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Rh2-enriched Korean ginseng (Ginseng Rh2+) inhibits tumor growth and development of metastasis of non-small cell lung cancer

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Background and objective: While there are multiple studies on the anti-tumoral effects of *Panax ginseng* as active ingredients (one or more ginsenosides derived from the extract) or as a whole plant extract, there is a lack of studies to assess the effects *Panax ginseng's* of active ingredients combined with the whole plant extract. Our aim was to study the effect of whole ginseng, enriched in the anti-tumoral Rh2 component and other ginsenosides (Ginseng Rh2+), on the metastatic capacity of non-small cell lung cancer (NSCLC). *Methods*: We evaluated the effects of Ginseng Rh2+ on survival, migration and motility, induction of apoptosis, and expression of its apoptosis-related proteins in non-small cell lung cancer (NSCLC) cells *in vitro* and on primary tumor growth and metastatic capacity in a syngeneic mouse lung cancer model *in vivo*. The effects of Ginseng Rh2+ on NSCLC cells were studied *in vitro* using: a colorimetric tetrazolium salt (XTT) assay, annexin V-FITC/PI, western blotting, wound healing motility assay, Transwell migration and cell adhesion assays. *In vivo*, mice were inoculated with Lewis mouse lung carcinoma cells subcutaneously to evaluate local tumor growth, or intravenously to evaluate the effects of Ginseng Rh2+ on development of experimental metastases. Mice were treated by intraperitoneal administration of Ginseng Rh2+ (0.005–0.5 g kg^{-1}) on days 6, 10, and 14 after tumor injection. *Results*: We found that Ginseng Rh2+ increased the apoptosis of NSCLC cells *in vitro*, demonstrating dose dependent down-regulation of the Bcl-2 anti-apoptotic gene and concurrent up-regulation of the Bax pro-apoptotic gene. Ginseng Rh2+ inhibited the tumor cells' capacity to attach to the ECM-related matrix and reduced cell migration. *In vivo*, Ginseng Rh2+ inhibited local tumor growth and reduced the development of experimental lung metastases. *Conclusion*: Our study suggests that Ginseng Rh2+ may potentially be used as a therapeutic agent for treatment of NSCLC.

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1. Introduction

Lung cancer is the leading cause of cancer related death worldwide.[1] Various therapies have improved the prognosis of lung cancer, yet the vast majority of non-small cell lung cancer (NSCLC) patients ultimately succumb to their disease.

Botanical medicines have recently been assessed as an adjuvant treatment for NSCLC[2,3] with ginseng showing promising anti-cancer activities.[4–6] Ginseng is widely used as a botanical medicine in East Asian countries, including Korea, China, and Japan. The ginseng plant is a deciduous perennial that belongs to the Araliaceae family. The various ginseng species belonging to the *Panax* genus, that are found only in the northern hemisphere and include the Asian ginseng (*Panax ginseng* C.A Meyer), the American ginseng (*Panax quinquefolius*), and the Japanese ginseng (*Panax japonicus* C A Meyer). They are well-characterized, and have been quantitatively and qualitatively characterized by broad biochemical profiling.[6]

The major active components of *Panax ginseng* are ginsenosides with more than 40 ginsenosides isolated. *Panax ginseng* and its ginsenosides have been shown to possess anti-cancer properties against lung cancer *in vitro*[5–7] and *in vivo*.[8,9] Their antitumor activity is related to various mechanisms including the inhibition of the nuclear translocation of NF-KB, induction of apoptosis through mitochondrial signaling pathways, and autophagy.[10–12] The Rh2 Ginsenoside, found in the red ginseng, has been shown to exert potent inhibition of cell proliferation, apoptosis, and resistance to chemotherapy.[13–17] In

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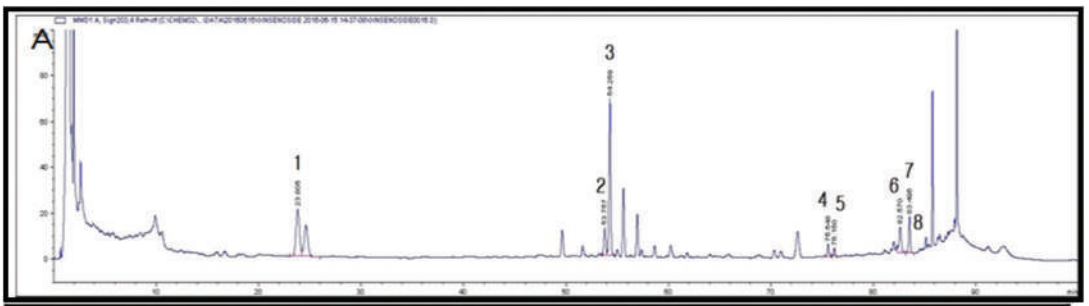
addition, Ginsenoside Rh2, as a single reagent, was previously shown to impede cell adhesion and to inhibit vessel formation.[18,19] To explore the properties of Rh2, we have developed an Rh2+ enhanced red ginseng extract, named hence, 'ginsenoside Rh2+'. It contains approximately 3.4 mg g^{-1} of Rh2 (S + R), considerably higher than the original red ginseng (Fig. 1).

The US FDA has issued the "Guidance for Botanical Drug Development" to support and facilitate the development of new standardized therapies from botanical sources.[20] This initiative is mainly based on the concept of botanical synergy ("entourage effect"), according to which botanical drugs (which usually contain plant toxins or protective substances) are often more efficacious than their isolated components,[21] similar to their use by the plant. For instance, in the case of cannabis, cannabidiol (CBD) or tetrahydrocannabinol (THC) alone was less effective in the treatment of pain, inflammation, or cancer palliation than the use of these molecules in the context of a whole plant extract.[22,23]

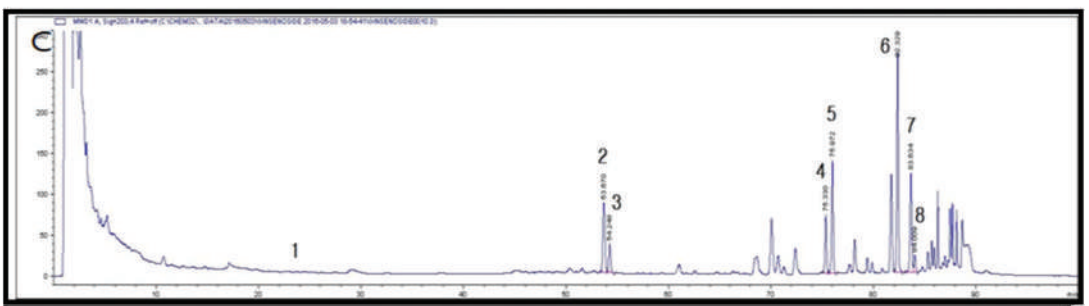
With the FDA-drafted initiative in mind we have developed a standardized Rh2-enriched Korean ginseng (Ginseng Rh2+) extract, offering both the entourage effect of whole ginseng, meanwhile enriching the anti-tumoral Rh2 component, which



B

G&V Ginseng Analysis (mg/g)

	Ginsenoside contents			Ginsenoside contents		Remarks
1	Rg1	3.27	5	Rg3(R)	0.20	This data was analyzed by G&V company
2	Rh1	0.96	6	Compound K	0.83	
3	Rb1	5.85	7	Rh2(S)	0.82	
4	Rg3(S)	0.32	8	Rh2(R)	0.00	



D

G&V Ginseng Analysis (mg/g)

	Ginsenoside contents			Ginsenoside contents		Remarks
1	Rg1	0.00	5	Rg3(R)	3.71	This data was analyzed by G&V company
2	Rh1	3.20	6	Compound K	5.89	
3	Rb1	1.50	7	Rh2(S)	2.95	
4	Rg3(S)	2.35	8	Rh2(R)	0.45	

Fig. 1 HPLC chromatograms of Red ginseng extract and Rh+ extract. (A) HPLC analysis of Red ginseng was performed using an Agilent 1200HPLC UV-VIS (MWD) detector by G&V Co. (Kangwon Do, Korea). (B) Main ginsenoside contents (mg g^{-1}): 1—Rg1, 2—Rh1, 3—Rb1, 4—Rg3(S), 5—Rg3(R), 6—Compound K, 7—Rh2(S), 8—Rh2(R). (C) HPLC analysis of Ginseng Rh2+ was performed using an Agilent 1200HPLC UV-VIS (MWD) detector by G&V Co. (Kangwon Do, Korea). (D) Main ginsenoside contents (mg g^{-1}): 1—Rg1, 2—Rh1, 3—Rb1, 4—Rg3(S), 5—Rg3(R), 6—Compound K, 7—Rh2(S), 8—Rh2(R).

is found only in trace amounts, by more than 100-fold, and other ginsenosides (detailed in Fig. 1). While there are multiple studies on the anti-tumoral effects of *Panax ginseng* as an active ingredient/s (different ginsenosides) or as a whole plant extract, there is a lack of studies to assess the effect of active ingredients (very rich in ginsenosides) combined with whole plant extract. Moreover, the majority of the studies have assessed the anti-cancer effects of ginseng on NSCLC cells *in vitro*, while there is a scarcity of studies evaluating the capacity of ginseng to inhibit the development of metastases in lung tumor models *in vivo*.

In this study we evaluated the anti-tumoral effect of Ginseng Rh2+ both *in vitro* on mouse and human NSCLC cells and *in vivo* using an experimental lung metastasis cancer model. We then investigated the biological processes which underlie the effects of Ginseng Rh2+ on tumor cells.

2. Methods

2.1. Cell lines and culture

Human NSCLC cells (H1299, H23, H520) and mouse Lewis lung cancer cells (3LL) were obtained from the American Type Culture Collection (Rockville, MD). The cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), antibiotics, glutamine, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate (all from Biological Industries, Beit HaEmeq, Israel). The cells were incubated at 37 °C with 5% CO_2.

2.2. Preparation of modified Korean ginseng extract (Rh2-enriched Korean ginseng)

Ginseng Rh2+ is a modified regular ginseng extract containing a number of reinforced ginsenosides from ginseng butanolic extract (GBX) following enzymatic treatments aiming to augment its anti-cancer effect. It was obtained from G&V Co. (Kangwon Do, Korea) and prepared as previously described.[24] Briefly, the roots of regular ginseng (4 years old) were purchased from the National Agricultural Cooperative Foundation (Chuncheongnam-do, Korea; Herbarium voucher number-2002-5761). A total of 20 g of pulverized ginseng roots were suspended in 380 ml of distilled water and sterilized at 121 °C for 15 min. The extract was fractionated by extraction with water, methanol and butanol. Sterilized laminarinase and pectinase (1 : 1, specific activity units) were added to the suspension, which was incubated at 40 °C for 2 days, after which it was evaporated to powder at 60 °C. The enzyme-modified ginseng powder was suspended in 400 ml of 80% (v/v) methanol. The suspension was sonicated and filtered through Whatman no. 2 filter paper (Whatman International Ltd, Maidstone, UK). The filtrates were combined and evaporated to dryness at 50 °C. The extract was dissolved in 200 ml distilled water, separated by a funnel and then extracted with 200 ml of butanol. The extract was dissolved to a concentration of 10% (w/v) in 70% ethanol. HPLC analysis of Rh2-enriched Korean ginseng has been performed by G&V Co. Kangwon Do, Korea using an Agilent 1200HPLC UV-VIS (MWD) detector. A comparison of the quantity of Rh2 including other ginsenosides (Rb1, Rg3, Rh1, and compound K) between red ginseng and Ginseng Rh2+ was carried out using an Acquity UPLC system (Waters, Milford, MA) with an Acquity BEH C18 high-performance liquid chromatography (HPLC) column as was described before. The concentrations of the prominent ginsenosides found in Ginseng Rh2+ are presented in Fig. 1.

2.3. Colorimetric XTT assay for cell survival *in vitro*

Cell survival was evaluated using an XTT assay (Biological Industries, Beit HaEmeq, Israel) described previously.[25] Briefly, the cells growing in 96-well microplates ($1.5–2 \times 10^3$ cells per well) were exposed to different concentrations of Ginseng Rh2+ for 72 h. A freshly prepared mixture of XTT (Colorimetric tetrazolium salt) and an activation reagent (peroxymonosulfate) was added to each well. Following 2 h of incubation at 37 °C, the plates were shaken and placed in a microwell plate spectrophotometer (plate reader, Sunrise, Switzerland). The optical density of the dye was read at 450 nm. Each plate included blank wells containing only media and XTT. The control wells contained non-treated cells and fresh medium. The experiments were performed in triplicate and repeated at least 3 times.

2.4. Wound healing assay for cell motility

Tumor cells were grown to confluence in 24-well microplates. A scratch in the monolayer was made using a sterile 1 ml pipette tip. The cells were then grown in full growth medium and Ginseng Rh2+ at different concentrations (0, 30, 60, 120 μg ml^{-1}). The width of the wounds was measured at 24 h using an ocular micrometer at ×40 magnification of an Olympus microscope. The scratch width in the non-treated cultures was used as the negative control. Differences between each group and the control group were considered statistically significant if the p value <0.05 ($*p < 0.05$ and $**p < 0.01$).

2.5. Transwell assay for cell migration

Tumor cell migration was measured by a Boyden chambers Transwell assay using inserts containing membranes with an 8.0 μm pore according to the manufacturer's recommendation (Greiner Bio-One GmbH, Germany). The lower Transwell chambers were filled with DMEM supplemented with 10% FCS. The upper chambers were filled with cells in serum-free DMEM containing Ginseng Rh2+ at different concentrations. The cells were incubated at 37 °C for 24 h. The culture media were discarded, and the top side of each Transwell chamber membrane was scraped with a wet cotton swab to remove non-migrated cells. The migrated cells were fixed with 70% ethanol and stained with Giemsa stain (Beckman Coulter Inc., Brea, CA). The average number of migrated cells was counted from 10 randomly selected microscopic fields at ×40 magnification. The migration index of the treated cells was calculated relative to controls. Each variant of the experiments was carried out in triplicate and each experiment was repeated at least 3 times. Differences between each group and the control group were

considered statistically significant if the p value <0.05 (*p < 0.05 and **p < 0.01).

2.6. Cell adhesion assay

The attachment capacity of the tumor cells to the extracellular matrix (ECM) was evaluated by seeding 5×10^4 cells on 24-well flat-bottom microplates covered with Matrigel (Merck, NJ, USA). Briefly, the wells were filled with DMEM supplemented with 10% FCS containing Ginseng Rh2+ at different concentrations (0, 15, 30, and 60 μM). After 24 h the media were removed and the plates were washed three times with PBS to remove floating cells. The attached cells were fixed with methanol for 15 min, stained with Giemsa solution for 20 min, and dried at room temperature. The average number of attached cells was determined by counting the cell number from 10 randomly selected fields at ×40 magnification under an Olympus microscope. Differences between each group and the control group were considered statistically significant if the p value <0.05 (*p < 0.05 and **p < 0.01).

2.7. Annexin V-FITC/PI assay for apoptosis

The cells (3×10^5 cells per well) were plated in 10 cm culture plates for 24 h, treated with different concentrations of Ginseng Rh2+ for 72 h, and then harvested. The adherent and non-adherent cells were collected and double-stained with Annexin V-FITC purchased from Biovision Inc. (San Francisco, USA) and propidium iodide (PI). Specific binding was performed by incubating the cells for 15 min at room temperature in a binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing 20 μg ml^{-1} PI and 200 μg ml^{-1} DNase-free RNase or a saturating concentration of annexin V-FITC. After incubation, the cells were pelleted and analyzed in a FACS Calibur (BD Bioscience, San Jose, CA, USA). Annexin-V-FITC-positive stained cells indicated an early apoptotic population, double-positive stained cells indicated a late apoptotic population, and PI-positive stained cells indicated the necrotic population. Data analysis was performed using FlowJo software (Tree Star, Inc., Oregon, USA). Each experiment was done in triplicate and the experiments were repeated at least twice.

2.8. Western blot analysis of selected proteins

The expression of selected proteins in cells was analyzed by western blot analysis according to the manufacturer's recommendations (BioRad, Hercules, CA, USA). The primary antibodies were rabbit or goat polyclonal IgG for mouse, or monoclonal IgG for Bax, Bcl-2, and β-actin (Santa Cruz, CA, USA). The secondary antibodies were either horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG depending on the primary antibody used. Band density was evaluated using an ECL kit (Biological Industries, Beit Haemek, Israel). Differences between each group and the control group were considered statistically significant if p < 0.05 (*p < 0.05 and **p < 0.01).

2.9. Animals

Eight-week-old C57BL/6J mice were obtained from Envigo (Harlan) (Jerusalem, Israel). All animal housing facilities were approved by the Local Ethics Committee of Laboratory Animal Use in accordance with the current regulations of the Ministry of Health of Israel (protocol #34-11-16). The number of mice in each treatment group was at least 10. All the mice survived until the end of the experiment.

2.10. Subcutaneous (s.c.) injection of cancer cells for simulation of local tumor growth

3LL cells (5×10^5cells in 0.2 ml of normal saline) were injected s.c. into the flanks of syngeneic C57BL/6J mice. Tumor size was measured with digital calipers every 2 days, and the animals were sacrificed when the tumors reached an average diameter of ~1.5 cm.

2.11. Intravenous (i.v.) inoculation of tumor cells for simulation of experimental lung metastasis

3LL cells (5×10^5 cells in 0.2 ml of normal saline) were injected into the lateral tail veins of C57BL/6J mice. The mice were monitored daily and sacrificed when they became moribund.

2.12. Treatment of tumor-bearing mice

Tumor-bearing mice were randomly divided into groups and treated with different concentrations of Ginseng Rh2+ (0.005, 0.025, 0.25, and 0.5 g kg^{-1}) injected i.p. on days 6, 10, and 14 after tumor cell injection. On day 18 of the experiment, all mice were sacrificed, and their internal organs and their s.c. tumors were harvested. Animal weight, tumor size, and the number of lung tumor colonies were measured. Tissue specimens were processed for histopathological examination.

2.13. Histopathological study

After fixation, the tissues were embedded in paraffin, and sections of 5 μm were stained with hematoxylin–eosin. Three sections per block were evaluated for the presence of cancer and other histopathological signs of toxicity related to Ginseng Rh2+ treatment. All histopathological studies were performed by a senior pathologist who was blinded to the animals' treatment.

2.14. Toxicity study

Tumor-bearing mice were treated with Ginseng Rh2+ as described above. The animals were weighed twice weekly and their clinical status was evaluated for the detection of cutaneous, neurological, gastrointestinal, and other pathological manifestations. On day 18, the animals were sacrificed and their organs were studied by gross and microscopic pathology.

2.15. Statistical analysis

The results for each variant in the different experimental *in vitro* designs were represented as an average of two to four experiments (performed in triplicate). Mean values and standard deviations were calculated for each point from the pooled

normalized data. Data were analyzed for significance by the Student's *t* test (two-tailed), and one-way analysis of variance (ANOVA) test (SPSS 26.0 software package, SPSS Inc., Chicago, IL, USA), including correction for family-wise error rate (FWER) analysis. We used reverse testing to assess sufficient power analysis to confirm the statistical difference between the treatment and the control group.[26] A p value <0.05 was considered statistically significant.

3. Results

3.1. The effect of Ginseng Rh2+ on the survival of lung cancer cells

We evaluated the capacity of Ginseng Rh2+ to inhibit the survival of several human and mouse lung cancer cells using an XTT assay (Fig. 2). All cells demonstrated a dose dependent survival inhibition. The most sensitive cell lines were the H1299 human lung adenocarcinoma and murine lung cancer cell lines – 3LL (IC_{50} = 120 μg ml^{-1} and 130 μg ml^{-1}, respectively) and the most resistant line was H520 human lung squamous cell carcinoma (IC_{50} = 250 μg ml^{-1}).

3.2. Ginseng Rh2+ induces apoptosis in H1299 cells

To evaluate the apoptotic capacity of Ginseng Rh2+ the H1299 cells were exposed to Ginseng Rh2+ at different concentrations (0–200 μg ml^{-1}) for 48 h. The treated cells were harvested by trypsinization and double-stained for Annexin V-FITC and PI. The fraction of apoptotic cells increased in response to Ginseng Rh2+ treatment in a dose-dependent manner: 0.18% of the untreated cells were apoptotic (annexin+), and 5.43% of cells treated with 100 μg ml^{-1} Ginseng Rh2+ and 8.09% of cells treated with 200 μg ml^{-1} Ginseng Rh2+ were apoptotic (Fig. 3a). The cells were either at the early apoptosis (Annexin V+/PI−) or at late apoptosis (Annexin V+/PI+) but did not die due to necrosis (Annexin V−/PI+), suggesting that the cytotoxicity of Ginseng Rh2+ in the H1299 cells is mainly driven by the induction of apoptosis. The 3LL cells treated with Ginseng Rh2+ were studied for apoptosis as described above demonstrating similar results to the human H1299 cells (not shown).

3.3. The effect of Ginseng Rh2+ on the expression of apoptosis-related genes

To assess the expression of pro (Bax) and anti-apoptotic (Bcl-2) proteins, protein concentrations were evaluated 24 h following culture with varying concentrations of Ginseng RH2+ using western blot analysis. Fig. 3b shows that the expression of the Bcl-2 protein decreased after 24 h incubation of human H1299 cells whereas the expression of Bax protein significantly increased. Quantification of at least 2 experiments was done using ImageJ object analysis (Fig. 3c).

3.4. The effect of Ginseng Rh2+ on the adhesion of 3LL cells to Matrigel

For *in vitro* cell experiments (adhesion to Matrigel, wound healing assay and Transwell migration capacity) we used mouse 3LL cells which were used in the subsequent *in vivo* experiments with C57Bl/6 mice-syngeneic to 3LL. The 3LL relates to the NSCLC category and the cells have the same sensitivity to Ginseng Rh2+ (Fig. 2). To evaluate the effect of Ginseng Rh2+ on the adhesion of 3LL cells to Matrigel, the cells were cultured for 24 h on Matrigel and then assessed for adhering cells. The number of cells adhering to the matrix decreased with increasing concentrations of Ginseng Rh2+ from 15 μg ml^{-1} to a peak at 60 μg ml^{-1} (Fig. 4), which was statistically significant compared to the control group. Cell

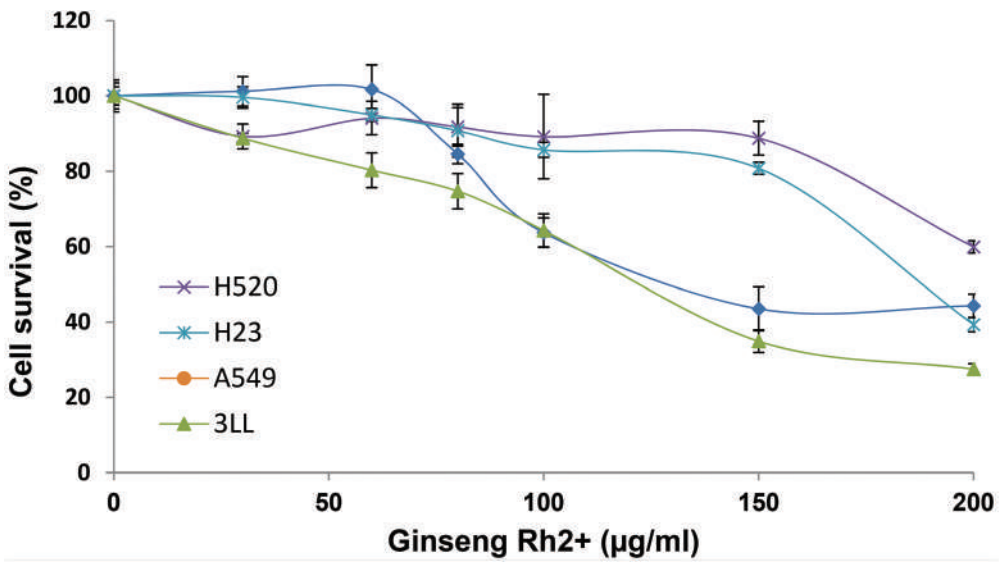


Fig. 2 Inhibitory effect of Ginseng Rh2+ on the survival of lung cancer cells. The cells were incubated with Ginseng Rh2+ for 72 h and cell survival was processed by an XTT assay. Mean values and standard deviations were calculated from three independent experiments each performed in triplicate *in vitro*.

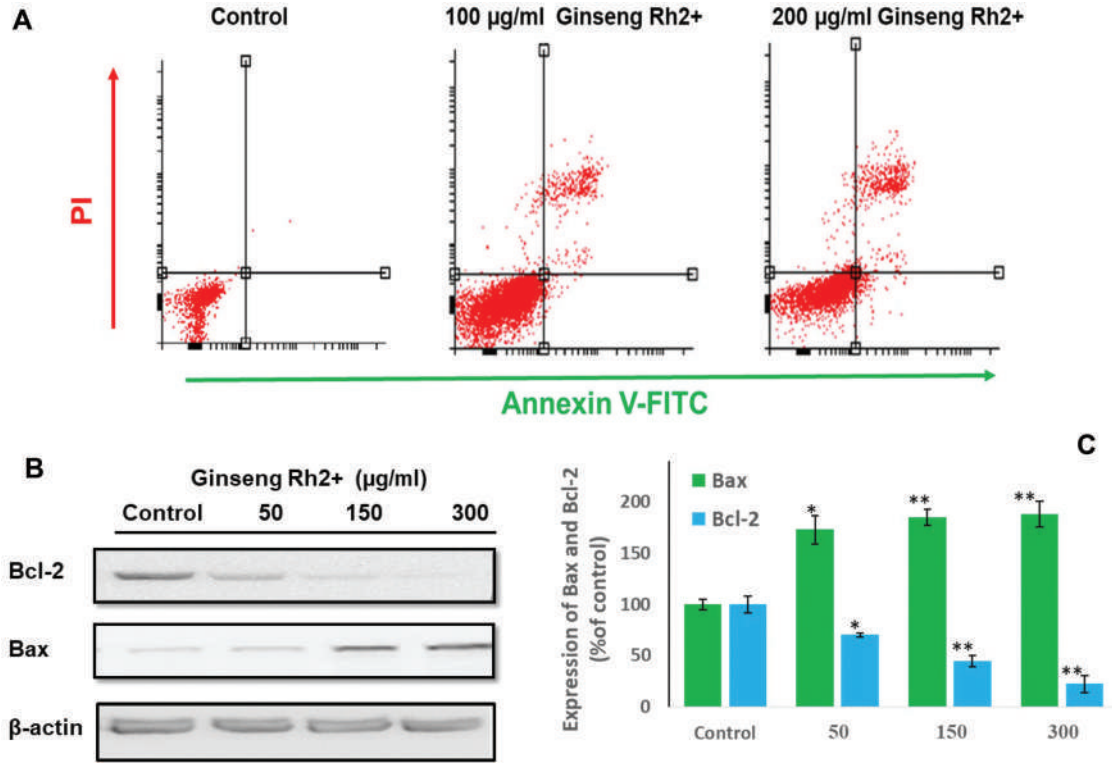


Fig. 3 Effect of Ginseng Rh2+ on the induction of apoptosis (A) and on the expression of Bcl-2 and Bax (B) including quantification of protein expression (C) in H1299 cells. The cells were treated with different doses of Ginseng Rh2+ for 48 h. The expression of apoptosis was analyzed by flow cytometry using PI and Annexin V-FITC staining (one representative experiment of three). Protein expression was evaluated by western blot analysis using monoclonal antibodies. Equal protein loading was confirmed by β actin staining. Quantification of at least 2 experiments was done using ImageJ object analysis. (C). Differences between each group and the control group were statistically significant (*$p < 0.05$ and **$p < 0.01$).

adherence to Matrigel correlates with the cancer cells' metastatic capacity.[27]

3.5. Ginseng Rh2+ inhibits cell motility evaluated using wound healing assay

To evaluate the effect of Ginseng Rh2+ on the cell motility, we conducted a wound healing assay using the 3LL cells. The cells were incubated in medium with or without Ginseng Rh2+ (30 to 120 µg ml^{-1}). Twenty-four hours later, the healing wound area was measured and compared to the initial value (at time zero). Fig. 5a demonstrates that Ginseng Rh2+ treatment inhibited the wound healing capacity of 3LL cells in a dose-dependent manner.

3.6. Ginseng Rh2+ inhibits the migration of lung cancer cells

To further evaluate the effect of Ginseng Rh2+ on the ability of cells to migrate, we assayed 3LL cells in the Transwell migration assay. The cells were plated in the upper part of a Transwell chamber with an 8 µm pore membrane and allowed to migrate for 24 h in response to FCS with or without varying concentrations of Ginseng Rh2+. Fig. 5b shows that the number of migrating cells decreased significantly in the pres-

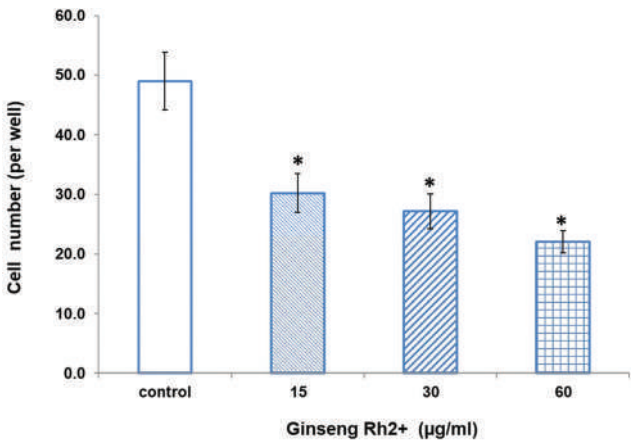


Fig. 4 Inhibitory effect of Ginseng Rh2+ on the attachment of 3LL cells to Matrigel. 5 × 10^4 cells per well were seeded on 24 well microplates covered with Matrigel and supplemented with DMEM and 10% FCS. Following 24 h of Ginseng Rh2+ treatment (0, 15, 30 and 60 µg ml^{-1}) the medium was removed, the cells were washed, and the attached cells were stained and counted. Mean values and standard deviations were calculated from two independent experiments each performed in triplicate. There was a statistically significant difference (*$p < 0.05$ and **$p < 0.01$) between each group and the control group.

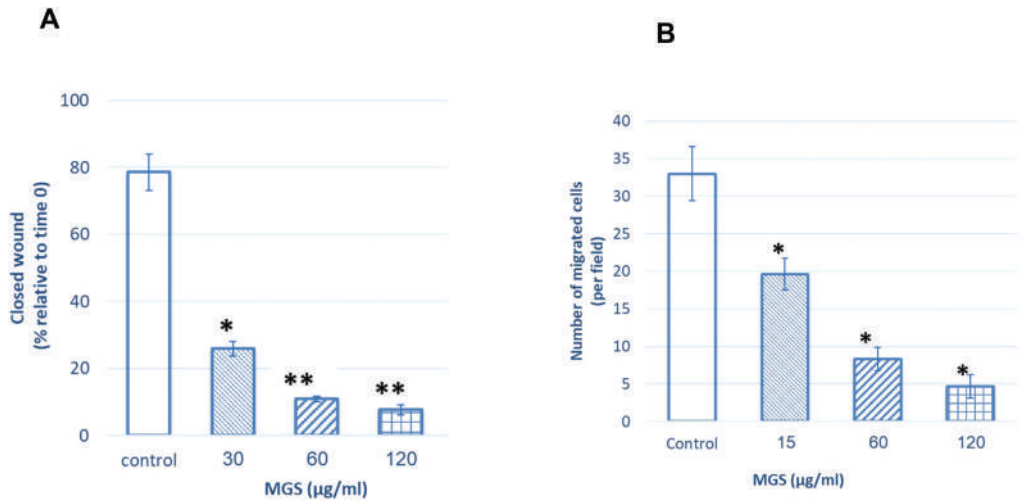


Fig. 5 Inhibitory effect of Ginseng Rh2+ on the motility (A) and migration activity (B) of 3LL cells. (A) Wound healing assay. Scratch wounds were formed by a sterile 1 ml pipette tip. Cells were grown in DMEM-10% FCS in the presence of various concentrations of Ginseng Rh2+. The wound width was evaluated at time 0 and 24 h after the scratching using an ocular micrometer at ×40 magnification. Mean values of closed wound (% relative to wound width at time 0) and standard deviations were calculated from two independent experiments performed in triplicate. (B) Transwell assay. The lower parts of Transwell chambers were filled with DMEM with 10% FCS, and 50 × 10^3 cells in serum-free DMEM with various concentrations of Ginseng Rh2+ were placed into the upper part of the chambers. The cells were fixed, stained, and dried after 24 h of incubation. The migrated cells were counted on 10 microscopic fields at ×40 magnification. The differences between each group and the control group were statistically significant (*p < 0.05 and **p < 0.01) in both assays.

ence of increasing concentrations of Ginseng Rh2+ starting from 0 to 120 μg ml^{-1} compared to the control group.

3.7. The effect of Ginseng Rh2+ on local tumor growth

To evaluate the effect of Ginseng Rh2+ on tumor growth *in vivo*, 3LL tumor cells were injected s.c. in the flanks of syngeneic mice. The tumor-bearing mice were treated with different concentrations of Ginseng Rh2+ (0.005–0.5 g kg^{-1}) on days 6, 10, and 14. Toxicity was not observed in any of the groups. The treatment with all four concentrations of Ginseng Rh2+ resulted in significant inhibition of tumor growth evaluated at day 18, with maximal tumor shrinkage observed at a drug concentration of 0.5 g kg^{-1} (Fig. 6).

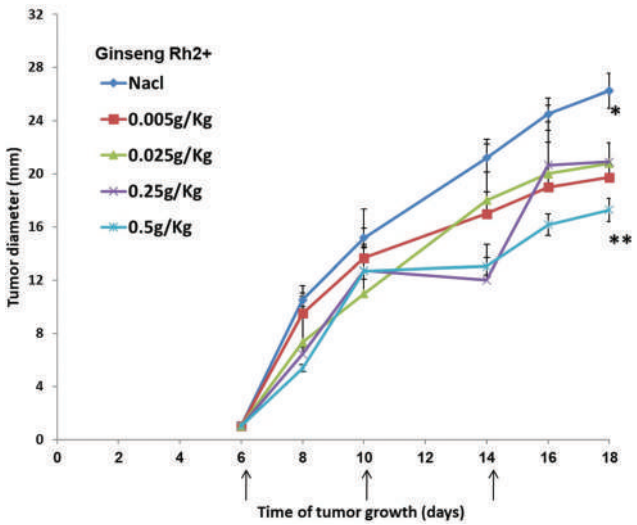


Fig. 6 Inhibitory effect of Ginseng Rh2+ on the growth of s.c. tumors produced by 3LL cells. 5 × 10^5 cells were inoculated s.c. in C57Bl/6 mice (10 mice per group). Ginseng Rh2+ was injected i.p. on days 6, 10 and 14. The mean tumor diameter was measured every other day using a digital caliper. Mean values and standard deviations were calculated from two independent experiments. The differences between each group and the control group were statistically significant (*p < 0.05 and **p < 0.01).

3.8. The effect of Ginseng Rh2+ on the development of experimental lung metastases

To evaluate the effect of Ginseng Rh2+ on the development of experimental metastases in the lungs, 3LL cells were injected in the tail vein and mice were treated with higher concentrations (0.25 g kg^{-1} and 0.50 g kg^{-1}) of Ginseng Rh2+. The treatment started at day 6 and continued in day 10 and 14, emulating a clinical scenario where the treatment is initiated only after a tumor is diagnosed. Small metastatic nodules could be detected as early as 10 days after i.v. tumor cell injection, and lungs were harvested at 18 days. All tested concentrations of Ginseng Rh2+ significantly inhibited the formation of visible tumor metastasis in the lungs, with maximal inhibition at a drug concentration of 0.5 g kg^{-1} (Fig. 7).

3.9. Ginseng Rh2+ toxicity study

To evaluate Ginseng Rh2+ toxicity, s.c. tumor-bearing mice were treated with different concentrations of Ginseng Rh2+ and monitored at the end of the experiment (day 18). Animals

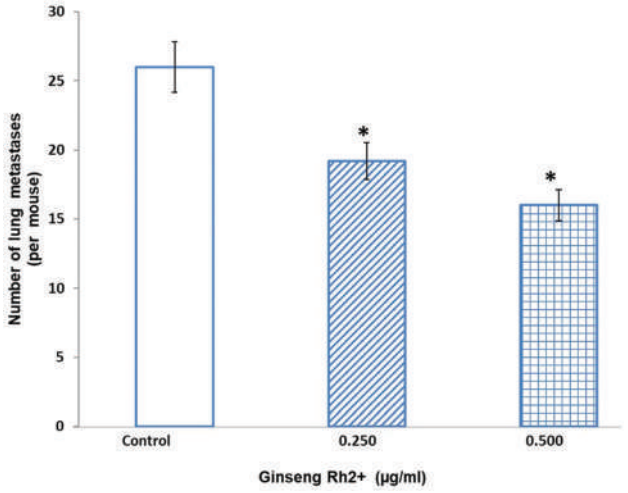


Fig. 7 Inhibitory effect of Ginseng Rh2+ on the development of experimental lung metastases. 3LL cells (2×10^5 cells per 0.2 ml) were injected i.v. into syngeneic mice. The mice were randomized in 10 mice per group. Ginseng Rh2+ (0.250 and 0.5 g kg^{-1}) was injected i.p. three times on days 6, 10 and 14. The mice were sacrificed and the number of metastatic nodules per both lungs was counted at the end of the experiment (day 18). Mean values and standard deviations were calculated from two independent experiments. The differences between each group and the control group were statistically significant (*$p < 0.05$).

were weighed three times per week. Animal weight was between 25 and 26 g both in the control and experimental groups. No significant weight change (15% from the initial weight) was recorded. In addition, detailed clinical observations were performed in all animals, once a week. No clinical signs of skin, gastrointestinal, or neurological toxicity (*e.g.*, changes in fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity) were detected.

Note that Ginseng extract at the concentration of 0.9–6 g kg^{-1} is widely used in Korea with reported health benefits and without reported toxicities.[28,29] Moreover the effective concentration of Rh2+ ginsenosides evaluated *in vitro* and *in vivo* is within the range of accepted safe and tolerable clinical use.[24] In summary, the therapeutic potential of Ginseng Rh2+ was demonstrated starting at 0.005 g kg^{-1}, which is within the range of a safe and tolerable dose for Ginseng.

4. Discussion

The findings of this study demonstrated that Ginseng Rh2+ has inhibitory effects on tumor growth and metastases of 3LL lung cancer cells. The inhibitory effects of Ginseng Rh2+ on local tumor growth, demonstrated in the s.c. mouse tumor model, were related to increased apoptosis regulated by Bax/Bcl-2. Evasion of programmed death is one of the most important mechanisms of tumor growth, resistance to immune responses and metastatic characteristics.[30,31]

Treatment with Ginseng Rh2+ at concentrations of 0.005–0.5 g kg^{-1} resulted in significant inhibition of tumor growth, with maximal tumor inhibition observed at a concentration of 0.5 g kg^{-1} (Fig. 6). In addition to the inhibition of local tumor growth, we showed that Ginseng Rh2+ reduced the development of experimental lung metastases (Fig. 7). Since treatment with Ginseng Rh2+ (0.25 g kg^{-1} and 0.50 g kg^{-1}) was initiated 6 days after i.v. cell injection, we suggest that Ginseng Rh2+ may suppress the development of visible metastases. This can be linked to the inhibition of tumor cells' survival and to the decrease of tumor cells' capacity to attach to an ECM-like substrate and to migrate. To produce a metastasis, tumor cells must complete a series of successive steps which include cell migration, invasion, survival in the blood turbulence, adhesion to the endothelium, extravasation, and distant growth.[32,33] Our *in vitro* results have demonstrated that at least two important steps of the metastatic process, specifically, the attachment to the invaded tissue and motility are impaired by Ginseng Rh2+. The Matrigel assay demonstrated that Ginseng Rh2+, at concentrations of 15–60 μg ml^{-1}, caused a significant reduction in cell attachment in a dose-dependent manner. The migratory capacity of 3LL cells was shown to be significantly suppressed by treatment with Ginseng Rh2+ at concentrations of 30 to 120 μg ml^{-1}.

A recent study examining the effect of administration of a modified single ginsenoside derivative, 20(S)-Rh2E2, to an LLC-1 xenograft mouse model showed that 20(S)-Rh2E2 effectively suppressed tumor growth and metastasis.[9] The authors found that 20(S)-Rh2E2 suppressed the expression markers of cell adhesion and cell invasion *in vitro* in H1299 cells, and the expression markers of metastasis and angiogenesis *in vivo* in the LLC-1 xenograft mouse model. Therefore, our *in vivo* findings, using a syngeneic model, support the results of Huang *et al.*[9] and add the biological processes which underlie the anti-cancer effects of *Panax ginseng*-enriched extracts on NSCLC tumor cells.

It was previously found that *Panax ginseng,* or the different ginsenosides separated from *Panax ginseng,* induce apoptosis and autophagy in lung cancer cells through different signaling pathways such as PI3K/Akt, NF-κB, EGFR, AMPK–mTOR, and JNK as well as activation of caspase-8, -9, and -3, through changes in mitochondrial membrane potential. Additional pathways included reduction of cyclin D1 and CDK4 expression, up-regulation of P21 expression, inhibition of cell proliferation and colony formation, and induction of cell cycle arrest in NSCLC cells.[5,7,8,34–38] In our study we evaluated the apoptotic capacity of Ginseng Rh2+ in H1299 cells. The treated cells were exposed to Ginseng Rh2+ at different concentrations (0–200 μg ml^{-1}) for 48 h, harvested by trypsinization, and double-stained with Annexin V-FITC and PI. The cells were either at early apoptosis (Annexin V+/PI–) or late apoptosis (Annexin V+/PI+) stage but they were not necrotic (Annexin V–/PI+), suggesting that cytotoxicity mediated by Ginseng Rh2+ in H1299 cells is mainly driven by the induction of apoptosis. Also, we found that the increase in apoptosis was regulated by Bax/Bcl-2 interaction. Laboratory data suggest that Bax and

Bcl-2 may impact the chemotherapy response of NSCLC cells at an early disease stage.[39] This supports our previous study which found that Ginseng Rh2+ induces autophagy-signaling by modulating AMPK-Ulk1 in A549 lung cancer cells, and suggests that Ginseng Rh2+ targets cell death by multiple pathways.[7]

Our standardized ginsenoside-enriched Ginseng Rh2+ is rich in the Rh2 ginsenoside (0.45 mg g^{-1}) in comparison with Korean ginseng (<0.01 mg g^{-1}). The effects of ginsenoside Rh2 may be further reinforced in an entourage-like effect, by ginsenosides other than Rh2+ found in ginseng including Rh1, Rg3(S), Rg3(R), and compound K that were found in the extract we have used – see Fig. 1. Previous studies have shown the cytotoxic potential of these ginsenosides against cancer cells.[35,40–42] The cumulative effects of ginsenosides other than Rh2 with regard to their individual effects and Rh2-supportive effects should be evaluated in future studies.

Novel technologies for standardization and for the enhancement of efficacy in botanical agents are a promising avenue for the development of novel functional foods, chemopreventive agents, and combined therapeutics.[43] We found no differences in weight reduction in control mice, and no microscopic changes in internal organs. These results resonate with those reported by Kim *et al.*[28] and other colleagues[29,44–46] on the safety profile of ginseng extract and its ginsenosides. Our previous case-series study showed the potential of ginseng treatment for NSCLC patients.[47] Further studies are warrented to evaluate ginseng as an adjunct therapy to anti-cancer drugs used in NSCLC treatments.[48]

In conclusion, the results of this study demonstrated that Ginseng Rh2+ inhibits lung cancer growth and metastases by targeting multiple pathological processes. The beneficial antitumoral effects of Ginseng Rh2+ delivered one week after tumor implantation were exerted without having systemic adverse effects. This indicates that Ginseng Rh2+ may potentially be used as a therapeutic agent for NSCLC. The methodology and findings on Ginseng Rh2+, in this study, may serve as a mechanistic infrastructure for the development of the field of botanical drugs.

Abbreviations

NSCLC	Non-small cell lung cancer
Ginseng Rh2+	Rh2-enriched Korean ginseng
CBD	Cannabidiol
THC	Tetrahydrocannabinol
GBX	Ginseng butanolic extract
s.c.	Subcutaneous
ANOVA	Analysis of variance

Conflicts of interest

The authors declare that there are no conflicts of interest.

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