A bioactive withanolide Tubocapsanolide A inhibits proliferation of human lung cancer cells via repressing Skp2 expression

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Abstract

Withanolides are generally defined as C_{28} steroidal lactones built on an intact or rearranged ergostane skeleton and have been shown to exhibit antiproliferative activity on various types of cancer cells. In this study, we investigated the effect of a new withanolide Tubocapsanolide A isolated from *Tubocapsicum anomalum* and addressed its molecular action. Tubocapsanolide A inhibited proliferation of A549, H358, and H226 human lung cancer cells via induction of G_1 growth arrest. We found that Tubocapsanolide A treatment led to up-regulation of cyclin E, p21, and p27, whereas other cyclins and cyclin-dependent kinases were not affected in A549 cells. Conversely, Skp2, the F-box protein that is implicated in the mediation of degradation of p21 and p27, was significantly down-regulated. Chromatin immunoprecipitation assay suggested that Tubocapsanolide A suppressed Skp2 expression by inhibiting the binding of Rel A to the nuclear factor-κB site of Skp2 gene promoter. In addition, we showed that inhibition of Skp2 is a critical step for the suppression of cell proliferation by Tubocapsanolide A because ectoexpression of Skp2 effectively reversed Tubocapsanolide A–induced p27 up-regulation and growth inhibition in human lung cancer cells. Collectively, we have identified Skp2 as a molecular target for Tubocapsanolide A and suggest that this withanolide may be useful for the prevention or treatment of cancer cells with Skp2 overexpression. [Mol Cancer Ther 2007;6(5):1572–8]

Received 12/31/06; revised 3/2/07; accepted 3/27/07.

Grant support: Kaohsiung Medical University Research Foundation (Q096012) and the National Sun Yat-Sen University-Kaohsiung Medical University Joint Research Center (H-C. Chang).

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doi:10.1158/1535-7163.MCT-06-0812

Introduction

Withania somnifera is one of the most important herbs used as a traditional remedy for several illnesses in Asian countries. This plant has been used as a constituent in more than a hundred herbal preparations to promote health and longevity in India for a long time. Its efficacy in many ailments has been confirmed by various pharmacologic experiments (1). The extract of the roots of the plant contains withanolides. These compounds are biologically active and may inhibit the enzymatic activity of cyclo-oxygenase-2 to suppress inflammation (2). In addition, recent studies showed that withanolides exhibit anticancer effect on human lung, colon, and breast cancer cells *in vitro* and exert immunopotentiating activity *in vivo* (3, 4). Moreover, these natural compounds also suppressed tumor angiogenesis and metastasis (5, 6). These results suggest that withanolides may be developed as a novel class of anticancer drugs. However, the molecular mechanism by which withanolides inhibit proliferation of human cancer cells is largely unknown.

Skp2 was originally identified as an associated protein of the cyclin A–Cdk2 complex in transformed cells (7). Subsequently, three independent studies showed that Skp2 binds to and mediates the ubiquitination of the cyclin-dependent kinase (CDK) inhibitor p27 (8–10), which was known to be degraded via the ubiquitin/proteasome pathway at the G_1 phase. The biochemical evidence that Skp2 may function as a specificity factor in p27 ubiquitination was reinforced by genetic evidence showing that p27 accumulates at high levels in mice that lack Skp2 (11, 12). Recent clinical investigations show that reduction of p27 protein is frequently found in various types of human cancer, including breast, lung, prostate, gastric, skin, colon, and ovarian cancer and is usually correlated with poor clinical outcome (13–19). Because Skp2 is a major player in the induction of p27 degradation, it is rational to speculate that amplification or overexpression of Skp2 may result in enhancement of p27 proteolysis and tumor formation. Indeed, recent works show that Skp2 is oncogenic and is overexpressed in human cancers (20, 21). Studies of primary tissues also show an inverse relationship between the expression of Skp2 and p27 (22–24).

In this study, we investigate the anticancer effect of a new withanolide Tubocapsanolide A isolated from *Tubocapsicum anomalum*, and our results indicate that Tubocapsanolide A

may suppress the transcription of *Skp2* oncogene and upregulate *p27* and *p21* to inhibit proliferation of human lung cancer cells.

Materials and Methods

Plant Material

The initial collection of *T. anomalum* (Solanaceae) was made on July 2003 near NanTao County and identified by Dr. Hsin-Fu Yen (National Museum of Natural Science, Taichung, Taiwan). A larger amount of the same plant was recollected at the Da-Han Mountain, Kaohsiung, on October 2004, and identified by Dr. Ming-Ho Yen (Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan). The samples were authenticated and deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University.

Extraction and Isolation

The air-dried stems and leaves (2.5 kg, part A) and roots (1.2 kg, part B) of *T. anomalum* were extracted separately with methanol at room temperature. The methanol extract of part A was partitioned between ethyl acetate/H_2O to yield ethyl acetate and H_2O extracts. The H_2O extracts were further partitioned with *n*-BuOH to give *n*-BuOH and H_2O extracts. These extracts were evaporated to give dark-green viscous residues. The residue from the ethyl acetate extract was further separated on a Si gel column (230–400 mesh, 5 × 20 cm) eluting with a gradient of *n*-hexane/$CHCl_3$/methanol to give 16 fractions (A1–A16). Fraction A8 (529.8 mg) was further purified on a silicon gel column using *n*-hexane-$CHCl_3$ (2:1) and $CHCl_3$ as eluents and recrystallized from methanol to give Tubocapsanolide A (42.5 mg).

Tubocapsanolide A

White powder: Mp. 233°C to 235°C. $[\alpha]_D^{24.4}$ +22.3° (*c* 0.1, methanol). UV (methanol) λ_{max} = 218 nm. CD [θ] +14,000 (256 nm). IR (neat):ν_{max} 3,403, 2,918, 1,688, 1,679, 1,380, and 1,132 cm^{-1}. For ^1H and ^{13}C nuclear magnetic resonance data, see Supplementary Table S1 to S3.[5] HRFAB-MS *m/z* 469.2594 $[M+Na]^+$ (calculated 469.2585). The purity is >95%.

Cell Culture

A549, H358, and H226 human lung cancer cell lines were obtained from the cell bank of the National Health Research Institute (Maoli, Taiwan). Cells were cultured in DMEM/F12 medium containing 10% heat-inactivated FCS and antibiotics (100 units/mL penicillin and 100 μg/mL streptomycin).

Reagents

A 50 mmol/L solution of Tubocapsanolide A was prepared with DMSO and stored as small aliquots at −20°C. Before use, Tubocapsanolide A was thawed and diluted in cell culture medium. Cyclin D1, E, A, B; CDK2, CDK4, CDK6; and p27 antibodies were purchased from Santa Cruz Biotechnology. Anti-p21 antibody was purchased from Cell Signaling Technology. Antiactin antibody was obtained from Chemicon. LipofectAMINE was obtained from Invitrogen. Luciferase activity assay system was obtained from Promega. Myc-tagged Skp2 expression vector was kindly provided by Dr. C.H. Lin (Academica Sinica, Taipei, Taiwan).

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay

Human lung cancer cells (5,000 per well) were seeded into 96-well culture plates. After 24 h, cells were incubated in 10% FCS medium containing vehicle (0.1% DMSO) or various concentrations of Tubocapsanolide A for 48 h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was done as described previously (25) to investigate the effect of Tubocapsanolide A on cell growth.

Cell Cycle Analysis

A549 cells were seeded at a density of 100,000 per well into six-well plates. After 24 h, cells were incubated in 10% FCS medium containing vehicle (0.1% DMSO) or various concentrations of Tubocapsanolide A for another 24 h and were harvested for staining of propidium iodide. Cell cycle distribution was analyzed by fluorescence-activated cell sorting flow cytometry (Becton Dickinson, Mountain View, CA) as previously described (26).

RNA Isolation and Reverse Transcription-PCR

Total RNA was isolated from cells and Skp2 mRNA expression was investigated by using the OneStep reverse transcription-PCR kit according to the manufacturer's protocol (Qiagen). *GAPDH* was used as an internal control to check the efficiency of cDNA synthesis and PCR amplification. cDNA synthesis was carried out at 50°C for 30 min and the condition for PCR was 30 cycles of denaturation (94°C/30 s), annealing (60°C/30 s), extension (72°C/45 s), and one cycle of final extension (72°C/10 min). The predicted sizes for PCR products for *Skp2* and *GAPDH* were 500 and 512 bp, respectively. The primers used were as follows: *Skp2*-forward: 5′-GTGTCAGTCGGCATTTGATG-3′, *Skp2*-reverse: 5′-TTCGAGATACCCACAACCCC-3′, *GAPDH*-forward: 5′-GAGTCAACGGATTTGGTCGT-3′, *GAPDH*-reverse: 5′-TGTGGTCATGAGTCCTTC CA-3′. After the reaction, PCR products were separated on a 2% 0.5× Tris-borate EDTA agarose gel, stained with ethidium bromide, and visualized under UV light.

Immunoblotting

For immunoblotting, vehicle (0.1% DMSO)– or Tubocapsanolide A–treated cells were harvested in a lysis buffer, and equal amount of cellular proteins was subjected to SDS-PAGE as described previously (27). Proteins were transferred to nitrocellulose membranes and the blots were probed with different primary antibodies followed by horseradish peroxidase–labeled secondary antibodies. Enhanced chemiluminescence reagent was used to depict the protein bands on the blots. For the detection of nuclear translocation of Rel A, nuclear proteins were extracted as described previously (28).

Analysis of Protein Stability

Protein half-life was measured by blocking protein synthesis with cycloheximide and harvesting the cells at various times. Cells were incubated with 10% FCS medium

[5] Supplementary data for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

containing vehicle or Tubocapsanolide A (0.5 μmol/L) for 24 h. Cells were treated with 10 μg/mL of cycloheximide and cellular proteins were harvested at various times. p21 and p27 expression was determined by Western blotting, and the protein level in cells collected at time zero was defined as 100%.

Promoter Activity Assay

Cloning and activity assay of *Skp2* gene promoter was done as described previously (27). In brief, cells were plated onto six-well plates at the density of 300,000 per well and grown overnight. Cells were transfected with 1 μg of Skp2 promoter-luciferase plasmid. After transfection, cells were treated with vehicle (0.1% DMSO) or various concentrations of Tubocapsanolide A in 10% FCS medium for 48 h. Promoter activity was determined by using a Firefly luciferase assay system (Promega) and normalized for the concentration of cellular proteins. Data of three independent experiments were expressed as mean ± SD. Paired results were evaluated by the Student's *t* test, and $P < 0.05$ was considered significant.

Chromatin Immunoprecipitation Assay

Vehicle- or Tubocapsanolide A–treated A549 cells were fixed with 1% formaldehyde at 37°C for 10 min. Cells were washed twice with ice-cold PBS containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, and 1 μg/mL pepstatin A), scraped, and pelleted by centrifugation at 4°C. Cells were resuspended in a lysis buffer [1% SDS, 10 mmol/L EDTA, and 50 μmol/L Tris-HCl (pH 8.1)], incubated for 10 min on ice, and sonicated to shear DNA. After sonication, lysate was centrifuged for 10 min at 13,000 rpm at 4°C. The supernatant was diluted in chromatin immunoprecipitation dilution buffer [0.01% SDS, 1% Triton X-100, 2 mmol/L EDTA, 16.7 mmol/L Tris-HCl (pH 8.1), 167 mmol/L NaCl, and protease inhibitors]. Anti–Rel A or nonimmune (negative control) antibodies were added to the supernatant and incubated overnight at 4°C with rotation. Chromatin immunoprecipitation assays were done as described previously (29). DNA fragments were recovered and were subjected to PCR amplification by using the primers specific for the detection of the −136/+132 region, which contained the nuclear factor-κB (NF-κB) site of human *Skp2* gene promoter. The sequences for the primers are as follows: sense 5′-ACATTTCCCAGTCAGCCGTA-3′ and antisense 5′-GCCTAGCAACGTTCCATCC-3′.

Statistical Analysis

Data of three independent experiments were shown as mean ± SD. *$P < 0.05$ when different groups with various treatments were compared.

Results

Tubocapsanolide A Potently Inhibits Proliferation of Human Lung Cancer Cells

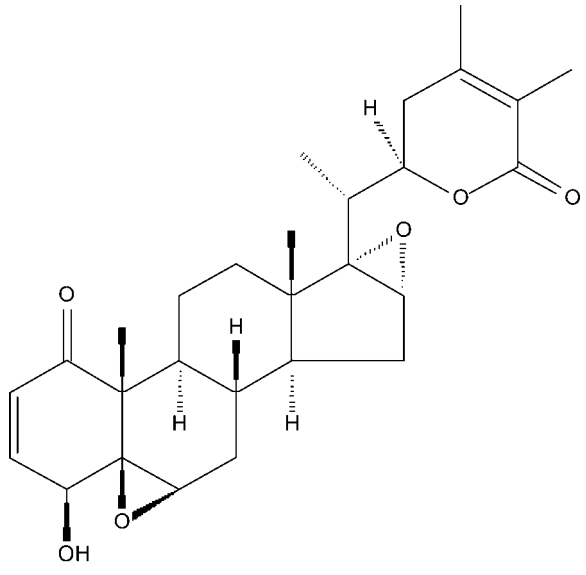
Tubocapsanolide A is a new bioactive withanolide isolated from *T. anomalum* and the chemical structure is shown in Fig. 1. We tested the effect of Tubocapsanolide A on the growth of various human lung cancer cell lines. Our results showed that Tubocapsanolide A potently inhibited proliferation of lung cancer cells in a dose-dependent manner (Fig. 2A). We also compared the growth-inhibitory activity between Tubocapsanolide A and cisplatin, a clinical chemotherapeutic drug. Our results showed that Tubocapsanolide A is more potent in the inhibition of proliferation of A549 lung cancer cells (Fig. 2B).

Tubocapsanolide A Blocks Cell Cycle Progression at the G_1-S Transition

We used A549 cells as a model to address the molecular mechanism by which Tubocapsanolide A inhibited cell growth. Flow cytometric analysis indicated that treatment of Tubocapsanolide A increased the cell number of G_0-G_1 phase (Fig. 3A). On the contrary, cell number of S phase was reduced. We next studied the alteration of G_1-associated cell cycle regulators in Tubocapsanolide A–treated cells. As shown in Fig. 3B, expression of CDK2, CDK4, and CDK6 was not affected by Tubocapsanolide A. Similarly, Tubocapsanolide A did not affect the expression of cyclin D1 and A. Conversely, the protein level of cyclin E was increased. We also examined the expression of CDK inhibitors p21 and p27 and found that these two inhibitory proteins were increased after Tubocapsanolide A treatment. Our data indicated that Tubocapsanolide A modulated the expression of cell cycle regulatory proteins to induce G_1 growth arrest.

Tubocapsanolide A Inhibits Skp2 Expression at the Transcriptional Level

An interesting observation is that Tubocapsanolide A increased the protein level of p21 and p27. Alteration of cell cycle regulators is mainly caused by change of protein



Tubocapsanolide A

($C_{28}H_{36}O_6$; MW 468.59)

Figure 1. Chemical structure of Tubocapsanolide A.

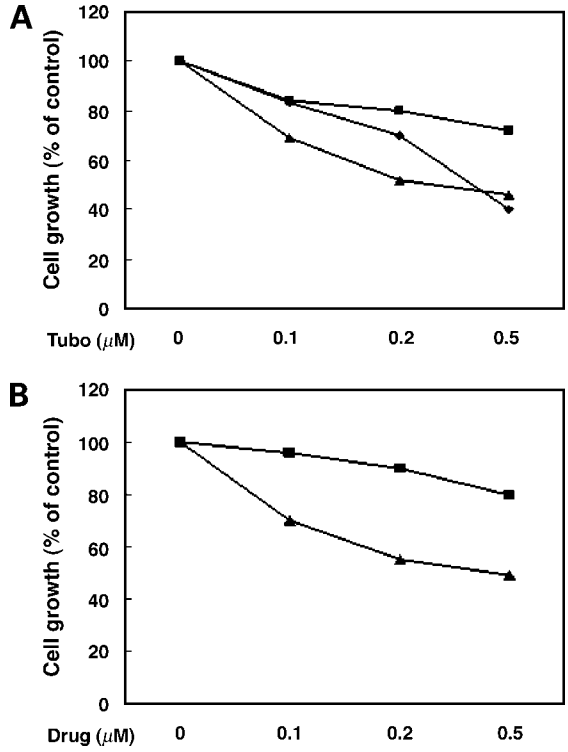


Figure 2. Effect of Tubocapsanolide A on growth of human lung cancer cells. **A,** H358 (■), A549 (▲), or H226 (♦) cells were cultured in 10% FCS medium containing various concentrations of Tubocapsanolide A (*Tubo*) for 48 h. MTT assay was done as described in Materials and Methods and the absorbance of cells treated without Tubocapsanolide A (0 μmol/L) was defined as 100%. **B,** A549 cells were treated with various concentrations of cisplatin (■) and Tubocapsanolide A (▲) for 48 h. MTT assay was done and the absorbance of cells treated without drugs was defined as 100%.

stability via posttranslational modification. Recent evidences indicated that p21 and p27 are degraded via the ubiquitin/proteasome pathway and the common F-box protein that mediated the degradation of these three proteins is Skp2 (8–10, 30). Therefore, we tested whether Tubocapsanolide A might regulate p21 and p27 protein stability and Skp2 expression in lung cancer cells. As shown in Fig. 4A, our results showed that the protein stability of p21 and p27 was increased in Tubocapsanolide A–treated cells. In addition, we found that Tubocapsanolide A down-regulated Skp2 protein level in a dose-dependent manner (Fig. 4B). Reverse transcription-PCR analysis showed that Tubocapsanolide A reduced Skp2 mRNA level in a dose-dependent manner (Fig. 4C). These results suggest that Tubocapsanolide A may inhibit Skp2 at the transcriptional level. We carried out promoter activity assay and our results showed that Tubocapsanolide A suppressed Skp2 promoter activity in a dose-dependent fashion (Fig. 4D).

Tubocapsanolide A Represses Skp2 Expression by Inhibiting Rel A – Mediated Transcription

We next addressed the signaling pathway by which Tubocapsanolide A inhibits Skp2 expression. A recent report has shown that NF-κB is a molecular target for other withanolides (31). Therefore, we investigated whether Tubocapsanolide A inhibited NF-κB activation. We found that the basal level of IκBα was low in A549 cells, which is in agreement with the reports that A549 cells exhibit constitutively active NF-κB (refs. 32, 33; Fig. 5A). Treatment of Tubocapsanolide A increased the protein level of IκBα. In addition, protein level of Rel A in the nucleus was significantly reduced in Tubocapsanolide A–treated cells (Fig. 5B). These data suggested that Tubocapsanolide A might attenuate NF-κB transcriptional activity to inhibit Skp2 expression. When our study was in progress, a report showed that Skp2 is a target for IKKα/NF-κB signaling pathway and plays an important role in the control of G_1-S phase progression (34). The authors also showed NF-κB binding sites are localized at the proximal region of human *Skp2* promoter. So, we tested whether Rel A is constitutively binding to *Skp2* promoter and whether Tubocapsanolide A affected this binding. Chromatin immunoprecipitation assay indeed showed that Rel A bound to *Skp2* promoter *in vivo* and Tubocapsanolide A

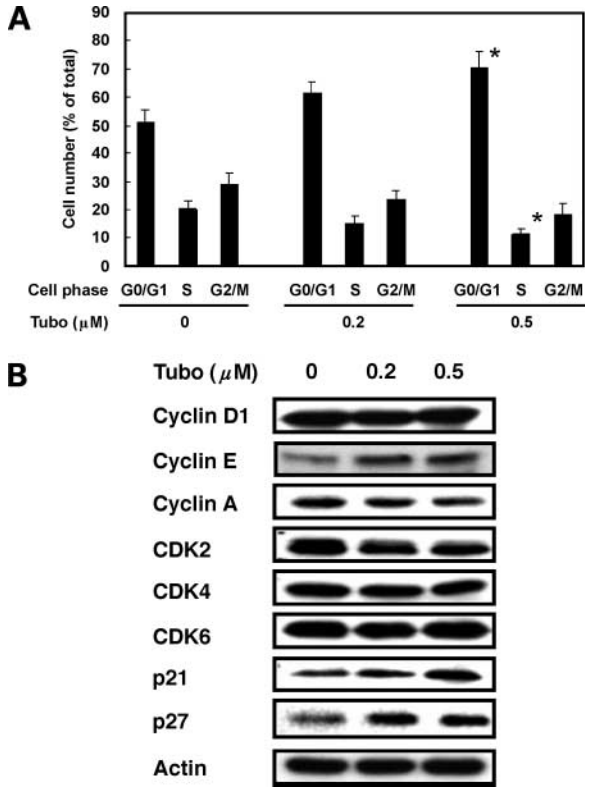


Figure 3. Tubocapsanolide A blocks the progression of cell cycle at the G_1 phase and up-regulates the expression of cyclin E, p21, and p27. **A,** A549 cells were treated with various concentrations of Tubocapsanolide A (*Tubo*) for 24 h. Cells were harvested, stained with propidium iodide, and the cell cycle distribution was analyzed by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$, when vehicle- and 0.5 μmol/L Tubocapsanolide A–treated groups were compared. **B,** cellular proteins were also harvested for the analysis of expression of cyclins, CDKs, and CDK inhibitors by Western blotting.

effectively attenuated this binding (Fig. 5C). The binding is specific because nonimmune immunoglobulin (as negative control) did not precipitate any protein-DNA complex. Collectively, these results suggest that Tubocapsanolide A represses *Skp2* expression by inhibiting Rel A–mediated transcription.

Ectoexpression of Skp2 Reversed Tubocapsanolide A–Induced p27 Up-regulation and Growth Inhibition

Our aforementioned results suggested that Tubocapsanolide A inhibited Skp2 expression, which led to increase of p21 and p27 proteins and inhibition of cell proliferation. To verify the functional importance of Skp2, we ectoexpressed this F-box protein and investigated the effect of Tubocapsanolide A on Skp2-overexpressing cells. As shown in Fig. 6A, ectoexpression of Skp2 effectively counteracted the up-regulation of p27 protein level by Tubocapsanolide A in A549 cells. Moreover, the growth-inhibitory action of Tubocapsanolide A was also reversed by ectoexpression of Skp2 (Fig. 6B).

Discussion

This is the first report to examine the effect of withanolides on *Skp2*, an oncogene that is involved in the tumorigenesis of various types of human cancer. Several previous studies have shown that withanolides exert anticancer effect on cancer cells (3–6). However, the molecular mechanism is largely known. We found that treatment of a new bioactive withanolide Tubocapsanolide A increased protein levels of p21 and p27 to inhibit proliferation of human lung cancer cells. These results led us to hypothesize that Tubocapsanolide A may affect the F-box protein Skp2, which controls the degradation of these two cell cycle regulators to enhance the protein stability and to increase the intracellular level of these two CDK inhibitors. Lines of evidence support our hypothesis. First, Tubocapsanolide A directly inhibits Skp2 via transcriptional repression. Second, ectoexpression of Skp2 reversed Tubocapsanolide A–induced down-regulation of p27. Third, ectoexpression of Skp2 counteracted the growth-inhibitory action of Tubocapsanolide A. Therefore, we conclude that Skp2 is a target for Tubocapsanolide A. Our conclusion is strengthened by the observation that expression of cyclin E was also increased in Tubocapsanolide A–treated cells. Original studies showed that Skp2 might be the F-box protein for cyclin E because cyclin E was significantly accumulated in $Skp2^{-/-}$ cells and ectoexpression of Skp2 induced polyubiquitination and degradation of cyclin E in cultured cells (11, 12). A subsequent study showed that degradation of cyclin E might be mediated by another F-box protein Fbw7 (35). However, Skp2 has been shown to involve in the degradation of cyclin E and knockdown of this F-box protein has been shown to increase the protein level of cyclin E in cells. The observation that cyclin E was increased in Tubocapsanolide A–treated cells further supported our conclusion that Skp2 is a major target for Tubocapsanolide A to suppress cell proliferation.

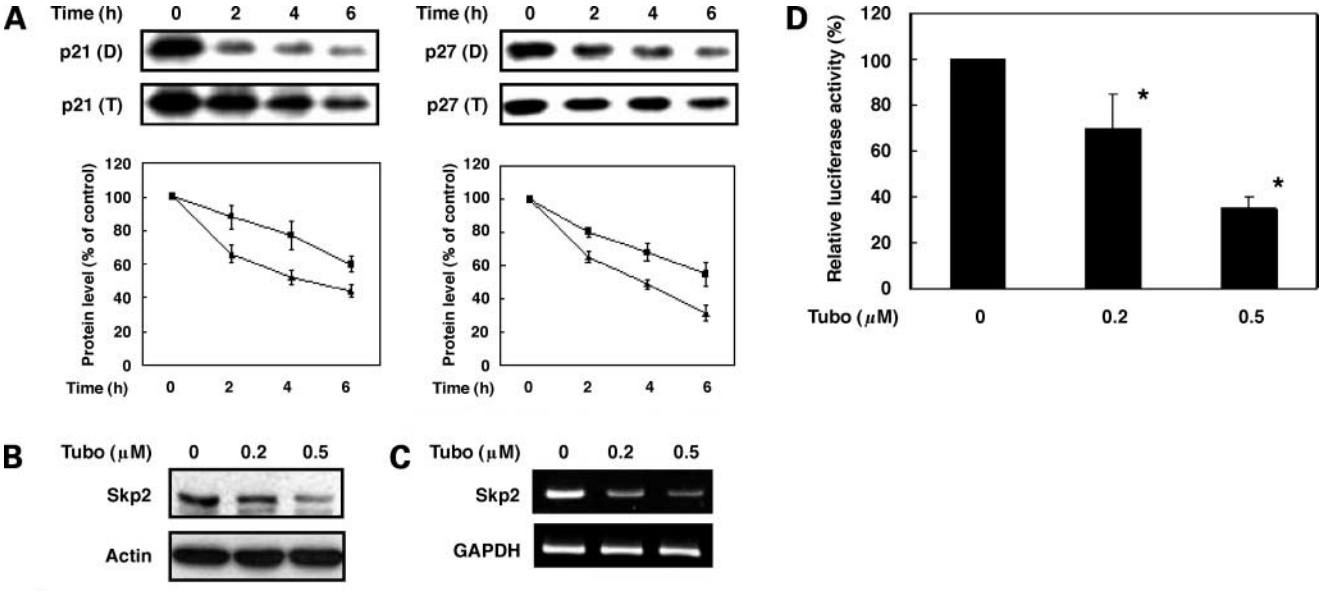


Figure 4. Tubocapsanolide A suppresses Skp2 expression and increases the protein stability of p21 and p27. **A,** A549 cells were cultured in 10% FCS medium containing vehicle (*D*, 0.1% DMSO) or Tubocapsanolide A (*T*, 0.5 μmol/L) for 24 h. Cells were treated with 10 μg/mL of cycloheximide and cellular proteins were harvested at various times. p21 and p27 expression was determined by Western blotting and the protein level in cells collected at time zero was defined as 100%. The relative protein level in cells harvested at different times was expressed as percentage (*bottom,* ▲, DMSO-treated cells; ■, Tubocapsanolide A – treated cells). **B,** cells were treated with various concentrations of Tubocapsanolide A for 24 h and Skp2 protein level was assayed by Western blot analysis. **C,** mRNA level of Skp2 in Tubocapsanolide A – treated cells was also studied by reverse transcription-PCR. **D,** cells were transfected with 2 μg of Skp2 promoter and treated with various concentrations of Tubocapsanolide A for 24 h. Luciferase activity was assayed and normalized to the concentrations of cellular proteins. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$ when vehicle- and Tubocapsanolide A – treated groups were compared.

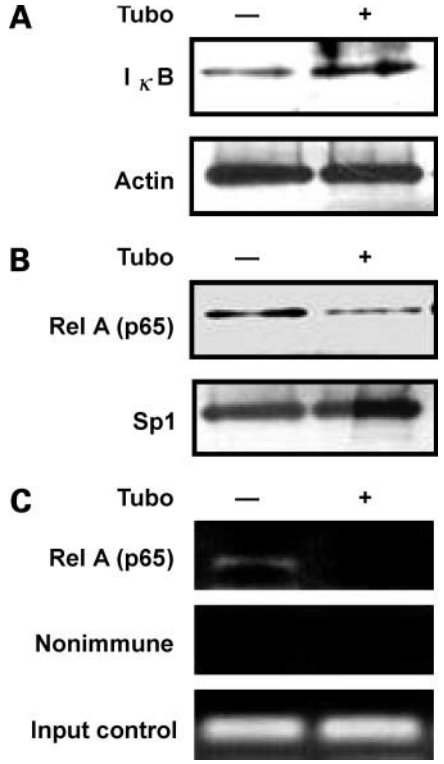


Figure 5. Tubocapsanolide A attenuates Rel A binding to Skp2 promoter. **A,** A549 cells were treated without (−) or with (+) 0.5 μmol/L of Tubocapsanolide A for 24 h. Cellular proteins were harvested and the protein level of IκB was investigated by Western blot analysis. **B,** cells were treated without (−) or with (+) 0.5 μmol/L of Tubocapsanolide A for 24 h. Nuclear proteins were extracted and the protein level of Rel A was assayed. Sp1 was used as an internal control for equal loading of nuclear proteins. **C,** cells were treated without (−) or with (+) Tubocapsanolide A for 24 h and fixed with 1% formaldehyde. Anti-Rel A or nonimmune (negative control) antibodies were added to precipitate the protein-DNA complex as described in Materials and Methods. DNA fragments were recovered and were subjected to PCR amplification by using the primers specific for the detection of the −136/+132 region, which contained the NF-κB site of human *Skp2* gene promoter.

The anticancer effect of several withanolides has been tested in a number of cancer cell lines (4). The authors focused on the apoptosis-inducing activity of the withanolides. However, we mainly addressed the molecular mechanism by which Tubocapsanolide A induced growth inhibition in cancer cells in this study. Therefore, it is not easy to directly compare the anticancer efficacy among these withanolides. However, our recent data indicated that Tubocapsanolide A triggered significant apoptosis at the concentrations of 0.5 to 1 μmol/L (0.24–0.48 μg/mL). This dose is close to the anticancer dose of the most effective withanolide, Withaferin A, tested in the previous study (4). Thus, Tubocapsanolide A is a bioactive withanolide with potent anticancer activity.

How Tubocapsanolide A or other withanolides inhibit NF-κB activation is still unclear. Although reduction of IKK activity and IκBα degradation are commonly observed in withanolide-treated cells, *in vitro* kinase assay indicated that IKK is not a direct target for withanolides (31, 36). These data suggest that one or more upstream kinases for IKK are potential candidates. Several kinases, including NF-κB–inducing kinase, NF-κB–activating kinase, mitogen-activated protein kinase kinase 1, mitogen-activated protein kinase kinase 3, and transforming growth factor-β activating kinase 1, have been shown to phosphorylate the IKK complex and induce NF-κB activation when overexpressed in cells (37–40). Whether these kinases are direct targets for withanolides need further studies. Also, it should be emphasized that IKK signaling pathway may not be the only mediator for withanolides. As shown in Fig. 5B, Tubocapsanolide A only partially reduced the protein level of nuclear Rel A. Therefore, we cannot exclude the involvement of other signaling pathways that may directly phosphorylate and activate Rel A. Our unpublished data indeed indicate that Tubocapsanolide A may affect IKK-independent signaling pathway to regulate expression of downstream target genes.

Previous studies have already confirmed that *Skp2* is an oncogene (20, 21). Investigations of primary tumor tissues also show that *Skp2* is overexpressed in human cancers (22–24). It is rational to hypothesize that natural or

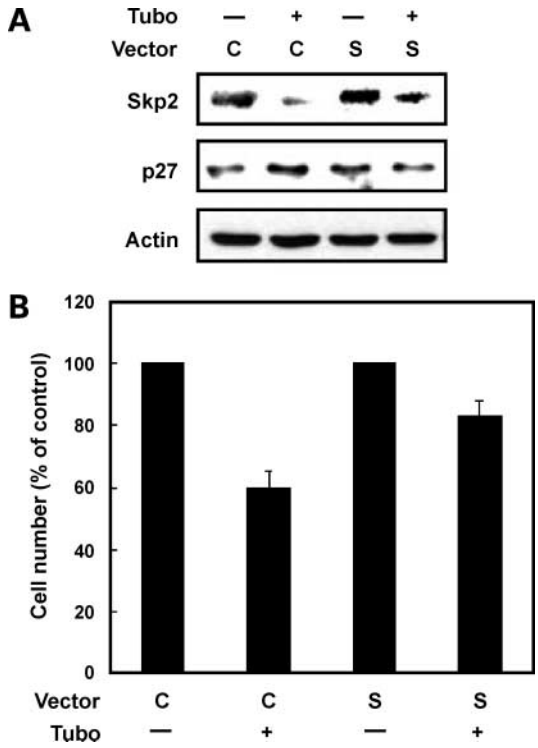


Figure 6. Ectoexpression of Skp2 reverses Tubocapsanolide A–induced p27 up-regulation and growth inhibition. **A,** A549 cells were transfected with control (*C*) or Skp2 (*S*) expression vectors. After 24 h, cells were treated without (−) or with (+) 0.5 μmol/L Tubocapsanolide A for another 24 h. Protein level of Skp2 and p27 was investigated, and actin was used as an internal control. **B,** cells were treated as described above and the cell number of each group was counted. Cell number of vehicle-treated (−) group was defined as 100%. Results of three independent experiments.

synthetic compounds that may directly inhibit Skp2 expression are potential anticancer drugs. Because the plant *W. somnifera* (which contains many bioactive withanolides) has been used as a constituent in more than a hundred herbal preparations in Asian countries for a long time, withanolides like Tubocapsanolide A may be developed as a novel class of chemopreventive or therapeutic drugs for the treatment of human cancers with *Skp2* overexpression.

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