A THESIS FOR THE DEGREE OF MASTER

Antitumor Effects of SB Injection, Extract from Phytopharmaceutical Preparations, in Canine Osteosarcoma and Melanoma Cell Lines

개의 골육종 및 흑색종 세포주에서의 자연 추출 약물 SB Injection의 항암 효과

2017년 2월

서울대학교 대학원

수의과대학 수의내과학 전공

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이 논문을 권경 수의학석사학위논문으로 제출함

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Abstract

Antitumor Effects of SB Injection, Extract from Phytopharmaceutical Preparations, in Canine Osteosarcoma and Melanoma Cell Lines

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The purpose of this study is to evaluate the effects of SB injection, an antitumor agent obtained from natural extracts, on canine cells. The antitumor effect of SB injection in D17 (canine osteosarcoma cells) and LMeC (canine melanoma cells) was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, cell cycle analysis, and annexin-V assay. Cell viability was inversely related to the concentration of chemical agents. The cell cycle of the affected cells was

arrested in the G2/M phase that led to an anti-proliferation effect. SB increased the rate of apoptosis dose-dependently. It is also used in combination with chemical drugs to reduce its dosage. SB showed no effect on the viability of PBMC (peripheral blood mononuclear cells) regardless of concentration, which suggested that SB does not suppress the activity of normal blood cells. This study suggests possibility of SB that can be considered as an alternative medication for canine osteosarcoma and melanoma in veterinary medicine.

Key words: SB Injection, Phytopharmaceutical Preparations, Antitumor agent, Canine Osteosarcoma, Canine Melanoma

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1. Introduction

There has been an increase in the number of tumor cases diagnosed in veterinary medicine. Although the outcome of chemotherapy in patients with tumor has improved, there are still various side effects including anorexia, depression, vomiting, haemorrhage, renal damage, hepatic damage, and bone marrow suppression, which could affect the lifespan of a patient. Therefore, studies on therapy using natural extracts as an alternative to chemotherapy is increasing (Bang et al., 2005; Chen et al., 2013; Choi et al., 2010; Gao et al., 2013; Hasegawa et al., 2011; Hong et al., 2012; Kim et al., 2010; Kim et al., 2004; Kim et al., 2002; Lee et al., 2005; Lee et al., 1999; Manosroi et al., 2006; Weng et al., 2012; Ye et al., 1996). Clients, in particular patients who have had serious chemotherapy-related side effects earlier and who do not have the financial condition to bear the cost of chemotherapy, do not want to opt for chemotherapy owing to various reasons.

Many natural extracts including SB have been reported to be effective agents against cancer cells in humans and mouse (Bang et al., 2005; Choi et al., 2010; Gao et al., 2013; Hong et al., 2012; Kim et al., 2010; Kim et al.,

2004; Kim et al., 2002; Lee et al., 2005; Lee et al., 1999; Weng et al., 2012; Ye et al., 1996). SB injection (SBP, Seoul, South Korea) which is extracted from pulsatilla saponin D, ginsenoside Rb, and glycine acid has been also considered as effective antitumor agent. 3-0-[O-α-L-rhamnopyranosyl-(l→2)-[0-β-D-glucopyranosyl-(l-→4)]-α-L-arabinopyranosyl] is main constituent of SB. It is an antitumor ingredient enriched in Korean Pulsatilla which is a kind of Saponin. It has antitumor effect which is activated by enzyme-reacting cascade. It inhibits formation of neoblood vessel and proliferation of tumor cells (Kim et al., 2005). Saponin has been proved to have antitumor activity against human and mice cancer cell lines. The human cancer cells reported include colon cancer (HCT116 and SW480), prostate cancer (PC3 and DUI145), breast cancer (MCF-7, MDA-MB-486, and MDA-MB-231), osteosarcoma (MG63 and 143B), myeloid leukaemia (HL-60), gastric adenocarcinoma (MKN-45), pulmonary adenocarcinoma (PC-14), and human hepatoma (HepG2) cell lines (Bang et al., 2005; Gao et al., 2013; Kim et al., 2010; Kim et al., 2002; Lee et al., 2005). The mouse cancer cells reported *in vitro* include leukaemia (B16 and P388) and lung carcinoma (LCC) cells (Hong et al., 2012; Lee et al., 1999).

However, there have not been many studies on applying SB to canine cells in veterinary medicine. In this study, we examined the antitumor effects of SB on D17 (canine osteosarcoma cell line) and LMeC (canine

melanoma cell line) by comparing with the effects of other commercial chemical agents.

2. Material and Methods

2.1. Cell cultures

Cell cultures. D17 cells (ATCC CCL-183), a canine osteosarcoma cell line gifted from the Department of Veterinary Pharmacology, Seoul National University, were cultured in High glucose-Dulbecco's Modified Eagle's Medium (H-DMEM; PAN Biotech, Aidenbach, Germany) containing 10% foetal bovine serum (FBS; PAN Biotech) and 1% 100 U/mL penicillin and 100 g/mL streptomycin (PS; PAN Biotech) (Bruserud et al., 2005; Gebhard et al., 2015; MINAMITANI, 2000). LMeC cells (Inoue et al., 2004) were cultured in RPMI-1640 with 10% FBS and 1% PS. The cells were incubated in 5% CO_2 at 37℃ (Lu and Kerbel, 1993; Rodeck and Herlyn, 1991).

2.2. Antitumor agents

SB is considered an antitumor agent with apoptotic and antioxidative activities. It was administered at doses of 0, 0.3125, 0.625, 1.25, 2.5 mg/kg according to previous studies (Bang et al., 2005; Gao et al., 2013; Hong et al., 2012; Kim et al., 2010; Kim et al., 2004; Kim et al., 2002; Lee et al., 2005; Lee et al., 1999; Weng et al., 2012). The other antitumor agents used to compare the efficiency of SB were cisplatin for D17 and LMeC cells and doxorubicin for D17 cells. Cisplatin was applied at doses of 0, 0.01, 0.05, 0.25 mg/kg (Alborzinia et al., 2011; Chun et al., 2005; Prabhakaran et al., 2013; Schmidt et al., 2016; Simon et al., 2001; Szewczyk et al., 2015) and doxorubicin was administered at doses of 0, 0.1, 0.5, 5, 10 μg/mL (Chun et al., 2005; Harisi et al., 2006; Schmidt et al., 2016; Simon et al., 2001; Smith et al., 2006; Szewczyk et al., 2015).

2.3. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

The cell viability was determined using MTT assay as reported in a previous study (Stockert et al., 2012). D17 and LMeC cells were seeded into 96-well plates at a density of 2,000 cells/100 µL/well each and incubated for 24, 48, and 72 h after treatment with the antitumor agents. The D17 cells were treated with different concentrations of SB (0, 0.3125, 0.625, 1.25, 2.5 mg/mL), cisplatin (0, 0.01, 0.05, 0.25 mg/mL), and doxorubicin (0, 0.1, 0.5, 5, 10 µg/mL) in 100 µL media, which was added to the cells in each well. The LMeC cells were also treated with different concentrations of SB (0, 0.3125, 0.625, 1.25, 2.5 mg/mL) and cisplatin (0, 0.01, 0.05, 0.25 mg/mL) in 100 µL media.

MTT solution of 5% v/v (Sigma, St. Louis) was prepared. After 24, 48, and 72 h, 20 µL MTT solution, which is 10% of the total working volume, was added to each well, and the cells were incubated for 4 h. The supernatant was removed by aspiration and 100 µL of DMSO (dimethyl sulfoxide) was added to each well to solubilize the formazan crystals. After shaking the plate for 15 min, the absorbance at 540 nm with a correction at 650 nm was measured by a spectrophotometer (680 Microplate Reader, Bio

rad®).

2.4. Cell cycle analysis

D17 and LMeC cells were dispensed at a density of 2×10^4/2 mL/well into 12-well plates and stabilized in 5% CO_2 at 37℃overnight. The supernatant was removed, and the culture media were replaced by media with different concentrations of SB. D17 cells treated with SB (0, 0.625, 2.5 mg/mL) and LMeC cells treated with SB (0, 0.3125, 0.625 mg/mL) were incubated in 5% CO_2 at 37℃ for 24 h.

The supernatant containing dead cells was collected in 15 mL conical tubes. After gently washing the well with 1 mL of PBS followed by suction drying, trypsinization with 400 μL/well trypsin was performed, and the cells were incubated in 5% CO_2 at 37℃ for 4 min. The live cells were detached and collected into the conical tubes in which the supernatant had been collected in the previous step. The samples were centrifuged at 850 rpm for 3 min, and the supernatant was removed. The cells were resuspended with 1 mL PBS to wash out the media and centrifuged at 850 rpm for 3 min.

After the removal of supernatant by suctioning, each pellet was resuspended with 0.3 mL PBS, and transferred into 1.5-mL Eppendorf tubes

to which 0.7 mL 100% ethanol was added and mixed gently by pipetting. The samples were centrifuged at 200 g, 4℃ for 5 min, and the supernatant was removed.

The cells were resuspended with 500 μL of 400 μg/mL RNase (final concentration would be 200 μg/mL) and kept for 30 min at room temperature. Five-hundred microliters of propidium iodide (PI; 100 μg/mL) was added to each sample and kept for another 30 min in a dark room at room temperature. The samples were measured by flow cytometry (FACS AriaII Automated High-speed Flow Cytometry System) (Chen et al., 2013; Choi et al., 2010; Gorczyca et al., 1993).

2.5. Annexin-V

D17 and LMeC cells were incubated in 5% CO_2 at 37℃ with 2×10^4/2 mL/well in the 12-well plates. The supernatant was collected in 15-mL conical tubes, and the wells were gently washed with 1 mL PBS. Trypsin at a concentration 400 μL/well was treated, and the cells were incubated in 5%

CO_2 at 37℃ for 4 min. The live cells were detached and collected in the same conical tubes containing the supernatant with dead cells. The samples were centrifuged at 850 rpm for 3 min, and the supernatant was removed. The cells were resuspended with 1 mL PBS to wash out any media and centrifuged at 850 rpm for 3 min. The supernatant was suctioned and discarded; the pellet was washed again with 1 × binding buffer. The cells were resuspended with 100 μL 1 × binding buffer. Fluorochrome-conjugated annexin V (5 μL) was added and the cells were incubated for 10 min at room temperature in the dark. The cells were washed with 2 mL 1 × binding buffer and resuspended with 200 μL binding buffer. Five microliters of PI was added and the absorbance was measured by flow cytometry (Chen et al., 2013; Choi et al., 2010; Kikuchi et al., 2005; Kim et al., 2007).

2.6. Combination with SB and other chemical agents

D17 and LMeC cells were seeded at a density of 2,000 cells/100 μL/well into 96-well plates and treated with various combinations of drugs in another 100 μL media. D17 cells were treated with 0.625 mg/mL of SB

mixed with different concentrations of cisplatin (0, 0.01, 0.05, 0.125 mg/mL) and doxorubicin (0, 0.1, 0.5, 5, 10 µL). LMeC cells were also treated with 0.625 mg/mL of SB mixed with cisplatin (0, 0.01, 0.05, 0.125 mg/mL). The cells were incubated in 5% CO_2, at 37℃ for 24, 48, and 72 h.

MTT solution (5% v/v) was prepared. Twenty microliters of MTT solution, which is 10% of the total working volume, was added to each well after 24, 48, and 72 h, and the cells were incubated for 4 h. The supernatant was removed by weak suctioning, and 100 µL DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured by spectrophotometry at 540 nm with a correction at 650 nm after shaking the plate for 15 min (680 Microplate Reader, Bio rad®).

2.7. Statistical analysis

All the experimental data were analyzed with GraphPad Prism (version 6) software (Graphpad Software Inc., San Diego, CA, USA.). All data are presented as mean±standard deviation (SD). The statistical significance of

mean values were examined using oneway ANOVA. p-Values <0.05 were considered to indicate statistical significance.

3. Results

3.1. MTT assay for D17 and LMeC cells

The absorbance of D17 cells treated with SB, cisplatin, and doxorubicin decreased with increasing concentration of the drugs. Compared to the negative controls, which were not treated with any antitumor agents, D17 cells treated with SB (0.3125, 0.625, 1.25, 2.5 mg/mL) showed gradually decreasing values, which indicated a reduction in cell viability at 24 h. In addition, D17 cells that were treated with low concentrations of SB showed a lower viability at 48 and 72 h (Fig. 2 A). At 48 h, the viability of cells treated with 0.312, 0.625, 1.25, 2.5 mg/mL of SB showed no significant differences. At 72 h, none of the values, except the negative control, showed any significant difference (Fig. 2 A).

Similar to the results of cells treated with SB, D17 cells treated with cisplatin and doxorubicin showed a gradual decrease in viability with increasing concentration of drugs. The viability of cells treated with lower concentrations also decreased over time in a similar manner as that of cells treated with higher concentrations of drugs. The cisplatin-treated cells had

almost the same viability at all concentrations after 48 h (Fig. 2 B). Doxorubicin-treated cells showed no significant difference in viability compared with cells treated with 1.25 and 2.5 mg/mL SB at 72 h (Fig. 2 C).

LMeC cells treated with SB and cisplatin exhibited significantly decreased viability compared with D17 cells at all time points, and the differences were apparent for each concentration (Fig. 3). Though there was no significant difference in viability at 0.3125 mg/mL of SB compared with that of the negative control, the viability of cells treated with doses higher than 0.625 mg/mL SB remarkably decreased at 24 h (Fig. 3 A). After 48 h, the cells treated with 0.3125 mg/mL SB exhibited significant suppression of viability (Fig. 3 B). The SB-treated LMeC cells expressed a consistent suppression until 72 h (Fig. 3 C).

3.2. MTT assay for PBMC (peripheral blood mononuclear cells)

To determine the safety of SB on normal cells, we treated PBMC sampled from a healthy Beagle dog with SB. The absorbance of SB-treated PBMC incubated for 24, 48, and 72 h was not significantly different from that of the negative control (Fig. 4). Concanavalin A (ConA) and

lipopolysaccharide (LPS) were added to stimulate PBMC infiltration. ConA is a lectin that has the ability to increase the synthesis of cellular products. LPS is an endotoxin, which induces a strong immune response in normal mammalian cells. The results showed that the viability of PBMC was not affected by SB treatment at concentrations up to 2.5 mg/mL until 72 h.

3.3. Cell cycle analysis

To determine the cell cycle phase affected by SB, we analysed D17 and LMeC cells after 24 h of SB treatment. The cell cycle is divided into G1 to M phase, which is further separated into sub-G1 (P2), G0/G1 (P3), S (P4), G2/M (P5), and M phase (P6). The normal D17 cell population was the highest in G0/G1 phase (P3) and the lowest in G2/M phase (P5). The P3/P5 ratio decreased with increasing SB concentrations, and it was less than 1 at 2.5 mg/mL of SB. This indicated an increase in the number of cells in the G2/M phase (P5) (Fig. 5 A). The P3/P5 ratio decreased remarkably with increasing concentrations of SB (Fig. 5 C).

Similar to D17 cells, the normal LMeC cell cycle consists of a large P3 population. As LMeC cell line is more sensitive to antitumor drugs

including SB when compared with the D17 cell line, the treatment concentration of SB for LMeC was rearranged. The P3/P5 ratio in LMeC cycle decreased with increasing concentrations of SB (Fig. 5 B). The gradient ratio of LMeC cell line across different SB concentrations was much higher than that of the D17 cell line. The P3/P5 ratio of the negative control was approximately double the ratio of cells treated with 0.3125 mg/mL of SB (Fig. 5 D). From these results, we could suggest that in both malignant cell lines, cell cycle arrest was induced by SB right before mitosis, which may inhibit cell proliferation.

3.4. Annexin-V for evaluation of cell damage

To analyse the relationship between SB concentration and malignant cell damage, we considered the number of annexin V-binding cells as an indicator of unstabilized cell membrane and early apoptosis detector (34, 35). The number of PI- and Annexin V-binding cells indicated that the number of damaged D17 and LMeC cells increased in an SB concentration-dependent manner (Fig. 6). Treatment of D17 cells with 0.625 mg/mL and 2.5 mg/mL of SB resulted in 18.2% and 51.5% apoptosis, respectively (Fig. 6 A). As LMeC cells were more sensitive to SB treatment, 0.3125 mg/mL

and 0.625 mg/mL of SB resulted in 31.4% and 50.9% apoptosis, respectively (Fig. 6 B).

The apoptosis rate increased with increasing concentrations of SB. In D17 cells, 0.625 mg/mL of SB resulted in two times more apoptosis than the negative control. The apoptosis rate in cells treated with 2.5 mg/mL SB was about 6 times higher than that of the negative control (Fig 6. C). LMeC also exhibited an increased rate of apoptosis with increasing concentrations of SB. At 0.3125 mg/mL and 0.625 mg/mL of SB, the apoptosis rate was 3 and 5 times more than that of the negative control, respectively (Fig. 6 D).

3.5. MTT assay - combination of chemical antitumor agents with SB

To reduce the dosage of chemical antitumor drugs, we combined chemical drugs preferable for each tumor cell with SB and determined the potency of the combination. When compared with cells treated with high-dose chemical agents alone, the combination required a much lower dosage of the chemical agent. D17 cells had no significant difference in viability between 0.25 mg/mL SB and a combination of 0.625 mg/mL SB and 0.01 mg/mL cisplatin, which is a much lower concentration, after 24 h (Fig. 7 A). D17

cell viability was similar in response to 10 μg/mL of doxorubicin and to a combination of 0.625 mg/mL of SB and 5 μg/mL of doxorubicin at 24 h and 72 h. In addition, D17 cell viability was similar in response to 10 μg/mL of doxorubicin and to a combination of 0.625 mg/mL of SB and 0.1 μg/mL of doxorubicin (Fig. 7 B).

LMeC cells treated with 0.625 mg/mL SB had similar viability for 0.25 mg/mL cisplatin and a combination of 0.625 mg/mL SB and 0.05 mg/mL cisplatin at 24 h. After 48 h, the cell viability for cells treated with 0.25 mg/mL cisplatin had no difference when compared with that of the combination of 0.625 mg/mL SB and 0.01 mg/mL cisplatin. In addition, after 72 h, the cells treated with a combination of 0.625 mg/mL SB and 0.05 mg/mL cisplatin had much lower viability than that of cells treated with 0.25 mg/mL SB alone.

4. Discussion

This is a study on SB, a natural extract from *Pulsatilla koreana*, *Panax gingseng,* and *Glycyrrhiza glabra.* SB consists of materials such as Saponin D that exert antitumor effects (Fig. 1). It has been known as a natural antitumor agent and used as an alternative medication for patients with severe side effects to chemotherapy, without good enough condition or without remarkable improvement after chemotherapy. There are a few studies on SB treatment for human and rodent malignant cell lines (Bang et al., 2005; Gao et al., 2013; Hong et al., 2012; Kim et al., 2010; Kim et al., 2004; Lee et al., 2005; Lee et al., 1999). Several studies about the use of agents from natural extracts for canine malignant cell lines are present (Bang et al., 2005; Gao et al., 2013; Hong et al., 2012; Kikuchi et al., 2005; Kim et al., 2010; Kim et al., 2007; Kim et al., 2004; Lee et al., 2005; Lee et al., 1999; Weng et al., 2012). However, natural extracts are not commonly used for treating cancers in veterinary medicine owing to a lack of studies about their effects on animals when compared with studies on humans. Therefore, in this study, we proved the antitumor effect of SB on canine osteosarcoma and melanoma cell lines as an initial step for considering SB as an alternative medication for cancers in veterinary medicine.

We examined two canine malignant cell lines, D17 and LMeC, for this study. We treated each cell line with SB and other commonly used commercial chemical agents. The concentration of the drugs tested in this study was based on previous studies including studies about SB for other mammalian cell lines including rats and humans (0.3 to 10 mg/mL) (Gao et al., 2013; Hong et al., 2012; Lee et al., 1999) and studies about cisplatin and doxorubicin for canine cell lines (Alborzinia et al., 2011; Chun et al., 2005; Harisi et al., 2006; Prabhakaran et al., 2013; Schmidt et al., 2016; Simon et al., 2001; Smith et al., 2006; Szewczyk et al., 2015).

D17 and LMeC cells were sensitive to SB; however, LMeC showed more sensitivity to SB. The viability of D17 cells was decreased significantly after 24 h by SB treatment at concentrations greater than 0.625 mg/mL and after 48 h by treatment at concentrations above 0.3125 mg/mL. The viability of LMeC had remarkably decreased on treatment of SB at concentrations greater than 0.625 mg/mL after 24 h. The viabilities of the two cell lines were approaching similar values, and it decreased remarkably over time regardless of the concentration of SB. Similar to SB, cisplatin and doxorubicin, which are the most popular chemical agents used to treat canine osteosarcoma, were applied to D17 cells. In addition, viability decreased and approached similar values in both cell lines treated with chemical agents. In the present study, it was observed that SB exerted

antitumor effects on canine malignant cell lines, and the effect was similar to those of other chemical agents. However, in this study, PBMC, which are normal blood cells, were not affected by SB concentration at any time point up to 72 h. This finding could be interpreted as a proof of the ineffectiveness of SB on normal cells, though it has a noticeable capability to suppress the malignant cell lines. To make sure the safety of SB it could be necessary to conduct experiments in vivo.

The cells treated with SB were damaged via induction of cell cycle arrest. The cell cycle population differed among D17 and LMeC cells treated with SB. In the absence of any treatment, the highest number of cells was present in the P3 phase (G0/G1 phase) in both cell lines. However, when the cells were treated with higher doses of SB, the number of cells in the P5 phase (G2/M phase) increased. It was observed that LMeC was more sensitive to lower doses of SB than D17. We suggested that the effect of SB on the malignant cells was caused by interrupting the cell cycle, especially P5 (G2/M phase), right before mitosis. SB inhibits the proliferation of two malignant cell lines, which could be a possible way of exerting antitumor effects.

The cell damage caused by SB could be determined by annexin-V assay. PI and Annexin-V were used for staining D17 and LMeC cells treated with

different concentrations of SB. The cells that were stained indicated dead cells, which were in the late stage of apoptosis. The apoptosis rate increased significantly with increasing concentrations of SB after 24 h of treatment. This implied that SB affected the cell membrane and induced apoptosis within 24 h. LMeC were more sensitive than D17 at low doses, approximately half of the concentration of SB. The increase in apoptosis rate was similar in both cell lines and was proportional to the concentration of drugs.

SB is expected to exert an antitumor effect with relatively less side effects. Simultaneously, for analysing the potency of other drugs, we applied a combination of existing commercial drugs and SB on the malignant cell lines. The combination exhibited the same or higher potency of antitumor effect for canine osteosarcoma and melanoma cells at a much lower concentration when applied with SB. The use of SB with chemotherapy agents can contribute to reduced risk of various side effects by lowering the concentration of chemical agents for achieving target remission. Thus, SB can be considered as an alternative clinical medicine for patients in these cancers. However, there is limitation with variety of cell lines and deficiency of data *in vivo*. It could be needed to confirm the effect on the other malignant cell lines which are not used in this study. To ensure the safety of SB, it would be necessary to conduct experiments *in vivo* and to

accumulate data applying to patients.

5. Conclusion

This study suggests that SB is expected as an effective antitumor agent with relatively less side effects on the normal blood cells. It has antitumor effect as other chemical agents by inducing cell cycle arrest and apoptosis proportionally to the concentration on canine osteosarcoma and melanoma cells. The use of SB with chemotherapy agents can be suggested that it could reduce risk of various side effects by lowering the concentration of chemical agents for achieving target remission. Thus, it could be considered as an alternative medicine and additional agent for the canine osteosarcoma and melanoma in veterinary medicine.

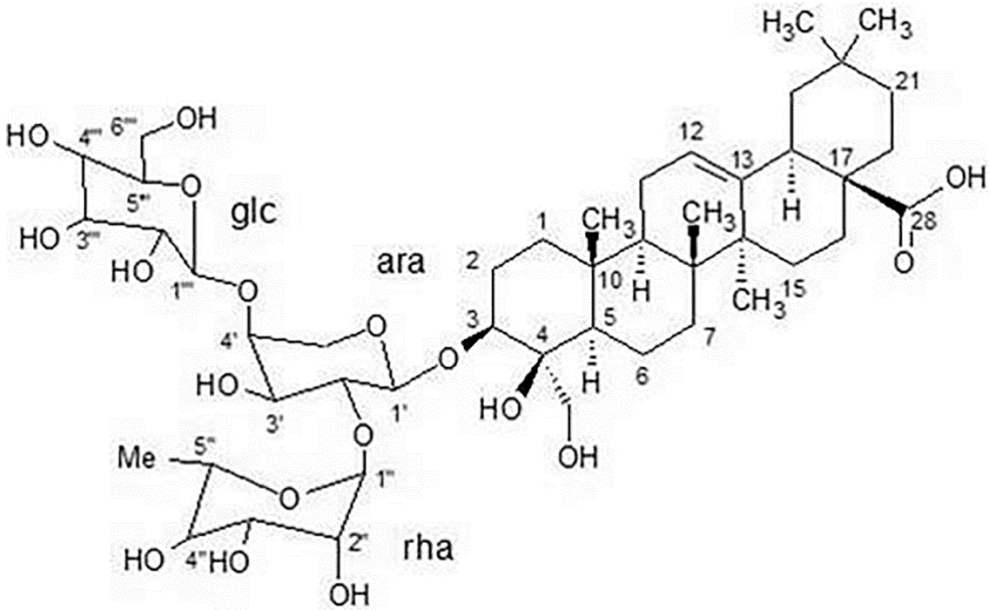

Figure1. The molecular structure of the major constituent of SB.

3-0-[O- α- L-rhamnopyranosyl-(l→2)-[0-β -D-glucopyranosyl-(l-→4)]- α - L- arabinopyranosyl] hederagenin (SBP, South Korea).

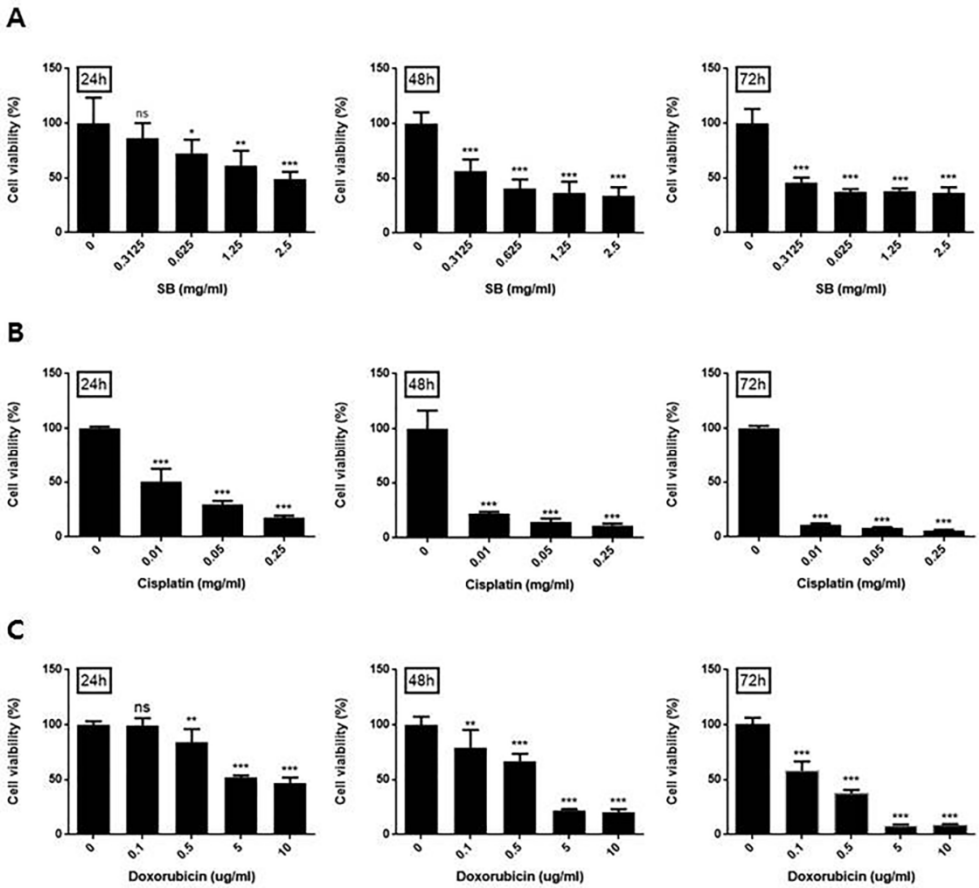

Figure 2. MTT assay for D17 cells treated with SB, cisplatin, and doxorubicin.

(A) The viability of D17 cells treated with different concentrations of SB. The viability of D17 cells decreased with increasing concentrations of SB. Although the viability differed with concentrations until 24 h, it showed a tendency to attain equilibrium regardless of the concentration of SB after 48 h. (B) The viability of D17 cells treated with cisplatin. The D17 cells treated

with cisplatin showed a rapid decrease in viability with concentration. (C) The viability of D17 cells treated with doxorubicin. Doxorubicin at different concentrations had a lower potency against D17 cells than cisplatin. All experiments were independently conducted in triplicate. Data are represented as mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

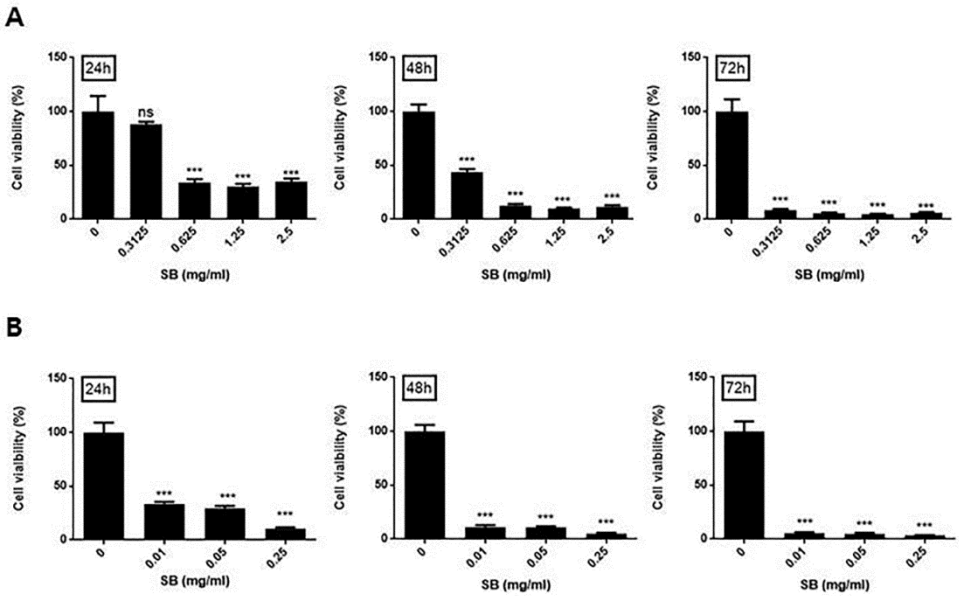

Figure 3. MTT assay for LMeC cells treated with SB and cisplatin.

(A) The viability of LMeC treated with SB. The viability of LMeC treated with SB decreased with increasing concentrations of SB, with significantly more viability suppression compared with that of D17. (B) The viability of LMeC treated with cisplatin. LMeC cells were more sensitive to cisplatin. There was no remarkable difference in sensitivity between concentrations after 48 h. All experiments were independently conducted in triplicate. Data are represented as mean ± standard deviation. *** $P < 0.001$

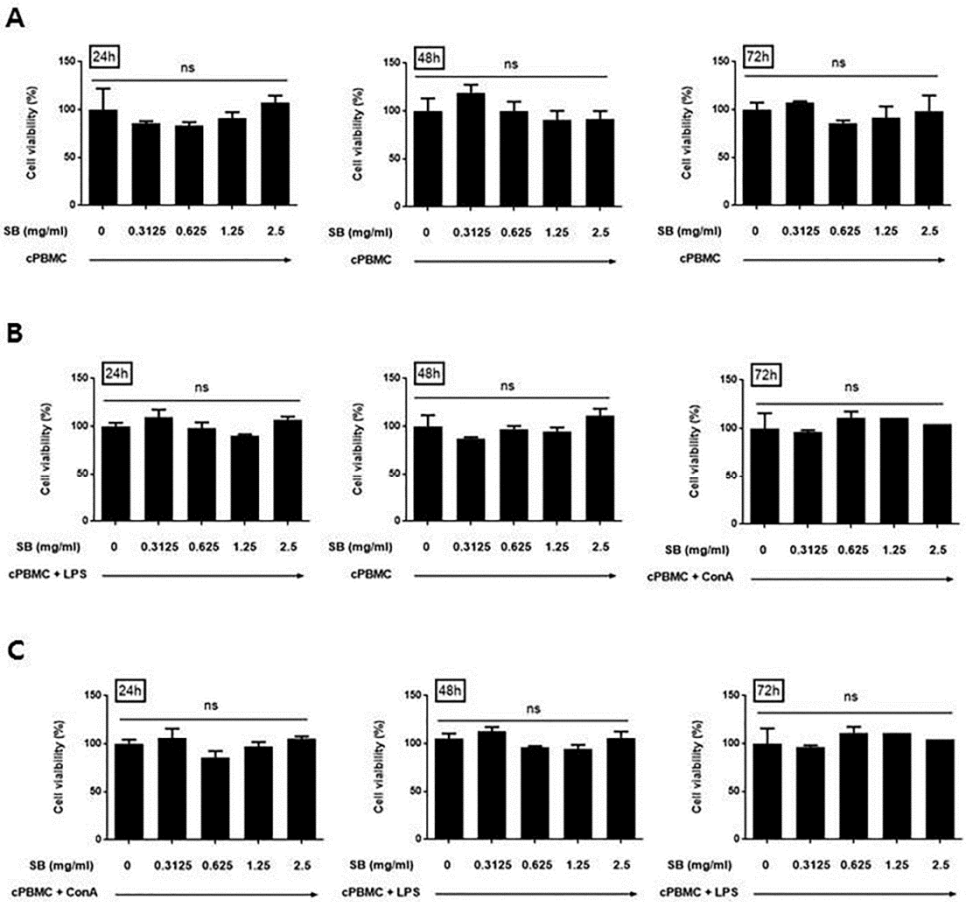

Figure 4. ***MTT assay for PBMC treated with various concentrations of SB.***

(A) The viability of PBMC treated with SB. There was no significant difference in viability at different SB concentrations compared with that of the negative control. (B) The viability of PBMC treated with SB on addition of ConA. On addition of ConA to stimulate the proliferation of PBMC, no

significant difference in cell viability was observed at different concentrations of SB. (C) The viability of PBMC treated with SB on addition of LPS. There was no remarkable difference in viability at different concentrations of SB compared with that of the negative control. All experiments were independently conducted in triplicate. Data are represented as mean ± standard deviation.

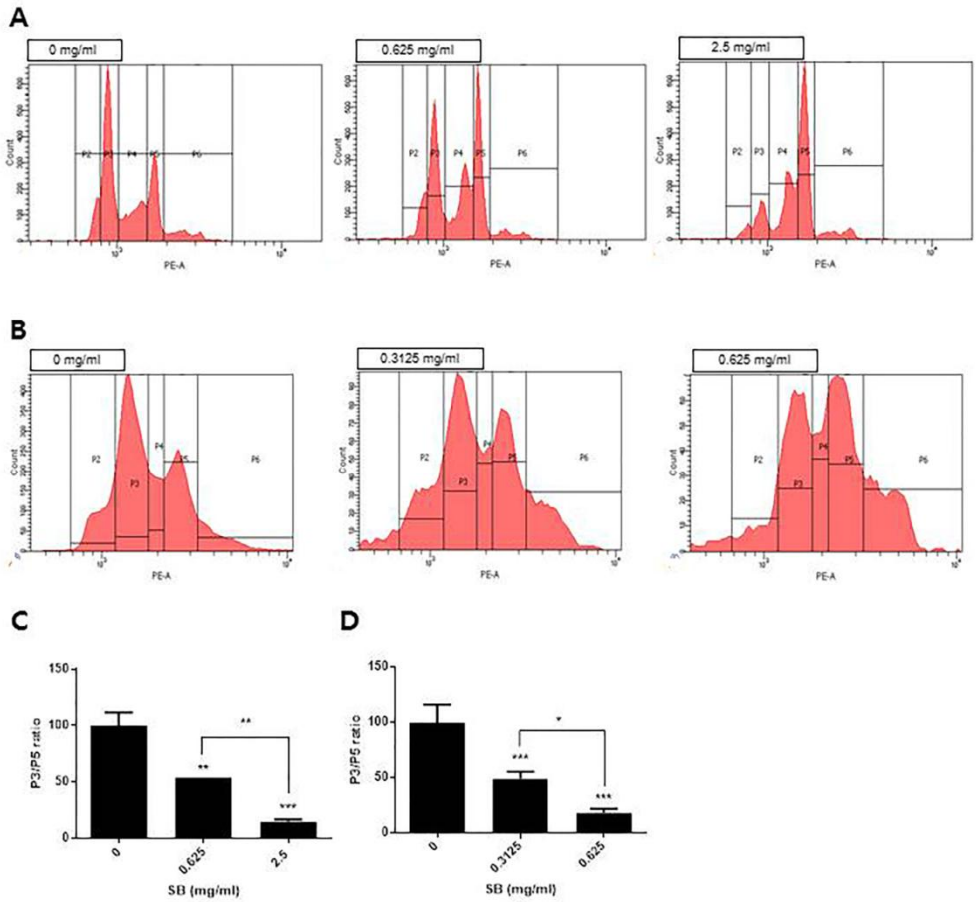

Figure 5. Cell cycle analysis of D17 and LMeC cells treated with SB after incubation for 24 h.

(A) The cell cycle of D17 treated with different concentrations of SB (0, 0.625, 2.5 mg/ml). Compared with the negative control, the number of SB-treated D17 cells in P3 was higher than that in the other phases. The number of cells in P5 increased with increasing doses of SB. At 2.5 mg/mL of SB,

increased number of cells was present in P5. (B) The cell cycle of LMeC treated with different concentrations of SB (0, 0.3125, 0.625 mg/ml). The number of cells in P3 was higher than that in P5 when compared with the negative control. When the concentration of SB was increased from 0.3125 mg/mL to 0.625 mg/mL, the number of cells in P5 was slightly higher than that in P3. The SB concentration at which the LMeC cell cycle was affected was lower than D17 cells. (C) P3/P5 ratio of D17 cells. The P3/P5 ratio of D17 cells significantly reduced with increasing SB concentrations compared with that of the negative control. The ratio for each concentration showed a significant difference. (D) The P3/P5 ratio of LMeC cells. The P3/P5 ratio of LMeC cells significantly reduced with an increase in SB concentration compared with that of D17 cells. All experiments were independently conducted in triplicate. Data are represented as mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

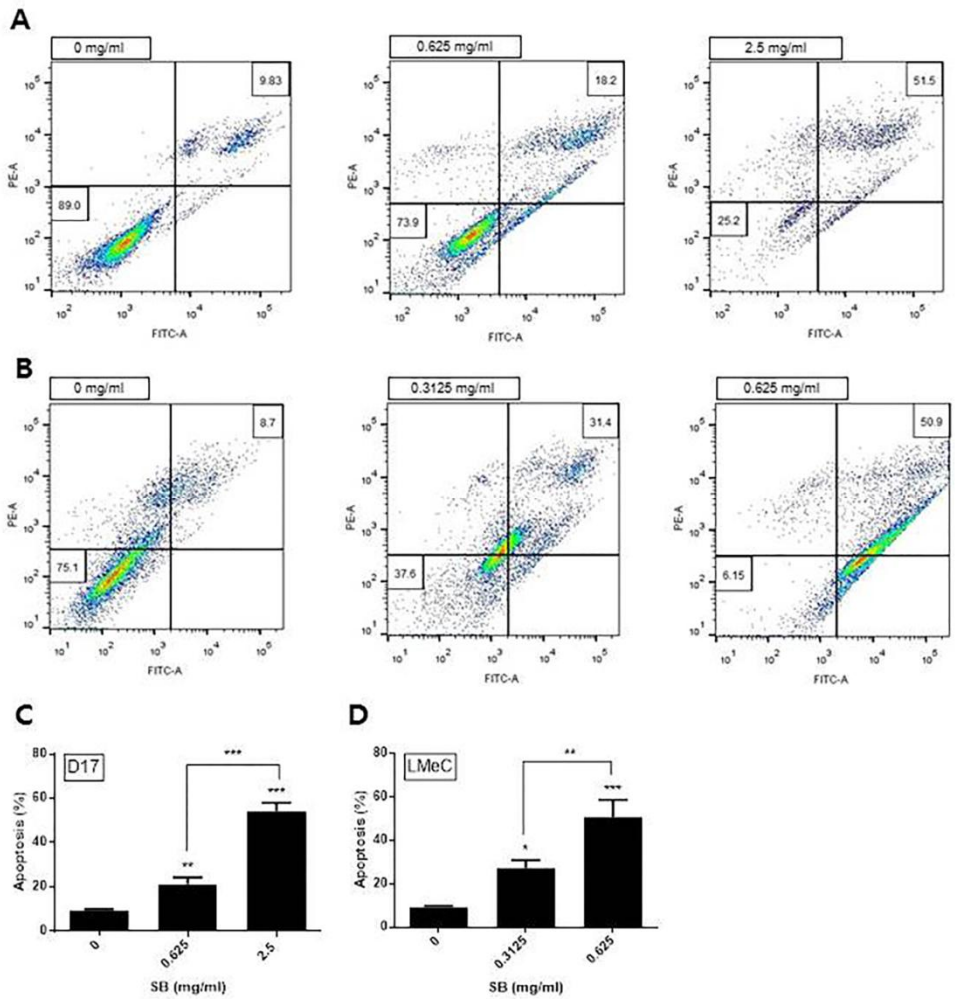

Figure 6. D17 and LMeC cells treated with SB incubated for 24 h and stained with PI and annexin V.

(A) Apoptosis of D17 cells treated with 0, 0.625, 2.5 mg/mL of SB. At 0.625 mg/mL of SB, the cell membrane of the D17 cells was damaged resulting in 18.2% apoptosis. When treated with 2.5 mg/mL SB, 51.5% apoptosis was

induced, which was remarkably higher than that at lower concentrations. (B) Apoptosis of LMeC treated with 0, 0.3125, 0.625 mg/mL SB. As LMeC was more sensitive to SB treatment, 31.4% apoptosis was induced by treatment with 0.3125 mg/mL SB and 0.625 mg/mL SB induced 50.9% apoptosis. (C) The rate of apoptosis of D17 after 24 h of treatment with SB. The apoptosis rate increased with increased concentrations (0.625 and 2.5 mg/mL). (D) The apoptosis rate of LMeC after 24 h treatment with SB. The apoptosis rate increased with increasing concentrations of SB (0.3125 and 0.625 mg/mL). All experiments were independently conducted in triplicate. Data are represented as mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

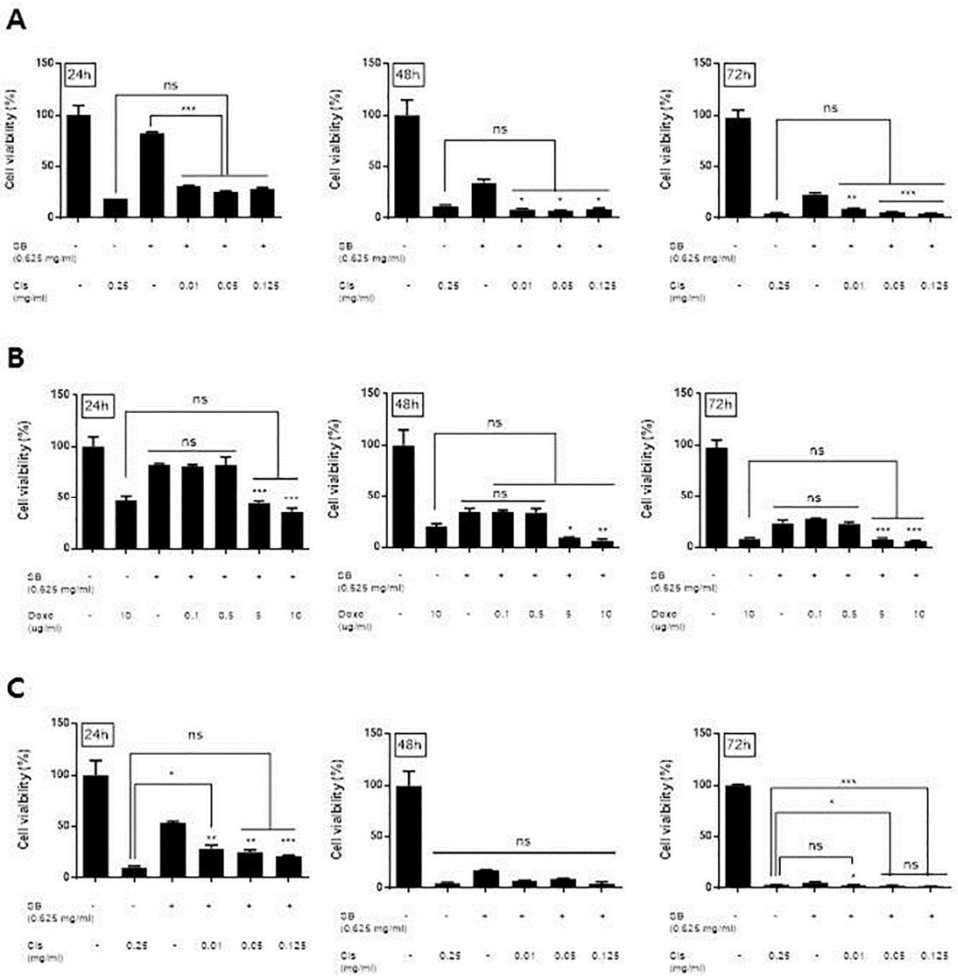

Figure 7. MTT assay for combination of drugs.

(A) MTT assay of D17 treated with a combination of SB and cisplatin. D17 cells showed no significant difference in cell viability at 0.25 mg/mL compared with the combination of 0.625 mg/mL SB and 0.01 mg/mL cisplatin after 24 h. (B) MTT assay of D17 cells treated with a combination of SB and doxorubicin. D17 cells showed similar cell viability for 10 μg/mL

doxorubicin compared with the combination of 0.625 mg/mL SB and 5 µg/mL doxorubicin at 24 and 72 h. In addition, D17 had similar cell viability for 10 µg/mL doxorubicin compared with the combination of 0.625 mg/mL SB and 0.1 µg/mL doxorubicin. (C) MTT assay of LMeC treated with a combination of SB and cisplatin. The viability of LMeC treated with 0.25 mg/mL cisplatin was similar to that of the combination of 0.625 mg/mL SB and 0.05 mg/mL cisplatin at 24 h. After 48 h, the viability for cells treated with 0.25 mg/mL cisplatin had no difference with that of the combination of 0.625 mg/mL SB and 0.01 mg/mL cisplatin. After 72 h, the viability of cells treated with the combination of 0.625 mg/mL SB and 0.05 mg/mL cisplatin was significantly lower than that of the cells treated with 0.25 mg/mL SB alone. The significance of the above-mentioned data was compared with that of the cells treated with 0.625 mg/ml SB only. All experiments were independently conducted in triplicate. Data are represented as mean ± standard deviation. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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국문 초록

개의 골육종 및 흑색종 세포주에서의 자연 추출 약물 SB Injection의 항암 효과

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권 경

이 연구의 목적은 자연 추출물 항암제제인 SB 주사제의 개의 악성 종양 세포에 대한 효과를 평가하는 것이다. D17(개의 골육종 세포주)와 LMeC(개의 흑색종 세포주)에 대한 SB 주사제의 항암 효과는 MTT assay, 세포 주기 분석 그리고 Annexin-V assay를 통하여 결정되었다. 세포

의 생존률은 항암제의 농도에 반비례하였다. 항암제에 영향을 받은 세포의 세포 주기는 G2/M기에 멈춤으로써 세포증식 억제 효과를 보였다. SB는 농도 의존적으로 세포 자가사멸율이 증가하였다. 이는 또한 다른 화학적 항암제제와 혼합하였을 때 그 농도를 감소시켰다. SB는 말초 혈액 단핵구에 대해서는 농도와 관계 없이 세포 생존율에 영향을 주지 않는 결과를 보여, SB가 정상 혈액 세포의 활성을 억제하지 않는다는 것을 제시하였다. 이상의 결과들은 SB의 개의 악성 골육종 및 흑색종에 대한 증식 억제 효과를 나타내고 있다. 이는 수의학의 암환자 치료에 있어 대체적인 치료 방법 연구를 위하여 시행되었으며 그 실험실적 효과 가치를 증명하였다. 이에 대한 치료적 도입을 위하여 다양한 개의 악성세포에 대한 추가 연구를 통해 약물의 활용 범위를 넓히고 실제 임상에서의 효과 및 부작용에 대한 데이터 축적이 필요할 것으로 보인다.

주요어: SB 주사제; 천연약물 재료; 항암제; 개의 골육종; 개의 흑색종

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