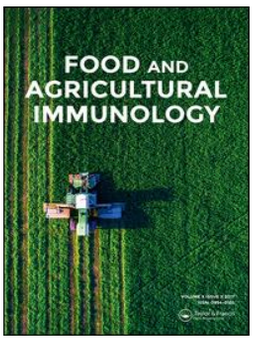

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Synergistic effect of combined protopanaxatiol and ginsenoside Rh2 on antiproliferative activity in MDA-MB-231 human breast cancer cells *in vitro*

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ABSTRACT

Breast cancer is the most common cancer in women worldwide. The antiproliferative activities of protopanaxatiol (PPT) and ginsenoside Rh2 was measured by evaluating the inhibition of MDA-MB-231 human breast cancer cell proliferation. The two-way combination of PPT and Rh2 was conducted. In the two-way combination, the EC_{50} values of PPT and Rh2 were 2.3- and 2.2-fold lower, respectively, than those of PPT and Rh2 alone. The combination index (CI) values were 0.55 ± 0.09, 0.65 ± 0.10, 0.79 ± 0.12, and 0.91 ± 0.14 at 50% and 95% inhibition rates. Combined PPT and Rh2 also increased the inhibition of cell invasion and migration compared with individual compounds tested in cell lines. Intracellular signalling array analysis demonstrated that phosphorylated BAD, p53, and p38 proteins were increased. The results indicate that combined PPT and Rh2 exhibits a synergistic effect in MDA-MB-231 cell proliferation.

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PPT; Rh2; synergy; breast cancer; antiproliferative activity

Introduction

Breast cancer is the second most common cancer in the world and by far the most common cancer among women. Approximately 1.67 million new breast cancer cases were diagnosed in 2012 (25% of all cancers); breast cancer is also the second most common cause of cancer-related death in developed regions (198,000 deaths, 15.4%) and the most frequent cause of cancer-related death in less developed regions (324,000 deaths, 14.3% of total) (Ferlay et al., 2015). Human epidermal growth factor receptor 2-negative, progesterone receptor-negative, and oestrogen receptor-negative characterize as triple negative breast cancer (Kreike et al., 2007). Triple-negative breast cancer, unlike other subtypes, lacks effective therapeutic and preventive strategies, which causes less progress in the treatment (Adams, Phung, Seeram, Li, & Chen, 2010). Furthermore, many reports have shown that natural products play a crucial role in the prevention

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and therapy of cancer, and more than 60% of the anticancer drugs are of natural products, including thousands of fruits, vegetables, and medical plants (Gan, Zeng, Liu, & Ye, 2015).

Ginseng is one of the best-selling herbal products in the world. There is now much interest in this product for its physiological functionalities, such as the ability to fight cardiovascular disease (Lee, Kim, Shin, & Yoon, 2014), anti-diabetes (Shishtar, Jovanovski, Jenkins, & Vuksan, 2014), and anti-central nervous diseases due to the ginsenosides constituents. There is also gained increasing attention in the treatment of anticancer activities associated with the proliferation of cancer (Chung et al., 2013), angiogenesis (Zeng et al., 2014), inflammation (Shin, Jung, Choi, & Lim, 2013), apoptosis (Li et al., 2014), and cell oxidative stress (Mao, Zhang, Wang, & Li, 2014). Previous studies showed that protopanaxatiol (PPT) possesses a series of pharmacological effects, such as antioxidant (Gao et al., 2015), anti-fatigue (Oh, Kim, Choi, Kim, & Kim, 2015), anti-obesity (Zhang et al., 2014), antidiabetic (Liu et al., 2013), and anti-adipogenesis properties (Han, Jung, Sohn, & Hwang, 2006). It was also shown that PPT exhibited an anticancer effect on HepG2 cells (Quan et al., 2015). PPT and Rh2 (protopanaxadiol-type) are the two major types of dammarane saponins. Previously, a study showed that Rh2 has beneficial impacts in breast cancer (Oh et al., 1999), hepatoma cells (Park, Kim, Kim, & Kang, 2012, glioma cells (Wu, Wu, Hu, Li, & Feng, 2011), lung cancer cells (Zhang, Yu, & Hou, 2011), and prostate cancer cells (Kim et al., 2004).

It is well known that chemotherapeutic combination methods have long been used to lower drug toxicity, to attenuate of the pathology of cancer cells and to achieve higher efficacy than using only one drug. Few studies have examined interactions between or among ginseng saponins involved in antiproliferative activity in MDA-MB-231 cells. The present study was therefore carried out to determine whether combined PPT and Rh2 has synergistic effects of antiproliferative activity in MDA-MB-231 human breast cancer cells.

Materials and methods

Chemicals

PPT and Rh2 were obtained from the National Institutes for Food and Drug Control (Beijing, China). Dimethyl sulfoxide (DMSO) and methylene blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hank's Balanced Salt Solution (HBSS), phosphate-buffered saline (PBS), foetal bovine serum (FBS) 2-(4-(2-hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (HEPES), and α-minimum essential medium (α-MEM) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA).

Cytotoxicity activity

The cytotoxicity of PPT or Rh2 toward MDA-MB-231 human breast cancer cells were measured by methylene blue assay as reported[40 41]. MDA-MB-231 cells in 100 μL fresh medium were plated in 96-well plate at a density of 4.0×10^4 cells/well and were incubated at 37°C in 5% CO_2 for 24 h. Then, the growth medium was removed and the cells were

treated with different concentrations samples or 100 μL fresh medium as control. After another 24 h incubation, the growth medium was removed from each well and the cells were washed with 100 μL phosphate-buffered saline (PBS). Cells were then stained with methylene blue solution (98% HBSS, 0.67% glutaraldehyde, 0.6% methylene blue) and incubated for 1 h. After that, the solution was removed and rinsed with deionized water three times. After the wells were air-dry, methylene blue stain in cells was eluted with the elution buffer [1% (v/v) acetic acid, 49% (v/v) PBS, and 50% (v/v) ethanol] by rotating on a bench shaker for 20 min. The absorbance was read at 570 nm by using a microplate reader (Bio-Rad, Ma., U.S.A.). Cytotoxicity was determined as percentage compared to the control [41]. All measurements were conducted in triplicate.

Antiproliferative activity

The antiproliferative activity of PPT and Rh2 towards MDA-MB-231 cells was analysed by the methylene blueassay according to a previously reported method (Felice, Sun, & Liu, 2009). Briefly, cells were grown and maintained in α-MEM containing 10% foetal bovine serum, 10 mM Hepes and 1% antibiotic-antimycotic in a humidified incubator at 37°C in a humidified atmosphere of 5% CO_2 (Jiang, Li, & Liu, 2016). MDA-MB-231 cells were seeded into 96-well plates with 2.5×10^6 cells per well. After 12 h, cells were treated with different concentrations of Rh2, PPT, or control. Then, 72 h later, cell proliferation was evaluated using the methylene blue assay, measuring absorbance at 570 nm via a microplate reader (Bio-Rad, Ma., U.S.A.).

Combination study

The two-way cell proliferation combination of Rh2 and PPT into MDA-MB-231 was tested. The EC_{50} levels of PPT or Rh2 were evaluated according to the dose-response curve. On the basis of the EC_{50} value, a variety of concentrations at different ratios of EC_{50} were analysed. For PPT and Rh2, the combined concentrations were $0.125 \times EC_{50}$, $0.25 \times EC_{50}$, $0.50 \times EC_{50}$, $0.75 \times EC_{50}$, $1.00 \times EC_{50}$, and $1.25 \times EC_{50}$. Finally, a variety of concentrations of PPT and Rh2 mixture were used to determine the dose-response curve in the MDA-MB-231 cell proliferation model.

A combination index (CI) was evaluated for the combinations of PPT and Rh2 according to CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA), which is based on the mass-action law and the Chou–Talalay equation (Chou, 2010). $CI < 1$ indicates a synergistic effect of a combination, $CI = 1$ indicates additive, and $CI > 1$ indicates antagonistic.

Wound-healing assay

Quantification of wound closure was carried out according to the kit assay (Ibidi, LLC, Munchen, Germany) using two individual wells for cell seeding. Every insert was placed in an individual dish, and 5×10^5 cells per millilitre of MDA-MB-231 cells were seeded and cultivated to form a confluent and homogeneous layer. After 24 h, cell seeding of a cell-free area was performed by discarded the culture insert. The wound was approximately 500 μm wide. Healing of the wound by migrating cells after PPT, Rh2 or PPT

plus Rh2 treatment was observed after 24 h by light microscopy (CX-2, Olympus) (Lee et al., 2012).

Invasion assay

The cell invasiveness was evaluated with an extracellular matrix (ECM) invasion assay kit (Millipore, Billerica, MA) following the manufacturer's instructions. Briefly, cells were suspended in 300 μL of serum free media at a density of 5×10^5 cells/mL and placed on the upper levels of an ECM-coated membrane insert. The invasion assay was terminated after 48 h with PPT, Rh2, PPT plus Rh2, or control. Then, the cells on the lower levels of the insert were stained with the solution in the kits, and the cells in the top levels were cleaned using a cotton applicator. The quantitation of invasive cells was measured using Matrigel invasion assays (Tapia, Argandona, Palomino, & Devoto, 2013).

Intracellular signalling array

A slide-based antibody array was evaluated with a PathScan Intracellular Signalling Array kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's protocol. MDA-MB-231 cells were treated with PPT, Rh2, control, and PPT plus Rh2 for 24 h. Total protein was extracted and adjusted to 0.5 mg/mL in an array diluent buffer contained in the kit. The detection antibody cocktail was mixed with the samples, antibodies were added to the samples for 4 h, and then, the HRP-linked streptavidin and substrate was added. Protein was visualized using UVP chemiluminescent detection and imaging system (Upland, CA, USA).

Statistical analysis

Data was analysed using SigmaPlot software version 11.0 (Systat Software, Inc. Chicago, IL, USA). Data were presented as the mean ± standard deviation (SD). Statistical analyses were carried out with Student's t test and analysis of variance (ANOVA) by SPSS software version 16.0. Differences were considered to be statistically significant at $p < 0.05$.

Results and discussion

Antiproliferative activity of MDA-MB-231

The antiproliferative activities of PPT, Rh2, and combined PPT and Rh2 towards human breast cell lines of MDA-MB-231 are shown in Figure 1. PPT showed antiproliferative activity at doses of 30–100 μM ($p < 0.05$), and the inhibition was dose-dependent. The

Table 1. EC_{50} Values of PPT, Rh2, and PPT in Combination with Rh2 in Inhibiting MDA-MB-231 Cell Growth.

Component	EC_{50} Single	Value Combined
PPT	62.44 ± 5.37 μM	27.74 ± 2.05 μM
Rh2	57.53 ± 3.97 μM	25.58 ± 3.25 μM

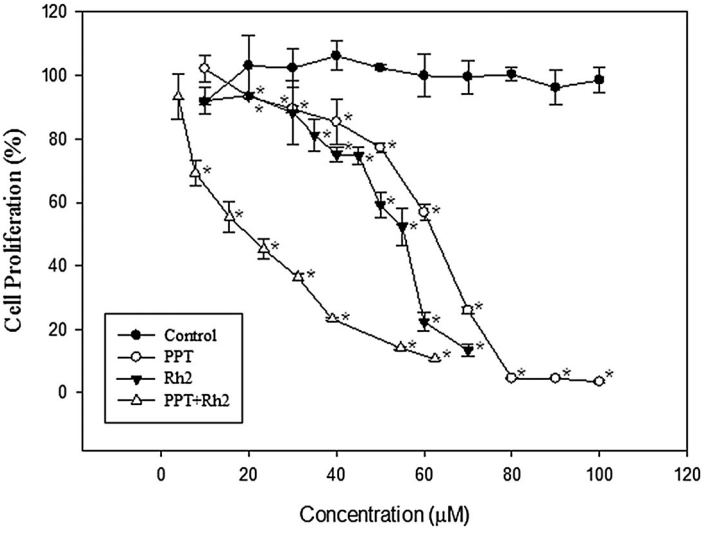


Figure 1. Synergistic interactions between PPT and Rh2 in inhibition of MDA-MB-231 human breast cancer cells proliferation (mean ± SD, $n = 3$). An asterisk (*) indicates a significant difference from the control ($p < 0.05$). Each value represents the mean ± SD of triplicates.

EC_{50} value of antiproliferative activities for PPT towards MDA-MB-231 cells was 62.44 ± 5.37 μM (Table 1). Rh2 also showed antiproliferative activity towards MDA-MB-231 cells in a dose-dependent manner at doses of 20–70 μM ($p < 0.05$) (Figure 1).

The EC_{50} value of antiproliferative activities for Rh2 was 57.53 ± 3.97 μM. Choi et al. showed that Rh2 treatment inhibited viability of MDA-MB-231 cells 28% and 85% at doses of 40 and 60 μM, respectively (Choi, Kim, & Singh, 2009).

Interest in drug combination is growing because it has the advantage of an increased anti-cancer effect, a lower drug dose, and reduced side effects. Compared to PPT and Rh2 alone, treatment with combined PPT and Rh2 significantly suppressed proliferation in the growth of MDA-MB-231 cells (Figure 1). The EC_{50} levels of PPT and Rh2 combination were 2.3- and 2.2-fold lower than those of PPT and Rh2 alone, which decreased to 27.74 ± 2.05 mg/mL and 25.58 ± 3.25 μM, respectively (Table 1). The CI levels of combined PPT and Rh2 at 50%, 75%, 90%, and 95% inhibition in MDA-MB-231 cell growth were 0.55 ± 0.09, 0.65 ± 0.10, 0.79 ± 0.12, and 0.91 ± 0.14, respectively, demonstrating that there was a strong synergistic effect at different concentrations. Xie et al. reported the synergistically effect of paclitaxel plus Rh2 in the LNCaP prostate cancer models both *in vitro* and *in vivo* and claimed that paclitaxel plus Rh2 showed synergistic effects in LNCaP cells to decrease 50% of effective dose (ED_{50}) and ED_{75} values. Administration of LNCaP tumours by Rh2 plus paclitaxel significantly reduced tumour growth and the serum prostate specific antigen. In addition, the proliferation markers in LNCaP tumours were significantly improved in immunohistochemical analysis (Xie et al., 2006). The nutrition function of food is similar with the additive and synergistic effects of phytochemicals rather than with a single compound alone. These compounds have differences in solubility, polarity, and molecular size, which may affect their distribution and bioavailability in different subcellular organs, tissues, cells, and organelles (Yang &

Liu, 2009). Furthermore, the purified phytochemicals may lose some of their bioactivity and behave differently in whole food (Liu, 2004).

Inhibition of cell migration

The important indicator of cancer metastasis is the migration and invasion of tumour cells. To further investigate the pharmacological activity of PPT, Rh2, and PPT plus Rh2 against cancer metastasis, we examined the effect on cell invasive and migratory ability in MDA-MB-231 cells. The effect of PPT, Rh2, and PPT plus Rh2 on migration was examined using the wound-healing assay. Figure 2 showed both PPT and Rh2 could inhibit the wound closure in the MDA-MB-231 cell. The combination of PPT

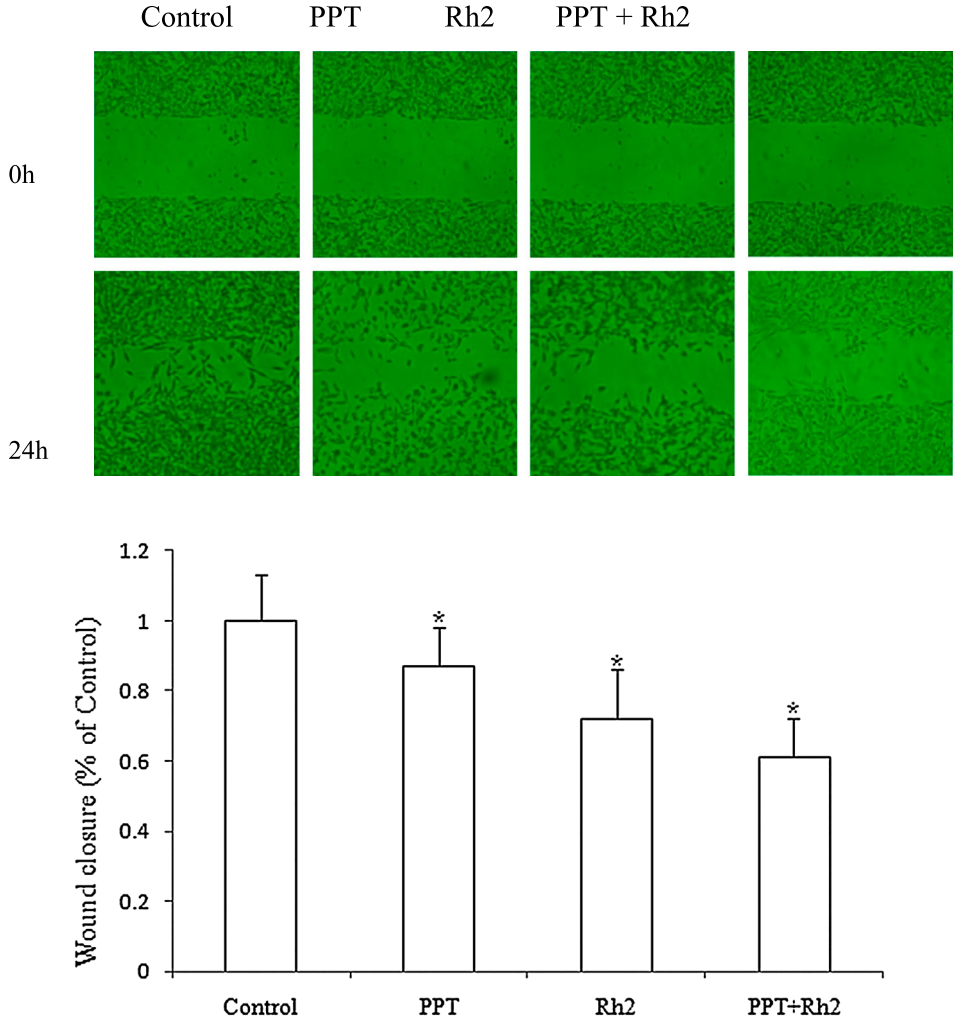


Figure 2. For the scratch assay, wounds were made when MDA-MB-231 cells were 90–100% confluent and after an overnight starvation. The cells were treated with vehicle control, 62.44 μm PPT, 57.53 μm Rh2, or 27.74 μm PPT + 25.58 μm Rh2 for 24 h. The closure of wounds were imaged and measured at 0 and 24 h. *Compared to control, #compared to PPT or Rh2 alone, $p < 0.05$.

and Rh2 significantly improved the inhibition abilities compared to PPT or Rh2 alone. The combination treatment decreased the wound closure by 35% compared to control. The effect of PPT, Rh2, and PPT plus Rh2 was tested using the transwell chamber assay for cell invasion. Figure 3 shows the mixture of PPT plus Rh2 significantly increased the inhibition invasion compared to PPT or Rh2 alone. The migration of MDA-MB-231 cells was inhibited by 58% with PPT plus Rh2 treatment compared to control. These results suggest that PPT, Rh2, and PPT plus Rh2 have strong invasive and migratory inhibition abilities in MDA-MB-231 cells. Additionally, the combination exhibited more effectiveness in these two assays.

Protein expression and signalling pathways

The intracellular signalling array analysis showed increased phosphorylation of multiple signalling molecules in MDA-MB-231 cells, including BAD (a pro-apoptic protein from Akt pathway), p53 (the tumour suppressor), and p38 (MAPK pathway) proteins (Figure 4). Rh2 indicated greater effects than PPT in increasing the phosphorylation of these proteins. The combination of PPT plus Rh2 showed a stronger ability to increase the inhibitory effect.

Liu et al. evaluated the antiproliferative activity of Rh2 in K562 and KG1-α human leukaemia cells *in vitro* and inhibition affection for the growth of human leukaemia xenograft tumours *in vivo* and reported that Rh2 has an antiproliferative effect on those cells by increase histone acetylation. In addition, Rh2 induced apoptosis through activation of the MAPK signalling pathway by significantly improved JNK, p-JNK, p38 and p-p38 protein expressions (Liu et al., 2015).

Choi et al., reported that Rh2 inhibited the MDA-MB-231 cells viability by reducing transcriptional ability of E2 promoter binding factor 1 and decreased the values of phosphorylated retinoblastoma protein in a luciferase reporter assay. In addition, Rh2

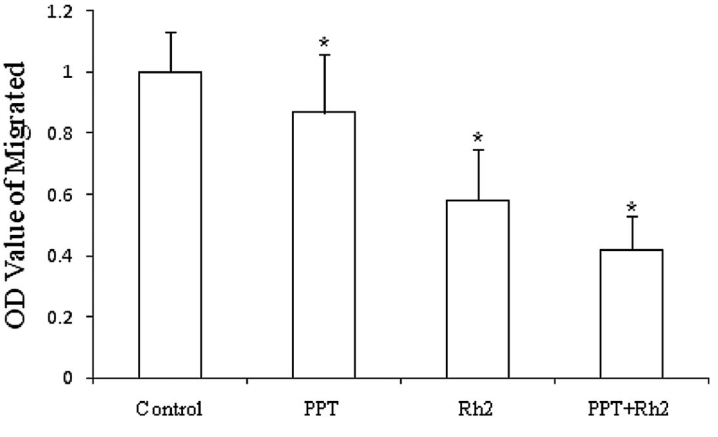
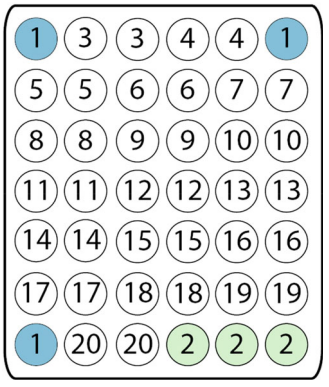


Figure 3. For transwell chamber assay, MDA-MB-231 cells were treated with vehicle control, 62.44 μm PPT, 57.53 μm Rh2, or 27.74 μm PPT + 25.58 μm Rh2 for 48 h. Cells suspended in serum-free medium were seeded on the upper membrane of transwell chamber and incubated for 48 h. Complete growth medium was added on the bottom. Cells on the lower membrane of chambers were counted. Data are presented as mean ± SD. *Compared to control, #compared to PPT or Rh2 alone, $p < 0.05$.



Intracellular Signaling

	Target	Phosphorylation Site	Modification
1	Positive Control	N/A	N/A
2	Negative Control	N/A	N/A
3	ERK1/2	Thr202/Tyr204	Phosphorylation
4	Stat1	Tyr701	Phosphorylation
5	Stat3	Tyr705	Phosphorylation
6	Akt	Thr308	Phosphorylation
7	Akt	Ser473	Phosphorylation
8	AMPKα	Thr172	Phosphorylation
9	S6 Ribosomal Protein	Ser235/236	Phosphorylation
10	mTOR	Ser2448	Phosphorylation
11	HSP27	Ser78	Phosphorylation
12	Bad	Ser112	Phosphorylation
13	p70 S6 Kinase	Thr389	Phosphorylation
14	PRAS40	Thr246	Phosphorylation
15	p53	Ser15	Phosphorylation
16	p38	Thr180/Tyr182	Phosphorylation
17	SAPK/JNK	Thr183/Tyr185	Phosphorylation
18	PARP	Asp214	Cleavage
19	Caspase-3	Asp175	Cleavage
20	GSK-3β	Ser9	Phosphorylation

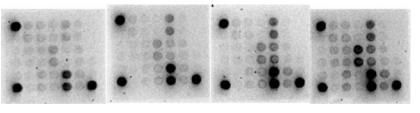


Figure 4. Modulations of intracellular signalling pathways in MDA-MB-231 human breast cancer cells using antibody array analysis. (A) MDA-MB-231 cells were treated with vehicle control (DMSO), 62.44 μm PPT, 57.53 μm Rh2, or 27.74 μm PPT + 25.58 μm Rh2 for 24 h. Total protein was extracted for the analysis. A slide-based antibody array was used for simultaneous detection of 18 signalling molecules (B) when phosphorylated or cleaved using a PathScan Intracellular Signaling Array kit. Each protein was arranged in duplicate. The names of the proteins with changes in phosphorylation are indicated on the images.

downregulation of cyclins and cyclin-dependent kinases (Cdk) resulted in enhanced recruitment of $p15^{Ink4B}$ and $p27^{Kip1}$ to cyclin D1/Cdk4 and cyclin D1/Cdk6 complexes and reduced interaction between cyclin D1 and Cdk4/Cdk6 (Choi et al., 2009). It has also been observed that the Rh2 induced apoptotic cell death by upregulation of Bax and activation of caspase-1 and caspase-3 in neuroblastoma cells (Kim & Jin, 2004). Ginsenoside Rg5 promoted breast cancer cell apoptosis by inducing G0/G1 cell cycle arrest in MDA-MB-453 and MCF-7 human breast cancer cell lines. The tumour suppressor p53 induces cell self-destruction through the exogenous death receptor pathway and endogenous mitochondrial pathway, which is called p53-dependent apoptosis (Kim & Kim, 2015). In addition, p53-dependent apoptosis is used to induce the expression of proapoptotic members. When the cells undergo DNA damage, p53 stops the cell cycle through p21 or it induces apoptosis. In response to cellular stress or DNA damage, p53 is stabilized by post-transcriptional modifications and the level of p53 increases (Laptenko & Prives,

2006). Activation and stabilization of p53 is responsible for cellular antiproliferative mechanisms such as cell senescence, apoptosis, and growth arrest (Brooks & Gu, 2003).

In conclusion, the combination treatment with PPT plus Rh2 synergistically improved the antiproliferative effect in MDA-MB-231 breast cancer cells, associated with increased regulations on multiple signalling proteins including BAD, p53, and p38. Therefore, the present study suggests that PPT plus Rh2 may be capable of becoming a treatment for alleviating triple-negative breast cancer.

Disclosure statement

No potential conflict of interest was reported by the authors.

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