24 h incubation and in S and G2/M phase arrest at the treatment for 48 h (Table 2). Therefore, the effect of antofine on cell cycle regulation might be various depending on the treatment time and cells used.

Since antofine moderately induced G0/G1 phase cell cycle arrest at 24 h incubation, we examined protein expression involved in the G0/G1 cell cycle progression by Western blot analysis. Antofine dose-dependently suppressed cyclin D1, cyclin E and CDK4 expression in HCT 116 cells, but the expression of proliferating cell nuclear antigen (PCNA), cyclin A and CDK2 were not much affected by treatment with antofine (Fig. 3(C)). In agreement with the results of cell cycle analysis, the effect of antofine on the expression of cyclin E and CDK4 was moderate. However, antofine markedly suppressed cyclin D1 expression in a concentration-dependent manner. Thus, the inhibition of cyclin D1 expression might be the primary contributor to the cell cycle arrest mediated by antofine.

3.4. Suppression of DNA synthesis and modulation of Wnt signaling activation by antofine treatment

To further explore the mechanisms of action to account for the profound anti-proliferative effect of antofine, we examined additional parameters involved in cancer cell growth. First, to examine the effect of antofine on DNA synthesis, we analyzed 5-bromo-2′-deoxyuridine (BrdU) incorporation into the cells by flow cytometry. As indicated in Fig. 4(A), control cells showed increased BrdU incorporation, whereas in antofine (100 nM)-treated cells the fluorescence intensity and BrdU incorporation were markedly decreased. This result indicates the involvement of DNA synthesis inhibition in the anti-proliferative effect of antofine in HCT 116 cells.

Since antofine suppressed cyclin D1 expression in HCT 116 cells, the effect of antofine on the activation of Wnt signaling pathway, an upstream signaling regulating cyclin D1 expression, was examined. It is known that dysregulation of the Wnt signaling pathway is closely involved in the colon cancer carcinogenesis [19]. In unstimulated cells, β-catenin is sequestered in the cytoplasm by the destruction complex consisting of adenomatous polyposis coil (APC), axin and GSK3β. GSK3β phosphorylates β-catenin and leads to its degradation by an ubiquitin-proteasome pathway. Upon stimulation of Wnt, however, the destruction complex is inactivated, and β-catenin is translocated to the nucleus and serves as a cofactor for Tcf/Lef transcription factor. This leads to the transcription of several target genes, such as cyclin D1, c-myc and MMP-7 [19]. To determine the effect of antofine on the activation of Wnt signaling pathway, we evaluated the effect on β-catenin/Tcf transcriptional activity in HCT 116 cells using a reporter plasmid containing the binding site of Tcf transcription factor (TOPflash) [20]. The β-catenin/Tcf transcriptional activity was constitutively activated in vehicle-treated control HCT 116 cells, and antofine down-regulated the elevated transcriptional activity in a concentration-dependent manner (Fig. 4(B)). Additionally employing SW480 human colon cancer cells that possess truncated mutant APC [21], the expression of several proteins

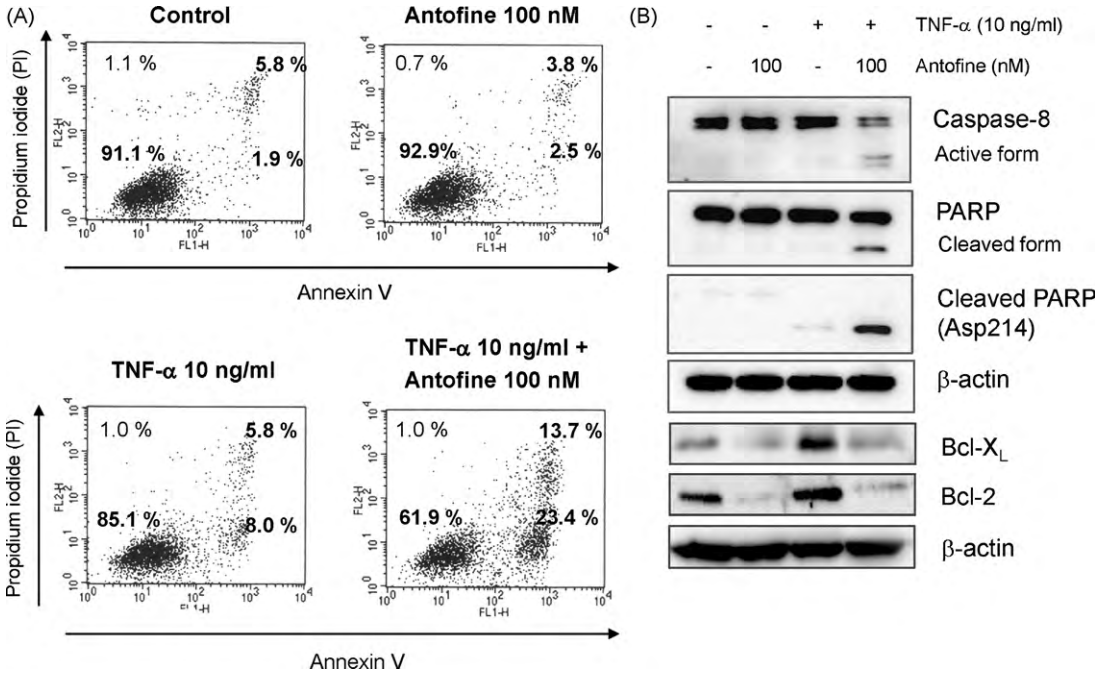

Fig. 5. Augmentation of TNF-α-mediated apoptosis by antofine in HCT 116 cells. HCT 116 cells were treated with antofine (100 nM) for 30 min prior to TNF-α stimulation, then TNF-α was added for an additional 24 h. (A) After harvesting, cells were stained with Annexin V and propidium iodide as described in Section 2, and immediately analyzed by flow cytometry. (B) Collected cells were lysed with 2X SDS sample buffer. Protein expression of caspase-8, PARP, Bcl-2 and Bcl-X_L was analyzed by Western blot analysis.

constituting the Wnt signaling cascade were also evaluated. As shown in Fig. 4(C), antofine reduced the expression of β-catenin and cyclin D1, one of the target genes of Wnt signaling, in a concentration-dependent manner in both SW480 and HCT 116 cells.

3.5. Enhancement of TNF-α-induced apoptotic cell death by treatment with antofine

Previous studies have demonstrated the dual role of TNF-α (tumor necrosis factor-α) in cancer cell proliferation [22,23]. TNF-α simultaneously stimulates signal transduction mediating cell death and survival by increasing caspase-mediated apoptosis and by activating NF-κB and its downstream cell survival signaling, respectively [22,23]. In recent study the inhibitory activity of phenanthroindolizidine alkaloids on NF-κB activation was reported [4,9,24]. Therefore, we hypothesized that antofine might have a potential to regulate TNF-α-mediated cell survival signaling, thereby enhancing TNF-α-induced apoptosis. HCT 116 cells were treated with antofine in the presence or absence of TNF-α (10 ng/ml) for 24 h. Microscopic observation revealed that the number of floating cells was more increased in cells treated with antofine (100 nM) and TNF-α (data not shown). Treatment with TNF-α alone increased apoptotic (annexin V-positive) cell population in an approximately 6.1% compared to vehicle-treated control, and antofine treatment markedly enhanced TNF-α-induced apoptotic cell death in an approximately 29.4% (Fig. 5(A)). In addition, to confirm the augmentation of TNF-α-mediated apoptosis by antofine, we also examined the effect of antofine on TNF-α induced caspase-8 activation and poly (ADP-ribose) polymerase (PARP) cleavage. As indicated in Fig. 5(B), antofine or TNF-α alone showed marginal effects on the cleavage of caspase-8 and PARP. However, in cells stimulated with TNF-α in the presence of antofine (100 nM) the cleavage and activation of caspase-8 as well as the cleavage of PARP were pronounced. Stimulation with TNF-α also markedly increased the expression of Bcl-2 and Bcl-X_L, pro-survival Bcl-2 family proteins, and these effects were remarkably attenuated in the presence of 100 nM antofine (Fig. 5(B)). These data suggest the enhancement of TNF-α-induced apoptosis by treatment with antofine in HCT 116 cells.

3.6. Antitumor effect of antofine on an in vivo tumor xenograft model bearing HCT 116 cells

Based on the potent anti-proliferative effect of antofine *in vitro*, we performed studies using an *in vivo* tumor xenograft model bearing HCT 116 cells to further determine the anticancer potential of antofine. When tumor size reached 95 to 100 mm^3, antofine (2 or 10 mg/kg) was administered i.p. to mice 3 times per week. Tumor volume in the control group was about 1800 mm^3 on day 35 after the drug treatment was started. However, treatment with antofine effectively suppressed tumor growth, and the inhibitory effect in the antofine-treated group (10 mg/kg) was about 50% compared with the control group (Figs. 6(A) and (B)). In addition, the body weight change by treatment with antofine was negligible (Fig. 6(C)), and no overt toxicity was found under these experimental conditions.

4. Discussion

Natural products have been considered to be valuable sources for developing therapeutic agents. In our continuing efforts to identify new entities for cancer chemotherapeutic agents from natural products, we found that a phenanthroindolizidine alkaloid, antofine, exhibited a potent inhibition of cancer cell growth *in vitro*. Here, we present additional information regarding the anti-proliferative mechanisms of action of antofine in human cancer cells.

In agreement with previously reported results on the inhibition of cancer cell proliferation [2,11,13], antofine exhibited potent anti-proliferative activities in various human cancer cells. These inhibitory effects were similar to or more potent than conventional anticancer agents, and the anti-proliferative effect of antofine was more pronounced compared to the effects of another phenanthroindolizidine alkaloid tylophorine which effect was previously described in the literature [4,9,24]. In addition, the anti-proliferative effect of antofine in drug-resistant cancer cells was similar to

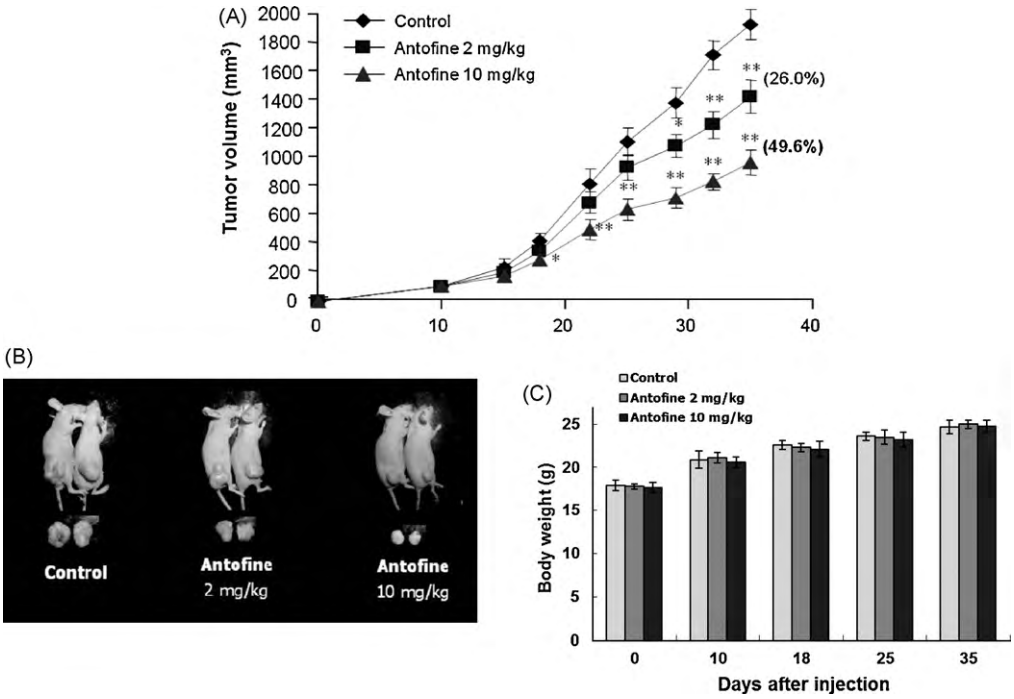

Fig. 6. Antitumor effect of antofine in a tumor xenograft model bearing HCT 116 cells. HCT 116 cells were injected subcutaneously into the right flank of each mouse. When tumor volume reached 95–100 mm^3 (10 days after inoculation), mice were administered antofine (2 or 10 mg/kg/day) by intra-peritoneal injections 3 times per week. To quantify tumor growth, 3 perpendicular diameters of the tumors were measured with caliper every 3–5 days (A). Tumors were excised from animals on day 35 after inoculation, and tumors were photographed (B). Body weights of the mice were monitored during the experiment for toxicity (C).

that in parental cancer cells (Table 1). Therefore, it is supposed that antofine might have a potential to overcome multidrug resistance but further investigation on the inhibitory effect in another drug-resistant cancer cells and the mechanistic studies remain to be elucidated.

To further investigate the mechanism of action of antofine on the regulation of cell proliferation, we primarily focused on determining whether antofine could induce cell death similar to conventional anticancer agents in HCT 116 cells, which was one of the most sensitive cells to antofine treatment. Although we treated with a high concentration (100 nM) of antofine, obvious cell death was not detected by microscopic observation as shown in Fig. 2(B). This was also confirmed by double staining with annexin V-FITC and PI. Treatment with antofine did not increase the number of cells stained with annexin V-FITC and/or PI (Fig. 2(C)). These results well coincide with a previous report in which a phenanthroindolizidine alkaloid, tylophorine, and its analogues exerted potent cytotoxic activities without inducing apoptosis up to 3 μM in HepG2 cells [4]. However, other reports showed that tylophora alkaloids induced apoptosis at the concentration of 3 or 30 μM in K562 human leukemia cells by inducing cytochrome c release and caspase-3 activation [7]. This discrepancy may be mostly due to differences of experimental conditions such as the treatment concentration, the used cell lines, and the other potential causes that might affect the experiments. Although we did not exclude the possibility that antofine might induce cell death at concentrations higher than 1 μM, considering the potency of antofine on the inhibition of cancer cell growth (less than 30 nM of IC_{50}s, in general), the induction of apoptosis at micromolar concentrations may not be meaningful to account for the anti-proliferative mechanism of antofine in cancer cells.

Since cell proliferation is finely regulated by promoting or blocking cell cycle progression, the effect of antofine on cell cycle progression was also examined. Data indicate that time- and dose-dependent modulation of cell cycle progression by treatment with antofine was not noticeable, suggesting modulation of cell cycle progression might partially contribute to its anti-proliferative effect of antofine (Table 2 and Figs. 3(A)–(C)). Interestingly, among proteins related to G0/G1 phase cell cycle progression, the expression of cyclin D1 was significantly down-regulated by treatment with antofine in HCT 116 cells (Fig. 3(C)). A recent study reports that cyclin D1 regulates cell cycle progression and mitochondrial function and size as well [25]. Therefore, although it is possible that cyclin D1 might be a main modulator associated with antofine-mediated cell cycle regulation, its additional effects might also contribute to the anti-proliferative action of antofine. Further studies would be necessary to elucidate the mechanism of action of antofine in terms of modulation of cyclin D1 expression.

According to these results, it is assumed that the mechanisms underlying antofine-mediated anti-proliferation are quite different from conventional anticancer agents, as it inhibits cell proliferation without inducing cell death and only weakly modulates cell cycle progression. In general, most anticancer agents suppress cell proliferation through induction of cell cycle arrest and subsequent apoptotic cell death in a time- or dose-dependent manner. Therefore, we further explored the effects of antofine on mechanisms regarding regulation of cell proliferation. On the basis of the inhibition of dihydrofolate reducase activity by a phenanthroindolizidine alkaloid [6], the effect of antofine on DNA synthesis was examined. As shown in Fig. 4(A), antofine markedly suppressed DNA synthesis, suggesting the inhibition of DNA synthesis might contribute to the anti-proliferative effect of antofine. In addition, cyclin D1 is known to be one of transcriptional targets upon activation of Wnt signaling [19]. We also found the significant inhibition of cyclin D1 expression in antofine-treated HCT 116 cells as shown in Fig. 3(C). Based on this information, to demonstrate the possible involvement of Wnt signaling regulation in antofine-mediated growth inhibition, we performed a reporter gene assay to determine the influence on regulation of β-catenin/Tcf transcriptional activity by antofine. As indicated in Fig. 4(B), antofine inhibited the hyper-activated β-catenin/Tcf transcriptional activity of HCT 116 cells in a

concentration-dependent manner. Further, it is known that SW480 cells possess wild-type β-catenin and practically activates Wnt signaling pathway [22,26]. The expression level of β-catenin and one of its transcriptional targets, cyclin D1, were suppressed by antofine in SW480 cells and HCT 116 cells. (Fig. 4(C)). Hence, these results suggest that the suppression of Wnt signaling might be also associated with antofine-mediated inhibition of cell proliferation and cyclin D1 expression, at least in part.

It is known that TNF-α mediates signal transduction involved in cell death and survival [22,23]. In this regard, the inhibition of TNF-α-mediated pro-survival pathway might enhance pro-apoptotic role of TNF-α and also increase its therapeutic potential. Based on previous reports on the suppression of NF-κB activation by phenanthroindolizidine alkaloids [4,9,24], we investigated the effect of antofine on the activation of TNF-α-mediated apoptosis. As indicated in the results, treatment with antofine markedly potentiated TNF-α-induced apoptosis, which was demonstrated by the increase of annexin V-positive cell population, the cleavage of PARP and caspase-8 and the decrease of TNF-α-induced Bcl-2 and Bcl-X_L expression (Figs. 5(A) and (B)). Thus, antofine might have a potential to enhance the pro-apoptotic effect of TNF-α. A recent report indicates that Bax, a protein belongs to the pro-apoptotic Bcl-2 farmily, plays an important role in the TNF-α-mediated apoptotic cell death [27]. As shown in Fig. 5(B), antofine attenuated the expression of anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-X_L. Thus, even if the effect of antofine on the expression of some pro-apoptotic proteins like Bax remains to be elucidated, it is suggested that modulation of pro- or anti-apoptotic proteins might be also an additional mechanism of action underlying potentiation of TNF-α-induced apoptosis by antofine. Moreover, it is also known that the inhibition of protein synthesis by cycloheximide sensitizes TNF-α-mediated apoptotic cell death [28,29]. Considering the suppressive effect of antofine on protein synthesis as reported in the previous literature [9], metabolic modulation by antofine might also account for its potential mechanism on the sensitization of the pro-apoptotic role of TNF-α. In addition to these suggestions, further studies would be required to investigate the detailed mechanism by which antofine potentiates TNF-α-mediated apoptosis induction.

We further confirmed the effectiveness of antofine in an *in vivo* tumor xenograft model. We found that compared to vehicle-treated control group, treatment with antofine suppressed tumor growth about 50% (Fig. 6). This *in vivo* effect appears to be less effective considering its potent anti-proliferative effect in human cancer cells *in vitro*. This might be associated with its mechanism of action, that is, inhibition of cell proliferation without inducing cell death unlike conventional anticancer agents. Nevertheless, this result might further support the potential of antofine as an anticancer agent.

In summary, the present study suggests that antofine has inhibitory effects on *in vitro* cancer cell proliferation and *in vivo* tumor growth. Inhibition of DNA synthesis and modulation of Wnt signaling activation might be considered as a possible mechanism of action for antofine in HCT 116 cells, Moreover, we also found antofine would enhance TNF-α-mediated apoptosis induction. These results collectively suggest the potential of antofine as an anticancer agent derived from natural products. Additional studies are in progress to discover the cellular targets of antofine and the general cellular changes mediated by antofine.

Acknowledgement

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD) (KRF-2006-311-E00590) (S.K. Lee).

References

[1] Gellert E. The indolizidine alkaloids. J Nat Prod 1982;45:50–73.
[2] Komatsu H, Watanabe M, Ohyama M, Enya T, Koyama K, Kanazawa T, et al. Phenanthroindolizidine alkaloids as cytotoxic substances in a Danaid butterfly, *Ideopsis similis*, against human cancer cells. J Med Chem 2001;44:1833–6.
[3] Stærk D, Lykkeberg AK, Christensen J, Budnik BA, Abe F, Jaroszewski JW. In vitro cytotoxic activity of phenanthroindolizidine alkaloids from *Cynanchum vincetoxicum* and *Tylophora tanakae* against drug-sensitive and multidrug-resistant cancer cells. J Nat Prod 2002;65:1299–302.
[4] Gao W, Lam W, Zhong S, Kaczmarek C, Baker DC, Cheng YC. Novel mode of action of tylophorine analogs as antutmor compounds. Cancer Res 2004;64:678–88.
[5] Huang MT, Grollman AP. Mode of action of tylocrebine: effects on protein and nucleic acid synthesis. Mol Pharmacol 1972;8:538–50.
[6] Rao KN, Venkatachalam SR. Inhibition of dihydrofolate reductase and cell growth activity by the phenanthroindolizidine alkaloids pergularinine and tylophoridine: the *in vitro* cytotoxicity of these plant alkaloids and their potential an antimicrobial and anticancer agents. Toxicol In Vitro 2000; 14:53–9.
[7] Ganguly T, Khar A. Induction of apoptosis in a human erythroleukemic cell line K562 by Tylophora alkaloids involves release of cytochrome c and activation of caspase 3. Phytomedicine 2002;9:288–95.
[8] Xi Z, Zhang R, Yu Z, Ouyang D, Huang R. Selective interaction between tylophorine B and bulged DNA. Bioorg Med Chem Lett 2005;15:2673–7.
[9] Shiah HS, Gao W, Baker DC, Cheng YC. Inhibition of cell growth and nuclear factor-κB activity in pancreatic cancer cell lines by a tylophorine analogue, DCB-3503. Mol Cancer Ther 2006;5:2484–93.
[10] Capo M, Saa JM. (−)-Antofine: a phenanthroindolizidine from *Vincetoxicum nigrum*. J Nat Prod 1989;52:389–90.
[11] Nam KA, Heo YH, Lee SK. Cytotoxic activity and G2/M cell cycle arrest mediated by antofine, a phenanthroindolizidine alkaloid isolated from *Cynanchum paniculatum*. Planta Med 2003;69:21–5.
[12] Nam KA, Lee SK. Evaluation of cytotoxic potential of natural products in cultured human cancer cells. Nat Prod Sci 2000;6:183–8.
[13] Fu Y, Lee SK, Min HY, Lee T, Lee J, Cheng M, et al. Synthesis and structure-activity studies of antofine analogues as potential anticancer agents. Bioorg Med Chem Lett 2007;17:97–100.
[14] Kim S, Lee J, Lee T, Park HG, Kim D. First asymmetric total synthesis of (−)-antofine by using an enantioselective catalytic phase transfer alkylation. Org Lett 2003;5:2703–6.
[15] Kim S, Lee T, Lee E, Lee J, Fan GJ, Lee SK, et al. Asymmetric total syntheses of (−)-antofine and (−)-cryptopleurine using (*R*)-(*E*)-4-(Tributylstannyl) but-3-en-2-ol. J Org Chem 2004;69:3144–9.
[16] Szakács G, Paterson JK, Ludwig JA, Booth-Centhe C, Gottesman MM. Targeting multidrug resistance in cancer. Nat Rev Drug Discov 2006;5:219–34.
[17] Willingham MC. Cytochemical methods for the detection of apoptosis. J Histochem Cytochem 1994;47:1101–9.
[18] Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 2006;24:1770–83.
[19] Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta 2003;1653:1–24.
[20] Korinek V, Barker N, Morin PJ, van Wichen D, de Weger R, Kinzler KW, et al. Constitutive transcriptional activation by a β-catenin-Tcf-complex in $APC^{-/-}$ colon carcinoma. Science 1997;275:1784–7.
[21] Ilyas M, Tomlinson IPM, Rowan A, Pignatelli M, Bodmer WF. β-Catenin mutations in cell lines established from human colorectal cancers. Proc Natl Sci Acad USA 1997;94:10330–4.
[22] van Horssen R, Ten Hagen TLM, Eggermont AMM. TNF-α in cancer treatment: molecular insights, antitumor effects, and clinical utility. The Oncologist 2006;11:397–408.
[23] Aggarwal BB. Signaling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol 2003;3:745–56.
[24] Gao W, Chen APC, Leung CH, Gullen EA, Fürstner A, Shi Q, et al. Structural analogs of tylophora alkaloids may not be functional analogs. Bioorg Med Chem Lett 2008;18:704–9.
[25] Sakamaki T, Casimiro MC, Ju X, Quong AA, Katiyar S, Liu M, et al. Cyclin D1 determines mitochondrial function in vivo. Mol Cell Biol 2006;26:5449–69.
[26] Kawada M, Seno H, Uenoyama Y, Sawabu T, Kanda N, Fukui H, et al. Signal transducers and activators of transcription 3 activation is involved in nuclear accumulation of β-catenin in colorectal cancer. Cancer Res 2006; 66:2913–7.
[27] LeBlanc H, Lawrence D, Varfolomeev E, Totpal K, Morlan J, Schow P, et al. Tumor-cell resistance to death receptor-induced apoptosis through mutational inactivation of the proapoptotic Bcl-2 homolog Bax. Nat Med 2002;8: 274–81.
[28] Nio Y, Zighelboim J, Berek J, Bonavida B. Cycloheximide-induced modulation of TNF-mediated cytotoxicity in sensitive and resistant ovarian tumor cells. Cancer Chemother Pharmacol 1990;26:1–8.
[29] Brown-Bryan TA, Leoh LS, Ganapathy V, Pacheco FJ, Mediavilla-Varela M, Filippova M, et al. Alternative splicing and caspase-mediated cleavage generate antagonistic variants of the stress oncoprotein LEDGF/p75. Mol Cancer Res 2008;6:1293–307.