Inhibitory effects of HangAmDan–B1 (HAD-B1) on A549 lung cancer cell proliferation and tumor growth in a xenograft model

ABSTRACT

HangAmDan-B1 (HAD-B1) was derived from HAD, which consists of six different herbal extracts. Our previous studies demonstrated the anti-angiogenic and anti-metastatic effects of HAD through in vitro cell-based assays and an in vivo animal model; therefore, HAD-B1 was expected to reveal anti-tumor function. In this study, the anti-tumor effects of HAD-B1 on solid tumor growth in nude mice bearing an A549 human lung cancer xenograft were investigated. HAD-B1 significantly inhibited A549 cell proliferation in a dose-dependent manner; however, there was no toxicity in NIH-3T3 normal fibroblast cells. Antibody microarray-based proteomic analysis demonstrated that the anti-proliferative effect of HAD-B1 on A549 cells was caused by down-regulation of STAT3 in the cells. HAD-B1 also markedly suppressed solid tumor growth as compared with vehicle- or cisplatin-treated control groups in an A549 cell xenograft mouse model, while there were no specific differences in the body weight and blood biochemical test. Taken together, these results suggest that HAD-B1 can be an anti-cancer agent without any toxicity.

Key words: A549 lung cancer cells, anti-cancer effect, HAD-B1, antibody microarray, tumor xenograft.

INTRODUCTION

HAD-B1 is composed of four materials (Panax notoginseng Radix, Cordyceps militaris, Panax ginseng C.A.Mey, and Boswellia carerii BIRDWOOD). HAD-B1 derived from HAD-B (Bang et al., 2000) was developed through multiple screening researches to fortify anti-cancer effects on lung cancer cell lines (Bang et al., 2010, 2011). HAD-B has since been prescribed for various cancer patients at the East West Cancer Center (EWCC), Dunsan Korean Medicine Hospital, Daejeon University, Korea (Kim et al., 2011, 2009). The results of several studies supported the efficacy of HAD-B in immune function, anti-angiogenesis and inhibition of cancer metastasis (Bang et al., 2011; Choi et al., 2011).

The anti-angiogenic function of HAD-B was caused by increases in the expression of SIRT1 and Rb2 proteins in HUVECs induced by basic fibroblast growth factor (bFGF) (Bang et al, 2010). HAD-B has also been shown to be safe through a toxicological study by Park et al. (2008) on HAD-B1 developed to overcome the problems of HAD-B such as high cost and high dosage required for long-term use can contribute to the reduction of the burdens of patients and enriched products. Because it has been manufactured using a new method, it is necessary to confirm the biological functions of HAD-B1. In this study, the anti-tumor function of HAD-B1 using an in vitro lung tumor cell-based proliferation assay and in vivo xenograft animal model was demonstrated.

MATERIALS AND METHODS

Preparation of HAD-B1

HAD-B1 was provided by the East West Cancer Center
Figure 1. HPLC profile of major components in HAD-B1. Water extract (1 mg/ml) of HAD-B1 was applied to a C18 column for HPLC and eluted by acetonitrile mixed with DW.

(EWCC), Dunsan Korean Medicine Hospital, Daejeon University, Daejeon, Korea. A voucher specimen (#HAD-B1-2014-10-HS) was deposited at the Institute of Traditional Medicine and Bioscience in Daejeon University. As shown in Figure 1A, the ingredients of HAD-B1 were soaked for 18 h at 60°C in a soaking bath. The fluid extracts were then dried twice using a rotary vacuum evaporator and a flat evaporator at 60°C, which produced powder (recovery ratio: 27.3%) that was used in the experiment. The HAD-B1 was dissolved in distilled water (DW).

**HPLC analysis of HAD-B1**

The HAD-B1 stock was prepared by extracting HAD-B1 powder with 1 mg of powder in 10 ml of DW at room temperature. The extract was then centrifuged at 1000 × g for 30 min and filtered and applied to the C18 column and eluted using acetonitrile mixed with DW. Figure 1B shows the results of HPLC of HAD-B1 fractions.

**Cell culture**

A549 Human lung cancer cells were cultured in Dulbeco’s Modified Eagle’s Medium (DMEM) containing 10% FBS and 1× antibiotics (Welgene, Daejeon, Korea). A549 cells were maintained routinely at 37°C under 5% CO₂.

**In vitro A549 cell proliferation assay**

A549 cells (2×10³ cells/well) were added to 96-well tissue culture plates coated with gelatin and allowed to adhere overnight. The cells were treated with HAD-B1 for 72 h, after which 10 µl of 5 mg/ml MTT solution was added to each well and the cells were incubated for 2 h at 37°C.
After the supernatants had been discarded, residual formazan crystals were dissolved in 100 μl of dimethyl sulfoxide (DMSO). The absorbance was measured at 595 nm on an enzyme-linked immunosorbent assay (ELISA) plate reader (Emax, Molecular Devices, USA), and all measurements were made in triplicate (Bang et al., 2010).

**In vivo tumor growth assay in Xenograft animal model of human A549 lung cancer cells**

A549 cells were injected subcutaneously to generate a Xenograft model in mice. Once the tumor size reached 100 mm³, HAD-B1 was administered orally once daily for 25 days, during which time the tumor size and body weight were checked daily. Blood biochemical tests were carried out using an automatic biochemical analyzer (HiTACHI, Tokyo, Japan). All care and handling of the animals was performed according to the Guide for the Care and Use of Laboratory Animals (HTRC-15-17(1)).

**Proteomic profiling in A549 cells treated with HAD-B1**

A549 were serum-starved by incubation in DMEM for 4 h. The cells were treated with or without HAD-B1. After 72 h of incubation, the cells were washed twice with phosphate buffer saline (PBS) and harvested in 5 mM ethylenediaminetetraacetic acid-PBS (EDTA-PBS). The cells were then centrifuged for 15 min at 1,800 rpm, after which the pellets were washed with PBS and re-centrifuged. A549 pellets were then extracted with Lysis-M™ (Roche, Germany) mammalian cell extraction buffer. Each protein extract (100 μg) was labeled with both cyanine 3 (Cy3) and cyanine 5 (Cy5) (GE Healthcare, UK) according to the manufacturer’s instructions. Free dyes were removed using Sigma Spin columns (S5059, Sigma, USA) and purified samples were stored at 4°C until use. Fluorescence-labeled cell lysates were applied to a prefabricated antibody micro-array (Bang et al., 2010, 2011) containing 48 distinct antibodies against proteins involved in cell proliferation (Hypromatrix, Worcester, MA, USA) and incubated for 1 h at 37°C in the dark. The slides were washed thrice with PBST, N₂-dried and analyzed using a fluorescence micro-array scanner. The antibody array slides were scanned using a GenePix 4100A microarray scanner (Axon Instruments, Union City, CA) with 532 and 635 nm lasers and image analysis of each spot was prepared using the manufacturer’s software package (GenePix 6.0, Axon Instruments). The internally normalized ratios (INRs) of all spots were calculated as previously described (Fumagalli et al., 1994).

**Immunoblot analysis**

Cells were harvested to extract proteins. The cell lysates were made in Lysis-M™ buffer (Roche, Germany) containing protease and phosphatase inhibitor cocktails (Roche, Germany), then clarified by centrifugation. Lysates containing 50 μg of protein were loaded into each well and separated through 12% SDS-gel electrophoresis. Gels were then soaked in buffer (16 mM Tris-HCl, 30 mM glycine, and 20% methanol), after which the proteins were transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked with 5% nonfat dry milk in PBST (137 mM NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 20 mM KH₂PO₄, 0.05% Tween 20, pH 7.4). The polyvinylidene difluoride membranes were then incubated with primary antibodies against signal transducer and activator of transcription 3 (STAT3) (1:10,000) and β-actin (1:8000) in phosphate-buffered saline/0.1% Tween 20 (PBST) containing 5% nonfat dry milk at 4°C overnight. Membranes were washed with PBST and incubated with secondary antibodies (anti-mouse-1:8000, anti-rabbit-1:8000). Finally, signals were developed using an ECL Western blotting detection kit and exposed to x-ray films (Bang et al., 2010).

**RESULTS**

**Inhibition of A549 cell proliferation in vitro by HAD-B1**

To examine the effects of HAD-B1 on lung tumors, we conducted a cell-based proliferation assay using A549 lung tumor cells. The results revealed that HAD-B1 inhibited the proliferation of A549 cells in a dose-dependent manner. Half-maximal inhibition of A549 cell proliferation by HAD-B1 and HAD-B was observed at a concentration of 108.27 ± 29.12 μg/ml and 215.73 ± 81.27 μg/ml, respectively (Figure 2A) (p<0.05, 0.01). HAD-B1 showed no toxicity toward normal fibroblast cells (NIH3T3 cells), although, HAD-B appeared to be more effective than HAD-B at suppressing A549 lung tumor cell proliferation without any toxicity against normal cells.

**Profiling of expression proteins in HAD-B1 treated A549 cells using antibody microarray**

The goal of this study was to investigate the molecular mechanism of the anti-proliferative effects of HAD-B1 on A549 lung tumor cells through proteomic analysis of cellular signaling proteins in the cells. To assess the expression pattern of endogenous signaling proteins in A549 treated with HAD-B1, a protein chip-based antibody microarray was employed. To conduct antibody microarray analysis, A549 cell lysates treated with either distilled water or HAD-B1 labeled with Cy3 or Cy5. The labeled lysates were mixed and spotted onto the antibody microarray, after which the fluorescence intensity of each
Figure 2. Inhibitory effects of HAD-B1 on the proliferation of A549 lung cancer cells. A549 cells (A) and NIH-3T3 fibroblast cells (B) were incubated with different concentrations of HAD-B1 for 72 h. After incubation, unbound cells were discarded with PBS and the remaining cells incubated for 2 h with 0.5 mg/ml MTT solution. Absorbance was measured at 595 nm using an ELISA reader. Tarceva and cisplatin were used as positive controls. Data shown are the means ± SEM and statistical analysis was performed using the Student’s t-test (n = 3) (* P<0.05 and ** P<0.01 versus control).

spot was measured and the differential protein expression pattern between both samples determined (Figure 3). Changed protein spots were then identified using graded virtual images showing the Cy5:Cy3 ratios of the tested spots and the normalized median values of the calculated Cy5:Cy3 ratios of the proteins (Figure 3A to C). The results revealed that HAD-B1 inhibited expression of STAT3 protein was confirmed by immunoblot analysis (Figure 3D).

In vivo inhibitory effect of tumor growth on A549 xenograft model

To further confirm the anti-lung cancer effect of HAD-B1 in vivo, we conducted a solid tumor growth assay using an A549 cell-xenograft mouse model. Briefly, $1.0 \times 10^6$ human lung cancer A549 cells were injected into the skin of nude mice and grown to a mass of approximately $100 \text{ mm}^3$. The tumor growth was notably inhibited in a dose dependent
Figure 3. Differential expression profiling of cell cycle proteins in A549 cells treated with HAD-B1 using the antibody microarray chip. (A) Graded virtual image of Cy5: Cy3 ratios from A549 cells treated with HAD-B1; (B) Map of the antibodies on ProteoChip; (C) Graphical representation of Cy5: Cy3 ratios on the antibody arrays in HAD-B1-treated A549 cells and (D) Validation of antibody microarray data by Western blot analysis.

manner when HAD-B1 was orally administered once daily to the mice (Figure 4A). Moreover, the mean tumor volume in the group treated with HAD-B1 (400 mg/kg) showed a greater reduction than those of the control groups treated with vehicle, Cisplatin (5 mg/kg), throughout the experiment, while the tumor volumes were nearly identical in the Tarceva (50 mg/kg) group and the HAD-B1 400 mg/kg treatment group (Figure 4A). The HAD-B1 treated group showed no changes in body weight or biochemical blood analysis (Figure 4B and C).

DISCUSSION

HAD-B1, which is the upgraded anti-cancer prescription of HAD-B, consists of four critical herbs that have positive effects on lung cancer. HAD-B1 differs from HAD-B in terms of production, extraction and enrichment methods. Previous reports demonstrated that HAD-B was effective against various cancers, particularly lung cancer (Kim et al., 2009; Park et al., 2010; Zheng et al., 2011). Against this backdrop, we developed HAD-B1, which is focused on lung
cancer treatment and examined its anti lung cancer effects in vitro and in vivo. The dose-dependent inhibition of A549 lung cancer cell proliferation by HAD-B1 was similar to that of HAD-B, indicating that these two compounds had similar anti-tumor activity despite differences in constituents.

Proteomic analysis of A549 cells treated with HAD-B1 revealed molecular mechanisms of anti-lung cancer activity of HAD-B1 with decreasing STAT3 protein expression. STAT3 is a member of a family of seven STAT proteins identified and designated STAT1, -2, -3, -4, -5a, -5b, -6 in mammals (Johnston and Grandis, 2011; Turkson and Jove, 2000; Xiong et al., 2014) that plays a central role in carcinogenesis by regulating the transcription of multiple key genes, including those involved in proliferation, differentiation, apoptosis, angiogenesis,
immune responses and metastasis (Johnston and Grandis, 2011; Xiong et al., 2014).

STAT3 degradation induced cell cycle arrest and enhanced cellular apoptosis (Johnston and Grandis, 2011). Taken together, our data demonstrated that the anti-proliferative activity of HAD-B1 in A549 cells was due to down-regulation of STAT3 followed by induction of cell apoptosis. The tumor growth experiment using A549 tumor xenograft mice confirmed the anti-lung cancer effect of HAD-B1. The growth of solid tumor in the HAD-B1 treated group was remarkably suppressed in a dose-dependent manner relative to the control groups. Tumor-bearing mice usually lose their weight due to cachexia; however, we found that HAD-B1 treated mice gained weight relative to the control groups.

Biochemical blood analyses of ALT, AST, ALP, BUN and CRE levels did not reveal any significant changes relative to the HAD-B1 treated group. These results demonstrated that HAD-B1 has potential for use as an inhibitor of solid tumor growth caused by A549 lung cancer cells without any side effects. Collectively, our data suggest that HAD-B1 could be an effective and safe drug for treatment of lung cancer.

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REFERENCES


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