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ORIGINAL PAPER

Ginsenoside Rp1 from *Panax ginseng* Exhibits Anti-cancer Activity by Down-regulation of the IGF-1R/Akt Pathway in Breast Cancer Cells

Ju-Hee Kang · Ki-Hoon Song · Jong-Kyu Woo · Myung Hwan Park · Man Hee Rhee · Changsun Choi · Seung Hyun Oh

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Abstract Cancer prevention is effective and reduces health care costs because cancer is often a preventable disease that can be affected by lifestyle factors. Therefore, researchers are interested in discovering natural compounds that have anticancer activities, such as delaying the development of cancer and preventing its progression. One such natural agent is ginseng (*Panax ginseng*), which is traditionally used in some parts of the world as a popular remedy for various diseases including cancer. We hypothesized that the ginsenoside Rp1, a component of ginseng, reduces cancer cell proliferation through inhibition of the insulin-like growth factor 1 receptor (IGF-1R)/Akt pathway. We first tested the efficacy of Rp1 against human breast cancer cell lines. Treatment with Rp1 inhibited breast cancer cell proliferation and inhibited both anchorage-dependent and -independent breast cancer cell colony formation. In addition, treatment with 20 μM Rp1 induced cycle arrest and apoptosis-mediated cell growth suppression. Our findings further indicated that Rp1 decreased the stability of the IGF-1R protein in breast cancer cells. Therefore, we suggest that Rp1 has potential as an anticancer drug and that IGF-1R is an important target for treatment and prevention of breast cancer.

Keywords Akt · Breast cancer · Ginseng · Ginsenoside Rp1 · IGF-1R

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Abbreviations

DMEM	Dulbecco's modified Eagle's medium
FBS	fetal bovine serum
Hsp90	heat shock protein 90
IGF	insulin-like growth factor
IGF-1R	IGF-1 receptor
IL	interleukin
MMP	matrix metalloproteinase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF	nuclear factor
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
RT	reverse transcriptase

Introduction

Breast cancer is the most common malignancy in women in the Western world and a major cause of death [1].

Chemotherapy, hormonal therapy, radiotherapy, and limited or radical surgery are all used in the treatment of patients with breast cancer [2]. Many anticancer agents have shown efficacy in extending the survival of breast cancer patients; however, the mechanisms by which these agents inhibit breast cancer progression are not clearly understood.

Because cancer is often a preventable disease that can be affected by lifestyle factors, cancer prevention strategies can be effective and reduce costs [3]. Consequently, researchers are interested in discovering natural compounds that have anticancer activities, such as delaying the development of cancer and preventing its progression [4]. For example, studies recently found that natural components of green tea and grape seeds may have anticancer effects [5–7]. In particular, natural agents posited to help prevent breast cancer include resveratrol, deguelin, ginseng, and epigallocatechin gallate [5–7].

Specifically, ginseng has been a popular and important natural remedy for various diseases in Asia for centuries [8]. Antioxidant, anticancer, and antidiabetic activities of ginseng have been described [9–12], and studies have shown that ginsenosides, which are active constituents of ginseng, are very effective at inhibiting the growth of cancer cells *in vitro* and *in vivo* [12, 13]. Moreover, ginseng was well tolerated and demonstrated disease control in patients with breast cancer [14]. Although the most commonly studied ginsenosides are Rb1, Rg1, Rg3, and Rh1, another ginsenoside, Rp1, exhibits the most potent chemopreventive and antimetastatic effects [15] and induces apoptosis and cell cycle arrest at the G1 phase that is accompanied by activation of caspase-3, -8, and -9 in HeLa cells [16]. Several laboratories have shown that Rp1 can act as a novel regulator of CD29-mediated cell adhesion events and as an inhibitor of interleukin (IL)-1β production by inhibiting the nuclear factor (NF)-κB pathway [17, 18].

The phosphoinositide 3-kinase (PI3K)-Akt pathway positively regulates NF-κB activity in cancer cells [19]. Akt is a proto-oncogene that is frequently amplified or activated in cancer [19]. Activated Akt can upregulate c-Myc expression through activation of the IκB kinase complex and NF-κB [20]. The PI3K/Akt pathway is also a downstream transducer of insulin-like growth factor (IGF)-1 receptor (IGF-1R) signaling [21]. Recent studies showed that the IGF-1R signaling pathway plays a major role in cell proliferation, survival, and apoptosis, and in tumor progression [22]. Consequently, researchers have designed molecules directed against the IGF-1/IGF-1R pathway and proven that some have antitumor activities [23].

Taken together, these results indicate that Rp1 may have potential as an anticancer drug, although its mechanism of action remains poorly understood. We hypothesized that Rp1 inhibits breast cancer cell proliferation by inhibiting the IGF-1R/Akt pathway. As described here, we tested the efficacy of Rp1 against the human breast cancer cell lines MDA-MB-231, MCF-7, T-47D, and doxorubicin-resistant MCF-7 (MCF-7/DOX). To investigate the molecular mechanisms underlying this anticancer activity, we studied the effects of Rp1 on cancer cell colony formation, cell cycle arrest and/or apoptosis, IGF-1R and phosphorylated Akt (pAkt) expression, and the half-life of IGF-1R.

Materials and Methods

Cells and Materials

MDA-MB-231, MCF-7, and T-47D were obtained from the American Type Culture Collection (Manassas, VA). MCF-7/DOX cells were derived from MCF-7 cells by continuous culture in the presence of step-wise increasing concentrations (0.1–1.0 μM) of doxorubicin (Sigma-Aldrich, St. Louis, MO) for more than three months. Breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (GIBCO Invitrogen, Grand Island, NY). Rp1 (purity 99%), a racemic mixture of *R*- and *S*-enantiomers at a 1.0:1.3 ratio, was obtained from Ambo Institute (Seoul, Korea) and prepared using established protocols [24, 25]. Rp1 was dissolved in 100% dimethyl sulfoxide (DMSO) and the cells were treated with the indicated dose of Rp1 or 0.1% DMSO (control). Antibodies against IGF-1Rβ, extracellular signal-regulated kinase 1, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against pAkt, Akt, and phosphorylated extracellular signal-regulated kinase 1/2 were purchased from Cell Signaling Technology (Danvers, MA).

MTT Assay

The inhibitory effect of Rp1 on the growth of breast cancer cell lines was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay. Breast cancer cells were plated in 96-well plates (3×10^3 cells/well) and cultured in medium containing various concentrations (5–20 μM) of Rp1. The cells were allowed to grow for a total incubation period of 72 h. After addition of 10 μl MTT (5 mg/ml) to the cultures and further incubation at 37 °C for 2 h, the medium was removed and 100 μl DMSO was added to each well. The optical density of each well at 562 nm was measured using a microplate spectrometer.

Cell Cycle Arrest and Apoptosis Analysis

The effect of Rp1 on cell cycle arrest and apoptosis in breast cancer cells was determined using flow cytometric

analysis. To synchronize the cell cycle of different breast cancer cells, the cells were cultured in serum-free medium for 30 h, then released from serum starvation, and treated with Rp1 (20 μM) for 48 h. The cells were harvested, washed, fixed in paraformaldehyde and 70% ethanol, and stained using an APO-BrdU kit (Biovision Inc., Mountain View, CA) according to the manufacturer's protocol. Apoptotic cells were stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (BD Biosciences, San Jose, CA). Briefly, 5×10^5 cells were washed with PBS and then labeled with Annexin V-FITC and propidium iodide according to the instructions provided by the manufacturer. Flow cytometric analysis was performed using a BD FACS Calibur flow cytometer (BD) equipped with a 488-nm argon-ion laser. Approximately 10,000 cells were evaluated for each sample.

Western Blot Analysis

Total cell extracts of human breast cancer cells were treated with Rp1 as indicated. Preparation of whole-cell lysates, protein quantification, gel electrophoresis, and Western blotting were performed as described previously [16]. Protein concentrations were measured using a bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL). Equal amounts of protein from cell lysates from each treatment group were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted with primary antibodies. Antibody binding to the membrane was detected using enhanced chemiluminescence Western blotting detection reagents obtained from GE Healthcare (Chalfont St. Giles, UK). To test the effect of Rp1 on the half-life of IGF-1R protein, cycloheximide (100 ng/ml) was added to the medium of Rp1-treated MDA-MB-231 or MCF-7/DOX cells for the indicated time, and whole cell extracts were prepared as described above.

Colony Formation Assay

The effect of Rp1 on breast cancer cell colony formation was tested using a colony formation assay. Cells were plated overnight in six-well plates at a density of 5×10^2 cells/well and treated with the indicated concentrations of Rp1 for two weeks. The cells were then fixed with methanol and stained with crystal violet. The colonies that formed in each well were counted, including all cell colonies containing 50 or more cells.

Soft Agar Colony Formation Assay

A soft agar colony formation assay was performed to assess anchorage-independent growth of breast cancer cells treated with Rp1. DMEM containing 10% FBS and 1% agarose was placed in 24-well plates as the base agar. Cells were seeded in the wells at a density of 1.5×10^3 cells/well in top agar, which consisted of DMEM with 10% FBS containing 0.5% agarose. The plates were then incubated at 37 °C in a humidified incubator for three weeks. During this time, cells were treated twice a week with cell culture media containing 5 or 20 μM Rp1. After incubation, the colonies in each well were counted.

Reverse Transcriptase-Polymerase Chain Reaction

To measure gene expression changes at the RNA level, reverse transcriptase (RT)-polymerase chain reaction (PCR) was performed. Total RNA was isolated from breast cancer cells using TRIzol reagent (Invitrogen, Carlsbad, CA). First, complementary DNA was synthesized from 2 μg of RNA extracted from the cells using an M-MLV RT kit (Invitrogen). RT-PCR was carried out with gene-specific primers for IGF-1R (forward, 5′-ACG CCA ATA AGT TCG TCC AC-3′; reverse, 5′-TCC ATC CTT GAG GGA CTC AG-3′). Primers amplifying a region of β-actin (forward, 5′-GTG GGG CGC CCC AGG CAC CA-3′; reverse, 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′) were used as internal controls. After PCR, the products were analyzed by separation on a 1.5% agarose gel in tris-acetate/ethylenediaminetetraacetic acid buffer.

Statistical Analysis

Data are shown as means ± standard deviations. Cell proliferation and colony formation data were analyzed by Student's *t*-test using Excel 2007 software (Microsoft Corp., Redmond, WA) to determine statistically significant differences between groups. All statistical tests were two-sided. A p value of less than 0.05 was considered statistically significant.

Results

Ginseng has long been a popular and important natural remedy for various diseases in Asia [8]. We first investigated whether the Rp1 component of ginseng has antiproliferative effects in cultured breast cancer cells. We treated the breast cancer cell lines MCF-7, MCF-7/DOX, MDA-MB-231, and T-47D with the indicated concentrations of Rp1 for 72 h and found that Rp1 inhibited breast cancer cell proliferation in a concentration-dependent manner in all cell lines (Fig. 1a). Similarly, the colony formation assay showed dose-dependent inhibition of colony formation by Rp1, further confirming the anticancer effects of Rp1. Long-term treatment with Rp1 (at concentrations of 10 μM in assays for anchorage-dependent colony formation and 5 μM in assays

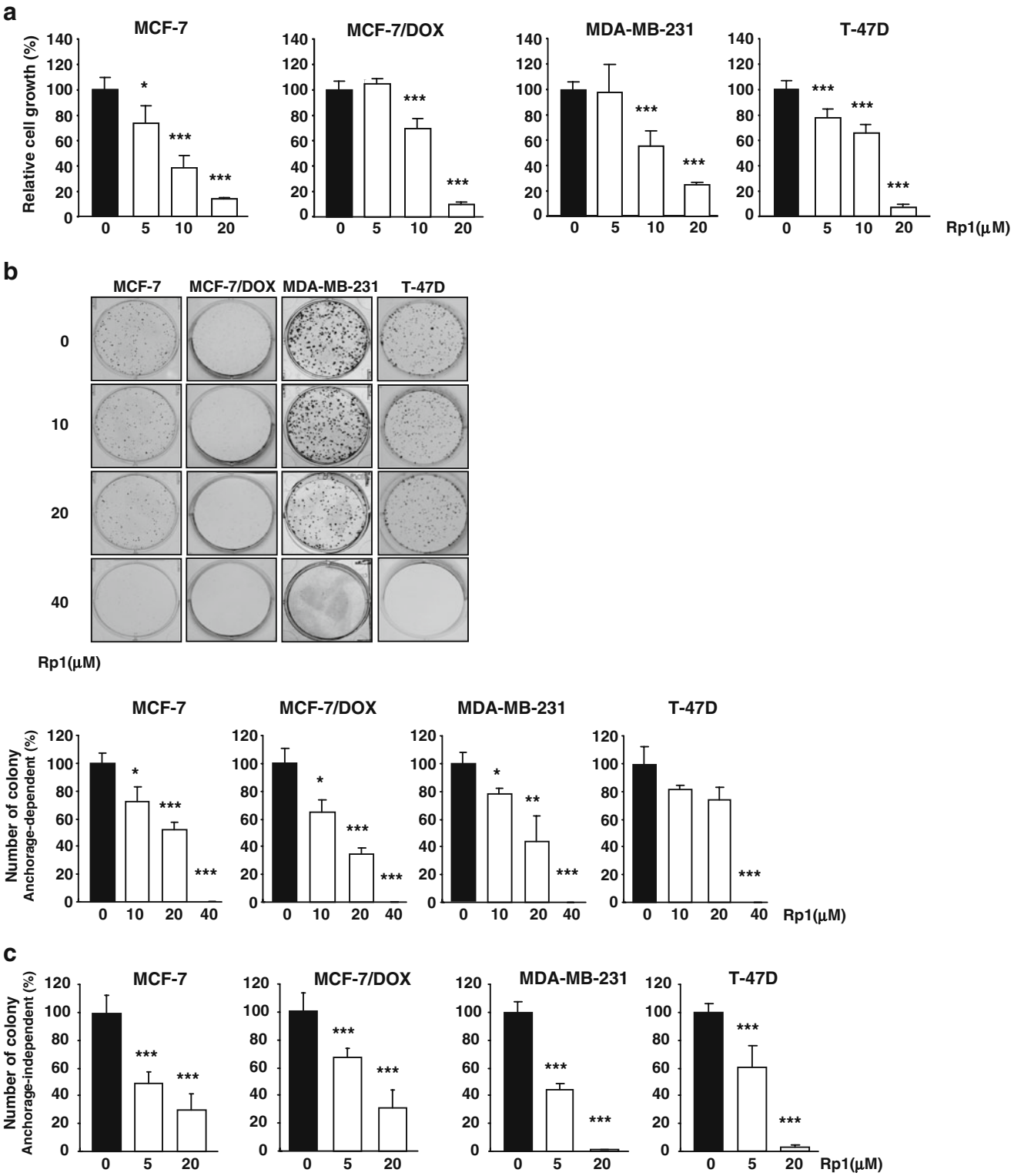
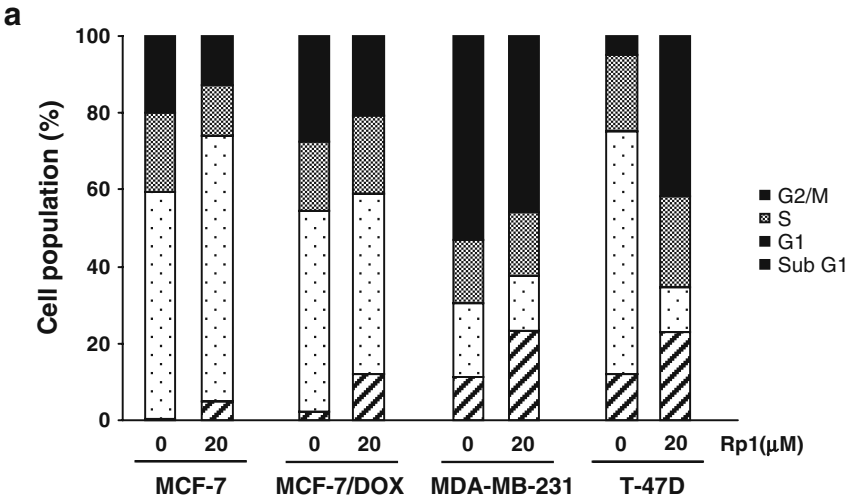


Fig. 1 Inhibition of cell proliferation by the ginsenoside Rp1 (μM). **a** Proliferation of MCF-7, MCF-7/DOX, MDA-MB-231, and T-47D breast cancer cells after 72 h treatment with Rp1 at the indicated concentrations. **b** Anchorage-dependent colony formation assay of breast cancer cells following treatment with Rp1 for two weeks. **c** Anchorage-independent colony formation assay of breast cancer cells following treatment with Rp1 for three weeks. The values shown are means ± standard deviation. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with the untreated group (Student's *t*-test)

for anchorage-independent colony formation) significantly reduced both anchorage-dependent and -independent colony formation in MCF-7, MCF-7/DOX, MDA-MB-231, and T-47D cells (Fig. 1b and c).

To determine whether the growth inhibition observed in these breast cancer cell lines was the result of apoptosis, we next analyzed cell-cycle arrest and apoptosis. As shown in Fig. 2a, Rp1 induced subG1 arrest in all the breast cancer



	MCF-7		MCF-7/DOX		MDA-MB-231		T-47D	
	con	Rp1(20μM)	con	Rp1(20μM)	con	Rp1(20μM)	con	Rp1(20μM)
sub-G1	0.50	4.70	2.31	11.41	8.77	16.97	11.94	20.61
G1	57.79	68.21	50.17	45.6	14.73	10.34	62.42	10.38
S	19.98	13.19	17.6	19.27	12.79	11.88	19.52	21.42
G2/M	19.59	12.59	26.43	20.03	40.84	33.43	4.96	37.46

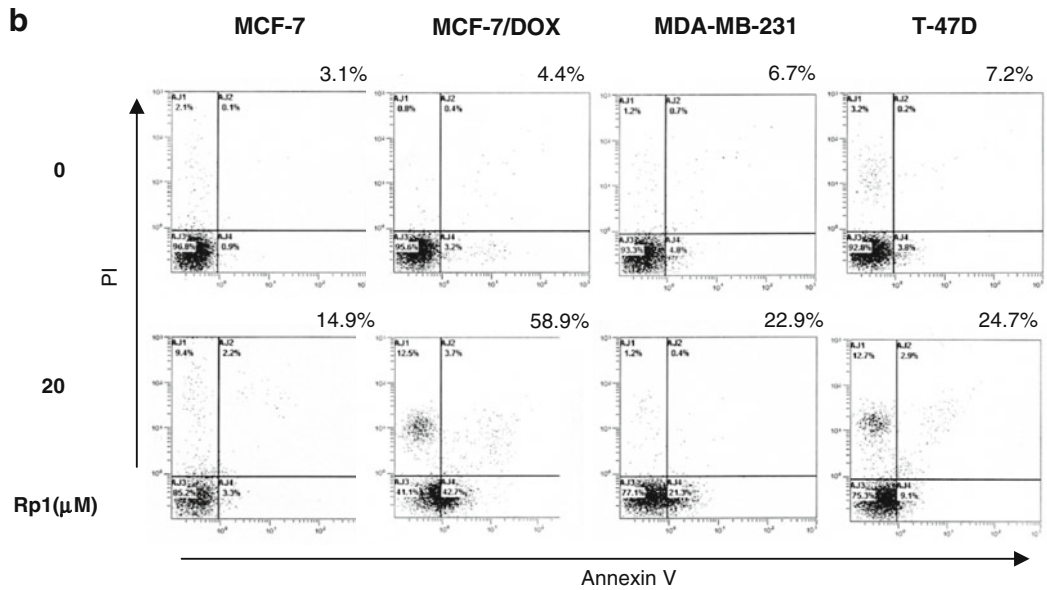


Fig. 2 Effect of Rp1 on cell-cycle arrest and apoptosis in MCF-7, MCF-7/DOX, MDA-MB-231, and T-47D breast cancer cells. **a** Breast cancer cells were grown in serum-free medium for 30 h and then released from serum starvation and treated with Rp1 (20 μM) for 48 h. Cells were stained with propidium iodide for cell cycle analysis using flow cytometry. **b** Apoptosis and/or necrosis in breast cancer cells was evaluated using annexin V-FITC and propidium iodide staining of breast cancer cells, and Rp1-induced cell death was analyzed using flow cytometry. The percentages of apoptotic and necrotic cells are shown in the top right corner of each panel

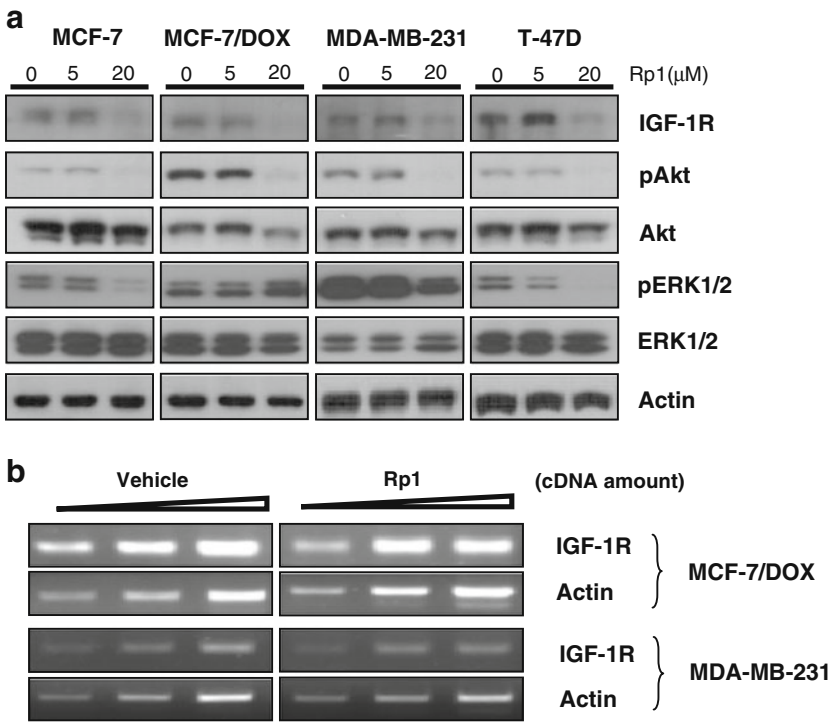


Fig. 3 Effect of Rp1 on the IGF-1R pathway in MCF-7, MCF-7/DOX, MDA-MB-231, and T-47D breast cancer cells. **a** Western blot analysis of breast cancer cells following treatment with Rp1 for 48 h. **b** RT-PCR analysis revealed that IGF-1R mRNA expression levels in MCF-7/DOX and MDA-MB-231 cells were not changed in response to treatment with Rp1

cell lines used in this experiment (Fig. 2a). FACS analysis after annexin V and propidium iodide staining showed that treatment with 20 μM Rp1 induced apoptosis (annexin V-positive or propidium iodide-positive cells) in all of the breast cancer cell lines tested; however, MCF-7/DOX cells had a stronger apoptotic response to Rp1 than the other cell lines (Fig. 2b). These results suggested that Rp1 induces cell-cycle arrest and apoptosis and that its anticancer effect varies according to the type of breast cancer cell. We next sought to clarify which molecule(s) were involved in the growth-inhibitory mechanism of Rp1 in breast cancer cells.

The IGF-1R signaling pathway is known to play a major role in cell proliferation, survival, and apoptosis, and in tumor progression [22, 26]. Additionally, phosphorylation of IGF-1R activates the PI3K/Akt pathway, which is known to positively regulate NF-κB activity [19]. It was recently reported that Rp1 regulates CD29-mediated cell adhesion events and suppresses IL-1β production by inhibiting the NF-κB pathway [17, 18]. Given that the IGF-1R/Akt pathway plays an important role in NF-κB signaling, we investigated the effect of Rp1 on the IGF-1R/Akt pathway in breast cancer cells. We observed that treatment with Rp1

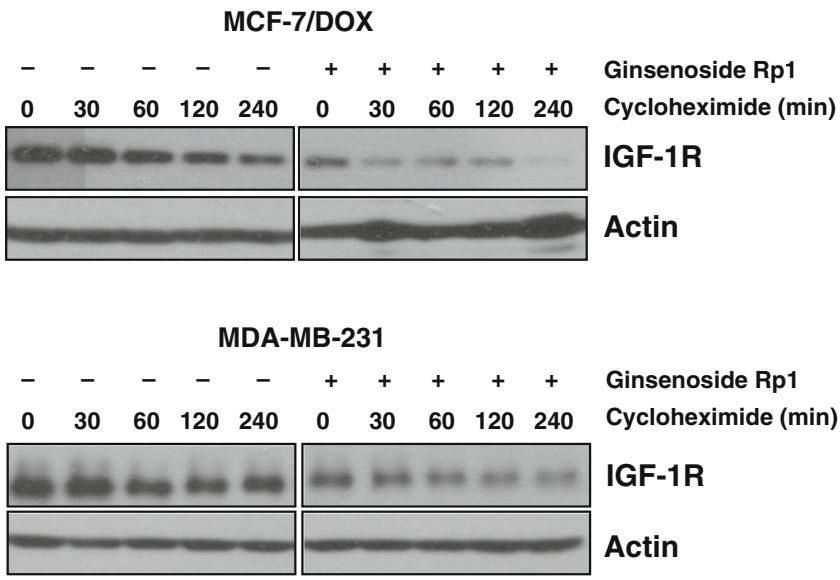


Fig. 4 Effect of Rp1 on IGF-1R protein stability in breast cancer cells. MCF-7/DOX and MDA-MB-231 cells were pretreated with Rp1 (20 μM) for 48 h and cycloheximide (100 ng/ml) was added to the medium for the indicated time to inhibit *de novo* protein synthesis. Cell lysates were analyzed by Western blotting

resulted in degradation of IGF-1R and reduced pAkt expression (Fig. 3a). RT-PCR analysis of the effects of Rp1 on IGF-1R mRNA expression in MCF-7/DOX and MDA-MB-231 cells revealed that the IGF-1R mRNA expression levels did not change in either cell line in response to treatment with Rp1 (Fig. 3b).

To identify the mechanism by which Rp1 reduces the level of IGF-1R protein expression in breast tumors, we incubated breast cancer cells with Rp1 and then added cycloheximide to block protein synthesis. We measured IGF-1R protein expression levels before and at various times during treatment with cycloheximide. The half-life of IGF-1R protein was significantly shorter in the Rp1-treated cells than in untreated cells (Fig. 4) indicating that Rp1 appears to act at least in part through decreasing the stability of IGF-1R protein in breast cancer cells.

Discussion

The ginseng active component Rp1 exhibits the most potent chemopreventive and antimetastatic effects of all ginsenosides and has been shown to induce cell-cycle arrest and apoptosis in cancer cells [15, 16]. Here, we show that Rp1 decreases the stability of IGF-1R protein in breast cancer cells. This is significant because the IGF-1R signaling pathway plays a pivotal role in cell proliferation and survival, apoptosis, and invasion [22, 26, 27].

PI3K and Akt, which are downstream transducers of the IGF-1R signaling pathway, positively regulate the expression of NF-κB, [19, 21] a transcription factor protein complex important for cell proliferation, angiogenesis, metastasis, tumor promotion, inflammation, and apoptosis suppression [28, 29]. Because recent results from several laboratories showed that Rp1 may inhibit the NF-κB pathway, and therefore act as a novel regulator of CD29-mediated cell adhesion events and a suppressor of IL-1β production [17, 18], we hypothesized that the mechanism by which Rp1 induces breast tumor cell-cycle arrest and apoptosis involves downregulation of the IGF-1R/Akt pathway in cancer cells, thus inhibiting the NF-κB pathway.

In this study, we examined the effect of Rp1 on the growth of breast cancer cells. Rp1 inhibited the proliferation of these cells in a concentration-dependent manner. We also showed that Rp1 inhibited both anchorage-dependent and -independent colony formation and that this inhibition of cancer cell growth was mediated by cell cycle arrest and apoptosis. Rp1 induced cell cycle arrest at the G1 phase in MCF-7 cells and at the G2/M phase in MDA-MB-231 and T-47D cells. Additionally, Rp1 significantly increased the number of dead cells (represented by the sub-G1 population) in MCF-7/DOX and T-47D cell lines. Consistent with these data, recent studies showed that treatment with Rp1 inhibited the proliferation of HeLa cells and upregulated the partial accumulation of these cells at the G1 phase [16]. Indeed, Rp1 appears to have strong antiproliferative and apoptotic activity in HeLa cells [16].

Interestingly, we observed that Rp1 treatment resulted in degradation of IGF-1R and reduced pAkt expression. RT-PCR analysis revealed that IGF-1R mRNA expression levels did not change in MCF-7/DOX or MDA-MB-231 cells in response to treatment with Rp1. We therefore measured IGF-1R protein stability and found that the half-life of IGF-1R protein in the Rp1-treated cells was significantly shorter than in untreated cells. Thus, Rp1 appears to decrease the stability of IGF-1R protein in breast cancer cells.

A recent report suggested that heat shock protein 90 (Hsp90), one of the major molecular chaperones, interacts with IGF-1R, Akt, epidermal growth factor receptor, and hypoxia-inducible factor-1α in cancer cells [30, 31]. Rp1 may therefore decrease the stability of IGF-1R by suppressing Hsp90 function, although this has yet to be studied. Western blot analysis showed that expression of epidermal growth factor receptor in MCF-7/DOX, MDA-MB-231, and T-47D cells was decreased by Rp1 treatment (data not shown). Additional ongoing studies are investigating the effects of Rp1 on Hsp90 function. Given that the IGF-1R/Akt pathway plays a major role in cell invasion and metastasis [27, 32], IGF-1R may have been the biologic target in the successful inhibition of lung metastasis in an Rp1-treated mouse model [15]. In this model, Rp1 appeared to have an antimetastatic effect, but did not show any inhibitory activity against matrix metalloproteinase (MMP)-2 [15]. We also confirmed that Rp1 had no effect on MMP-2 or MMP-9 activity (data not shown). Therefore, investigation of how metastasis may be inhibited by Rp1 would be worthwhile.

In conclusion, Rp1 induced cell cycle arrest and apoptosis and decreased the stability of the IGF-1R protein in breast cancer cells. We suggest that Rp1 has potential as an anticancer drug and that IGF-1R is an important target in the treatment of breast cancer.

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