Buddlejasaponin IV induces apoptotic cell death by activating the mitochondrial-dependent apoptotic pathway and reducing $\alpha_2\beta_1$ integrin-mediated adhesion in HT-29 human colorectal cancer cells

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Abstract. Colon cancer is one of the most frequent malignant neoplasms worldwide. Epidemiological studies suggested that the development of colon cancer can be prevented by plant-derived ingredients. In the present study, the chemopreventive activity of buddlejasaponin IV (BS-IV), isolated from the aerial part of *Pleurospermum kamtschaticum*, was investigated using cell viability, DNA fragmentation, caspase-3 activity, anoikis, cell adhesion, and flow cytometry assays and a murine lung metastasis model. Protein expression levels were detected by western blotting. Treatment with BS-IV significantly reduced cell viability and caused DNA fragmentation in HT-29 human colorectal cancer cells. BS-IV increased the ratio of Bax to Bcl-2 by significantly inhibiting Bcl-2 expression levels. BS-IV reduced expression levels of procaspase-9, procaspase-3, and full-length poly (ADP-ribose) polymerase (PARP) and increased cleaved PARP and nonsteroidal anti-inflammatory drug activated gene-1 expression levels and caspase-3 activity. In addition, BS-IV decreased the attachment of HT-29 cells to the extracellular matrix proteins collagen type I and IV and downregulated cell surface expression of $\alpha_2\beta_1$ integrin by inhibiting its glycosylation. BS-IV also reduced the expression and phosphorylation levels of focal adhesion kinase (FAK) and Akt, and the reduced FAK and Akt levels were rescued by treatment with a caspase-3 inhibitor Z-VAD-FMK. Furthermore, orally administered BS-IV inhibited the formation of tumor nodules in Balb/C mice intravenously injected with CT-26 murine colorectal cancer cells. Collectively, these findings indicated that BS-IV induces apoptosis via the mitochondrial-dependent pathway by increasing the ratio of Bax to Bcl-2 and activating caspases. BS-IV also induces anoikis by inhibiting $\alpha_2\beta_1$ integrin-mediated cell adhesion and signaling and inhibits the lung metastasis of colon cancer cells. Therefore, BS-IV may serve as a promising cancer chemopreventive agent.

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Introduction

Colon cancer remains one of the most prevalent forms of cancer worldwide (1). Despite the development of preventive and therapeutic strategies for reducing morbidity and increasing survival, the 5-year survival rate for patients with colon cancer varies from 92% for stage I to 11% for patients with metastatic cancers (2). The higher survival rate of colon cancer in earlier stages offers the potential that prevention as well as early detection and treatment for colon cancer can improve patient overall survival. Chemoprevention is considered one of the most promising approaches for preventing colon cancer and diminishing its threat and is defined as the administration of agents to prevent the induction of cancer and to inhibit or delay the development of invasive cancer (3). Due to their chemical diversity, low toxicity, and ability to modulate a variety of signal transduction pathways and cell processes, natural products, particularly plant-derived substances, have drawn increasing attention as new chemopreventive candidates (4-6).

Buddlejasaponin IV (BS-IV) (Fig. 1A) is one of the active components of the aerial part of *Pleurospermum kamtschaticum* (*P. kamtschaticum*) (Hoffmann; *Umbelliferae*), which is a perennial edible herb distributed in the fields and mountains of Asian countries that has been traditionally used

to treat cold, arthritis, atherosclerosis, and impotence (7). BS-IV has been shown to exhibit a remarkable protective effect against D-galactosamine-induced hepatotoxicity but not carbon tetrachloride-induced hepatotoxicity (8), and inhibit hypercholesterolemia and hyperlipidemia through extrinsic and intrinsic inducers by reducing oxidative stress in high cholesterol diet-induced rats (9). BS-IV has been revealed to inhibit the production of proinflammatory mediators, including nitric oxide, prostaglandin E_2, tumor necrosis factor-α, interleukin (IL)-1β, and IL-6, and the expression levels of cyclooxygenase-2, inducible nitric oxide synthase, and nuclear factor-κB in lipopolysaccharide-stimulated RAW 264.7 macrophages (10-12). The anti-inflammatory and analgesic effects of BS-IV have also been observed in animal models (12). Regarding its antitumor activity, the methanolic extract of *P. kamtschaticum* induced apoptosis in colon cancer cells by activating mitochondrial-dependent apoptotic signaling and upregulating nonsteroidal anti-inflammatory drug activated gene-1 (NAG-1) apoptotic protein and inhibited the lung metastasis of colon cancer cells in mice (13). BS-IV induced cell cycle arrest at the G2/M phase and apoptosis via both mitochondrial-dependent and death receptor-mediated pathways in immortalized human oral keratinocytes and human oral squamous cell carcinoma cells (14,15).

In the present study, the apoptosis-inducing capability of BS-IV and the underlying molecular mechanism were investigated and it was found that the inhibition of integrin-mediated survival signaling, as well as the activation of mitochondrial-dependent apoptotic signaling, are implicated in BS-IV-induced colon cancer cell death.

Materials and methods

Reagents and antibodies. BS-IV was generously provided by Professor Hee-Jun Park (Sangji University, Wonju, Korea) (10). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), 1% antibiotic-antimycotic mixture, phosphate-buffered saline (PBS), and trypsin-EDTA were purchased from Gibco; Thermo Fisher Scientific, Inc. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), Triton X-100, bovine serum albumin (BSA), and Tween-80 were purchased from Sigma-Aldrich; Merck KGaA and protease inhibitor cocktail was purchased from Roche Diagnostics GmbH. Z-VAD-FMK (cat. no. 627610), MG132 (cat. no. 474790), and calpeptin (cat. no. 03-34-0051) were purchased from Calbiochem; Merck KGaA. Antibodies were obtained from the following sources: Bax (cat. no. sc-7480) and Bcl-2 (cat. no. sc-7382) antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies from Santa Cruz Biotechnology, Inc.; caspase-3 (cat. no. 14220), caspase-9 (cat. no. 9508), poly (ADP-ribose) polymerase (PARP) (cat. no. 9542), Akt (cat. no. 4685) and phosphorylated (p)-Akt antibodies (cat. no. 4060) from Cell Signaling Technology, Inc.; NAG-1/GDF15 antibody (cat. no. ab180929) from Abcam; integrin $\alpha_2\beta_1$ (cat. no. MAB1998), α_2 (cat. no. AB1936) and β_1 (cat. no. AB1952) antibodies from Chemicon International; Thermo Fisher Scientific, Inc.; focal adhesion kinase (FAK; cat. no. 610088) and p-FAK (Tyr397; cat. no. 611807) antibodies from BD Transduction Laboratories; β-actin antibody (cat. no. A1978) from Sigma-Aldrich; Merck KGaA; and secondary fluorescein isothiocyanate (FITC)-conjugated antibody (cat. no. 6400-08) from SouthernBiotech. All reagents used in the present study were of analytical grade.

Cell culture. Human colorectal cancer HT-29 cells (cat. no. 30038) and murine colorectal cancer CT-26 cells (cat. no. 80009; both from Korea Cell Line Bank) were cultured in DMEM containing 10% FBS and a 1% antibiotic-antimycotic mixture in a humidified 5% CO_2 incubator at 37˚C. Both cell lines were authenticated by STR profiling and screened periodically for mycoplasma contamination using a Mycoplasma Detection kit (MycoAlert™; Lonza Group, Ltd.).

MTT assay. HT-29 cells ($5x10^3$ cells/well) were seeded into 96-well plates and incubated in DMEM with various concentrations of BS-IV for 2 h at 37˚C. Cell viability was measured using an MTT assay as previously described (13). The purple formazan product was dissolved with DMSO and absorbance was detected at 570 nm.

DNA fragmentation assay. HT-29 cells were treated with 4, 5 and 6 μM of BS-IV. After 2 h, the cells were harvested, and DNA was extracted as previously described (13). Extracted DNA was separated on a 1.8% agarose gel containing ethidium bromide at 50 V and then visualized at 300 nm by ultraviolet transillumination.

Western blot analysis. HT-29 cells ($1x10^6$ cells) were treated for 2 h with 4, 5 and 6 μM of BS-IV. HT-29 cells were also treated for 2 h with proteasome inhibitor MG132 (5 μM), calpain inhibitor calpeptin (10 μM), caspase-3 inhibitor Z-VAD-FMK (5 μM) in the absence or presence of 6 μM BS-IV. Cells were lysed using RIPA buffer containing protease inhibitor cocktail (Santa Cruz Biotechnology, Inc.), and the protein concentration was determined using a BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) as previously described (13). Protein from the cell lysates (50 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (MilliporeSigma). Membranes were blocked in 5% skim milk in Tris-buffered saline (10 mM Tris, pH 8.0 and 150 mM NaCl) with 0.1% Tween-20 (TBS-T) for 2 h at room temperature and then incubated with primary antibodies (1:1,000) against target molecules overnight at 4˚C. After washing, the blots were incubated with a 1:5,000 dilution of the respective HRP-conjugated secondary antibodies for 1 h at room temperature. The targeted proteins were visualized using an enhanced chemiluminescence detection kit (Amersham; Cytiva) according to the manufacturer's protocol. The Band intensities were quantified using an ImageJ analyzer (version 1.8.0; National Institutes of Health) and normalized to the intensity of β-actin, a loading control.

Caspase-3 activity assay. HT-29 cells were treated with 4, 5 and 6 μM of BS-IV for 2 h, and caspase-3 activity was measured using a commercially available caspase activity assay kit (cat. no. E-CK-A311; Enzo Life Sciences) according to the manufacturer's instructions. Absorbance was measured at 405 nm using an ELISA plate reader.

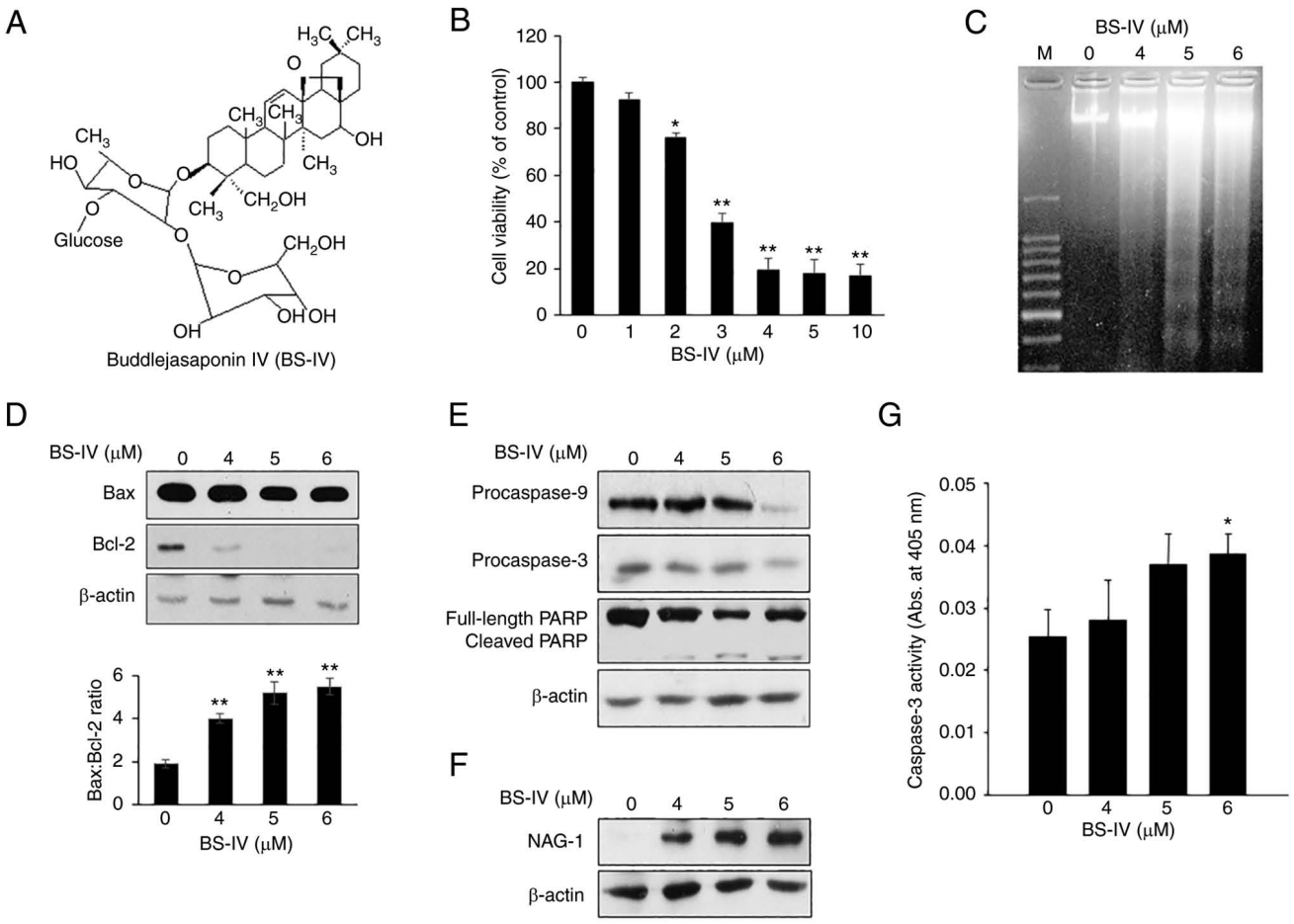

Figure 1. BS-IV induces apoptotic cell death via a mitochondrial-dependent pathway and the induction of NAG-1 in HT-29 colorectal cancer cells. (A) Chemical structure of BS-IV. (B-G) HT-29 cells were treated with the indicated concentrations of BS-IV for 2 h. (B) Cell viability was measured using an MTT assay. (C) Fragmented DNA was measured by agarose gel electrophoresis. The protein levels of (D) Bax, Bcl-2, (E) procaspase-9, procaspase-3, full-length and cleaved PARP and (F) NAG-1 were determined by western blotting using specific antibodies. After normalization to the intensity of β-actin using an image analysis program, the ratio of Bax to Bcl-2 protein was determined. (G) Caspase-3 activity was determined using a commercially available caspase activity assay kit. *P<0.05 and **P<0.01 vs. untreated HT-29 cells. BS-IV, buddlejasaponin IV; NAG-1, nonsteroidal anti-inflammatory drug activated gene-1; PARP, poly (ADP-ribose) polymerase.

Anoikis assay. HT-29 cells (3x10^6 cells/well) were plated in culture dishes coated with solution of 1% polyHEMA (Sigma-Aldrich; Merck KGaA) in 95% ethanol and treated with 4, 5 and 6 μM of BS-IV for 2 h. The attached cells and suspended cells in culture media were harvested and stained with 0.4% trypan blue solution. After 3 min incubation at room temperature, cells were observed and counted under a light microscope at a magnification of x10.

Cell adhesion assay. HT-29 cells (7.5x10^4 cells) were seeded into CytoMatrixTM cell adhesion strips coated with human collagen type I (cat. no. ECM104) or IV (cat. no. ECM105; both from Sigma-Aldrich; Merck KGaA) and treated with 4, 5 and 6 μM of BS-IV or anti-$\alpha_2\beta_1$ integrin antibody. Cell adhesion to BSA-coated strips served as a negative control. After 1 h of incubation at 37˚C, attached cells were stained with 0.2% crystal violet in 10% ethanol for 5 min at room temperature. The stained cells were gently washed with PBS, and the cell-bound stain was completely solubilized using solubilization buffer (a 50/50 mixture of 0.1 M NaH_2PO_4, pH 4.5 and 50% ethanol) for 5 min at room temperature. Absorbance was measured at 570 nm on a Benchmark microplate reader (Bio-Rad Laboratories, Inc.).

Flow cytometric assay. HT-29 cells were treated with 4, 5 and 6 μM of BS-IV for 2 h. The cells were collected, washed with PBS containing 1% BSA, and incubated with anti-$\alpha_2\beta_1$ integrin antibody (10 μg/ml) for 1 h at room temperature. After washing, the cells were resuspended in wash buffer (Mg^{2+} and Ca^{2+}-free PBS containing 1% BSA) and incubated with a 1:50 dilution of FITC-conjugated secondary antibody for 30 min at 4˚C. The cells were suspended in 200 ml of wash buffer and finally analyzed using a FACSCalibur equipped with CellQuest software (version 7.5.3; Becton-Dickinson, Inc.).

Detection of deglycosylated β_1 integrin subunit. HT-29 cells (1x10^6 cells) were treated with 5 μM BS-IV for 2 h. Cell lysates were treated with peptide: N-glycosidase F (PNGase F; cat. no. P0704S; New England BioLabs, Inc.) according to the manufacturer's instructions and then incubated overnight at 37˚C. The cell lysates were subjected to western blotting using a monoclonal antibody against β_1 integrin to assess the effects of deglycosylation.

Murine lung metastasis. Animal studies were approved (approval no. 2020-0193) by the animal ethics committee of

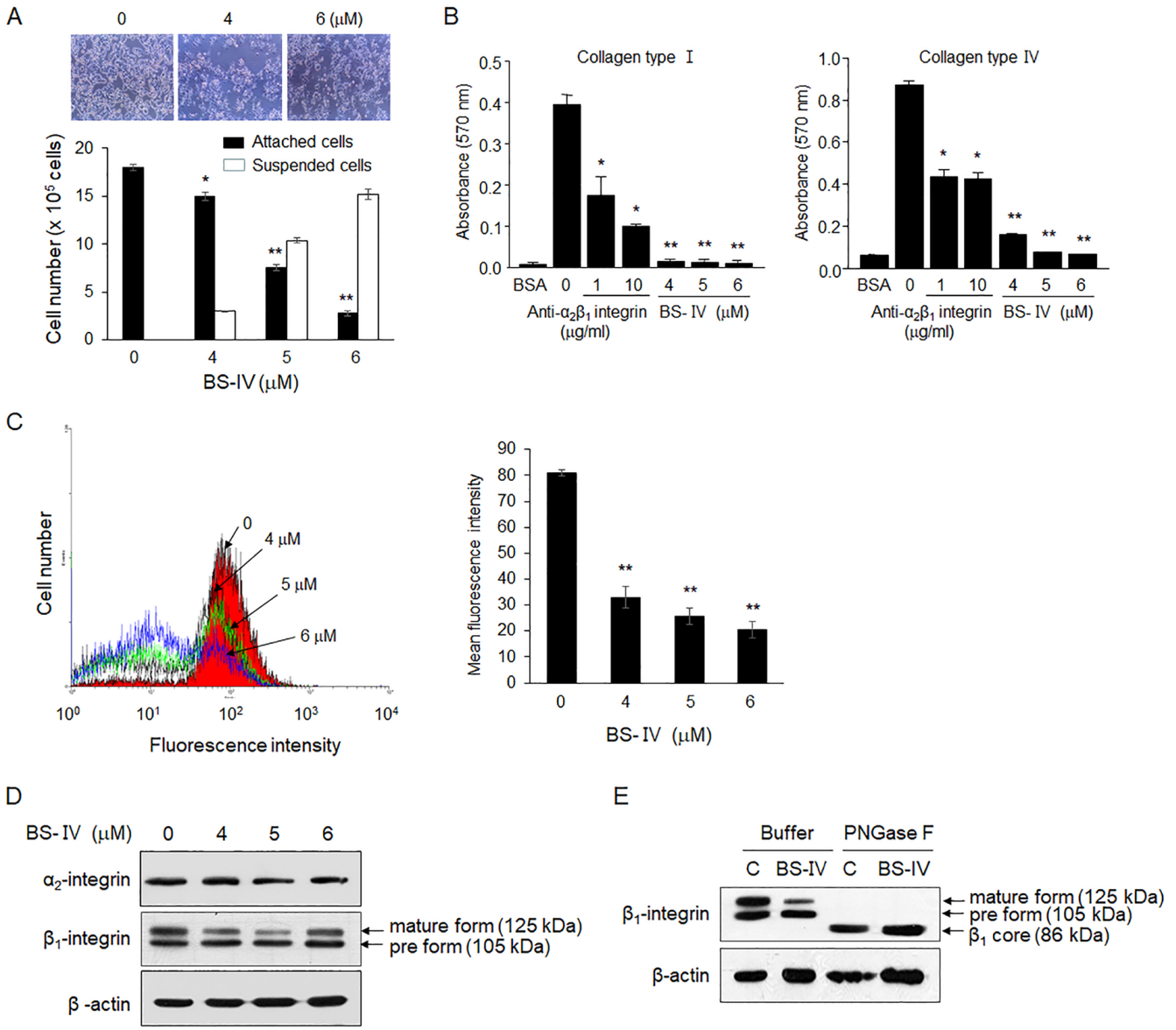

Figure 2. BS-IV inhibits the adhesion of HT-29 colorectal cancer cells on collagen type I- and type IV-coated plates and cell surface expression of $\alpha_2\beta_1$ integrin. (A) Anoikis assay of HT-29 cells treated with the indicated concentrations of BS-IV for 2 h. Cell morphology was observed with a microscope (magnification, x100). (B) Cell adhesion assay of HT-29 cells treated with the indicated concentrations of BS-IV or anti-$\alpha_2\beta_1$ integrin antibody in CytoMatrix™ cell adhesion strips coated with human collagen type I or IV for 1 h. Cell adhesion to BSA-coated strips served as a negative control. (C) Flow cytometric analysis of HT-29 cells treated with various concentrations of BS-IV for 2 h. The collected cells were incubated with anti-$\alpha_2\beta_1$ integrin antibody for 1 h at room temperature. (D) Western blot analysis of expression levels of the α_2 and β_1 integrin subunits in HT-29 cells treated with the indicated concentrations of BS-IV for 2 h. (E) Western blot analysis of expression levels of the β_1 core protein and glycosylated forms in HT-29 cells treated with the indicated concentrations of BS-IV in the absence or presence of PNGase F for 2 h. *P<0.01 and **P<0.005 vs. untreated HT-29 cells. BS-IV, buddlejasaponin IV; PNGase F, peptide: N-glycosidase F.

Yonsei University College of Dentistry (Seoul, Korea) and conducted in accordance with the approved guidelines of the regional authorities according to Yonsei University animal care regulations. Male BALB/c mice (n=30, 5 weeks-old, 18±3 g; Orient Co. Ltd.) were provided a standard laboratory diet and water *ad libitum* and were maintained at a temperature of 22±2˚C under a 12 h light/dark cycle. Male BALB/c nude mice were randomly divided into 5 groups, with 5 mice per group. Mouse colorectal cancer CT-26 cells (1x10^5 cells/200 μl PBS) were injected into the tail vein of Balb/c male mice (n=5) using a 27-gauge needle. Vehicle (PBS containing 1% DMSO and 1% Tween-80) and BS-IV at 0.01, 0.1, 1, and 2 mg/kg body weight (BW) were intraperitoneally administered to mice 30 min before cancer cell injection and once daily for 14 days after injection. Control mice received PBS and vehicle. The mice were euthanized by rapid cervical dislocation, and the lobes of the lungs were separated and fixed in Bouin's solution for 4 h at room temperature (Sigma-Aldrich; Merck KGaA). The number of tumor nodules on the lung surfaces was counted under an ocular micrometer, and the lung weights were also recorded.

Statistical analysis. Data are expressed as the mean ± standard error (SE) of three independent experiments. Student's t-test was used to compare the differences between two groups and one-way ANOVA followed by a Turkey's post hoc test was

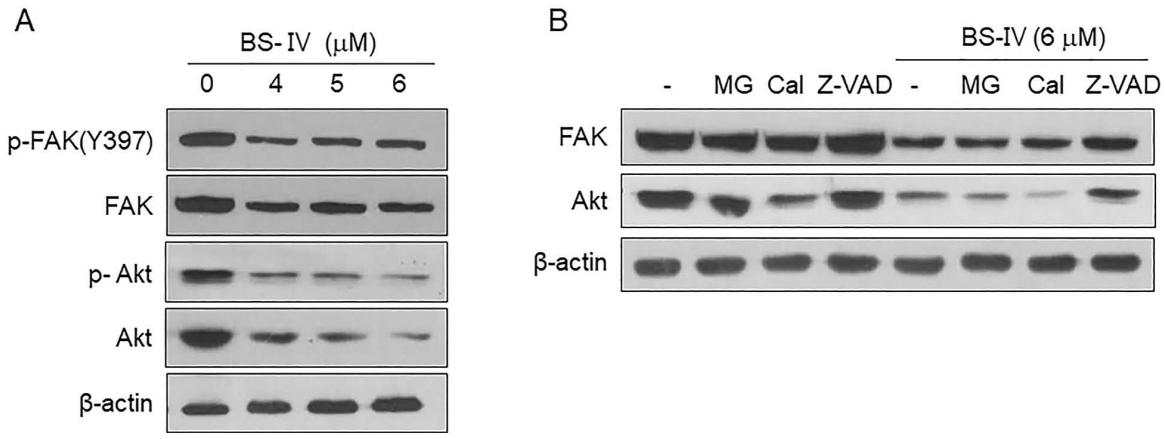

Figure 3. BS-IV reduces the expression and phosphorylation levels of FAK and Akt. (A and B) HT-29 cells were treated with various concentrations of BS-IV for 2 h (A) with or (B) without MG, Cal, or Z-VAD. Expression and phosphorylation levels of FAK and Akt were detected by western blotting. BS-IV, buddlejasaponin IV; FAK, focal adhesion kinase; MG, MG132 proteasome inhibitor; Cal, calpeptin calpain inhibitor; Z-VAD, Z-VAD-FMK caspase-3 inhibitor.

used to compare differences between multiple groups using SPSS version 19.0 (IBM Corp.). P<0.05 was considered to indicate a statistically significant difference.

Results

BS-IV induces apoptosis in HT-29 cells. Treatment with BS-IV for 2 h inhibited the viability of HT-29 colorectal cancer cells by 23.6% at 2 μM, 60.3% at 3 μM, and 80.5% at 4 μM (Fig. 1B). Treatment with BS-IV for 2 h displayed a characteristic ladder pattern of discontinuous DNA fragmentation, a distinct biochemical hallmark of apoptosis (Fig. 1C). Western blot analysis showed that treatment with BS-IV for 2 h weakly reduced pro-apoptotic Bax expression but significantly suppressed antiapoptotic Bcl-2 expression, resulting in an increased ratio of Bax to Bcl-2 in HT-29 cells in a dose-dependent manner (Fig. 1D). Expression levels of procaspase-9, procaspase-3, and full-length PARP as a substrate of caspase-3 were reduced, and cleaved PARP level was increased in BS-IV-treated HT-29 cells (Fig. 1E). In addition, BS-IV treatment induced dose-dependent increase in NAG-1 expression (Fig. 1F) and increased caspase-3 activity (Fig. 1G). These results indicated that BS-IV induces apoptosis via a mitochondrial-dependent pathway and expression of NAG-1 in HT-29 human colorectal cancer cells.

BS-IV decreases cell adhesion and expression levels of $\alpha_2\beta_1$ integrin in HT-29 cells. It was also detected that the number of attached cells decreased and the number of floating cells increased in BS-IV-treated HT-29 cells (Fig. 2A). It was next investigated whether BS-IV affected the attachment of HT-29 cells to the extracellular matrix (ECM). The attachment of HT-29 cells was significantly increased in collagen type I- or type IV-coated plates compared with BSA-coated plates. BS-IV treatment reduced the attachment of HT-29 cells to collagen types I and IV to the level of cell attachment to BSA, whereas the addition of anti-$\alpha_2\beta_1$ integrin antibody at 10 μg/ml blocked cell attachment to collagen types I and IV by 75 and 51%, respectively (Fig. 2B). The molecular mechanism by which BS-IV inhibits the attachment of HT-29 cells to collagen types I and IV was further explored. Flow cytometric analysis demonstrated that treatment with BS-IV inhibited cell surface expression of $\alpha_2\beta_1$ integrin in HT-29 cells in a dose-dependent manner (Fig. 2C). Western blot analysis indicated that treatment with BS-IV reduced cellular levels of the α_2 subunit and β_1 subunit mature form (Fig. 2D). The β_1 integrin subunit is synthesized as an 86 kDa polypeptide and sequentially glycosylated to a precursor form of 105 kDa followed by a mature form of 125 kDa in the endoplasmic reticulum and Golgi apparatus. The maturation of β1 integrin is important for its transport to the cell surface and/or binding to ECM ligands. Cell surface α integrins are transported to the cell surface after binding to β_1 integrin in cells (16). Treatment with BS-IV and then PNGase F, which completely removes oligosaccharide chains from glycoproteins, converted both the precursor and mature forms of β_1 integrin to the β_1 integrin core of an 86 kDa polypeptide. β_1 integrin core levels were not reduced by BS-IV treatment (Fig. 2E). These results indicated that BS-IV inhibits the adhesion of HT-29 cells on ECM components and that the reduced cell adhesion to collagen types I and IV in response to BS-IV treatment may be attributed to reduced levels of $\alpha_2\beta_1$, the cellular receptor of collagen types I and IV, which appears to occur by reducing expression of the α_2 subunit and blocking the maturation of β_1 integrin precursors via glycosylation.

BS-IV decreases the expression and phosphorylation of FAK and Akt. It was further examined whether BS-IV affected the activation of FAK and Akt, major signaling molecules that play important roles in numerous fundamental cellular functions, including cell adhesion (17). Treatment with BS-IV reduced the expression and phosphorylation of Akt, as well as the expression and Tyr^{397} phosphorylation of FAK (Fig. 3A). The reduced FAK and Akt expression levels were recovered in the presence of the caspase-3 inhibitor Z-VAD-FMK but not the proteasome inhibitor MG132 or the calpain inhibitor calpeptin in HT-29 cells treated with 6 μM BS-IV (Fig. 3B). These results indicated that caspase-3 activated in response to BS-IV treatment may reduce the expression and phosphorylation levels of Akt and FAK by degrading them.

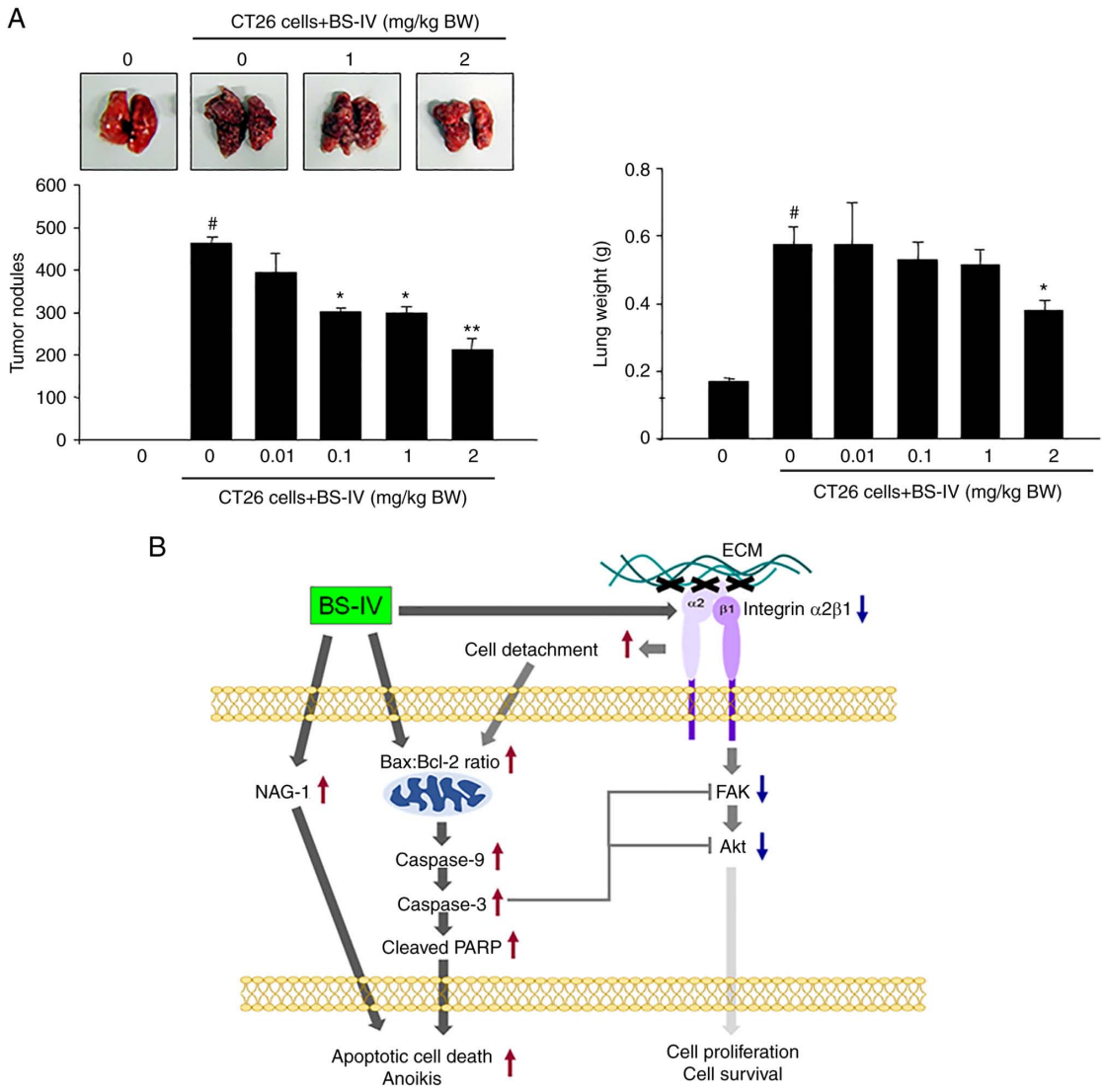

Figure 4. BS-IV inhibits lung metastasis of CT-26 murine colorectal cancer cells. (A) CT-26 cells ($1x10^5$ cells/0.2 ml) were injected into the tail vein of BALB/c male mice (n=5). The indicated doses of BS-IV were intraperitoneally administered 30 min prior to inoculation of CT-26 cells and once daily for 2 weeks after cell inoculation. The tumor nodules in the lungs were counted, and lung weight was measured in the sacrificed mice. $^{\#}P<0.05$ vs. vehicle-treated mice; $^{*}P<0.05$ and $^{**}P<0.01$ vs. mice injected with CT-26 cells alone. (B) Proposed molecular mechanism underlying cell death-inducing activity of BS-IV. BS-IV induces apoptotic cell death by inducing NAG-1 expression and activating the mitochondrial-dependent pathway. In addition, BS-IV induces anoikis and inhibits integrin-mediated antiapoptotic signaling by downregulating cell surface expression of $\alpha_2\beta_1$ integrin. BS-IV-induced caspase-3 also contributes to blockade of $\alpha_2\beta_1$ integrin-mediated antiapoptotic signaling by degrading FAK and Akt. BS-IV, buddlejasaponin IV.

BS-IV administration inhibits in vivo lung metastasis. Intravenous injection of CT-26 murine colorectal cancer cells into mice caused the formation of tumor nodules in the lung, resulting in a significant increase in lung weight. Intraperitoneally injected BS-IV significantly reduced the number of lung tumor nodules and lung weight in a dose-dependent manner (Fig. 4A). Administration of BS-IV at 2 mg/kg BW reduced the formation of lung tumor nodules by 54% and lung weight by 33%. These results indicated that BS-IV suppresses lung metastasis of murine colon carcinoma CT-26 cells.

Discussion

Apoptosis is a critical process for organism development and the maintenance of tissue homeostasis and is tightly regulated by mitochondrial-dependent and death receptor-mediated apoptotic signaling pathways. Cancer cells lose the ability to undergo apoptosis and lead to uncontrolled proliferation (18). The induction of apoptosis in cancer cells can be an attractive target for cancer chemoprevention and treatment. In a previous study, it was reported that the methanol extract of *P. kamtschaticum* induced apoptosis via mitochondrial-dependent apoptotic signaling and the induction of the NAG-1 apoptotic protein in colon cancer cells (13). In the present study, it was found that BS-IV is an apoptosis-inducing compound of *P. kamtschaticum* by confirming DNA fragmentation, increased Bax/Bcl-2 ratio, activation of caspase-3 and caspase-9, cleavage of PARP, and induction of the NAG-1 apoptotic protein in HT-29 colorectal cancer cells. NAG-1, a member of the transforming growth factor-β superfamily, can be induced either in a p53-dependent or p53-independent manner, and overexpression of NAG-1 is correlated with growth inhibition and apoptosis induction in cancer cells (19). Furthermore, it was revealed that BS-IV induces anoikis, a special type of apoptotic cell death, and the molecular mechanism by which BS-IV reduces

cell adhesion and induces anoikis in HT-29 colorectal cancer cells was investigated.

Anoikis is a crucial cell death program induced in the absence of cell attachment to the ECM or by cell adhesion to inappropriate locations. Anoikis resistance endows cancer cells to survive under suspension conditions or to proliferate at ectopic sites. This dysregulation in anoikis execution is considered a hallmark of cancer cells and contributes to distant metastasis (20). Thereby, anoikis-inducing phytochemicals may be promising candidates for anticancer agents. Cell attachment to the ECM is mediated via integrins, cell surface receptors for ECM ligands. Integrin binding of ECM promotes cancer cell proliferation, migration, and metastasis and protects cancer cells from apoptosis and anoikis by eliciting diverse signaling pathways (21). $\alpha_2\beta_1$ integrin has been implicated in the adhesion, proliferation, and metastasis of colon cancer cells (22-24). The present study indicated that BS-IV significantly inhibited the attachment of HT-29 cells to collagen types I and IV, and this inhibition resulted from the downregulated cell surface expression of $\alpha_2\beta_1$ integrin by reducing protein levels of the α_2 integrin subunit and maturation of the β_1 integrin subunit via glycosylation. These results suggested that disruption of the $\alpha_2\beta_1$ integrin-ECM interaction in HT-29 cells is at least a partial inducer of BS-IV-mediated death signaling.

Cell adhesion to the ECM triggers pro-survival pathways through the activation of downstream molecules and suppresses anoikis through the intrinsic and extrinsic apoptotic pathways of cell death (19). FAK is one of the most important integrin signaling molecules recruited into focal adhesions upon cell-ECM contact and promotes cell survival, proliferation, and motility. Upon integrin ligation and clustering, FAK auto phosphorylates at Tyr 397, enhancing intermolecular FAK kinase activity, and association with FAK and phosphoinositide 3-kinase leads to the phosphorylation of Akt (25,26). Akt activation promotes cell survival by releasing Bcl-2 and directly inhibiting the caspase cascade (27). In the present study, BS-IV reduced protein expression levels of FAK and Akt, resulting in their decreased phosphorylation levels. FAK and Akt protein levels were rescued by treatment with a caspase-3 inhibitor. These findings suggested that the degradation of FAK and Akt by BS-IV-activated caspase-3 can also contribute to inhibiting integrin-mediated signaling in HT-29 colorectal cancer cells.

Metastasis is a sequential process in which tumor cells detach from their site of primary growth, invade through the surrounding host tissue into the circulation, disseminate to distant organs, and extravasate and proliferate to form metastatic foci. Intraperitoneal administration of BS-IV, which induced anoikis in HT-29 colorectal cancer cells, inhibited the lung metastasis of CT-26 murine colorectal cancer cells.

In conclusion, BS-IV induces apoptotic cell death, including anoikis, in colon cancer cells by activating the mitochondrial-dependent pathway, inducing NAG-1 expression, and inhibiting integrin-mediated antiapoptotic signaling (Fig. 4B). Therefore, BS-IV may serve as a beneficial chemopreventive and anti-metastatic agent with potent proapoptotic and anoikis-inducing potential. BS-IV is a triterpene saponin and has been reported to show a poor bioavailability due to the poor permeability and significant hepatic first-pass effect (28). For clinical application of BS-IV, further studies shall be conducted by the authors to improve its limitations, including water solubility, absorption, and bioavailability.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JEK, SKL and WYC conceived the study, designed and performed experiments, analyzed the data, interpreted the results and wrote the manuscript. SKL and WYC confirm the authenticity of all the raw data. JP, MJJ, SEA and HJY contributed to the conception of the study, analyzed the data and reviewed the results. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Animal studies were approved (approval no. 2020-0193) by the animal ethics committee of Yonsei University College of Dentistry (Seoul, Korea) and conducted in accordance with the approved guidelines of the regional authorities according to Yonsei University animal care regulations.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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