

Anti-metastatic Potential of Ginsenoside Rp1, a Novel Ginsenoside Derivative

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Ginsenoside Rp1 (G-Rp1) is a novel ginseng saponin with a chemopreventive action. In this study, we examined the anti-metastatic activities of G-Rp1 using relevant *in vitro* assays and *in vivo* metastasis models. Using a U937 cell-cell adhesion assay, we found that exogenously added G-Rp1 down-regulates β 1-integrin (CD29) activation at concentrations between 10 to 40 μ M and suppresses the *in vitro* tube formation of human umbilical vein endothelial cells (HUVECs). Furthermore, this compound directly blocked cell viability of cancer cells such as A549 and HCT15 cells. In agreement with *in vitro* findings, G-Rp1 strongly inhibited the metastatic lung transfer of B16-F10 melanoma cells, which have a high surface level of β 1-integrins, without altering body weight. Therefore, these results suggest that G-Rp1 may act as an anti-cancer agent by strongly inhibiting cell viability and metastatic processes, presumably by inhibiting the adhesion of tumor cells and vessel formation.

Key words ginsenoside Rp1; anti-cancer effect; metastasis; tube formation; cell adhesion

Tumor invasion is an important step in the sequential process of metastasis and is subdivided into three steps, including tumor cell adhesion, migration and enzymatic degradation of the extracellular matrix and basement membrane.¹⁾ Tumor metastasis is a major cause of death in cancer patients.²⁾ Therefore, the discovery of agents with anti-metastatic activity, which would enable cancer patients to live longer, is a popular research objective.³⁾

Ginsenoside Rh1 (G-Rh1), G-Rh2, G-Rg3 and G-Rg5 are all minor saponin components with anti-tumor activity that are derived from Red ginseng.⁴⁻⁶⁾ Of these components, G-Rg5 is reported to have the most potent anti-tumor activity, but this compound is chemically unstable, which limits its potential for development into a potent anti-cancer agent.⁷⁾ Ginseng-derived saponins have no *in vivo* toxicity according to safety tests. Nonetheless, the development of biologically-active ginsenosides for food or medicinal purposes is limited and unqualified for patent status, since the components are widely known. Because of the stated limits of plant-derived ginsenosides, we aimed to synthesize novel ginsenoside-originated compounds with improved stability and anti-cancer activity without increasing toxicity. Given these goals, G-Rp1 was prepared on a large scale from crude ginsenosides (e.g., G-Rg5 and G-Rk1) by means of a reduction with hydrogenation. Indeed, G-Rp1 proved to be stable, had improved solubility.^{8,9)} Furthermore, the fact that G-Rp1 displays a chemopreventive action⁸⁾ drove us to evaluate its anti-tumor activity in terms of anti-metastatic potential. Therefore, in this study, we examined the inhibitory activity of G-Rp1 on aspects of tumor metastasis using relevant *in vitro* and *in vivo* assays.

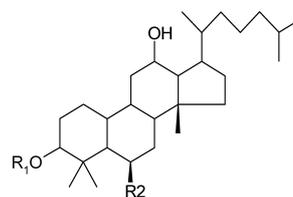
MATERIALS AND METHODS

Materials G-Rp1 (Fig. 1) of 97% purity was prepared using established protocols.⁹⁾ Matrigel was purchased from

BioCoat (Horsham, PA, U.S.A.). (7-Methoxycoumarine-4-yl)-acetyl-Pro-Leu-Gly-Leu-(3-[2,4-dinitrophenyl]-L-2,3-diamino-propionyl)-Ala-Arg-NH₂ (dyfluorescence-labeled Mca-peptide) was purchased from Bachem (Bubendorf, Switzerland). Matrix metalloproteinase (MMP)-2 was obtained from Boehringer Mannheim (Mannheim, Germany). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) and 4-aminophenyl-mercuric acetate (APMA) were purchased from Sigma (St. Louis, MO, U.S.A.). Antibodies to CD29 (MEM 101A, IgG1)¹⁰⁾ and CD43 (161-46, IgG1)¹¹⁾ were used in the cell-cell adhesion assays.

Animals Four-week-old male C57BL/6 mice were purchased from the Korea Research Institute of Chemical Technology (Daejeon, Korea). They were maintained under conventional conditions. Laboratory pellet chow (Samyang, Inc., Daejeon, Korea) and water were administered *ad libitum*.

Cell Line and Culture The mouse melanoma cell line (B16-F10), human umbilical vein endothelial cells (HUVECs), A549 and HCT15 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). A549, HCT15, B16-F10 and U937 cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, U.S.A.) and



R₁ = Glc-Glc-
R₂ = Glc

Fig. 1. The Chemical Structure of G-Rp1

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an antibiotic cocktail (100 U/ml of penicillin and 100 $\mu\text{g/ml}$ of streptomycin) and subcultured by trypsinization every three to four days. HUVECs were subcultured in EGM-2 medium (BD Bioscience, Lake Placid, NJ, U.S.A.) every three to four days and were used for experiments between passages 2 and 7. Cells were grown at 37 °C and 5% CO₂ in humidified air.

CD29-Mediated Cell–Cell Adhesion Assay The CD29-mediated cell–cell adhesion assay was performed as reported previously.¹⁰ Briefly, U937 cells, pre-treated with G-Rp1, were seeded at a density of 2×10^5 cells/well on a 96-well plate and incubated at 37 °C for 1 h followed by further incubation with aggregative antibodies (1 $\mu\text{g/ml}$) for 1 to 2 h in flat-bottomed micro-well plates. Images of the cells were captured with an inverted phase contrast microscope attached to a video camera using NIH image software.

Capillary-Like Tube Formation Assay HUVECs (2×10^4 cells/well) were dispensed into Matrigel-coated 24-well plates in 0.5 ml of EGM-2 media containing various concentrations of G-Rp1 (0 to 40 μM) and incubated for 24 h. The cells were then visualized by microscopy, and tube formation was scored by counting whether or not tubes formed, as reported previously.¹²

MMP-2 Enzymatic Activity Assay MMP-2 enzymatic activity was assayed with MMP-2 (gelatinase A) and fluorescence-labeled Mca-peptide as reported previously.¹³ MMP-2 was activated by 2 mM APMA at 37 °C for 30 min and incubated with various concentrations of G-Rp1. After a 30-min preincubation at 37 °C, 10 μM of Mca-peptide was added and the plates incubated for 6 h at 37 °C. Reading was then performed by a Spectrofluor Plus fluorimeter, set at excitation and emission wavelengths of 325 nm and 393 nm, respectively. Substrate degradation in the presence of inhibitor at a given concentration was calculated as the percentage fluorescence in relation to control wells.

MTT Assay Direct cytotoxicity of G-Rp1 was evaluated by measuring the extent of cell proliferation after 48 h incubation by MTT assay as described previously.¹⁰

Experimental Lung Metastasis Model Experimental lung metastasis of B16-F10 cells were assessed following intravenous injection of B16-F10 cells into syngeneic C57BL/6 mice using an established protocol.¹⁴ Briefly, the B16-F10 cells were harvested from monolayer culture by trypsinization. Cell viability was determined using the trypan blue assay and the cell density was adjusted to 2.5×10^6 in 0.2 ml 0.85% saline. The cells were injected into the lateral tail vein of mice. After 4 h, the mice were orally administered a G-Rp1 dose of 3 mg/kg every other day for 13 d. The mice were sacrificed 14 d after tumor inoculation. Their lungs were separated and fixed in Bouin's solution. Lung tumor colonies were counted under a dissecting microscope. The body weight was measured at 0, 3, 6, 9, 12, and 14 d, respectively.

Statistical Analysis Data are expressed as mean \pm S.E.M. For statistical comparison, results were analyzed using the ANOVA test and Student's *t*-test. A *p* value of <0.05 was considered a statistically significant difference. All statistical tests were carried out using the computer program STATISTICA, version 4.5 (StatSoft Inc., Microsoft Corporation, Oklahoma, U.S.A.).

RESULTS AND DISCUSSION

Tumor invasion is an important step in the sequential process of metastasis. This process requires tumor cell adhesion, migration and enzymatic degradation of the extracellular matrix and basement membrane.¹ Since blockade of the metastatic potential of cancer cells helps cancer patients live longer,³ there is a great effort to develop new drugs with anti-metastatic potential.

With the goal of developing novel anti-metastatic therapies, we established a quantitative CD29-mediated cell–cell adhesion assay using U937 cells, which has a high surface level of CD29, and its agonistic antibody MEM101A.¹⁰ The MEM101A antibody strongly induces cell–cell adhesion and a blocking antibody to CD29 (MAR4) remarkably suppresses the cell clustering, indicating functional activation of CD29. Since numerous cancer cells are reported to express high levels of CD29 and show increased CD29-mediated adhesion,^{15–17} we employed this assay as a primary screening method for developing novel anti-metastatic agents. In addition, tumor metastasis requires the formation of new blood vessels for maintaining tumor survival and growth. A capillary-like tube formation assay using HUVEC plated in Matrigel is a simple and currently-accepted *in vitro* method used to test the inhibitory activity of various compounds on blood vessel formation.¹² Therefore, we employed these two cell-based assays to screen novel anti-cancer drugs with anti-metastatic potential. Interestingly, we found that G-Rp1 was able to significantly inhibit CD29-mediated cell–cell adhesion up to 90% in a dose-dependent manner (Fig. 2A) and without altering cell viability (data not shown), suggesting that this compound may block the functional activation of CD29. Moreover, G-Rp1 completely blocked tube formation of HUVEC at a 40 μM concentration and partially blocked HUVEC tube formation at a 10 μM concentration (Fig. 2B), suggesting that this compound may modulate the formation of new blood vessels during tumor metastasis. Finally, we examined direct cytotoxic effect of G-Rp1 using several cancer cell lines such as A549 and HCT15 cells, since several anti-metastatic agents were also reported to directly block the growth of tumor cells. Indeed, G-Rp1 showed a much strong cytotoxicity with IC₅₀ values ranged from 5 to 10 μM , compared to G-Rg3 (Table 1).

How can G-Rp1 modulate those cellular events, cell adhesion, tube formation and direct cytotoxicity, at the same time is not fully elucidated in this study. However, the facts that 1) G-Rp1 was able to suppress actin cytoskeleton rearrangement (Cho *et al.*, submitted) and 2) actin cytoskeleton plays a critical role in cell–cell adhesion and migration of HUVECs as well as cell proliferation led us to assume that modulation of actin cytoskeleton by G-Rp1 might be a potential pharmacological mechanism. Considering in particular that G-Rp1 seems to both block activation of CD29, which plays a central role in tumor cell migration and metastasis, and inhibit the formation of new blood vessels, which are required for survival and growth of the migrated tumor cells, we further evaluated the effects of G-Rp1 in an *in vivo* tumor metastasis model.

To determine if G-Rp1 administration could inhibit tumor metastasis, we employed a mouse model of lung metastasis. To more strongly correlate our *in vitro* and *in vivo* results, we

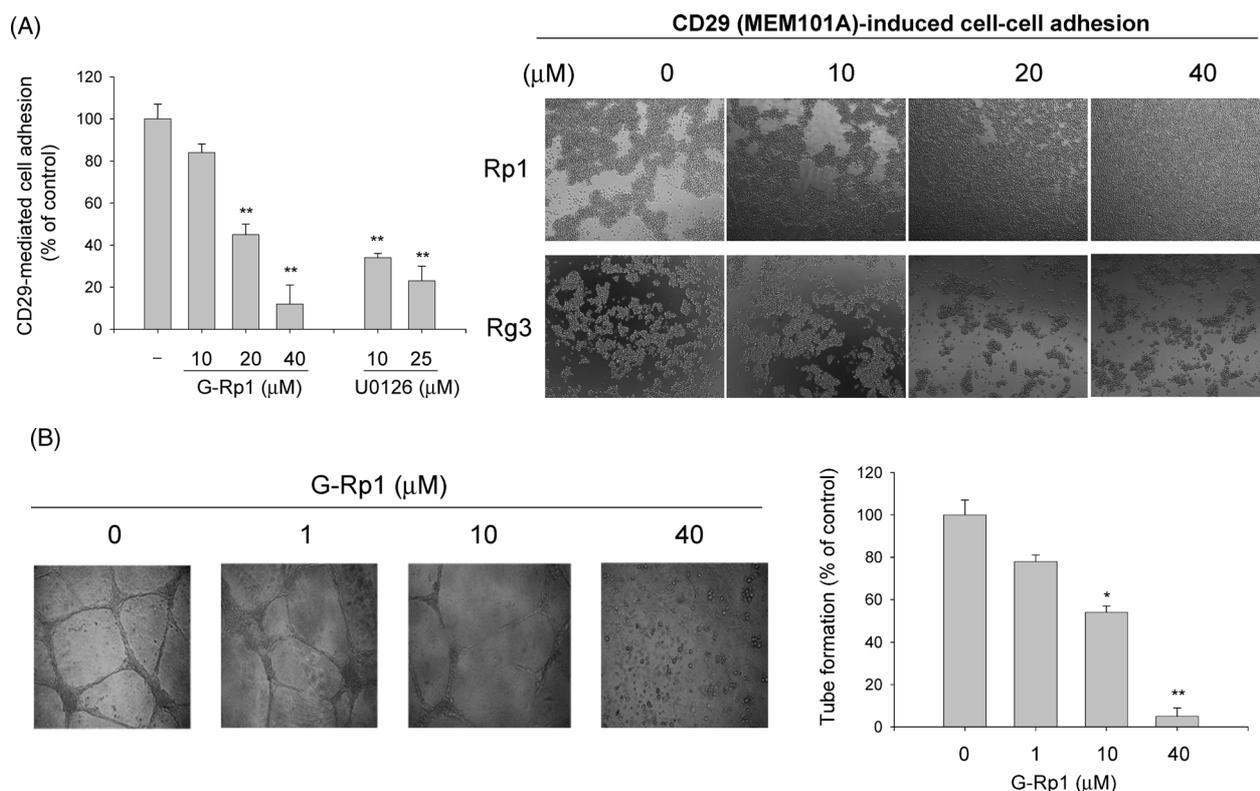


Fig. 2. The Effect of Exogenous G-Rp1 on CD29-Induced Cell-Cell Adhesion and Tube Formation by HUVECs

(A) U937 cells were incubated with the indicated concentrations of G-Rp1, G-Rg3 or U0126 in the presence or absence of pro-aggregative (activating) antibody [antibodies (1 μg/ml each) to CD29 (MEM 101A)] for 2 h. Left panel: Cell-cell adhesions were determined using a quantitative U937 cell-cell adhesion assay as described in Materials and Methods. Right panel: The images of the cells in culture were captured using an inverted phase contrast microscope attached to a video camera. (B) HUVECs were plated at 2×10^4 cells/well in Matrigel-coated 24-well plates and then exposed to various concentrations of G-Rp1. After 24 h, the culture supernatants were removed and the cells were fixed with 10% formalin. Left panel: Images of tube formation obtained with G-Rp1 treatment. Right panel: The areas and numbers of the formed tubes were calculated. Data are presented as the mean \pm S.E.M. of three independent observations, performed in triplicate. * $p < 0.05$ and ** $p < 0.01$ compared to control.

Table 1. Direct Cytotoxicity of G-Rp1 against Several Cancer Cell Lines

Cell line	Cytotoxicity [IC ₅₀ value (μM)]	
	G-Rp1	G-Rg3
A549	7.2 \pm 0.5	76.4 \pm 2.3
HCT15	7.7 \pm 1.2	89.1 \pm 9.9

used B16-F10 melanoma cells as the “metastatic” cell type, since they have a high surface level of CD29 and show increased CD29-mediated cell adhesion properties.^{15,17} Furthermore, dose of G-Rp1 was initially decided to 3 mg/kg, since a fatty acid (oleoyl derivative)-conjugated form of propanaxatriol showed significant effect at concentrations ranged between 3 to 15 mg/kg.¹⁸ In agreement with the *in vitro* data, orally-administered G-Rp1 displayed strong anti-metastatic potential in this model (Fig. 3). Specifically, this compound diminished the pulmonary metastatic colony numbers of B16-F10 melanoma cells up to 47.4% (Fig. 3B), compared with mice that did not receive G-Rp1, without altering normal body weight (data not shown). Similarly, a well-known standard control drug, adriamycin,¹⁹ also significantly blocked lung metastasis of injected cancer cells up to 37% of untreated control mice (Fig. 3B), indicating that our experiment was well performed. In an effort to determine if G-Rp1 inhibits the matrix degradation aspect of metastasis, we tested if G-Rp1 could inhibit the activity of MMP-2

(gelatinase), a proteolytic enzyme that promotes neoplastic cell invasion and metastasis by degrading extracellular network proteins.²⁰ We found that G-Rp1 did not show any MMP-2 inhibitory activity (Fig. 4), suggesting that modulatory role of MMP-2 by G-Rp1 can be excluded as a potential pharmacological target. Therefore, our data suggest that G-Rp1 may represent an anti-metastatic agent that acts by blocking the activation of CD29, inhibiting tube formation and suppressing the viability of cancer cells.

Using *in vitro* screening methods, a CD29-mediated cell adhesion assay, a HUVEC tube formation assay, and a direct cytotoxicity assay, and an *in vivo* metastatic lung transfer model with B16-F10 melanoma cells, we conclude that G-Rp1 could be developed as a new anti-cancer agent with anti-metastatic potential. Ginseng root has been used medicinally for a long time and its curative and protective efficacies in numerous diseases have been proven. Additionally, the ginseng-derived new saponin G-Rp1 seems to be advantageous in clinical trials. Like other ginseng-derived saponins, G-Rp1 did not show *in vivo* toxicity.⁹ Considering that G-Rp1 has both direct cytotoxic activity on cancer cells and anti-metastatic potential *via* inhibiting functional activation of β 1-integrins and tube formation of HUVECs, we therefore came to the conclusion that G-Rp1 could be clinically developed as a novel multi-potent anti-cancer agent.

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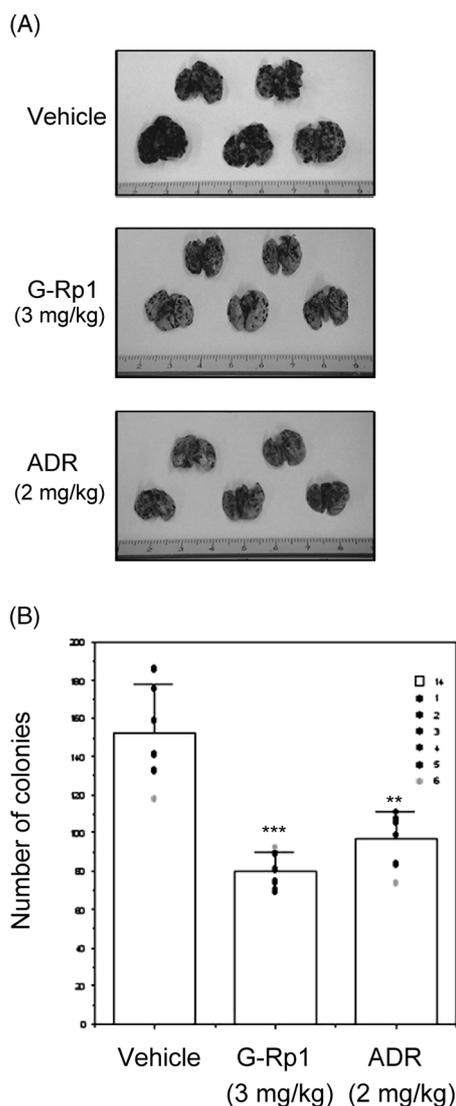


Fig. 3. The Inhibitory Effects of Oral G-Rp1 Administration on Pulmonary Metastasis in C57BL/6 Mice i.v. Injected with B16-F10 Cells

Seven mice per group were intravenously injected with 7×10^4 B16-F10 cells. G-Rp1 or adriamycin (ADR) were orally administered at doses of 2 to 3 mg/kg every other day for 14 d. The mice were sacrificed on day 14 following tumor inoculation and the lungs were separated and fixed in Bouin's solution. Lung tumor colonies were counted under a dissecting microscope. (A) Images of lung metastatic colonies in control, G-Rp1 or ADR-treated groups. (B) The tumor metastatic colonies in the lungs were counted. Data are presented as the mean \pm S.E.M. of three independent observations, performed in triplicate. ** $p < 0.01$ and *** $p < 0.001$ compared to control.

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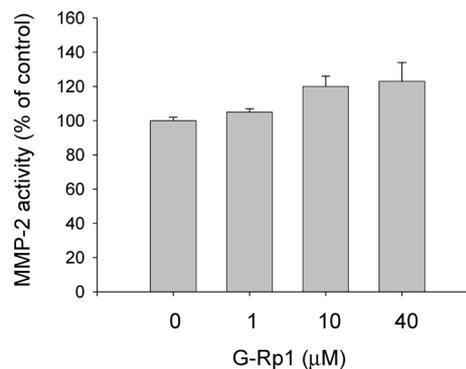


Fig. 4. Effect of G-Rp1 on MMP-2 Activity

Activated MMP-2 was incubated with G-Rp1 and Mca-peptide ($10 \mu\text{M}$) for 6 h at 37°C . Reading was then performed by a Spectrofluor Plus fluorimeter. Substrate degradation in the presence of inhibitor at a given concentration was calculated as the percentage fluorescence in relation to control wells. Data represent mean \pm S.E.M. of three independent observations performed in triplicate.

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