Ginsenoside from *Panax ginseng* Meyer Enhances the Cytotoxic and Apoptotic Effect of Cisplatin in A549 Human Lung Cancer Cells

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Park, *et al*.: Ginsenoside Rf Enhances CPP Effect in Cancer Cells

Ginsenosides from *Panax ginseng* Meyer have been used in combination with cisplatin to enhance anticancer potential of cisplatin. However, the combined effects of ginsenoside Rf and cisplatin has not been studied so far. Thus, we evaluated the anticancer activity of ginsenoside Rf alone and combined with cisplatin by using A549 cell line. Our results showed that cytotoxicity, reactive oxygen species generation and apoptotic effect of cisplatin at 1 µg/ml was enhanced by ginsenoside Rf along with the increase of p53 expression at protein and gene level, as well as reduction of the mRNA expression levels of Bcl-2 and Bax was higher for the combined treatment. Further, phosphorylation of epithelial growth factor receptors induced by cisplatin alone was decreased after exposing the cells to the combined treatment. Similarly, the motility of the cells was higher decreased after combining cisplatin and ginsenoside Rf than single drug treatment. In this study, ginsenoside Rf increased the anticancer effect of cisplatin on A549 cells.

Key words: *Panax ginseng*; Cisplatin; Apoptosis; EGFR; Lung cancer

Cisplatin is a well-known chemotherapeutic drug used for the treatment of different human cancers, such as the bladder, lung, ovarian, and testicular cancer. The root of cisplatin to induce cytotoxicity in cancer cells has been linked to its ability to interfere with DNA

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repair mechanism and with this induce DNA damage in cancer cells[1,2]. However, several studies reported drug resistance and several side effects observed in patients during the treatment[1]. Among this, the search for new therapeutic agents with the capability to decrease the side effects and overcome the drug-resistance of cisplatin has been increasing over the years.

Over the years, numerous natural compounds had been used as anticancer therapeutic agents[3]. *Panax ginseng* Meyer, a traditional herbal medicine used for thousands of years in East Asian countries, showed a variety of anticancer properties in several studies[4]. Ginsenosides isolated from *P. ginseng*, have been reported to enhance the anticancer activity of cisplatin[5]. However, the activity of the unique ginsenoside Rf (Rf), isolated only from *P. ginseng* root, and its interaction with cisplatin has not been reported so far. Previously, Rf has demonstrated to induce G2/M phase cell cycle arrest and apoptosis in human osteosarcoma, MG-63 cell line, through the mitochondrial pathway[6]. Thus, we hypothesize that Rf alone might have an effect on cell motility, induces cytotoxicity and apoptosis, as well as enhanced the anticancer activity of cisplatin in A549 lung cancer cells.

The ginsenoside Rf, a unique compound from *P. ginseng*; was received from ginseng bank, Kyung Hee University (South Korea) in powdered with a purity of ≥95%. Cisplatin (Platosin) was obtained at 1 mg/ml from Pharmachemie B. V. (GA, Netherlands). RPMI-1640 culture media was purchased from GenDEPOT Inc. (TX, USA). Fetal bovine serum (FBS) and the antibiotics, 100 UI/ml penicillin and 100 µg/ml streptomycin, from Gibco-Brl (MD, USA).

Non-small lung carcinoma cells (A549), were obtained from Korean Cell Line Bank (Seoul, South Korea). The cells were grown in RPMI-1640, supplemented with 10% FBS and 1% of antibiotics. The cells were maintained in an incubator at 37° with a humidified atmosphere of 5% CO_2. The evaluation of cell toxicity was done by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyletrazolium bromide) assay. The cells were exposed to cisplatin (1 µg/ml) in the presence or absence of ginsenoside Rf for 72 h. Ten microlitres of MTT assay solution (5 mg/ml) was added to each well and incubated for 3 h after the treatment had finished. Then, old media was replaced by 100 µl of DMSO and incubated for 30 min. The amount of formazan formed by viable cells was measured by a multi-model plate reader (Bio-Tek Instrument, Winooski, VT) at a test wavelength of 570 nm with a reference wavelength of 630 nm[7]. All experiments were repeated in triplets.

A549 cells were cultured in a 12-well plate (2.5×10^5 cells per well) and incubated for 24 h at 5% CO_2 and 37° humidified atmosphere. After a complete confluence was reached, old medium was replaced with serum-free growth media for 24 h. Then, a 10 µl sterile pipette tip was used to make a scratch in A549 cell cultures layer. In order to remove dead or floating cells, each well was washed twice with PBS. The cells were stimulated with epidermal growth factor (EGF, 20 ng/ml) and treated according to our schedule: 2% FBS, 2% FBS+EGF, 2% FBS+EGF+cisplatin 1 µg/ml, 2% FBS+EGF+Rf 100 µM and 2% FBS+EGF+cisplatin 1 µg/ml+Rf 100 µM. The scratch gap width at 24 h in each treatment group was measured at two different positions and compared to the gap width at 0 h. The analysis of the images, taken at x10 under an optical microscope Eclipse ME600L (Nikon Instruments, Melville, NJ), was done by T-scratch program[8]. The motility of the cells in response to the treated cells was determined relative to the EGF vehicle control.

A549 cells were exposed to cisplatin (1 µg/ml) in the presence or absence of ginsenoside Rf (100 µM) for 48 h. Cells were washed twice with 1x PBS and fixed with 3.7% (v/v) formaldehyde for 5 min at room temperature and washed twice with PBS. In order to dye the nucleus, the cells were stained with Hoechst 33258 solution (2 µg/ml) for 30 min in dark condition at room temperature. Nuclear morphologies of the Hoechst-positive cells were observed and photographed under a fluorescence microscope (x400, Optinity, Korean Labtech) for further analysis.

Total RNA was isolated from cultured cells using TriZol LS reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The first-strand cDNAs was synthesized by using Thermo Scientific cDNA synthesis kit (Onebio, Lithuania EU)[9,10]. Initial denaturation at 95° for 3 min followed by a PCR cycle of denaturation at 95° for 45 s, annealing at 58° for 1 min and strand extension at 72° for 1 min. The number of cycles was 30. The final step included incubation at 72° for 10 min. The resultant PCR products were electrophoresed on a 0.8% agarose gel and analyzed with Image J software[11]. SYBR Green qPCR Super Mix UDG kit (Invitrogen, Carlsbad, CA) was used for quantitative real-time polymerase chain reaction (qRT-PCR) amplification

in a R-Corbett Rotor-Gene Model 6000 (Mortlake, NSW 2137, Australia). Amplifications were performed at an initial temperature of 95° for 10 min, followed for 40 cycles at 95° for 10 s, 60° 15 s, and 72° for 20 s. The analysis was done by follow the delta cycle threshold (Ct) method.

Cells were plated at a density of 1×10^4 cells per wells in 96 well plate, allowed to attach overnight and exposed to treatment for 72 h. The cells were stained with 10 µM H_2DCFDA for 30 min at 37°, and the fluorescence intensity of the cells was determined using multi-model plate reader.

A549 cells were seeded in 100 mm dish culture plate at 5×10^6 cells per dish. After 24 h incubation, the cells were subject to serum starvation for 20 h following by 48 h of treatment. External EGF stimulation was done at 20 ng/ml for 30 min prior protein isolation. After stimulation and the treatment time was finished, the cells were rinsed twice with ice-cold PBS. The total proteins were solubilized with 2X sodium dodecyl sulphate (SDS) loading buffer (100 mM Tris-Cl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% glycerol and 200 mM β-mercaptoethanol) for 5 min at room temperature (RT). Then, the protein was denatured at 95° for 10 min and storage at –20°[12].

For immunoblotting, proteins of total cell lysates were loaded and resolved in 10% SDS-polyacrylamide gel electrophoresis and run at 120 V. The proteins were then transferred to nitrocellulose membranes (Millipore) at 100 V for 2 h. The membranes were blocked at room temperature (RT) for 1 h with 5% skim milk. After blocking, the blots were incubated with specific antibodies (Phospho-EGF Receptor (Tyr1068), EGF receptor, p53, and β-actin) overnight at 4°. The blots were then washed seven times with TBS-T, followed by goat antimouse or antirabbit IgG secondary antibody for 2 h at RT. Immunolabelling was visualized by enhanced chemiluminescence detection (EMD Millipore). Band densities were measured using ImageJ software[11].

The statistical analyses were performed using GraphPad 6.04 software (La Jolla, CA 92037, USA). Results are expressed as mean±SEM. The statistical significance of differences between values was evaluated by one-way ANOVA. The differences were considered significant at P≤0.05.

In the present study, we investigate the ability of ginsenoside Rf (Rf) to increase the anticancer activity of cisplatin. The effect was associated with the

inhibition of cell growth, cell motility; and induction of apoptosis at *in vitro* level. Previously, was reported the ability of compounds derived from medicinal plants to enhance the cytotoxicity of cisplatin in A549 lung cancer cells[13]. Furthermore, it had been reported that ginsenosides from *P. ginseng* enhanced the anticancer activity of cisplatin[13,14]. In this study, as a result of the treatment with Rf at 100 µM in the presence of cisplatin at 1 µg/ml, a significant reduction in the cell viability compared to individual Rf and cisplatin treatments was observed (fig. 1). In addition, the combined treatment significantly enhanced the ROS generation compared to the individual treatments (fig. 2). This result indicated that ginsenoside Rf may enhance the cytotoxicity of cisplatin through increasing the generation of reactive oxygen species.
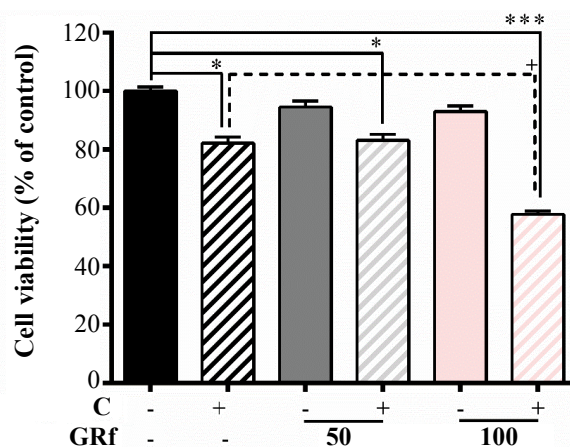

Fig. 1: Cytotoxicity and pharmacological interaction of ginsenoside Rf and cisplatin
Results are representative of three independent experiments. Data are shown as mean±SEM. ***P<0.05 vs. control. *P<0.05 vs. cisplatin alone. G Rf: Ginsenoside Rf (µM) and C: cisplatin (1 µg/ml)
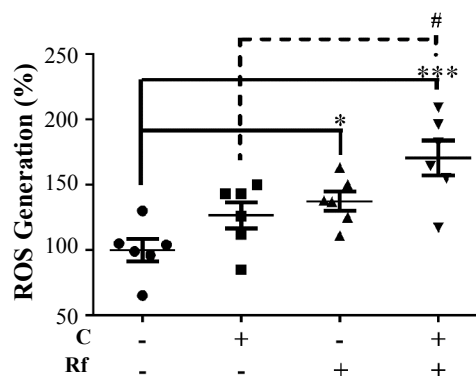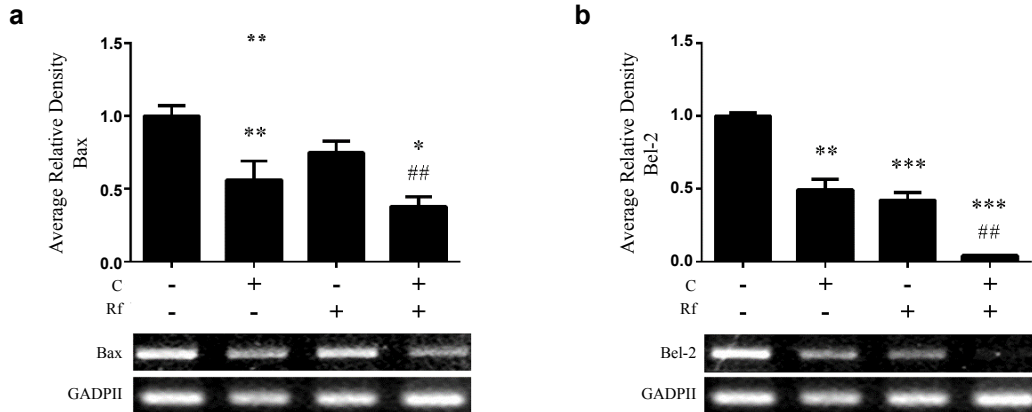

Fig. 2: Intracellular ROS generation induced by ginsenoside Rf and cisplatin
The production of intracellular ROS in treated A549 cells was detected by using H2DCFDA. Data are shown as mean±SEM. ***P<0.05 vs. control. #P<0.05 vs. cisplatin alone. ROS: reactive oxygen species; Rf: ginsenoside Rf (100 µM) and C: cisplatin (µg/ml)
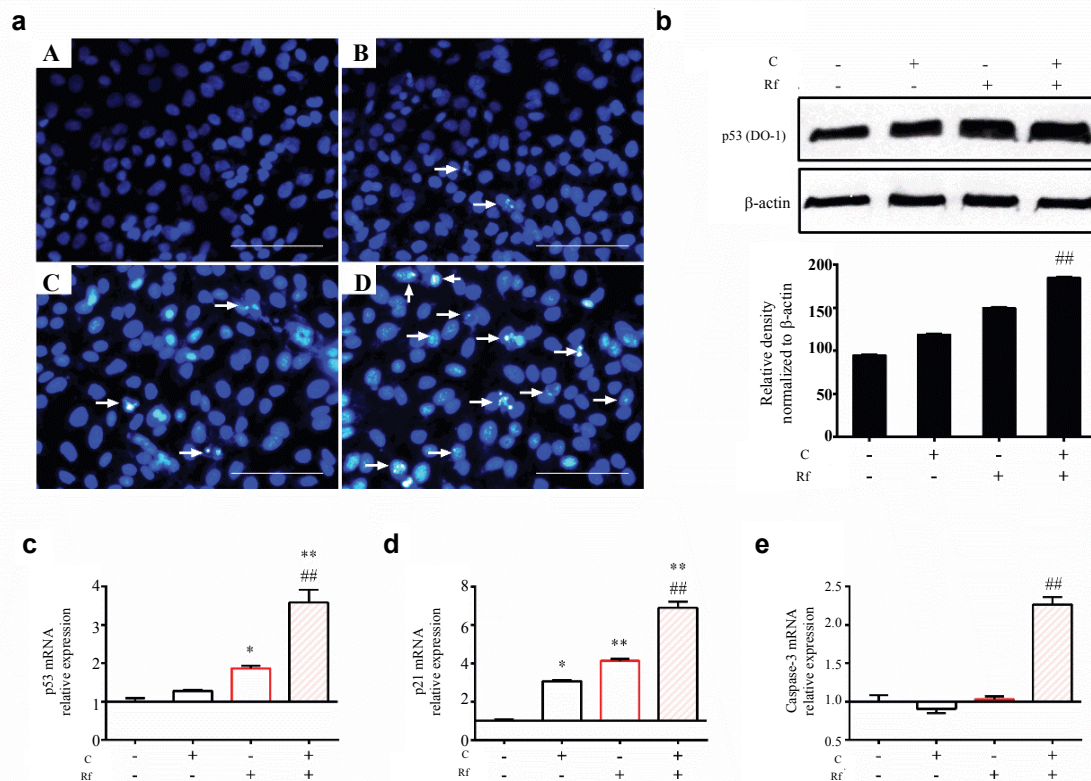
The induction of apoptosis by cisplatin through induction of DNA damage had been well documented[15,16]. Besides, the induction of apoptosis by Rf in osteosarcoma cells had been already reported[1,15-17]. Thus, in order to determinate whether the cytotoxicity of the combined treatment of Rf and cisplatin can be related to the induction of apoptosis, we evaluated the effect of the single and combined treatment on the expression of apoptotic markers as well as evaluate its effect on the nucleus morphology. A higher reduction in mRNA expression levels of Bax (fig. 3a) and Bcl-2 (fig. 3b) was observed in the combined treatment group than the single ones. In addition, highly number of apoptotic cells was observed in the present of cisplatin and RF than these drugs alone (fig. 4a). Further, protein expression analysis of p53 protein showed a

Fig. 3: Expression of pro- and antiapoptosis genes in A549 cells
The density of PCR bands of relative gene expression of apoptotic genes was done by ImageJ software. Data are shown as mean±SEM. ***P<0.05 vs. control. Rf: ginsenoside (100 µM); C: cisplatin (1 µg/ml)
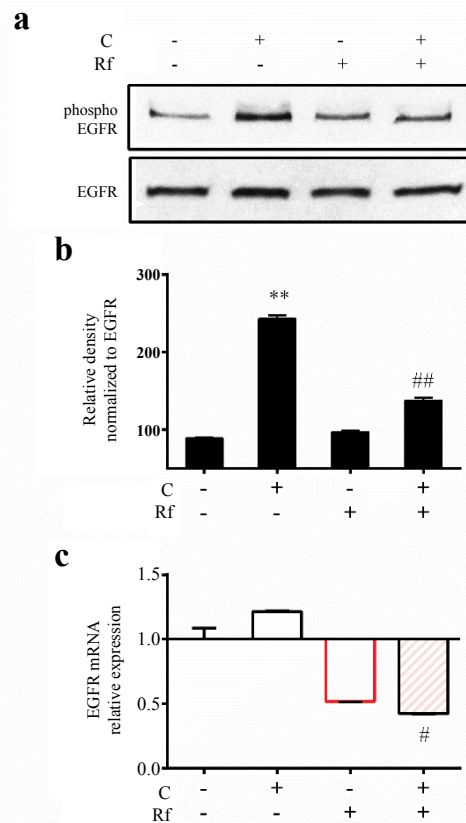

Fig. 4: Analysis of apoptotic activity of the single and combined drug treatment in A549 cells
(a) Morphological changes in the nucleus were observed by Hoechst 33258 assay after the following treatment: A. untreated cells, B. Rf at 100 µM, C. cisplatin at 1 µg/ml and D. cisplatin at 1 µg/ml + Rf at 100 µM. Apoptotic cells are indicated with arrows. Scale bar: 10 µm. (b) Western blot analysis of expression of the pro-apoptotic p53 protein. (c-e) mRNA expression analysis of apoptotic related genes normalized to GAPDH. Results are representative of three independent experiments. Data are shown as mean±SEM. **P<0.05 vs. control. ##P<0.05 vs. cisplatin alone. Rf: ginsenoside (100 µM); C: cisplatin (1 µg/ml)

significant increase in the presence of the combined treatment (fig. 4b). Also, mRNA expression of p53, p21 and caspase 3 genes was higher in the combined group (fig. 4c-e). In previous studies, was reported that the genes, Bcl-2 and Bax, can be capable of independent regulation of a common apoptotic pathway[18]. Besides, some studies indicated that a decrease in the expression levels of Bax is associated with cisplatin resistance and Mutation of p53 gene[19]. This mutation of the p53 gene was previously reported in A549 cells[20]. For this reason, we suggest that the independent regulation of Bax and Bcl-2 genes and increase in the pro-apoptotic markers in A549 cells observed during the combined treatment with Rf and cisplatin might be involved in the induction of morphological changes of the nucleus visualized through Hoechst 33258 staining.

The activation of epithelial growth factor receptors (EGFR) by cisplatin had been reported previously[21]. This phosphorylation of EGFR leads to the activation of different pathways and with this the initiation of other processes, such as migration and invasion[22]. Since metastasis represent the major problem in the treatment of cancer and involves multiple processes such cell migration[23], evaluation of the ability of the combined treatment with Rf and cisplatin to reduce the phosphorylation of EGFR and cell migration was evaluated. It was found that Rf (alone) did not modify the expression of phospho-EGFR. On the other hand, the combined treatment with Rf and cisplatin reduced the expression of phospho-EGFR previously enhanced by cisplatin (fig. 5). Also, was observed that the combined drug treatment induces a higher decreased on cell migration than single drug treatment (fig. 6a and b). Next, in order to define if the scratch assay result were related to cell migration and not to the, evaluation of mRNA levels of cadherin, snail and slug genes, which are related to epithelial-mesenchymal transition (EMT) process because of its relation to wound healing and cancer progression[24]. Our results have shown the increase in the expression of e-cadherin gene (fig. 6c) and the decrease of snail and slug genes (fig. 6d-e) for the combined drug treatment, this effect was significantly different in comparison with the cisplatin treatment alone. This finding suggested an antimigratory activity of Rf in the presence of cisplatin though a possible shift of motile mesenchymal cells, EGF-stimulated A549 cells; into epithelial cells.

This study exposed for the first time the effect of

Fig. 5: Analysis of epithelial growth factor receptor (EGFR) pathway
(a) Immunoblotting of phospho-EGFR and EGFR proteins in presence or absence of treatment. (b) Density analysis of PCR bands was done by Image J software. (c) mRNA expression of EGFR gene normalized to GAPDH. Data are shown as mean±SEM. **P<0.05 vs. control. Rf: ginsenoside (100 µM); C: cisplatin (1 µg/ml)

ginsenoside Rf (Rf) in the combination of cisplatin against A549 a lung-carcinoma cell line. It was observed that Rf at 100 µM enhanced the cytotoxicity, apoptotic and the effect on cell motility of cisplatin at 1 µg/ml. It is envisaged that further studies are needed by the use of other cancer cell lines to determinate whether the effect is observed in other cancer cells or if is specific to A549 lung cancer cells.
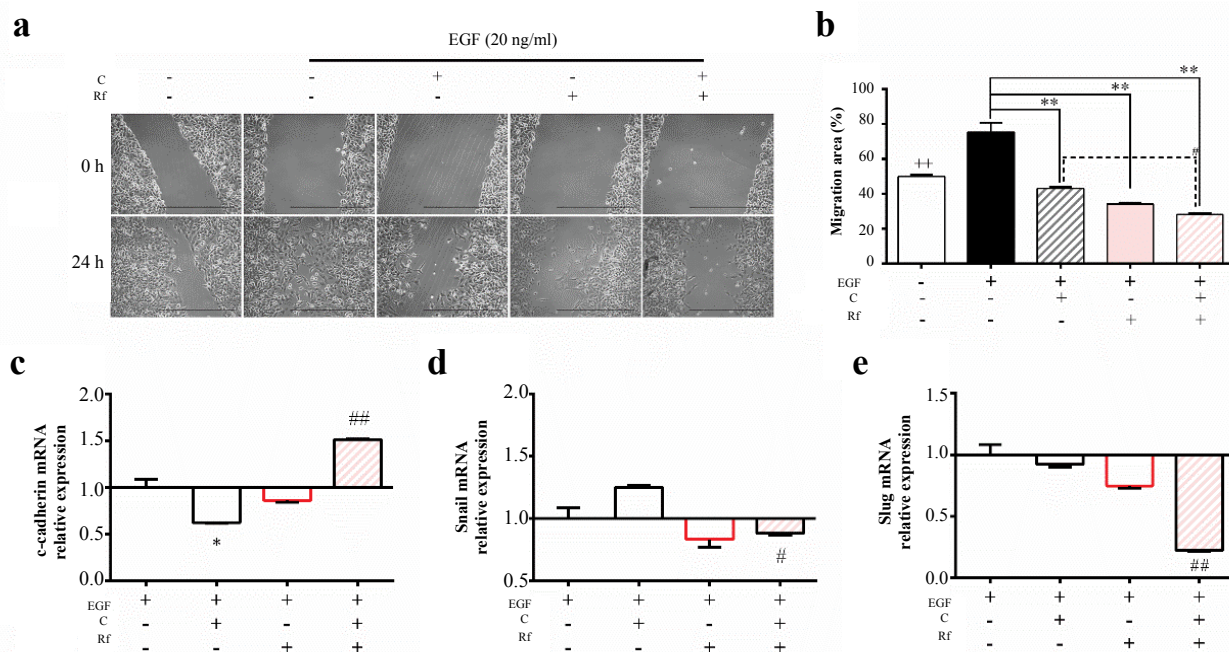
Conflict of interest:

The authors report no declarations of interest.

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Fig. 6: Ginsenoside Rf and cisplatin reduce cell migration in A549 cells
(a) Photographs of A549 cells were taken at the beginning of the scratch assay and after 24 h. Scale bar: 50 μm. (b) Percentage of the scratch gap width after 24 h. (c-e) Evaluation of the mRNA levels for epithelial and mesenchymal genes. Each column represents the mean±SEM. [++]P<0.05 Control versus EGF-Control. [**]P<0.05 versus EGF-control. [##]P<0.05 versus cisplatin alone. EGF: epidermal growth factor (20 ng/ml); Rf: ginsenoside (100 μM); C: cisplatin (1 μg/ml)

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