

Inhibitory effect of snake venom toxin from *Vipera lebetina turanica* on hormone-refractory human prostate cancer cell growth: induction of apoptosis through inactivation of nuclear factor κ B

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Abstract

We investigated whether the snake venom toxin (SVT) from *Vipera lebetina turanica* inhibits cell growth of human prostate cancer cells by inducing apoptosis and also studied possible signaling pathways involved in this cell death. SVT inhibited growth of PC-3 and DU145 cells, androgen-independent prostate cancer cells, but not LNCaP cells, a human androgen-dependent prostate cancer cell. Cells were arrested in the G₂-M phase by SVT with a concomitant decrease in the expression of the G₂-M phase regulatory protein cyclin B1 and were also arrested in the G₁-S phase with decreasing expression of cyclin-dependent kinase 4, cyclin D1 and cyclin E. In addition to the growth-inhibitory effect, SVT increased the induction of apoptotic cell death. Untreated PC-3 cells show high DNA binding activity of nuclear factor κ B (NF- κ B), an antiapoptotic transcriptional factor, but this was inhibited by SVT and accompanied by a significant inhibition of p50 translocation into the nucleus, as well as phosphorylation of inhibitory κ B. Consistent with the induction of apoptosis and inhibition of NF- κ B, this toxin increased the expression of proapoptotic proteins such as p53, Bax, caspase-3, and caspase-9, but down-regulated

antiapoptotic protein Bcl-2. However, SVT did not show an inhibitory effect on cell growth and caspase-3 activity in cells carrying mutant p50 and inhibitory κ B kinase plasmids. Confocal microscopy analysis showed that SVT is taken up into the nucleus of the cells. These findings suggest that a nanogram concentration range of SVT from *V. lebetina turanica* could inhibit hormone-refractory human prostate cancer cell growth, and the effect may be related to NF- κ B signal-mediated induction of apoptosis. [Mol Cancer Ther 2007;6(2):675–83]

Introduction

Prostate cancer is the most common cancer, as well as the second leading cause of cancer-related deaths, in men of western countries (1). Current therapies for prostate cancer, such as surgery, radical prostatectomy, and radiation therapy, are effective in many patients with locally advanced disease, but many of these patients eventually have recurrence due to the high resistance of prostate cancer cells against chemotherapeutic agents (1–3). In normal prostate, organ homeostasis is maintained by a dynamic balance between the rate of cell proliferation and the rate of programmed cell death (apoptosis; ref. 4). Failure to undergo apoptosis has been implicated in tumor development and resistance to cancer therapy. Promotion of apoptosis in prostate cancer cells may lead to the regression of cancer cells and improved prognosis of refractory disease (5). Thus, any agents that induce apoptosis may be useful for chemotherapy against prostate cancer (6).

Recently, several studies have reported that nuclear factor κ B (NF- κ B) is constitutively activated in human prostate cancer tissue, androgen-insensitive human prostate carcinoma cells and prostate cancer xenografts (7–9). Therefore, agents capable of suppressing the NF- κ B pathway may also be potentially useful in the prevention and management of prostate cancer growth and resistance via the induction of apoptotic prostate cancer cell death.

Snake venom toxins (SVT) are greatly feared, but many researchers believe natural SVTs are useful biological resource, containing several pharmacologically active components that could be of potential therapeutic value (10–13). We also recently found that SVT from *Vipera lebetina turanica* inhibited NF- κ B activation and target gene expression through its interaction with signal molecules (p50 and inhibitory κ B kinases [IKK]) in the NF- κ B pathway (14).

In this study, we conducted an analysis to evaluate the prostate cancer cell response to SVT from *V. lebetina*

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turanica, to determine the ability of this venom toxin to act as a therapeutic agent to suppress prostate cancer cell growth and chemotherapeutic resistance by inducing apoptotic cell death, and to determine possible mechanisms related to the inactivation of NF- κ B signals.

Materials and Methods

Materials

SVT from *V. lebetina turanica* was purchased from Sigma (St. Louis, MO). The expression plasmid encoding IKK β K44A-Flag was obtained from Dr. Warner C. Greene (University of California, San Francisco, CA; ref. 15), and the expression plasmids encoding IKK α C178A-Flag and IKK β C179A-Flag were obtained from Dr. Dae-Myung Jue (The Catholic University of Korea, Seoul, Korea).

Cell Culture

The PC-3, DU145, and LNCaP prostate cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI 1640 (HyClone, South Logan, UT) supplemented with 10% fetal bovine serum (HyClone) and penicillin/streptomycin (100 unit/mL, HyClone). Cell cultures were then maintained at 37°C in a humidified atmosphere with 5% CO₂. RAW 264.7 macrophage cells (16) and primary neuronal cells (17) were prepared and cultured as previously described.

Cell Viability Assay

Cells were plated in 96-well plates, and subconfluent cells were subsequently treated with SVT (0.25 to 2 μ g/mL) or vehicle (distilled water) for 24 h. After treatment, cell viability was measured by Cell Counting Kit-8 (CCK-8) system (Dojindo Laboratory, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, CCK-8 solution (10 μ L per 100 μ L of medium in each well) was added, the plates were then incubated at 37°C for 1 h, and the absorbance of each well was read at 450 nm using a microplate reader.

Cell Cycle Analysis

To examine the effect of SVT on cell cycle distribution of asynchronous populations of prostate cancer cells, replicative DNA synthesis and DNA content were analyzed using bivariate flow cytometric analysis. SVT-treated/untreated PC-3 cells were harvested by trypsin-EDTA release and fixed in ice-cold 70% ethanol. At least 1 to 2 h before flow cytometric analysis, cells were resuspended in a 1-mL aliquot of modified Vindelov's DNA staining solution (10 μ g/mL RNase A and 5 μ g/mL propidium iodide in PBS). Flow cytometric analysis was done with flow cytometry system (FACS Calibur-S System, BD Bioscience, San Jose, CA). Cells in the G₁, S, and G₂-M phases of the cell cycle were determined with Modfit LT (Verity House Software, Top-sham, ME).

Apoptosis Evaluation

Apoptosis assays were done by observing morphologic changes and by the terminal nucleotidyl transferase-mediated nick end labeling (TUNEL) assay. In short, PC-3 cells were cultured on eight-chamber slides. After treatment with SVT (0.25 to 2 μ g/mL) or vehicle for 24 h, the

cells were washed twice with PBS, and then fixed and processed for 4',6-diamidino-2-phenylindole and TUNEL staining assay as previously described (18).

Immunofluorescence Staining

PC-3 cells were plated in the chamber slides at a density of 5 \times 10³ cells per chamber. The cells were then cultured with SVT (0.25 to 2 μ g/mL) or vehicle. Twenty-four hours later, the cells were washed once with PBS and fixed with 4% paraformaldehyde for 20 min, membrane-permeabilized by exposure to 0.1% Triton X-100 for 2 min in PBS, and placed in blocking serum (5% bovine serum albumin in PBS) at room temperature for 2 h. The cells were then exposed to primary rabbit polyclonal antibody for active caspase-3 (1:50 dilution, Cell Signaling Technology Inc., Beverly, MA) overnight at 4°C. After washes with ice-cold PBS, followed by treatment with an antirabbit secondary antibody labeled with Alexa Fluor 568 (1:100 dilution, Molecular Probes Inc., Eugene, OR) for 2 h at room temperature, immunofluorescence images were acquired using a confocal laser scanning microscope (TCS SP2, Leica Microsystems AG, Wetzlar, Germany) equipped with a 630 \times oil immersion objective.

Cellular Uptake Assay

To determine whether SVT could be taken up into the cells, cells (5 \times 10³ cells per chamber) were cultured in chamber slides and then treated with SVT labeled with the Alexa Fluor 488 Microscale Protein Labeling Kit (Molecular Probes) according to the manufacturer's instructions. Cells were incubated for 24 h at 37°C and fixed in 4% paraformaldehyde; the membranes were then permeabilized by exposure to 0.2% Triton X-100 in PBS for 5 min, and the cells were placed in blocking serum (5% horse or goat serum in PBS). Immunofluorescence images were acquired using a confocal laser scanning microscope with a 630 \times oil immersion objective.

Western Blot Analysis

Western blot analysis was done as described previously (16). The protein transfer membrane was incubated for 5 h at room temperature with specific antibodies for cyclin-dependent kinase 4 (CDK4), cyclin E, cyclin D1, cyclin B1, p53, Bax, Bcl-2, caspase-9, caspase-3, p65, p50 antibody (1:1,000), inhibitory κ B (I κ B) (1:500) or phosphorylated I κ B (1:500; Santa Cruz Biotechnology, Santa Cruz, CA). The blot was then incubated with the corresponding anti-rabbit/goat immunoglobulin G-horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.). Immunoreactive proteins were detected with the Enhanced Chemiluminescence Western blotting detection system (Amersham Pharmacia Biotech, Inc., Buckinghamshire, United Kingdom). The relative density of the protein bands was scanned by densitometry using MyImage (Seoulin Bioscience Inc., Seoul, Korea) and quantified by Labworks 4.0 software (UVP Inc., Upland, CA).

Preparation of Nuclear Extracts and Electromobility Shift Assays

The DNA binding activity of NF- κ B was determined using an electrophoretic mobility shift assay done according to the manufacturer's recommendations (Promega, Madison, WI).

In brief, PC-3 cells were cultured on 100-mm culture dishes. After treatment with SVT (0.25 to 2 $\mu\text{g}/\text{mL}$) or vehicle for 24 h, the cells were washed twice with PBS, followed by the addition of 1 mL of PBS, and the cells were scraped into a cold Eppendorf tube. Nuclear extracts were prepared and processed for electrophoretic mobility shift assay as previously described (18). The relative densities of the DNA-protein binding bands were scanned by densitometry using MyImage (SLB) and quantified by Labworks 4.0 software (UVP Inc.).

Statistical Analysis

Experimental results are expressed as mean \pm SE. One-way ANOVA was used for multiple comparisons followed by Dunnett's test. Differences with P values of <0.05 were considered statistically significant.

Results

Inhibition of Cell Growth and Induction of Apoptosis

To evaluate an effect of SVT from *V. lebetina turanica* on the cell growth of human prostate cancer cells, we analyzed cell viability using the CCK-8 assay. SVT inhibited androgen-independent human prostate cancer cell line PC-3 (Fig. 1A) and DU145 cells (Fig. 1B) growth with IC_{50}

values of 1.7 and 1.8 $\mu\text{g}/\text{mL}$, respectively. To determine the inhibitory effect of SVT in androgen-dependent human prostate cancer cell, we treated LNCaP cells with the same doses of SVT. SVT inhibited LNCaP cell growth (Fig. 1C); however, the magnitude of the inhibitory effect was much stronger in PC-3 or DU145 cells than that in the LNCaP cells (IC_{50} value of 9.1 $\mu\text{g}/\text{mL}$). Therefore, in the following investigation of the mechanism, we used only androgen-independent prostate cancer cells, especially PC-3 cells, to determine how SVT inhibits prostate cancer cell growth. To discriminate the susceptibility of cancer cells from that of normal cells, we explored the effects SVT in macrophages (murine macrophagelike cell line RAW 264.7 cells). We also treated primary cultured rat embryonic neuronal cells with SVT because this toxin can act through the nicotinic acetylcholine receptor. However, SVT did not reduce cell viabilities in RAW 264.7 cells (Fig. 1D) and neuronal cells (Fig. 1E) at doses below 2 $\mu\text{g}/\text{mL}$. To delineate whether the inhibition of cell growth by the SVT was due to an increase in the induction of apoptosis, we evaluated changes in the morphology of human prostate cancer cells using phase-contrast microscopy (Elclipse TE-300; Nikon Instech Co., Kawasaki, Kanagawa, Japan).

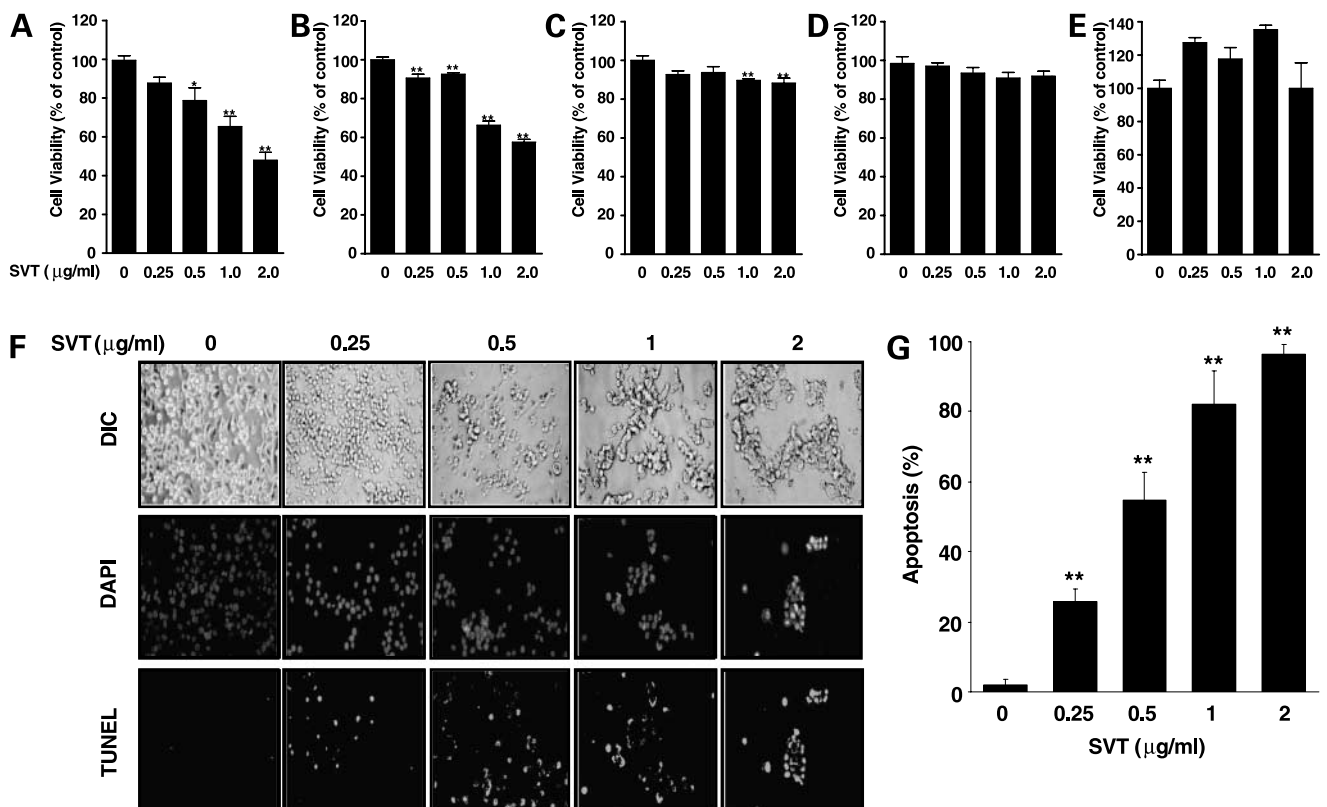


Figure 1. Cell viability in response to SVT. Cell viability was determined by the CCK-8 assay in PC-3 cells (A), DU145 cells (B), LNCaP cells (C), RAW 264.7 cells (D), and neuronal cells (E). PC-3 cell morphologic changes were observed under phase contrast microscope (F, top; magnification, 200 \times), and apoptotic cells were examined by fluorescence microscopy after TUNEL staining (F, bottom; magnification, 100 \times). Total number of cells in a given area was determined with 4',6-diamidino-2-phenylindole nuclear staining (F, middle; magnification, 100 \times). The apoptotic index (%) was calculated as the number of TUNEL-positive stained cells divided by the total cell number (G). Columns, means of three experiments, with triplicates of each experiment; bars, SE. *, $P < 0.05$; **, $P < 0.01$, significantly different from SVT-untreated cells.

Under the phase-contrast microscope, cells treated with SVT (0.25 to 2 $\mu\text{g}/\text{mL}$) and vehicle for 24 h presented with cytoplasmic blebbing, cell shrinkage, cytoplasmic condensation, and irregularity in shape (Fig. 1F, *top*). We also evaluated PC-3 cell apoptosis by TUNEL assay. TUNEL-positive cells dose-dependently increased in SVT-treated PC-3 cells, and the majority of cells were TUNEL positive in the cells treated with 2 $\mu\text{g}/\text{mL}$ of SVT (Fig. 1F, *bottom*, and G). These results show that SVT treatment strongly induced apoptosis in human prostate cancer cells.

Cell Cycle Arrest at the G₀-G₁ and G₂-M Phase

To evaluate whether the arrest of the PC-3 cells in specific cell cycle could be related to cell death, we analyzed cell cycle after treatment with SVT. With increasing concentrations of SVT, the number of cells distributed in the S phase decreased significantly compared with cells in other phases (Fig. 2).

Involvement of NF- κ B Signaling Pathway in the Apoptotic Cell Death by SVT

SVT had been shown to negatively regulate the nuclear transcription factor NF- κ B by means of protein-protein interaction (14). NF- κ B is known to be an inhibitory transcription factor of apoptosis. To investigate whether SVT can inactivate NF- κ B, and thereby hinder its anti-apoptotic ability, ultimately causing the cells to undergo apoptosis, we assessed NF- κ B activity in PC-3 cells treated with various concentrations of SVT for 24 h. NF- κ B was highly activated in PC-3 cells; however, the constitutive activation of NF- κ B was gradually reduced by culturing the cells in the presence of SVT (Fig. 3A). The NF- κ B DNA complex in the treated cells was competed out with an unlabeled, but not irrelevant, oligonucleotide (SP-1) and was supershifted by anti-p50 and p65 antibodies, which show the specificity of the NF- κ B band (data not shown). Translocation of p50 into the nucleus (less extensive translocation of p65) also decreased significantly, as shown by both immunocytochemistry and Western blotting (Fig. 3B

and C, *top*). The decrease in p50 translocation was accompanied by a significant decrease in the phosphorylation of I κ B in the cytosol (Fig. 3C, *bottom*).

Because SVT interacts with NF- κ B signal molecules by means of protein-protein interactions with the cysteine residues in NF- κ B signal molecules (14), we also conducted a transient transfection assay using a fusion gene containing the pCMV promoter and p50 or IKK mutants. PC-3 cells were transfected with these mutant gene constructs. Cell viability was then measured after stimulating the cells with SVT (1 $\mu\text{g}/\text{mL}$) for 24 h. In contrast to significant inhibition of cell viability in cells transfected with vector alone, SVT did not inhibit cell viability (Fig. 4A and B) or alter the number of caspase-3-reactive cells in PC-3 cells transfected with p50 (C62S), IKK α (C178A), or IKK β (C179A) mutants (Fig. 4C and D). However, SVT inhibited cell growth in PC-3 cells transfected with an IKK β mutant (K44A; data not shown). Moreover, the interaction between SVT and mutant p50 or IKKs was significantly abolished (data not shown), suggesting that the removed cysteine residues are the targets of SVT.

Expression of Cell Cycle and Apoptosis Regulatory Proteins

Expression of cell cycle regulatory proteins was also altered in cells treated with SVT. Consistent with cell cycle arrest in the G₁ and G₂-M phases, SVT decreased the expression of G₂-M phase regulating protein cyclin B1 and proteins (CDK4, cyclin D1 and E) regulating G₁ phase in the cells (Fig. 5A). Execution of apoptosis occurs through the activation of Bax and the caspase proteases and inactivation of Bcl-2. Caspases are a family of proteases that are expressed as inactive proenzymes in normal cells and, upon activation, are capable of cleaving structural and functional proteins involved in key cellular processes (19). The increase of apoptotic action was confirmed by the ability of SVT to induce caspase-3 and caspase-9 activations. Figure 5B reveals a Western blot analysis of Bax, Bcl-2, caspase-3, and caspase-9 expressions in PC-3 cells

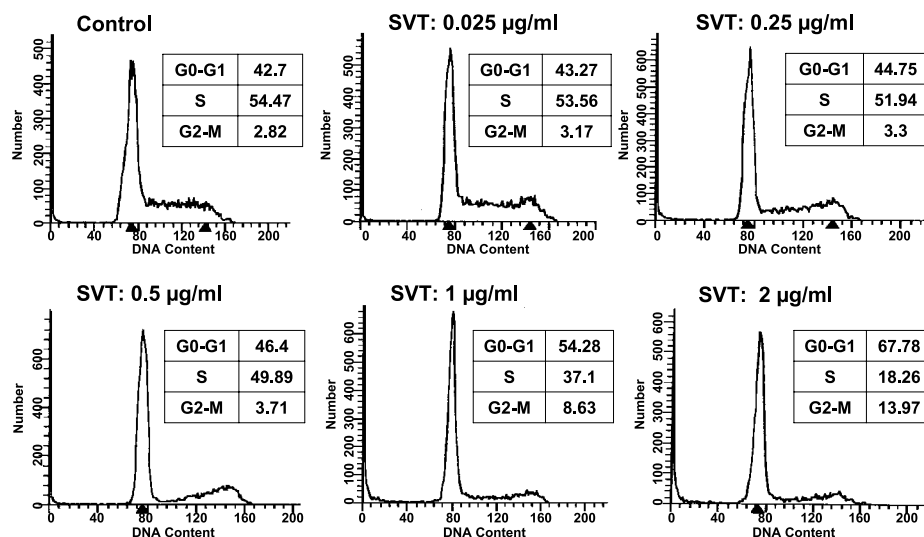
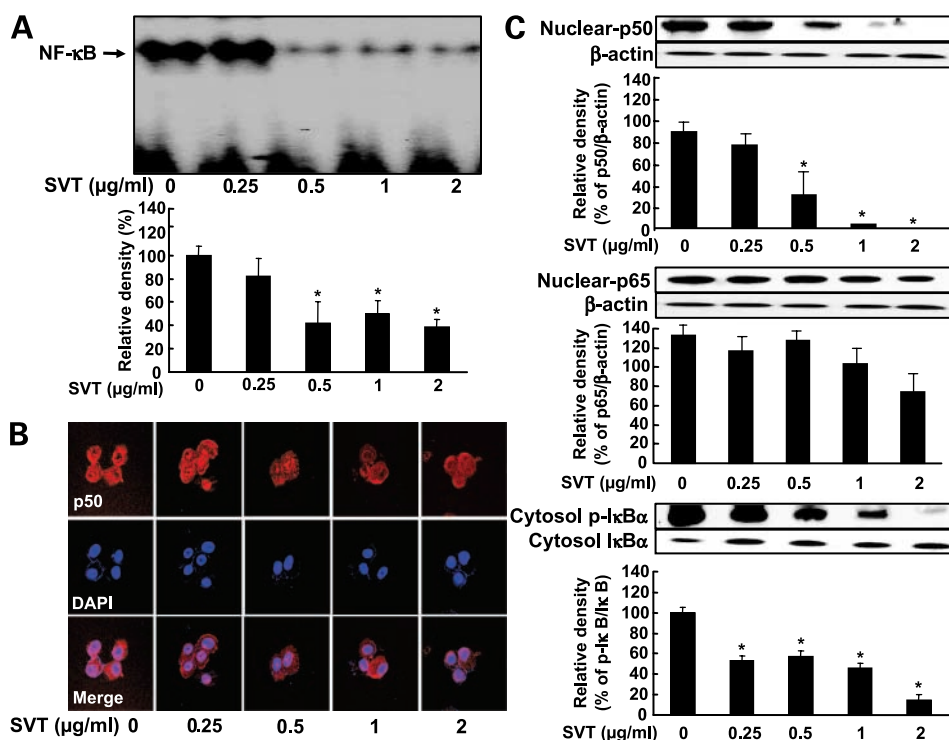


Figure 2. Cell cycle analysis of PC-3 cells treated with various doses of SVT. DNA content was analyzed by flow cytometry as described in Materials and Methods. PC-3 cells were treated with 0 to 2 $\mu\text{g}/\text{mL}$ SVT for 24 h. Representative of three similar experiments with triplicates.

Figure 3. Effect of SVT on NF- κ B activation in PC-3 cells. **A, top**, NF- κ B DNA binding activity was determined; **bottom**, quantification of band intensities from three independent experimental results was determined by densitometry. *, $P < 0.05$, statistically significant differences from the untreated group. **B**, PC-3 cells were treated with 0.25 to 2 μ g/mL SVT for 24 h, and then the intracellular location of p50 was determined by immunofluorescence confocal scanning microscope (magnification, 630 \times). Double staining (Merge) with p50 and 4',6-diamidino-2-phenylindole staining shows the localization of p50 in the nucleus. **C**, cytosolic and nuclear proteins were used to determine the expression of p50, p65, I κ B, and β -actin (internal control) proteins. For the three experiments, *columns*, mean values of the relative densities of each band; *bars*, SD. Each experiment was done in triplicates. *, $P < 0.05$, significantly different from SVT-untreated cells.



treated with various doses of SVT. Expression of the tumor suppressor gene, p53, and the active forms of caspase-3 and caspase-9 increased, but Bcl-2 expression decreased in a dose-dependent manner in the cells treated with SVT for 24 h (Fig. 5B). Immunocytochemical analysis further supports that the number of caspase-3-reactive cells increased following treatment with SVT (Fig. 5C). To investigate whether SVT can be taken up into the nucleus and thereby cause the inactivation of NF- κ B and apoptotic cell death regulatory gene expression, we determined the location of SVT by treating cells with SVT labeled with a fluorescent dye. The uptake of the labeled SVT into the cells was shown under a confocal laser scanning microscope. As seen in Fig. 5D, SVT was taken up into the membranes and nuclei of cells. The translocation into the nucleus was evidenced by the merging of 4',6-diamidino-2-phenylindole staining of the nucleus and Alexa Fluor 488 dye-labeled SVT.

Discussion

The central and novel finding in the present study is the identification of the anticancer efficacy of SVT from *V. lebetina turanica* against advanced human prostate carcinoma cells. In the case of advanced prostate cancer, the cancer cells become resistant to apoptosis and do not respond to cytotoxic chemotherapeutic agents (3). Therefore, additional agents that induce apoptotic cell death in prostate cancer cells could be useful in controlling this malignancy (20).

It has been well established that NF- κ B is an important element in regulating cell growth or apoptosis of tumor

cells, including androgen-independent prostate cancer cells (21). The constitutive activation of NF- κ B has been reported in the androgen-independent PC-3 and DU145 cells, but not in the androgen-dependent LNCaP cell (22, 23). Our results show that SVT more selectively inhibited PC-3 and DU145 cell growth than LNCaP cell growth (Fig. 1). These may be caused by differences in the cellular levels of NF- κ B between the cell lines. These data suggest that the loss of NF- κ B signaling may be a significant contributor in SVT-induced androgen-independent prostate cancer cell death. In fact, our results show that down-regulation of NF- κ B signals by SVT in the PC-3 cell is consistent with cell growth inhibition (Fig. 3A and B). At the molecular level, SVT inhibits constitutively activated NF- κ B signaling by impairing I κ B α phosphorylation with the inhibition of p50 translocation (Fig. 3C). Consistent with the present finding, we previously found that SVT binds with NF- κ B, IKK α , and IKK β , resulting in the down-regulation of NF- κ B activity in RAW 264.7 cells and astrocytes (15). Moreover, reduced SVT-induced apoptotic cell death was found in cells transfected with mutant p50, IKK α , and IKK β , in which critical cysteine residues were replaced with other amino acids (Fig. 4A and B). These data therefore suggest that SVT-induced PC-3 cell death could involve blocking NF- κ B activation, and this effect may be due to the direct binding of SVT to NF- κ B signal molecules that have sulfhydryl groups in their active sites. Direct inhibition of the DNA binding activity of NF- κ B in the nucleus is also possible because we found that SVT translocated into the nuclei in which p50 or p65 could bind to the I κ B-binding elements of target genes. Consequently, the reduced

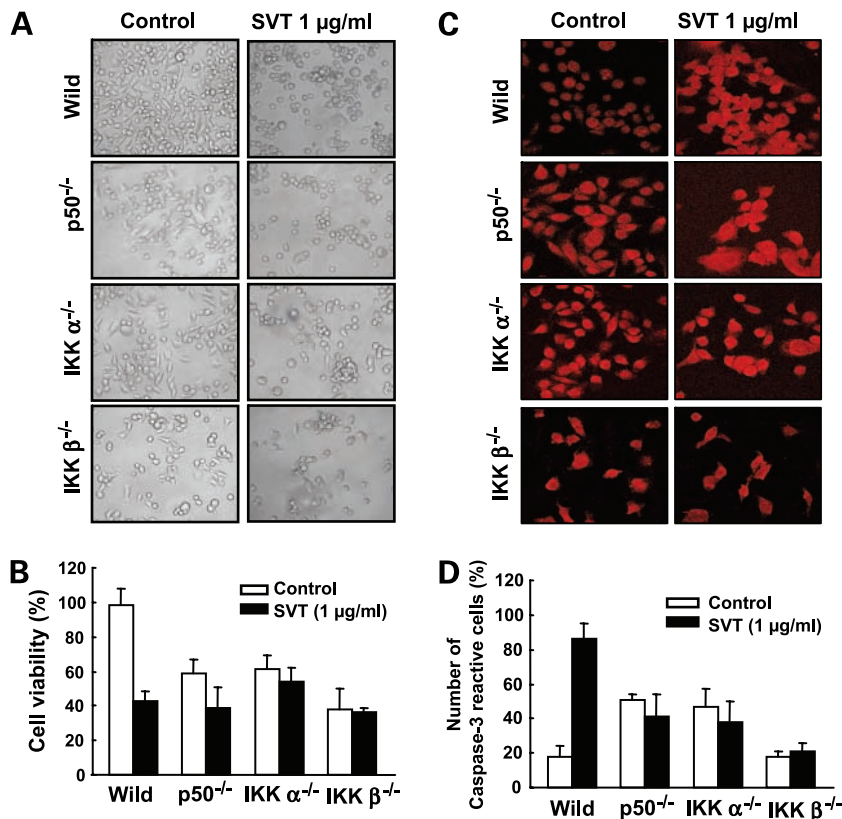


Figure 4. Cell viability and caspase-3-reactive cell number in PC-3 cells transfected with the p50 and IKK mutant plasmids. **A**, transfected PC-3 cells were incubated with 1 µg/mL SVT for 24 h, and morphologic changes were observed under microscope (magnification, 200×). **B**, transfected PC-3 cell viabilities were determined by CCK-8 assay. **C**, PC-3 cells were treated with 1 µg/mL SVT for 24 h. The active caspase-3-reactive cell number was determined by immunofluorescence using an anti-active caspase-3 antibody. Pictures were taken with a confocal scanning microscope (magnification, 630×). **D**, the caspase-3-reactive cell index (%) was calculated as the number of caspase-3-positive stained cells divided by the total cell number. *Columns*, mean of two experiments with triplicates of each experiment; *bars*, SD. Each figure is representative of three similar experiments.

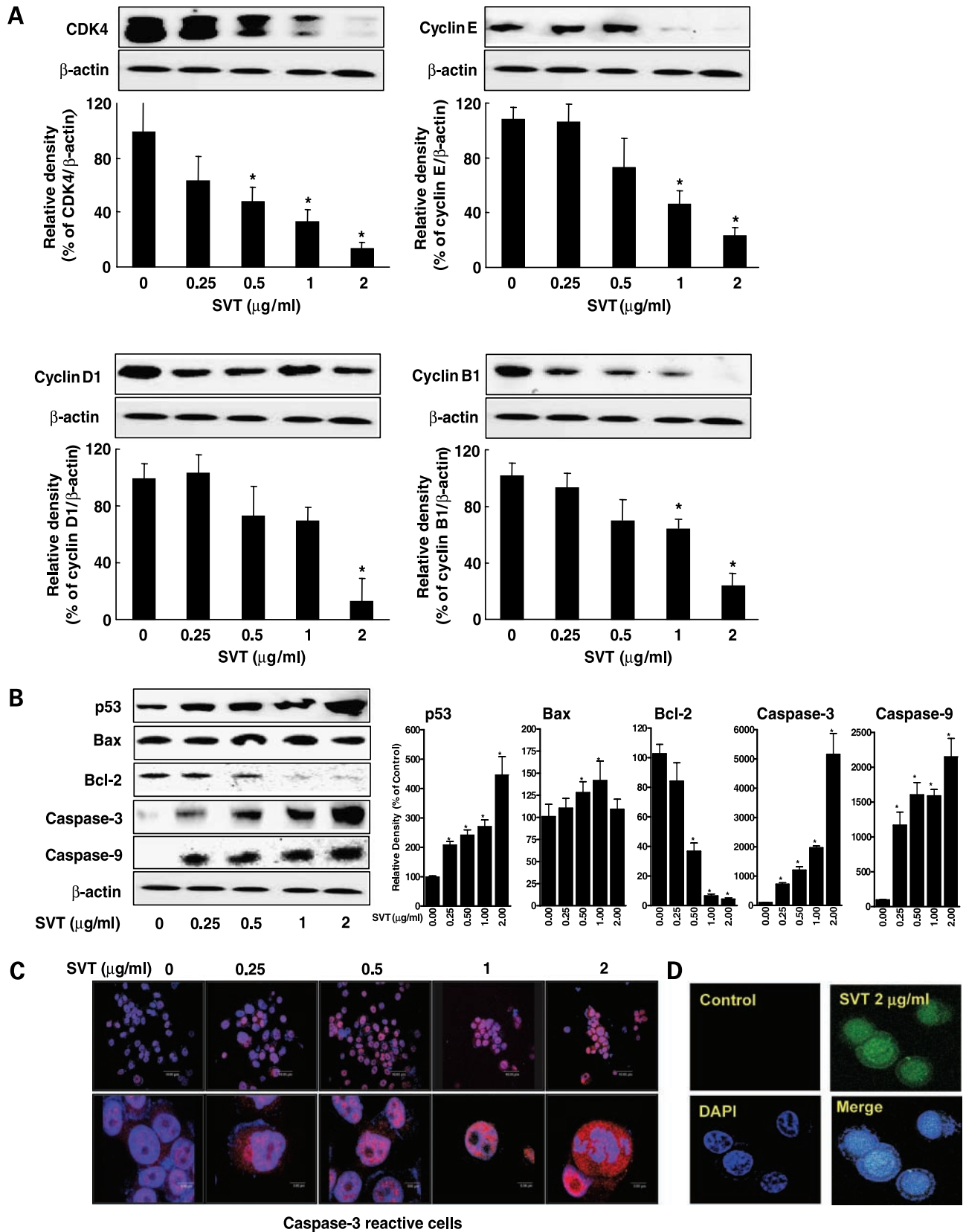
nuclear translocation of NF-κB proteins was associated with the down-regulation of the constitutively overexpressed or NF-κB-dependent antiapoptotic proteins.

Several genes (proteins), such as Bcl-2, and/or up-regulated apoptotic genes, such as Bax, caspase-3 and caspase-9, and p53, are regulated by NF-κB. Expression of proapoptotic proteins p53, Bax, and caspase-9 was significantly activated by SVT, whereas the expression of antiapoptotic protein Bcl-2 was down-regulated. Moreover, our results showed that caspase-3 was most potently activated by SVT, and this activation was attenuated in cells transfected with mutant p50, IKKα, and IKKβ (Fig. 4C and D). These data suggest that the activation of caspase-3 is critical in SVT-induced PC-3 cell death.

Cell cycle analysis data showed that SVT caused a strong G₀-G₁ cell cycle arrest and a weak G₂-M arrest in prostate cancer cells (Fig. 2). Furthermore, mechanistic investigation showed that SVT-induced G₀-G₁ and G₂-M phase arrest in PC-3 cells is mainly mediated by the down-regulation of

CDK4, cyclin D1 and E1 (at G₀-G₁ checkpoint) and cyclin B (at the G₂-M checkpoint; Fig. 4A). CDKs, CDK inhibitors, and cyclins play essential roles in the regulation of cell cycle progression (24). CDK inhibitors are tumor suppressor proteins that down-regulate the cell cycle progression by binding active CDK-cyclin complexes and thereby inhibiting their kinase activities (24, 25). Cyclin D1 is rate limiting for the progression of the cell cycle from the G₁ phase to the S phase, during which DNA replication occurs (26). NF-κB is essential for cyclin D1 expression (27) and regulates mitogenic signaling through transcriptional induction of cyclin D1 (28). Moreover, down-regulation of cyclin D1 through antisense treatment led to the apoptosis of melanoma cells and caused tumor shrinkage of xenotransplants in nude mice (29). Together, these results suggest that down-regulation of cyclin D1 through regulation of NF-κB activity is sufficient to trigger apoptosis in PC-3 cells. Consistent with this notion, the inhibition of NF-κB activity by SVT led to the down-regulation of cyclin

Figure 5. Effect of SVT on expression of cell cycle and apoptosis-related proteins in PC-3 cells. Equal amounts (50 µg) of whole cell lysates were subjected to electrophoresis and analyzed by Western blotting for cell cycle regulatory molecules (**A**, CDK4, cyclin D1, cyclin E, and cyclin B1) and apoptosis regulatory molecules (**B**, p53, Bax, Bcl-2, caspase-3 and caspase-9). The relative density was analyzed by densitometry. Similar patterns of protein expression were obtained from three experiments. *Columns*, mean of three experiments; *bars*, SD (**A** and **B**). **C**, PC-3 cells were treated with SVT for 24 h. Cells were washed and fixed, and the active caspase-3 reactive cell number was determined by immunofluorescence using an antiactive caspase-3 antibody. Pictures were taken with a confocal scanning microscope (original magnification, 630×). Each figure is representative of three similar experiments. **D**, PC-3 cells were treated with Alexa Fluor 488 dye-labeled SVT for 24 h. Cells were washed, and the uptake of labeled SVT was shown by confocal scanning microscopy (magnification, 630×). Double staining (Merge) with fluorescence-labeled SVT and 4',6-diamidino-2-phenylindole staining shows the localization of SVT in the nucleus. Each figure is representative of three similar experiments.



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D1 in PC-3 cells. Similar results were found in another recent study that showed that acetyl-boswellic acids inhibit cyclin D1, as well as the antiapoptotic Bcl protein expression in the culture of prostate cancer cells; thus, this compound resulted in a reduction in tumor growth and invasiveness in PC-3-bearing nude mice (8). Consequently, SVT-induced G₀-G₁ and G₂-M phase arrest, accompanied by the reduction of cell distribution into the S phase, is a possible mechanism for cell growth inhibition leading to apoptotic cell death. Therefore, the present data indicate that SVT causes apoptotic cell death of PC-3 cells, and its effect may be related to the inactivation of NF- κ B and the activation of caspase-3.

The possible usage of natural toxins as pharmaceutical applications has been shown with several toxins, including SVT, in various *in vitro* or animal models, as well as in clinical studies (14, 30, 31). Divergence in the biological activities of various proteins isolated from snake venom has been reported. Siigur et al. isolated fibrolytic enzymes from the *V. lebetina* snake venom (32). Saxatilin, isolated from a Korean snake (*Gloydius saxatilis*), has been shown to inhibit platelet aggregation, human umbilical vein endothelial cell proliferation, and smooth muscle cell migration (31). Salmosin, a disintegrin purified from a Korean snake (*Agkistrodon halys brevicaudus*) venom, interacts with integrin $\alpha(v)\beta(3)$ and induces apoptotic cell death by competing with the extracellular matrix for direct binding to integrin $\alpha(v)\beta(3)$ on the cell surface (33). Thus, our present findings, which showed the *in vitro* anticancer efficacy of SVT and mechanistic rationale (cell cycle arrest and apoptosis induction) against advanced human prostate cancer cells, warrant further *in vivo* efficacy studies in preclinical human prostate cancer models, as well as the estimation of pharmacologically achievable doses having biological significance in *in vitro* studies. We have done a preliminary *in vivo* experiment using PC-3 cell-inoculated nude mice (Supplementary Fig. S1).⁶ SVT (0.4 mg/kg injected intradermally every 2 days for a period of 16 days) significantly inhibited tumor growth by ~40% to 50% (Supplementary Fig. S1).⁶ Furthermore, this preliminary result also showed that SVT (0.4 mg/kg, which corresponds to a dose of 2 μ g/mL *in vitro*) did not induce any serious health problems, such as eruption, swelling, weight loss, or death in the animals. The LD₅₀ value for SVT in mice is 2.5 mg/kg (13). Therefore, the calculated therapeutic Index (LD₅₀/ED₅₀) for SVT in prostate cancer is 6.25, suggesting that SVT could be safely used in a therapeutic application. The positive outcomes of larger numbers of *in vivo* animal studies would confirm a basis for the development of SVT as a novel agent for human prostate cancer prevention and/or intervention. Therefore, our results suggesting that extremely low concentrations (*in vitro*; below 2 μ g/mL, *in vivo*; 0.4 mg/kg, *in vivo*) of the natural toxin SVT could be useful as an anti-prostate cancer agent.

⁶ Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

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