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Research article

Anticancer effect of joboksansam, Korean wild ginseng germinated from bird feces

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ABSTRACT

Background: Joboksansam, Korean bird wild ginseng, is an artificially cultivated wild ginseng germinated from bird feces. Although numerous pharmacologic activities of wild ginsengs have been reported, the beneficial effect of joboksansam in cancer has not been elucidated. In this study, we investigated the *in vivo* and *in vitro* anticancer activities of joboksansam powder.

Methods: To evaluate the *in vivo* anticancer activity of joboksansam, we established a xenograft mouse model bearing RMA cell-derived cancer. Direct cytotoxicity induced by joboksansam powder was also investigated *in vitro* using (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The inhibitory activity of this powder on the activation of cell survival signaling involving Akt and Src was examined with immunoblot analysis.

Results: Joboksansam powder displayed strong inhibitory activity against the increased tumor size, increased weight of total body and cancer tissues, and mortality of tumor-bearing mice. Joboksansam powder also suppressed the activation of survival regulatory enzymes Akt and Src, as assessed by phosphorylation levels in the immunoblot analysis of tumor tissues. Interestingly, the viability of RMA cells *in vitro* was directly decreased by joboksansam treatment.

Conclusion: Overall, our results strongly suggest that joboksansam powder has the potential to protect against cancer generation by direct cytotoxic effects on cancer cells resulting from suppression of cell survival signaling.

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1. Introduction

The root of Korean ginseng (*Panax ginseng* Meyer) has been used for 20 centuries in Korea, China, and Japan, and its beneficial activities, including antioxidative, anti-inflammatory, antidiabetic, antiobesity, and anticancer effects, have been widely demonstrated [1,2]. Recent systemic studies have improved our understanding of the pharmacologic mechanisms and active components in *P. ginseng* roots. The efficacy value of wild ginseng was indicated to be higher than that of cultivated ginseng, although the production

rate of wild ginseng was much lower. To increase the production rate of wild ginseng under cultivated conditions, we recently developed new cultivation methods for wild ginseng and obtained increased germination rates by preparing digested seeds in the feces of birds that eat ginseng berries, as in the natural process of the wild ginseng life cycle. Under these conditions, joboksansam displayed higher levels of germination than another artificially cultivated wild ginseng, sanyangsansam [3]. However, the pharmacologic value of joboksansam has not been fully elucidated, even though cultivation of this ginseng is very similar to the natural

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product. In this study, our aim was to test the pharmacologic activity of joboksansam by examining its anticancer activity in a xenograft mouse model bearing RMA (murine T cell lymphoma) cell-derived cancer.

2. Materials and methods

2.1. Materials

Joboksansam (10 yr old) was cultivated in Namyangju (Kyunggi, Korea). Sodium carboxymethylcellulose (Na CMC), dimethyl sulfoxide (DMSO), and (3-4-5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Standard ginsenosides were purchased from Ambo Institute (Daejeon, Korea). Fetal bovine serum and RPMI 1640 were purchased from Gibco (Grand Island, NY, USA). RMA cells used in the present experiments were obtained from the American Type Culture Collection (Rockville, MD, USA). All other chemicals were from Sigma. Total or phospho-specific antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Plasmid constructs containing Src, AKT, and β -actin were used as reported previously [4–6].

2.2. Preparation of joboksansam powder

An ultrafine air mill (Turbo Mill, HKP-05; Korea Energy Technology, Seoul, Korea) was used to obtain ultrafine powder as reported previously [7,8]. The coarse powders prepared previously were pulverized into ultrafine powder particles with sizes in the range of 0.1–50 μ m in a milling chamber with a hot jacket (180°C). Feeding rate and circumferential velocity of the impeller in the grinding zone were fixed at 3 kg/h and 100 m/s, respectively. Simultaneously, a centrifugal air classification system separated the powders according to particle size. The ultrafine Joboksansam powder obtained was stored in a desiccator until use.

2.3. Cell culture

RAW264.7 and HEK293 cells were cultured in RPMI 1640 and Dulbecco's modified Eagle's medium, respectively, supplemented with 10% heat-inactivated fetal bovine serum and 1% antibiotics (penicillin and streptomycin) at 37°C under 5% CO₂ [9].

2.4. Drug treatment

For *in vivo* experiments, joboksansam powder was suspended with 0.5% Na CMC. For *in vitro* cytotoxicity tests, joboksansam powder was suspended in 100% DMSO at a concentration of 100 mg/mL, and the solution was filtered prior to dilution with culture medium as reported previously [10,11].

2.5. Xenograft mouse model experiments

All animal experiments were carried out in accordance with the National Research Council's Guidelines (IACUC, Korea) for the Care and Use of Laboratory Animals. The experimental protocol was approved by the Animal Experiments Committee of Sungkyunkwan University. C57BL/6 mice (female, 5 wk old; Orient-bio, Sungnam, Republic of Korea) were used as our xenograft animal model. Mice were housed individually on a 12-h day/12-h night cycle at 23–27°C and had access to food and water. Mice were randomly divided into two groups ($n = 14$ /group): (1) a vehicle control group ($n = 10$), wherein animals received oral administration of 0.5% Na CMC; (2) a joboksansam powder treatment group ($n = 14$), wherein animals received oral administration of joboksansam powder

(400 mg/kg). To produce tumors, each mouse was implanted with RMA cells (1×10^6 cells per animal) subcutaneously in the back next to the right hind leg. Joboksansam powder or vehicle was administered orally from Day 1 to Day 14. On the indicated days, the tumors were identified and measured with a standard caliper. Tumor volume was calculated as follows: tumor volume (mm^3) = [tumor length (mm) \times tumor width (mm)²]/2. Mice were sacrificed at Day 33.

2.6. Cell cytotoxicity

The cytotoxicity of joboksansam powder against RMA cells was evaluated using a conventional MTT assay as previously described [12]. Briefly, the cells were plated in 96-well plates at a density of 5×10^4 cells/well and treated with different concentrations of joboksansam powder (0, 200, 400, and 800 μ g/mL) for 24 h. Absorbance was measured at 540 nm using a microplate reader.

2.7. Immunoblot analysis of total lysate from tumor

Total lysates prepared from cancer tissue were subjected to Western blot analysis of total or phospho-forms of Src and AKT as reported previously [13]. Total or phosphorylated forms of Akt, Src, and β -actin were visualized as described previously [14].

2.8. High-performance liquid chromatography analysis

For determination of ginsenosides in joboksansam powder, high-performance liquid chromatography (HPLC) was conducted as stated previously. The exact conditions of HPLC are described in Table 1.

2.9. Statistical analysis

All data presented in this paper are the mean \pm standard deviation of an experiment performed with 14 (Fig. 1) or three (Figs. 2–4) replicates. For statistical comparisons, the results were analyzed using analysis of variance/Scheffe *post hoc* test and Kruskal–Wallis/Mann–Whitney tests. A $p < 0.05$ was considered statistically significant. All statistical tests were carried out using the computer program SPSS (Version 22.0, 2013, IBM Corp., Armonk, NY, USA).

Table 1

Instrument and working conditions for ginsenoside analysis by high-performance liquid chromatography (HPLC)

Instrument		Shimadzu LC-10AT HPLC system	
Column		YMC AM303, 4.6 \times 250 mm	
Detector		UV/VIS detector (203 nm)	
Solvent A		H ₂ O	
Solvent B		Acetonitrile	
Injection volume		20 μ L	
Flow rate		1 mL/min	
Gradient elution system		%A	%B
Time (min)		0	80
		30	20
		35	80
		60	65
		65	45
		65	20
		70	80

UV/VIS, Ultraviolet/visible.

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