



SB365, *Pulsatilla* saponin D suppresses the proliferation of human colon cancer cells and induces apoptosis by modulating the AKT/mTOR signalling pathway

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ABSTRACT

Pulsatilla koreana has been used as a traditional medicine for the treatment of several diseases. The purpose of this study was to determine if SB365, *Pulsatilla* saponin D isolated from the root of *P. koreana* inhibits the progression of colon cancer. We found that SB365 strongly suppressed the growth and proliferation of colon cancer cells and induced their apoptosis. Also, SB365 showed anti-angiogenic activity by decreasing the expression of HIF-1 α and VEGF. These results were confirmed by an *in vivo* study showing that SB365 significantly inhibited tumor growth by the induction of apoptosis and inhibition of angiogenesis with stronger anticancer activity than 5-FU. When further examined for its anticancer mechanism, SB365 effectively suppressed the AKT/mTOR pathway both *in vitro* and *in vivo*. Taken together, our study demonstrated that SB365 inhibits the AKT/mTOR pathway, leading to the suppression of tumor growth and angiogenesis together with induction of apoptosis. Therefore, SB365 is a good candidate as a natural product for use in the treatment of colon cancer.

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1. Introduction

Human colon cancer is a leading cause of cancer-related death in most economically developed countries and the second most prevalent cancer worldwide. In the USA, the estimated number of new colon cancer cases diagnosed in 2008 was 148,810 with 49,960 deaths (Jemal et al., 2008). Half of all patients diagnosed with colorectal cancer eventually die from the disease. Less than 10% of patients with metastatic colorectal cancer survive more than five years after diagnosis. Current treatment of colon cancer is surgical resection combined with chemotherapy using cytotoxic drugs and radiation therapy (Matsui, Omura, Kawakami, Morita, & Sakamoto, 2006; Oehler & Ciernik, 2006). Colon tumors develop through several stages and progress over a protracted period because of increased genomic instability leading to the up-regulation of oncogenes and the down-regulation of tumor suppressor genes (Fang et al., 2006; Samowitz & Slattery, 2002). Many anticancer drugs have been clinically applied for the treatment of colon cancer. Of these drugs, 5-fluorouracil (5-FU) has been one of the most widely prescribed drugs for the treatment of colon cancer, especially metastatic colon cancer. Studies have shown that, for the treatment of metastatic colon cancer, higher doses of 5-FU produced greater adverse effects, but were no more effective than

lower doses (Delval & Klastersky, 2002; Meregalli et al., 1998). Hence, more effective and safer therapeutic strategies for the treatment of colon cancer are urgently needed.

A number of studies have proven that various herbal extracts and compounds possess antitumor activities (Mandy, Chi, Kelvin, Anthony, & Joshua, 2007). One of these is *Pulsatilla koreana*, which belongs to the family Ranunculaceae. Its root has been widely used in traditional medicine for the treatment of several diseases, in particular, malaria and amoebic dysentery (Bae, 1999). It also has been reported to possess anti-inflammatory and anti-parasitic effects (Ye et al., 1995). This plant includes many effective components such as saponins, ranunculin, anemonin, protoanemonin and triterpenes (Ye et al., 1996). In particular, there are 17 saponins in *P. koreana*, of which, saponin D has been reported to demonstrate cytotoxicity against lung cancer cells (Bang et al., 2005; Kim, Bang, Lee, & Ahn, 2004). Thus, *Pulsatilla* saponin D (here after designated SB365) was selected from among the many kinds of saponins isolated from *P. koreana* and evaluated for its anticancer effects in colon cancer cells.

The phosphatidylinositol 3-kinase/AKT/mammalian target of rapamycin (PI3K/AKT/mTOR) signaling axis plays a critical role in the proliferation of colon cancer cells including their resistance to apoptosis, and the angiogenesis and metastasis that is central to the development and maintenance of colon cancer (Cantley, 2002; Kobayashi et al., 1999; Philp et al., 2001). The result of the activation of PI3K on tumor growth and progression is thought to be mediated by AKT, a downstream effector of PI3K (Shaw & Cant-

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ley, 2006). A variety of downstream targets are regulated by AKT including mTOR, which promotes protein translation, growth, metabolism and angiogenesis (Guertin & Sabatini, 2007). Recent evidence has suggested that p70S6 kinase (S6K) also regulates phosphorylation of this residue in response to both mitogen- and nutrient-derived stimuli (Chiang & Abraham, 2005). PI3K has been shown to be activated in 32% of colon cancer cases (Samuels et al., 2004). In particular, the AKT gene has been reported to be overexpressed in 62% of colon cancer patients (Yoshifumi et al., 2010). Moreover, it has been reported that AKT phosphorylation in human colon cancer correlates with cell proliferation and apoptosis inhibition, as well as various clinicopathologic parameters such as invasion grade, vessel infiltration, metastasis to lymph nodes, and tumor stage (Itoh et al., 2002; Khaleghpour, Li, Banville, Yu, & Shen, 2004). Also, phosphorylation of mTOR and its downstream target, p70S6K, were detected in 61 and 57% of human colon cancer cases, respectively (Wang et al., 2011).

In this study, we isolated SB365 from *P. koreana* and investigated whether SB365 has the ability to inhibit cancer cell growth and angiogenesis. Here, we find that SB365 induces apoptosis by modulating the Akt/mTOR signalling pathway in colon cancer cells.

2. Material and methods

2.1. Cells and materials

Human HT-29 and LoVo colon cancer cells were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). These cells were cultured in Roswell Park Memorial Institute Media 1640 (RPMI-1640), supplemented with 10% foetal bovine serum (FBS) and 1% penicillin/streptomycin. The FBS, and all other agents used in the cell culture studies were purchased from Invitrogen (Carlsbad, CA). The cultures were maintained at 37 °C in a CO₂ incubator with a controlled humidified atmosphere composed of 95% air and 5% CO₂. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), and proteinase K were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Preparation of SB365

The SB365 was isolated from the roots of *P. koreana* collected from Kyeryong Mountain near Daejeon, Korea. The powder roots (50 g) were extracted three times using 50% aqueous ethanol (500 ml), and the resulting extracts were combined and concentrated *in vacuo* to yield a light brown residue. The residue was suspended in 300 ml of acetone and centrifuged. The resulting supernatant was removed to produce a brown precipitate. The precipitate was suspended in water and filtered in order to remove the insoluble portion. The filtrate was concentrated to produce a brown mass. The fraction was chromatographed using a Sephadex LH-20 column (200 g, 60 × 4 cm) with an 80:20 mixture of methanol and H₂O, resulting in four fractions; SPX1 (139 mg, 24.8%), SPX2 (344 mg, 61.4%), SPX3 (61 mg, 10.9%), and SPX4 (15.7 mg, 2.8%). The eluents were detected using thin layer chromatography (Butanol–acetic acid–H₂O = 4:1:1, Si gel, 0.25 mm). After heating, the chromatogram was sprayed with 10% H₂SO₄. The third fraction, which exhibited the most potent activity, was again chromatographed by solid phase high performance liquid chromatography (HPLC; solid phase; RP-C18, 250 × 10 mm, mobile phase; MeOH:H₂O (82:20) as the mobile phase, 210 nm, 1 ml/min) to yield three major fractions. Of these, SPX3 exhibited the most potent activity and was subsequently purified by HPLC to yield SB365, a saponin D. In order to generate a more exact analysis of the purified SB365, we also used mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy.

2.3. Measurement of cell proliferation

Cell viability was performed using the MTT assay. Briefly, HT-29 and LoVo colon cancer cells were plated at a density of $1-3 \times 10^4$ cells/well in 96-well plates and then incubated for 48 h. The media was then removed and the cells were treated with either DMSO, as a negative control, or various concentrations of SB365. The final concentration of DMSO in the media was $\leq 0.1\%$ (v/v). After the cells were incubated for 48 h, 20 μ l of MTT solution (2 mg/ml) was added to each well and the cells were incubated for another 4 h at 37 °C. The formazan crystals that formed were dissolved in DMSO (200 μ l/well) with constant shaking for 5 min. The plate was then read on a microplate reader at 540 nm. Three replicate wells were used for each analysis. The median inhibitory concentration (IC₅₀, defined as the drug concentration at which cell growth was inhibited by 50%) was assessed using the resulting dose–response curves.

2.4. Immunodetection of incorporation of BrdU

The HT-29 colon cancer cells were plated onto 18-mm cover glass in RPMI-1640 medium and grown to ~70% confluence for 24 h. The cells were then treated with 10 μ M of SB365 for 2 h and then pulse-labelled (4 h) with 10 μ M 5'-bromo-2'-deoxyuridine (BrdU). After labelling, the cells were washed twice with phosphate buffered saline (PBS) and fixed in ice-cold 1% paraformaldehyde. Fixed cells were washed with PBS to remove the organic solvent. The FITC-labelled anti-BrdU antibody, diluted with PBS buffer containing 0.1% Triton X-100, was used to measure BrdU incorporation by fluorescence microscopy.

2.5. Western blot assay

The cells were washed three times with ice-cold PBS before lysis using a buffer containing 1% Triton X-100, 1% Nonidet P-40, and the following protease and phosphatase inhibitors: aprotinin (10 mg/ml), leupeptin (10 mg/ml) (ICN Biomedicals, Asse-Relegem, Belgium), phenylmethylsulfonyl fluoride (1.72 mM), NaF (100 mM), NaVO₃ (500 mM) and Na₄P₂O₇ (500 mg/ml) (Sigma-Aldrich). Equal amounts of protein were separated using 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The protein transfer was checked using Ponceau S staining solution (Sigma-Aldrich). Immunostaining of the blots was performed using the primary antibodies, followed by the secondary antibody conjugated to horseradish peroxidase with detection using enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). Restore Western blot stripping buffer (Pierce, Rockford, IL) was used to strip the immunostained blot prior to restaining for the analysis of a second protein. Primary antibodies were purchased as followings; Bcl-2, Bax, VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), β -actin (Abcam, Cambridge, UK), total AKT, phospho-AKT, total mTOR, phospho-mTOR, total p70S6K, phospho-p70S6K, cleaved caspase-3, and PARP-1, (Cell Signaling Technology, Beverly, MA). The secondary antibodies were purchased from Amersham Biosciences. The bands were visualised with the ECL plus system (Amersham Biosciences).

2.6. Diamidino-2-phenylindole (DAPI) staining and terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) assay

HT-29 colon cancer cells were plated onto 18-mm cover glass in RPMI-1640 medium and grown to ~70% confluence over for 48 h. The cells were treated with SB365 at a dose of 10 μ M for 24 h. They were fixed in ice-cold 1% paraformaldehyde, washed with PBS and then stained with 2 μ g/ml of 4,6-diamidino-2-phenylindole (DAPI)

for 20 min at 37 °C. The stained cells were examined for a fluorescence of nuclear fragmentation. Terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) was performed using the TUNEL kit (Millipore, Billerica, MA).

2.7. Enzyme-linked immunosorbent assay (ELISA)

Serum VEGF concentration was measured using an ELISA with the Quantikine kit (R&D Systems, Minneapolis, MN). Following the manufacturer's instructions, samples were pipetted into a 96-well polystyrene microplate pre-coated with a monoclonal antibody specific for VEGF. After washing, an enzyme linked polyclonal antibody specific for VEGF was added to the wells. After additional washings, a substrate solution was layered into each well and produced a colorimetric reaction. The intensity of the colour, which was proportional to the amount of VEGF bound in the initial step, was measured using a multiplate spectrophotometer reader at an optical density of 450 nm.

2.8. Tumor xenograft study

To establish the HT-29 tumors in mice, the HT-29 colon cancer cells were grown in culture, detached by trypsinization, washed,

and then resuspended in PBS. Six weeks old athymic BALB/c nude mice (Orient Bio) were injected with 5×10^6 cells in the right flank to initiate tumor growth. After the tumor volume reached 50–100 mm³, they were randomly divided into three groups of eight mice. Mice were fed with either SB365 or 5-FU at a dose of 10 mg/kg by oral gavage 3 times a week for 3 weeks, respectively. The control group was fed 0.2 ml saline. Body weight and tumor size were recorded every three days. The tumor size was calculated using the formula $0.5 \times \text{long axis} \times (\text{short axis})^2$.

2.9. Immunohistochemistry

After being blocked with normal goat serum (Vector Laboratories, Burlingame, CA) for 1 h, frozen tissue sections were incubated for 1 h at room temperature in dilutions of 1:100 of Ki-67, VEGF, CD 34, p-AKT, and p-mTOR antibodies. The sections were visualised by an avidin–biotin peroxidase complex solution using an ABC kit (Vector Laboratories). The sections were washed in PBS and developed with a diaminobenzidine tetrahydrochloride substrate for 15 min and then counterstained with hematoxylin.

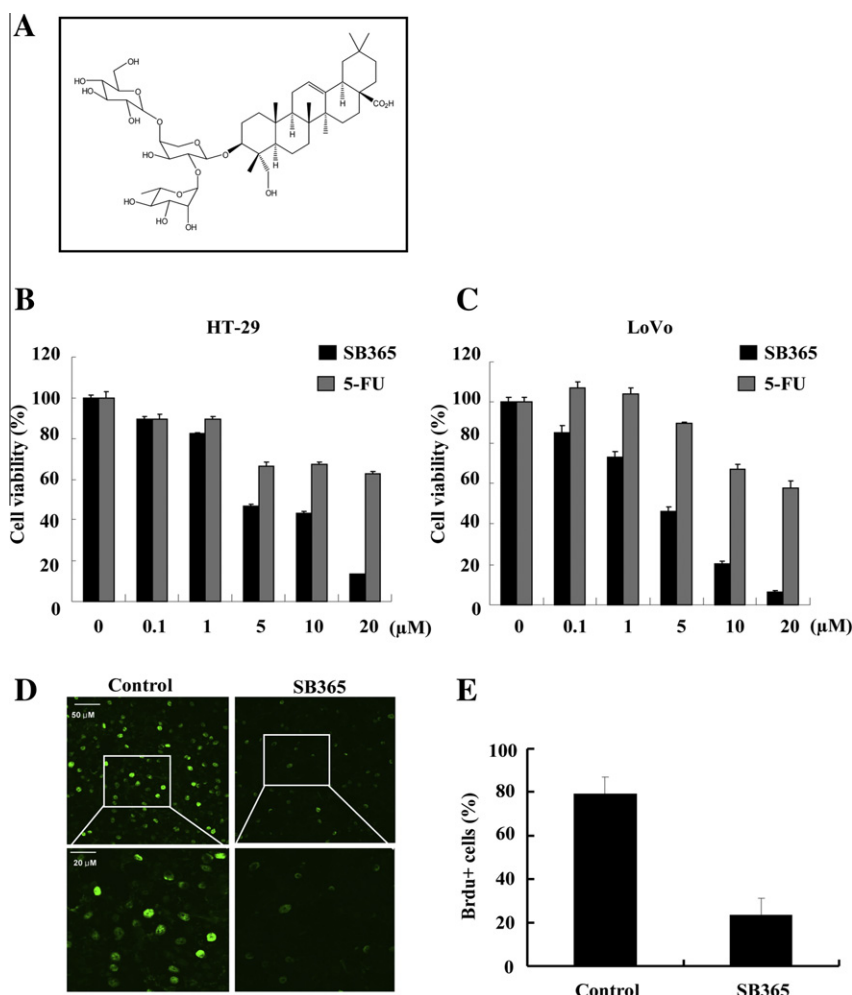


Fig. 1. Chemical structure of SB365 (*Pulsatilla saponin D*) and its effect on the proliferation of human colon cancer cells. (A) Chemical structure of SB365. Cytotoxic effects of SB365 and 5-fluorouracil (5-FU) on (B) HT-29 and (C) LoVo colon cancer cells were measured using the MTT assay. Colon cancer cells were seeded in 96-well culture plates. After incubation for one day, the cells were treated with various concentrations of SB365 or with 0.1% dimethyl sulfoxide (DMSO) as a negative control. After incubation for 48 h, the cells were subjected to the MTT assay. (D) Effect of SB365 on proliferation of HT-29 colon cancer cells were measured by BrdU staining, which were photographed at 200× and 400× magnification. (E) Results are expressed as percent cell proliferation relative to the proliferation of control. Data are represented as mean ± S.D. from triplicate wells.

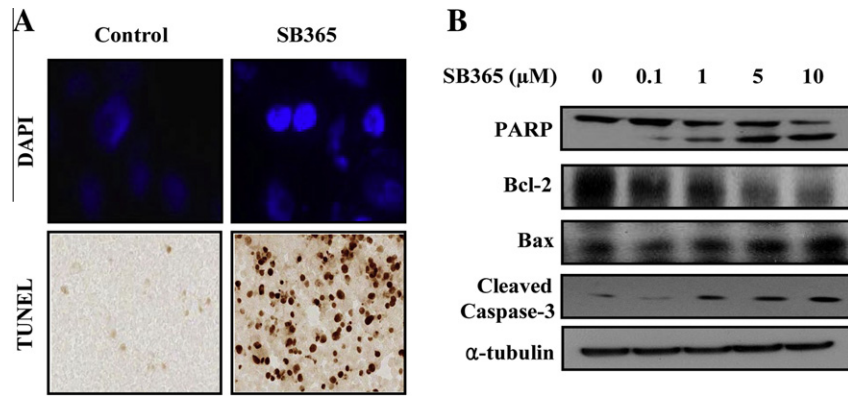


Fig. 2. Effect of SB365 on apoptosis of HT-29 colon cancer cells. HT-29 cells were treated with SB365 (0, 0.1, 1, 5, and 10 μM) for 24 h. (A) The induction of apoptosis by treatment with 10 μM of SB365 was evaluated by DAPI and TUNEL staining, which were photographed at 400 $\times$ magnification. (B) The expression of PARP, Bcl-2, Bax, and cleaved caspase-3 were determined by Western blotting in cells treated with SB365 at the indicated doses for 24 h.

2.10. Statistical analysis

Data were expressed as mean $\pm$ S.D. Statistical analysis was performed using ANOVA. A p -value of 0.05 or less was considered statistically significant. Statistical calculations were performed using SPSS software for the Windows operating system (Version 10.0; SPSS, Chicago, IL).

3. Results

3.1. Effect of SB365 on the proliferation of human colon cancer cells

To evaluate the anticancer properties of SB365, we first compared the cell growth of two colon cancer cell lines (HT-29 cell and LoVo), which had been treated with SB365 and 5-FU. As shown

in Fig. 1B and C, the cells were exposed to various concentrations (0, 0.1, 1, 5, 10 and 20 μM) of SB365 for 48 h. The results revealed that SB365 treatment inhibited cell growth in a dose-dependent manner. SB365 had more potent anticancer activity than 5-FU. Indeed, IC₅₀ values of SB365 were 1.9 and 1.8 μM in HT-29 and Lovo cells, whereas IC₅₀ values of 5-FU were >20 μM in both HT-29 and Lovo cells. It induced a reduction in cell growth rate at a dose of 1 μM in colon cancer cells and strongly inhibited 40–80% of cell growth at doses of 5 and 10 μM . To confirm the effect of SB365 on cell proliferation, the incorporation of BrdU into DNA was measured using a DNA strand break assay. HT-29 cells were treated with 10 μM of SB365 for 2 h, followed by BrdU labelling for 4 h. Immunodetection using FITC-labelled BrdU antibody was then carried out. An increase in DNA breakage was indicated by the presence of higher FITC-labelled anti-BrdU antibody (Fig. 1D and E).

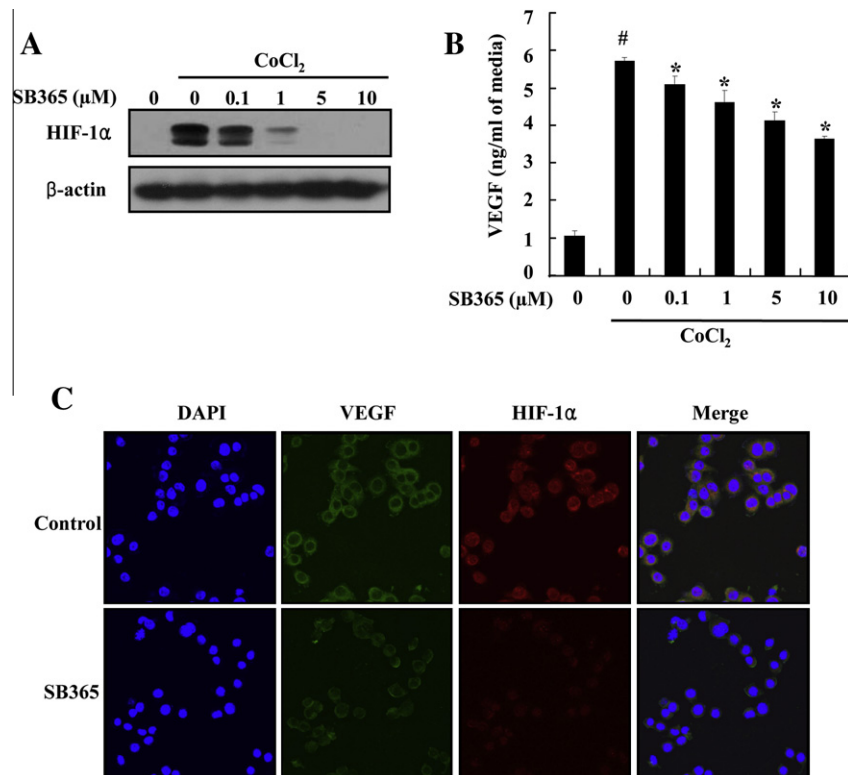


Fig. 3. Effect of SB365 on the angiogenesis of HT-29 colon cancer cells. (A) Expression of HIF-1 α by SB365 in hypoxia-induced HT-29 cells (CoCl₂, 100 μM). (B) Production of VEGF in hypoxia-induced HT-29 cells. (C) Immunofluorescent imaging of the expression of HIF-1 α and VEGF by SB365 (10 μM) in hypoxia-induced HT-29 cells (CoCl₂, 100 μM). Data are represented as mean $\pm$ S.D. from the triplicate wells. # p < 0.01 compared to control, * p < 0.05 compared to CoCl₂ group.

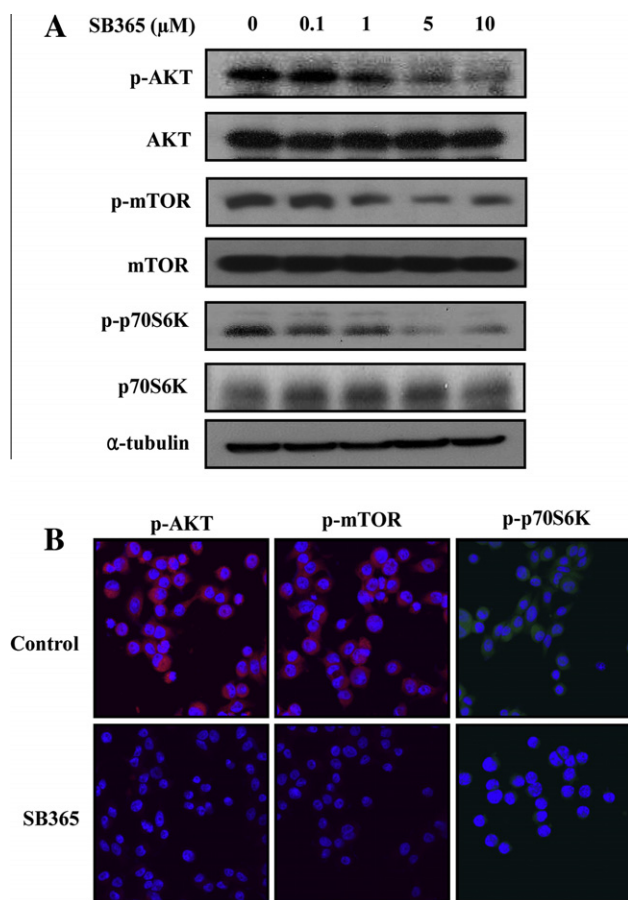


Fig. 4. Effect of SB365 on the AKT/mTOR pathway signaling in HT-29 colon cancer cells. (A) Cells were treated with SB365 at various doses (0.1–10 μM). Western blotting experiments for p-AKT, p-mTOR, and p-p70S6K were performed with the cell lysates obtained after SB365 treatment. (B) Immunofluorescent imaging results for the target proteins of the PI3K/AKT/mTOR pathway after SB365 (10 μM) treatment. For labelling, anti-rabbit antibodies against p-AKT, p-mTOR, and p-p70S6K were used. DAPI was used to counterstain the nucleus. 400× magnification.

The figures highlighted that the proliferation of HT-29 colon cancer cells was inhibited by SB365 treatment.

3.2. Effects of SB365 on apoptotic cell death in HT-29 colon cancer cells

To identify the pro-apoptotic effect of SB365 in HT-29 cells, we performed DAPI staining, TUNEL assay, and Western blot analysis. As shown in Fig. 2A, the cells treated with 10 μM of SB365 presented the morphological features of apoptotic cells, such as bright nuclear condensation and perinuclear apoptotic bodies, visualised by DAPI staining. SB365-induced apoptosis was confirmed by detection of DNA fragmentation using TUNEL staining.

After SB365 treatment at a dose of 1 μM, the protein expression using the Western blot method revealed evidence that caspase-3 and PARP started to cleave and showed that the Bcl-2/Bax ratio changed rapidly (Fig. 2B). These results revealed that SB365 induced cell apoptosis in colon cancer cells.

3.3. Effects of SB365 on the expression of HIF-1α and VEGF in HT-29 colon cancer cells

HIF-1α is the main transcriptional modulator of angiogenic factors such as VEGF. Thus, it was appropriate to investigate the effect of SB365 on the expression of hypoxia-induced HIF-1α and VEGF. HT-29 cells were treated with various concentrations of SB365

(0.1–10 μM) under a hypoxia-mimic condition induced by treatment with 100 μM CoCl₂ for 12 h. As shown in Fig. 3A, the HIF-1α expression was increased under the hypoxic condition and SB365 treatment inhibited the hypoxia-induced HIF-1α expression in a dose-dependent manner. When cells were treated with 0.1–10 μM of SB365 under the hypoxia condition, the detection of VEGF they secreted into the media was performed by ELISA. The VEGF level in the medium at 12 h was significantly increased under the hypoxia condition compared to the control, whereas the treatment of SB365 under hypoxia inhibited the secretion of the VEGF protein at dose concentrations from 0.1 to 10 μM (Fig. 3B). As shown in Fig. 3C, expression of HIF-1α and VEGF by the treatment of SB365 was decreased in immunofluorescent images when compared with the control.

3.4. Effects of SB365 on the AKT/mTOR/p70S6K pathway

In order to study whether SB365 inhibits cell growth at a molecular level, we performed Western blotting. AKT/mTOR plays an important role in regulating critical cellular functions, including cell growth and metabolism. We investigated the effects of SB365 on the AKT/mTOR pathway in HT-29 colon cancer cells. When HT-29 cells were treated with various concentrations of SB365, the phosphorylation levels of AKT and its downstream factor, mTOR, were effectively suppressed. The mTOR activation resulted in the phosphorylation of effectors, such as p70S6K, which lead to mTOR dependent gene transcription regulating cell proliferation, and protein synthesis. Therefore, we further identified the effect of SB365 on the expression of p70S6K. As expected, SB365 inhibited phosphorylation of p70S6K in a dose-dependent manner (Fig. 4A). As shown in Fig. 4B, phosphorylation of AKT, mTOR, and p70S6K were down-regulated by treatment with SB365 as compared with the control.

3.5. Inhibition of colon cancer growth by SB365 in mouse xenograft model

We examined the effects of SB365 and 5-FU using athymic BALB/c nude mice implanted with human colon cancer cells. We found that tumor volume and tumor weight were significantly suppressed in mice treated with 5-FU doses of 10 mg/kg or SB365 doses of 10 mg/kg (Fig. 5). Of particular note, SB365 treatment suppressed tumor growth more than 5-FU treatment. Moreover, SB365 and 5-FU treatment produced little change body weight during the experiments (Fig. 5B), suggesting the compounds have little toxicity at the tested concentrations.

3.6. Immunohistochemistry by SB365 treatment in colon cancer mouse xenograft model

From the histopathological analysis using H&E staining, we observed a greater degree of tumor apoptosis and necrosis in the SB365 treated group as compared to the control and 5-FU treated groups. An immunohistochemistry was performed to evaluate the expression of PCNA, a representative marker of proliferation. PCNA was also highly expressed in the control group, but poorly expressed in the SB365-treated groups. The results of the TUNEL assay and immunohistochemistry of cleaved caspase-3 showed that oral administration of SB365 induced apoptosis of colon cancer cells. Immunohistochemical analysis was performed to confirm the anti-angiogenic effect in a mouse xenograft model. Cells positive for vascular endothelial growth factor VEGF, and blood vessel marker, CD 34 were much less prominent in the SB365-treated group. These results suggested SB365 had an anti-angiogenic effect on colon cancer xenografts. Furthermore, SB365 decreased the

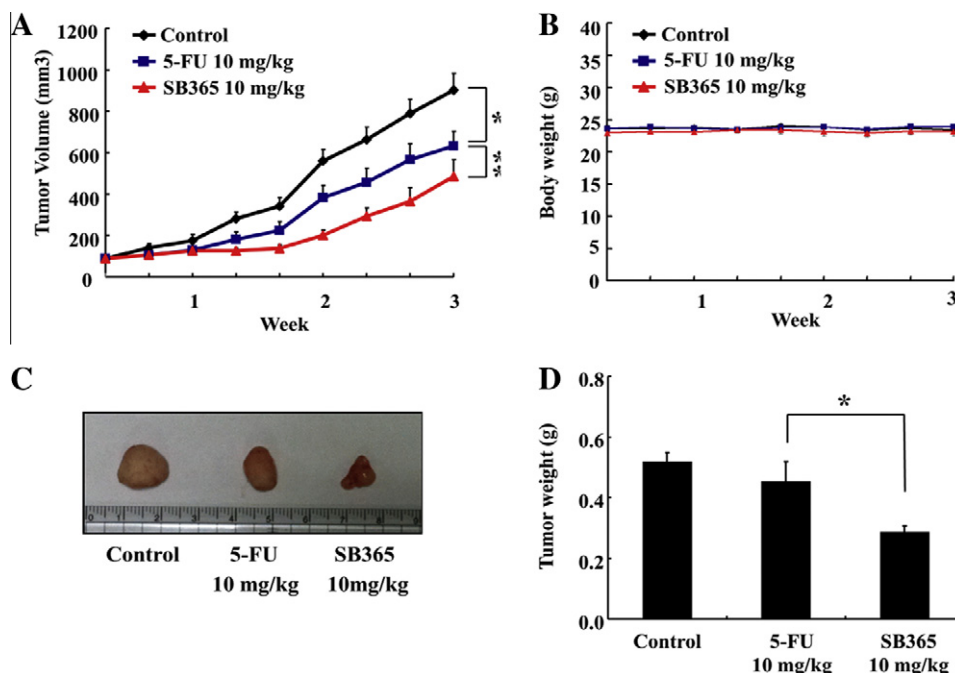


Fig. 5. *In vivo* anticancer activity of SB365 in the colon cancer mouse xenograft model. All mice were implanted by subcutaneous injection of HT-29 cells (5×10^6 cells) on the flank. After the tumors reached 50–100 mm³ in size, the mice received an oral administration of SB365 or 5-FU (10 mg/kg) 3 times a week for 3 weeks. (A) Tumor growth in the HT-29 mouse xenograft model. (B) Average body weight of nude mice. (C) Isolated tumor size and (D) Tumor weight from the HT-29 mouse xenograft. Data is represented by mean $\pm$ S. D ($n = 6$). * $p < 0.05$ and ** $p < 0.01$, compared to control.

phosphorylation of AKT and mTOR, thus regulating many different events involved in cell survival and proliferation (Fig. 6).

4. Discussion

Despite recent advancement in the understanding the carcinogenic processes of colon cancer, the increasing patient populations and relatively low remission rates associated with chemotherapy have urged the scientific community to establish more effective treatment regimens by adopting novel and innovative approaches. The discovery and use of active medicinal compounds from herbal/natural sources have provided alternative treatment choices for patients (Smith & Boon, 1999). In this study, we obtained SB365, a saponin D from *P. koreana*, and explored its anti-cancer effects and mechanism against colon cancer cells. SB365 effectively suppressed cell growth/proliferation, angiogenesis, and the induction of apoptosis through the modulation of the AKT/mTOR pathway in colon cancer cells. Furthermore, SB365 showed more potent anti-cancer efficacy, inhibiting cell proliferation and tumor growth as compared to 5-FU. This is the first report that clearly characterises the anti-tumor properties of SB365 and identifies its mechanism in both colon cancer cells and a tumor xenograft model.

Anti-cancer drug-induced cell death and the induction of apoptosis are used to inhibit cancer cell growth/proliferation (Dowsett et al., 1999; McKnight, Gray, O'Kane, Johnston, & Williamson, 2005). We found that SB365 inhibited cell growth/proliferation in colon cancer cell lines, inhibiting 40–80% of the growth of colon cancer cells at concentrations from 1 to 10 μ M. These results were consistent with previous reports in which many kinds of saponins inhibit cell growth in various cancer cells (Chen, Shih, Huang, & Cheng, 2011; Li, Fernandez, Rajendran, Hui, & Sethi, 2010; Peng, Zhou, Kong, & Zhang, 2010; Tin, Cho, Chan, James, & Ko, 2007). Interestingly, SB365 produced a lower IC₅₀ (at a dose of 2 μ M) than other saponins in colon cancer cells (Kim et al., 2008; Tong et al., 2010). The inhibitory effect of saponins on colon cancer cell growth

has been demonstrated in the different species and sources. For instance, steroidal saponin from *Astragalus membranaceus* and saponin from *Platycodon grandiflorum*, inhibited cell growth by 50% at doses of 39.8 and 37.1 μ g/ml, respectively (Kim et al., 2008; Mandy et al., 2007). More importantly, SB365 showed a greater efficacy than 5-FU in treating colon cancer cells. SB365 led to, not only inhibition of cell growth/proliferation, but also to apoptosis. DNA fragmentation and nuclear chromatin condensation have been demonstrated in HT-29 cells treated with SB365. Moreover, the observation of caspase-3 activation and PARP cleavage also confirms that promotion of apoptosis by SB365 involves a caspase dependent pathway. Expression of members of the Bcl-2 family was determined to provide better insight into the apoptotic signaling involved in SB365-treated HT-29 cells. Bcl-2 is an anti-apoptotic protein that prevents mitochondrial permeability transition pore opening and the release of cytochrome C following DNA damage (Reed, 1999). Downregulation of Bcl-2 expression was found in the present investigation along with the upregulation of the pro-apoptotic member Bax. These findings suggest that induction of apoptosis in SB365-treated HT-29 cells could be associated with the caspase-dependent cascade that involves the activation of the mitochondrial pathway initiated by the inhibition of Bcl-2. These events were supported by *in vivo* results, showing that SB365 treatments of 10 mg/kg increased the expression of cleaved caspase-3 and DNA fragmentation by TUNEL, and led to inhibition of tumor growth in colon cancer xenograft models. These results indicate that the induction of apoptosis and inhibition of cell growth/proliferation induced by SB365 may contribute to the suppression of tumor growth.

Given the importance of tumor angiogenesis in the growth of colon cancer, the inhibition of angiogenic pathways is an alternative for targeting cancer cell proliferation. VEGF is a potent inducer of angiogenesis and HIF-1 α is the major regulator of VEGF transcriptional activation (Jiang, Rue, Wang, Roe, & Semenza, 1996; Xia, Meng, Cao, Shi, & Jiang, 2006). In the present study, SB365 obviously inhibited the expression of HIF-1 α and VEGF under hy-

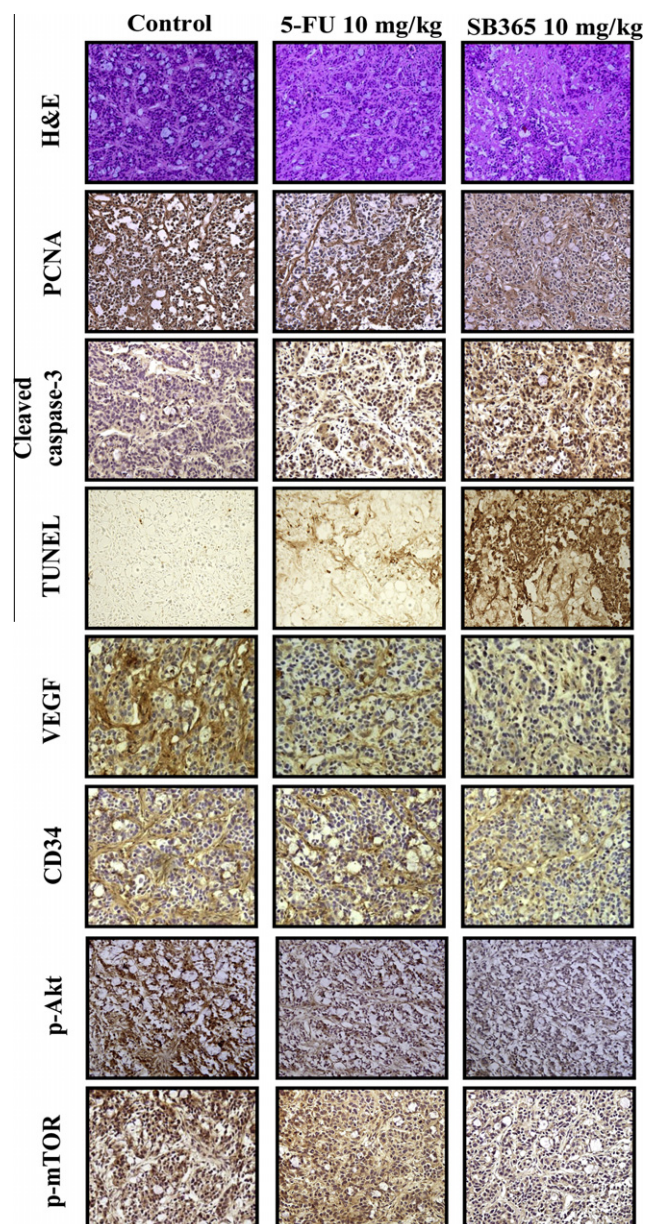


Fig. 6. Immunohistochemistry for proliferation, apoptosis, and angiogenesis. Tumors were excised and processed for immunostaining for PCNA, cleaved caspase-3, TUNEL, VEGF, CD34, p-AKT, and p-mTOR including H&E staining. 400× magnification.

poxia and also decreased the secretion of VEGF in HT-29 cells, showing that SB365 inhibited hypoxia-induced angiogenesis. Besides, its anti-angiogenic effect was supported by decreased expression of CD34 and microvessel endothelial marker, and in decreased VEGF expression in tumor tissue from the xenograft animal model. From our *in vitro* and *in vivo* results, we concluded that SB365 has potent anti-angiogenic activity by inhibiting VEGF and HIF-1 α . Indeed, these results were similar to those presented in previous reports that various saponins inhibited angiogenesis by inhibiting the growth and decreasing the expression of VEGF in cancer cells (Arai et al., 2011; Chen, Zhang, & Wang, 2010; Chen et al., 2011; Tian et al., 2005).

Several signaling pathways, such as mitogen-activated protein kinase and AKT/mTOR, have been implicated in the cellular hypoxic response. An inhibitor of mTOR signaling has been reported to have antiangiogenic activities by decreasing vessel density in

several tumor models, which is linked to a decrease in VEGF production and inhibit vascular endothelial cell response to stimulation by VEGF (Brugarolas, Vazquez, Reddy, Sellers, & Kaelin, 2003; Hudson et al., 2002). Although many individual saponins have been isolated from natural/herbal plants, studies on the anti-cancer mechanism of these compounds have been insufficient. Thus, we investigated the effect of SB365 on the AKT/mTOR pathway in colon cancer cells. As expected, SB365 inhibited the AKT/mTOR pathway and effectively suppressed the expression of HIF-1 α and VEGF. As several studies demonstrated that one of the mechanisms by which mTOR controls protein synthesis is through phosphorylating downstream substrates such as p70S6K, an effector of mTOR in colon cancer cells, we evaluated its expression. Likewise, SB365 effectively inhibited the phosphorylation of p70S6K in colon cancer cells. Overall, our results indicate that SB365 was able to inhibit cell growth/proliferation and apoptosis through the AKT/mTOR pathway. Inhibition of the AKT/mTOR pathway has been reported to follow the induction of the downstream mitochondrial apoptotic pathway by alteration in the ratio of Bcl-2/Bax and the activation of caspase-3 (Paz-Ares, Blanco-Aparicio, Garcia-Carbonero, & Carnero, 2009; Van-Blerk & Levine, 1975). With these previous results in mind, we showed that SB365 increased expression of Bax, suppressed that of Bcl-2, and activated caspase-3, which was accompanied by inhibition of the AKT/mTOR pathway. Thus, our study results suggest that SB365-induced apoptosis may be mediated through the suppression of the AKT/mTOR pathway. Also, it has been addressed that the AKT/mTOR pathway regulates VEGF and HIF-1 α expression through activation of p70S6K (Jiang & Liu, 2008). Therefore, the inhibition of VEGF and HIF-1 α expression by SB365 may signify that SB365-induced angiogenesis is regulated by the AKT/mTOR pathway.

In summary, SB365 suppressed the growth of human colon cancer cells and the angiogenesis process *in vitro* and *in vivo*. It also strongly induced apoptosis in human colon cancer cells. This study may provide useful data for a future clinical trial of SB365 in human colon cancer patients.

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