Paclitaxel resistance in MCF-7/PTX cells is reversed by paeonol through suppression of the SET/phosphatidylinositol 3-kinase/Akt pathway

WEIPENG ZHANG[1], JIANGXIA CAI[1,2], SIYING CHEN[1], XIAOWEI ZHENG[1], SASA HU[1], WEIHUA DONG[1], JUN LU[1], JIANFENG XING[3] and YALIN DONG[1]

[1]Department of Pharmacy, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, Xi'an, Shaanxi 710061; [2]Department of Pharmacy, The People's Hospital of Bayingol Mongolian Autonomous Prefecture, Korla, Xinjiang 841000; [3]Department of Pharmacy, College of Medicine, Xi'an Jiaotong University, Xi'an, Shaanxi 710061, P.R. China

Received May 13, 2014; Accepted February 5, 2015

DOI: 10.3892/mmr.2015.3468

Abstract. Breast cancer is one of the most prevalent types of malignant tumor. Paclitaxel is widely used in the treatment of breast cancer; however, the major problem contributing to the failure of chemotherapy in breast cancer is the development of drug resistance. Therefore, it is necessary to identify novel therapeutic targets and reversal agents for breast cancer. In the present study, the protein expression levels of SET, protein phosphatase 2A (PP2A) and phosphatidylinositol 3-kinase (PI3K)/Akt pathway were determined in MCF-7/PTX human breast carcinoma paclitaxel-resistant cells using western blot analysis. Small interference RNAs (siRNAs) were used to knock down the gene expression of *SET* in MCF-7/PTX cells and the cell viability was assessed following treatment with paclitaxel, using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays and flow cytometry. In addition, western blot analysis was used to determined PI3K/Akt pathway activity following *SET* knockdown. Furthermore, the reversal effects of paeonol on paclitaxel, and its underlying mechanisms of action, were investigated using western blot analysis and reverse transcription-quantitative polymerase chain reaction. The results demonstrated that increased levels of SET and PI3K/Akt pathway proteins were present in the MCF-7/PTX cells, compared with normal MCF-7 cells. Knockdown of *SET* significantly sensitized MCF-7/PTX cells to paclitaxel and induced cell apoptosis. In addition, the expression levels of the adenosine triphosphate binding cassette (ABC) transporter proteins were significantly reduced in the MCF-7/PTX cells compared with the normal MCF-7 cells. SET-induced paclitaxel resistance was found to be associated with the activation of the PI3K/Akt pathway. Paeonol significantly reduced the mRNA and protein expression levels of SET in the MCF-7/PTX cells. Furthermore, paeonol significantly sensitized the MCF-7/PTX to paclitaxel via regulation of ABC transporters, B cell lymphoma-2 (Bcl-2) and Bcl-2-associated X protein. In addition, paeonol inhibited SET-mediated paclitaxel resistance by attenuating PI3K/Akt pathway activity in the MCF-7/PTX cells. In conclusion, the results of the present study demonstrated that SET was associated with paclitaxel resistance in MCF-7/PTX cells, and that paeonol reversed paclitaxel resistance in MCF-7/PTX cells by downregulating the activity of the SET/PP2A/Akt pathway.

Introduction

Breast cancer is one of the most prevalent types of malignant tumor, which has a severe impact on the physical and mental health, and can be life-threatening. In addition, the incidence rates of breast cancer have increased by at least double, almost triple, in the past few decades in Asian countries (1). Multidrug resistance in breast cancer is one of the primary obstacles leading to the clinical failure of chemotherapy (2). Paclitaxelas, a first-line treatment with significant antitumor activity, is widely used in the treatment of breast cancer; however, its frequent use can lead to resistance (3). The potential mechanisms associated with paclitaxel resistance have been reported in several studies, and include the dysregulation of the P-glycoprotein (P-gp) drug efflux pump, variations in tubulin structure, altered signal transduction and inhibition of the activation of apoptotic pathways (4-8). However, the intricate mechanisms of drug resistance have been associated with multiple targets and pathways in tumor cells, therefore, it is necessary to identify novel therapeutic molecular targets and signal transduction networks for the treatment of breast cancer (1,2). In addition, effective chemotherapy reversal agents, which may reduce paclitaxel resistance in breast cancer remain to be elucidated. Traditional Chinese Medicines have been reported to be important in sensitizing cancer cells

Correspondence to: Professor Yalin Dong, Department of Pharmacy, The First Affiliated Hospital of Medical College, Xi'an Jiaotong University, 277 Yanta West Road, Xi'an, Shaanxi 710061, P.R. China
E-mail: dongyalin@mail.xjtu.edu.cn

Key words: SET, breast cancer, paclitaxelresistance, paeonol, phosphatidylinositol 3-kinase/Akt

to chemotherapy and overcoming drug-resistance in clinical treatment (9). Therefore, herbs used in Traditional Chinese Medicine may offer promise in identifying efficient multidrug resistance reversal agents with low toxicity.

The peony phenol, 4-methoxy-2-hydroxyacetophenone (paeonol), is derived from Traditional Chinese Medicine and is the primary active ingredient of cortex moutan from the root bark of *Paeoniasuffruticosa andrews* and the grass of the ricinuscommunissecco plant, XuChangqing (*Pycnostel mapaniculatum* k. schum) (10). Previous studies have demonstrated that paeonol has numerous pharmacological properties, including antioxidant and anti-inflammatory activities as well as the inhibition of allergic reactions and immune regulation (11-14). It has been reported that paeonol is also involved in defense against tumors and the reversal of multidrug resistance in tumor cells. Xu *et al* (15) demonstrated that paeonol has a significant growth-inhibitory effect on the human HepG2 hepatoma cell line by inducing cell apoptosis and arresting the cell cycle in the S phase. In addition, Kim *et al* (16) reported that paeonol significantly inhibits the proliferation and migration of tumor cells, the mechanism of which involved, a least in part, inhibition of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway and the activity of matrix metalloproteinase. Furthermore, paeonol reverses endoplasmic reticulum stress-induced doxorubicin resistance in human hepatocellular carcinoma cells by targeting the cycloxygenase (COX)-2-mediated inactivation of PI3K/Akt/CCAAT-enhancer-binding protein homologous protein (17). These studies indicated that, due to its significant antitumor and chemotherapy sensitization effects, paeonol may be a novel therapeutic reversal agent for use in the treatment of drug-resistant breast cancer.

The SET protein is a member of the nucleosome assembly protein family, characterized by a nitrogen end structure domain, a nucleosome assembly structure domain and a carboxylic acid structure of domain (18). SET, which is distributed and expressed in multiple organs and tissues, has a wide variety of biological functions, which are involved in controlling cell cycle, nucleosome assembly, DNA transcription, cell apoptosis, cell migration and histone binding (19-23). A previous study demonstrated that the SET may be a potential molecular antitumor target. Boqun *et al* (24) reported that the overexpression of SET in polycystic ovary syndrome led to a poor prognosis. Another study found that the expression levels of SET were over two times higher in uterine, stomach, colon and rectal cancer tissues compared with those in corresponding normal tissues (25). SET contributes to tumorigenesis, at least in part, by inhibiting endogenous protein phosphatase 2A (PP2A), a cellular phosphatase, which negatively regulates multiple pro-growth/prosurvival signaling pathways associated with the progression of cancer, including Akt, β-catenin and c-Myc (26). Taken together, SET is important in facilitating cellular growth and proliferation, and interacting with pathways that promote tumorigenesis and metastasis.

In our previous study, the protein profiles between paclitaxel-resistant MCF-7/PTX and sensitive MCF-7 cells were analyzed using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization time of light mass spectrometry (MALDITOF-MS), in which SET was one of the most significantly altered proteins (27). Therefore, it was hypothesized that SET may be important in the occurrence of drug-resistance in the development of breast cancer. The present study aimed to detect whether the SET protein was associated with drug resistance in paclitaxel-resistant MCF-7/PTX human breast carcinoma cells. In addition, whether paeonol partially reversed drug resistance in MCF-7/PTX cells, and the reversal mechanism by which this may proceed, was examined to determine the potential use of SET inhibitors to sensitize breast cancer to therapeutic drugs.

Materials and methods

Materials. Paclitaxel was purchased from Nanjing Luye Sike Pharmaceutical Co., Ltd (Nanjing, China). Paeonol was obtained from Ningbo Tianzhen Pharmaceutical Co., Ltd (Zhejiang, China). Verapamil was obtained from China Pharmaceutical Biological Products Analysis Institute (Beijing, China). Verapamil is a non-specific P-gp inhibitor, which acts an an efficient reversal agent for overcoming drug resistance (28). Verapamil has been used as a positive control in numerous studies regarding chemoresistance (28-30). Furthermore, in our previous study verapamil was able to overcome paclitaxel resistance in MCF-7/PTX cells (31). Therefore, verapamil was used as a positive control in the present study. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethyl sulphoxide (DMSO) was purchased from Sigma-Aldrich (St. Louis, MO, USA). A Lipofectamine 2000™ transfection reagent kit and annexin-V FITC staining kit was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Rabbit polyclonal primary antibodies against Akt (cat. no. 9272; 1:1,000 dilution), phophorylated (p)-Akt (cat. no. 9271; 1:1,000 dilution), B cell lymphoma (Bcl-2)-associated X protein (Bax; cat. no. 2772; 1:2,500 dilution), Bcl-2 (cat. no. 1876; 1:2,500 dilution), caspase 9 (cat. no. 9501; 1:2,000 dilution), caspase 3 (cat. no. 9661; 1:2,000 dilution) and anti-poly adenosine diphosphate-ribose polymerase (PARP; cat. no. 9542; 1:2,000 dilution) were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary rabbit polyclonal β-actin (cat. no. bs-0061R; 1:800 dilution) antibody was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Rabbit polyclonal breast cancer resistance protein (BCRP; cat. no. sc-25822; 1:500 dilution) and multidrug resistance-associated protein 1 (MRP1; cat. no. sc-13960; 1:500 dilution) antibodies were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Rabbit polyclonal SET (cat. no. CTX106342; 1:2,000 dilution), P-gp (cat. no. GTX108370; 1:500 dilution) and PP2A (cat. no. GTX101690; 1:5,000 dilution) antibodies were obtained from GeneTex (Irvine, CA, USA). Horseradish-peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. CW0103; 1:20,000 dilution) was purchased from CW biotech (Beijing, China).

Cell culture generation. The human MCF-7 breast carcinoma cell line was obtained from the Chinese Academy of Science (Shanghai, China). The paclitaxel-resistant MCF-7/PTX cells were established, as previously described (32). Briefly, the MCF-7/PTX cell line was established after a continuous induction from 2 to 30 nM paclitaxel in a stepwise escalating

Table I. Primer sequences for reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)
SET	GGAGGAAGATGAAGAGGCAT	TGGCTTTATTCTGCGTTTGAC	242
MDR1	GAGCCCATCCTGTTTGACTG	GCTGCCCTCACAATCTCTTC	92
BCRP	AGCAGGGACGAACAATCATC	GCCAATAAGGTGAGGCTATCA	82
MRP1	AAGGTGGACGAGAACCAGAA	AACAGGGCAGCAAACAGAAC	110
β-actin	TGACGTGGACATCCGCAAAG	CTGGAAGGTGGACAGCGAGG	205

MDR1, multidrug resistance gene 1; BCRP, breast cancer resistance protein; MRP1, multidrug resistance-associated protein 1.

concentration manner. The half maximal inhibitory concentration (IC_{50}) values of paclitaxel for MCF-7/S and MCF-7/PTX cells were 20±0.085 nM and 2291±125 nM, respectively. The reversal fold (RF) was 115. The MCF-7 cells were cultured in 4 ml RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL) and 1% penicillin/streptomycin (Qilu Pharmaceutical Co., Ltd., Jinan, China) at 37˚C under a humidified atmosphere of 5% CO_2. Furthermore, 100 μl culture medium was added to the control wells, and each group included four replicates. The culture conditions for the MCF-7/PTX cells were the same to those of the MCF-7 cell line, with the exception of the addition of 30 nM paclitaxel.

MTT cell viability assay. In order to determine cell viability, the cells were plated at $1x10^4$ cells per well in 96-well plates in volumes of 100 μl RPMI-1640 medium. Following culture for 24 h at 37˚C and 5% CO_2, the medium was removed and 100 μl culture medium containing a series of concentrations of paeonol (15, 30, 60, 120, 250, 320 and 400 μM) or paclitaxel (0.75, 1.5, 2.0, 2.5, 3.0, 3.5and 4.0 μM) was added to each well. A total of 100 μl culture medium was added to the control wells and each group included four replicates. Following incubation for 48 h, 20 μl (0.5 mg/ml) MTT was added to each well for an additional 4 h. The blue MTT formazan precipitate was then dissolved in 100 μl DMSO and the culture plates were gently agitated for 15 min. Subsequently, the density of formazan was measured using a plate reader (ELx808; BioTek, Winooski, VT, USA) at a wavelength of 492 nm in each well. The IC_{50} values were determined using GraphPad Prism5.0 software (GraphPad Sotware, Inc., La Jolla, CA, USA). The RF values, which indicated the potency of reversal, were calculated as the IC_{50} of the cytotoxic drug / IC_{50} of the cytotoxic drug with test-drug pretreatment.

Small interference RNA (siRNA) synthesis and transient transfection of cells. Double strand siRNA oligonucleotides encoding human *SET* (*SET* siRNA1, siRNA2 and siRNA3) were designed by Shanghai GenePharma Co., Ltd (Shanghai, China). The negative control was scrambled siRNA, and the transfection efficiency was compared to that of siRNA nucleotides targeting *β-actin*. For transient transfection, the MCF-7/PTX cells were seeded into a six-well plate at a density of $6x10^5$ cells per well for 24 h at 37˚C. After 24 h, the cells were transfected with the siRNAs targeting *SET* for 48 h at a final concentration with Lipofectamine 2000™ reagent, according to the manufacturer's instructions. The scrambled siRNA was used as a negative control in the transfection assay. After 48 h, the mRNA and protein expression levels of SET were confirmed using reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunoblotting with cellular extracts, and the cells were seeded for proliferation assays.

RT-qPCR analysis. Total RNA was isolated using an RNA Fast 2000 kit (Shanghai Fastagen Biotechnology Co., Ltd., Shanghai, China). RT-qPCR was performed using a Prime Script RT Master Mix Perfect Real Time kit (cat. no. DRR036A; Takara Bio, Inc., Dalian, China) and SYBR Premix Ex Taq II (Takara Bio, Inc.), according to the manufacturer's instructions. The primer sequences and product lengths are listed in Table I. The cycle conditions for RT-qPCR were as follows: 40 cycles of 95˚C for 30 sec, 95˚C for 5 sec, various annealing temperatures for 30 sec, depending on the target gene (58˚C for *SET*; 60˚C for *MDR1*; 58˚C for *MRP1* and 58˚C for *BCRP*), followed by 60˚C for 30 sec for cooling. The mRNA expression levels in each sample were normalized to that of *β-actin*.

Western blot analysis. The cells from each treatment group were collected, at a density of $2x10^5$ cells/ml, and lysed in 120 μl radioimmunoprecipation assay lysis buffer (Beyotime Institute of Biotechnolgy, Haimen, China), and the protein concentrations were determined using bicinchoninic acid reagent (Beyotime Institute of Biotechnolgy). Subsequently, the lysates were subjected to 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (Beyotime Institute of Biotechnolgy) and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Prior to incubation with specific antibodies overnight at 4˚C, the blots were blocked with 5% non-fat milk for 4 h at room temperature. The blots were then labeled with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1:20,000 dilution), visualized using BeyoECL Plus Detection system (Beyotime Institute of Biotechnology). All experiments were performed independently at least three times.

Flow cytometry assay. The cells from each treatment group were double stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) in the dark for 30 min at room temperature using an annexin V-FITC/PI apoptosis detection kit, according to the manufacturer's instructions. Any cells, which were annexin V^+/PI^- were in early apoptosis,

Table II. Effects of paeonol on the cytotoxicity of paclitaxel on MCF-7/PTX cells.

Group	Paeonol (μM)	IC_{50} of paclitaxel (nM)	RF
Control	0	2290.87±125.2	-
Paeonol	15	688.90±5.13	3.32
	30	389.15±2.64	5.88
	60	280.13±4.15	8.18
Verapamil	10	225.28±2.24	10.17

Effects of paeonol on the sensitivity of MCF-7/PTX cells to paclitaxel were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. The MCF-7/PTX cells were treated with various concentrations of paclitaxel (0.75, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 μM) in the presence of paeonol at the indicated concentration for 48 h. Verapamil was used as positive control drug. The IC_{50} of paclitaxel and RF values were calculated. Values are presented as the mean ± standard deviation of three independent experiments. MCF-7/PTX cells, paclitaxel-resistant MCF-7 human breast carcinoma cells; IC_{50}, half maximal inhibitory concentration; RF, reversal fold.

whereas cells in the late apoptotic stage were Annexin V^+/PI^+. Analyses of the apoptosis profiles were performed using Coulter Elite 4.5 Multi cycle software (Beckman Coulter, Brea, CA, USA). Experiments were performed independently in triplicate.

Statistical analyses. The values are expressed as the mean ± standard deviation, unless otherwise indicated. Statistical analyses were performed using a one-way analysis of variance with SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

SET/PP2A/Akt pathway is significantly activated in MCF-7/PTX cells. According to a previous study, the activation of the PI3K/Akt pathway and drug resistance are closely associated (33). As a key upstream negative regulator of the PI3K/Akt pathway, PP2A reduces PI3K/Akt pathway activity and inhibits apoptosis in numerous types of cancer cell (26). In addition, SET is a potent physiological inhibitor of PP2A (19). In order to clarify whether the SET, PP2A and PI3K/Akt signaling pathways are activated in MCF-7/PTX cells with the development of acquired resistance to paclitaxel. Western blot analyses were performed to detect the protein expression levels of the SET, PP2A, PI3K/Akt signaling pathway and the downstream apoptosis-associated factors of Akt, including Bax and Bcl-2. As shown in Fig. 1, the protein expression levels of SET, p-Akt and Bcl-2 were markedly increased in the MCF-7/PTX cells compared with that of normal MCF-7 cells, whereas the protein expression levels of PP2A and Bax were reduced. The expression of total Akt was not altered between the two cell lines (Fig. 1). These results suggested that the SET/PP2A/Akt pathway may be involved in paclitaxel resistance in breast cancer.

SET knockdown using siRNAs significantly attenuates paclitaxel resistance in MCF-7/PTX cells. SET may be associated with paclitaxel resistance in breast cancer. In order to detect whether the knockdown of *SET* affected the sensitivity of MCF-7/PTX cells to paclitaxel, siRNAs targeting *SET* were transfected into the MCF-7/PTX cells, and the transient transfection efficiencies were quantified using RT-qPCR. As shown in Fig. 2A, at 48 h post-transfection, the mRNA expression of SET was decreased significantly. In addition, 72 h post-transfection, western blot analysis was used to detect the protein expression of SET, which was markedly reduced compared with that of the untransfected and siRNA control-transfected MCF-7 cells (Fig. 2A). Growth inhibition was determined using an MTT assay; the results of which revealed that, following 48 h paclitaxel treatment, knockdown of *SET* in the MCF-7/PTX cells sensitized the cells to paclitaxel (Fig. 2B).

The effects of *SET* knockdown on cell apoptosis in the MCF-7/PTX cells were evaluated by flow cytometric analysis. As shown in Fig. 2C, following 48 h treatment with 600 nM paclitaxel, the apoptosis rates were 9.18% in the parental MCF-7/PTX cells and 9.94% in the MCF-7/PTX cells transfected with control siRNA ($P>0.05$), whereas the apoptotic cells were 16.02% in MCF-7/PTX SET siRNA cells ($P<0.05$). This result demonstrated that MCF-7/PTX cells with downregulation of SET were more sensitive to paclitaxel compared with the control group. However, compared with parental MCF-7/PTX cells, the number of apoptotic cells was markedly increased in the *SET*-knockdown MCF-7/PTX cells (Fig. 2C). Overall, these results suggested that the knockdown of *SET* contributed to the sensitization of MCF-7/PTX cells to paclitaxel.

SET knockdown markedly suppresses the PI3K/Akt signaling pathway. In order to further examine the potential mechanisms underlying *SET* knockdown-induced paclitaxel resistance reversal in MCF-7/PTX cells, western blot analysis was used to detect the expression levels of ABC transporter proteins and the activity of the PI3K/Akt signaling pathway in MCF-7/PTX cells transfected with *SET* siRNA. The results revealed that the mRNA and protein levels of classic multidrug resistance proteins, P-gp, BCRP and MRP1 in the *SET-knockdown* MCF-7/PTX cells were significantly reduced compared with those in the untransfected or control siRNA-transfected MCF-7/PTX cells (Fig. 3A). As shown in Fig. 3B, the *SET*-knockdown MCF-7/PTX cells had significantly increased protein expression of PP2A. These results demonstrated that knockdown of *SET* led to the reversal of paclitaxel resistance, which was closely associated with the expression of PP2A. As an important downstream factor of PP2A, Akt is important in tumor cell survival and drug resistance (26). Western blot analysis revealed that, in the *SET*-knockdown MCF-7/PTX

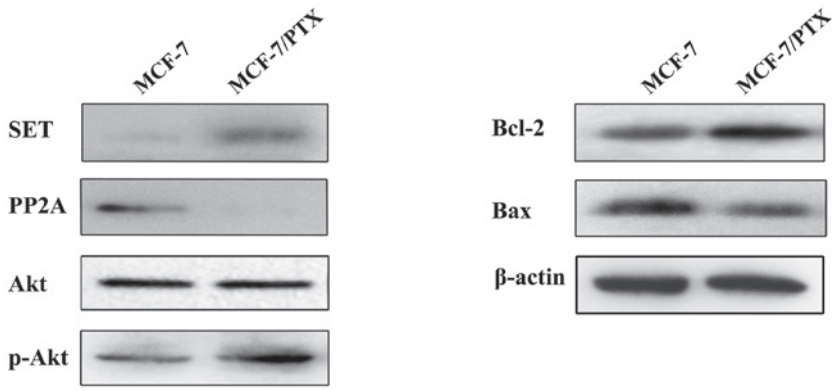

Figure 1. Expression levels of the SET/PP2A/Akt signaling pathway proteins in MCF-7 and MCF-7/PTX cells were determined using western blot analysis. Results are representative of three independent experiments. β-actin was used as an internal control. PP2A, protein phosphatase 2A; p-, phosphorylated; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; MCF-7/PTX, paclitaxel-resistant MCF-7 human breast carcinoma cells.

cells, the protein expression of p-Akt was significantly reduced (Fig. 3B). In addition, the protein expression of Bax was significantly increased, whereas that of Bcl-2 was decreased (Fig. 3B). These results suggested that SET mediated cellular apoptosis through the activation of the PI3K/Akt signaling pathway in the MCF-7/PTX cells.

Intrinsic cytotoxicity of paeonol in the MCF-7 and MCF-7/PTX cells. The MCF-7 and MCF-7/PTX cells were treated with various concentrations of paeonol (15, 30, 60, 120, 250, 320 and 400 μM) for 48 h, and the intrinsic cytotoxicity of paeonol was determined using an MTT assay. As shown in Fig. 4, paeonol inhibited the growth of MCF-7 and MCF-7/PTX cells in a dose-dependent manner. According to the cell viability curves (Fig. 4A), three doses of Paeonol were identified (15, 30 and 60 μM), which had the lowest cytotoxic effects on the MCF-7/PTX cells and the inhibitory concentration was <5%. Therefore, to investigate the effect of paeonol on reversal efficiency, with minimal effects on cell vitality, the concentrations of 15, 30 and 60 μM were selected for use. Verapamil was used as a positive control. MTT assays were performed and RF values were determined to examine whether paeonol reversed the resistance of MCF-7/PTX cells to paclitaxel. As shown in Table II, after 48 h of treatment with paeonol (15, 30 and 60 μM), the RF values were 3.3, 5.9 and 8.2, respectively. RF>1 indicated that the drug sensitized the MCF-7/PTX cells to paclitaxel; RF=1 indicated no reversal effect; and RF<1 indicated that paeonol desensitized the cells to paclitaxel. The results demonstrated that paeonol significantly reduced the concentration of paclitaxel required to obtain 50% growth inhibition, and reversed paclitaxel resistance in the MCF-7/PTX cells.

Paeonol potentiates apoptosis in MCF-7/PTX cells. In order to investigate the mechanisms of sensitization induced by paeonol in the MCF-7/PTX cells, the expression levels of apoptosis-associated proteins were investigated following paeonol treatment. As shown in Fig. 4B, the results indicated that paeonol markedly increased the cleavage of full length caspase-9, caspase-3 and PARP in the MCF-7/PTX cells 72 h after treatment with paeonol compared with the untreated cells, and this occurred in a dose-dependent manner. These results suggested that paeonol promoted cell apoptosis in the MCF-7/PTX cells.

Paeonol suppresses the actvity of the PI3K/Akt signaling pathway through inhibition of SET. In order to detect whether SET and its downstream targets were modulated by paeonol, the MCF-7/PTX cells were treated with 15, 30 and 60 μM paeonol for 48 h. RT-qPCR and western blot analysis revealed that paeonol significantly decreased the mRNA and protein expression of SET in the MCF-7/PTX cells, in a dose-dependent manner (Fig. 5A). In addition, the mRNA and protein levels of P-gp, MRP1 and BCRP, which were previously found to be overexpressed in the MCF-7/PTX cells (Fig. 3A), were also significantly reduced (Fig. 5A). To further investigate the potential reversal mechanism of paeonol, the protein levels of PP2A, p-Akt and Akt were detected by western blot analysis, following treatment with paeonol in the MCF-7/PTX cells. The results demonstrated that, 72 h after treatment with 15, 30 and 60 μM paeonol in the MCF-7/PTX cells, the protein expression of PP2A was significantly increased and those of p-Akt were significantly decreased in a dose-dependent manner (Fig. 5B). Furthermore, following treatment with increasing concentrations of paeonol, the protein expression of Bax was significantly increased and that of Bcl-2 was significantly decreased (Fig. 5B). These results demonstrated that paeonol inhibited the PI3K/Akt pathway, enhancing the sensitivity to paclitaxel, possibly through down-regulating SET in MCF-7/PTX cells.

Discussion

As a novel anticancer drug, paclitaxel is widely used for chemotherapy in the treatment of breast cancer. However, drug resistance is one of the primary obstacles leading to the failure of chemotherapy in breast cancer (34). Previous studies have reported numerous mechanisms, which may be involved in paclitaxel resistance, including differences in the expression of drug efflux pump ABC transporter proteins, tubulin mutation and inhibition of the apoptotic pathway (4-8). The *SET* gene was first identified in patients with acute undifferentiated leukemia, and its biological function involves histone acetylation, apoptosis, transcription regulation, nucleosome assembly and other post-translational modifications (18). In head and neck squamous cell carcinoma, SET inhibits the expression of its downstream tumor suppressor factor, PP2A, and activates the PI3K/Akt signaling pathway (35). Activation of the

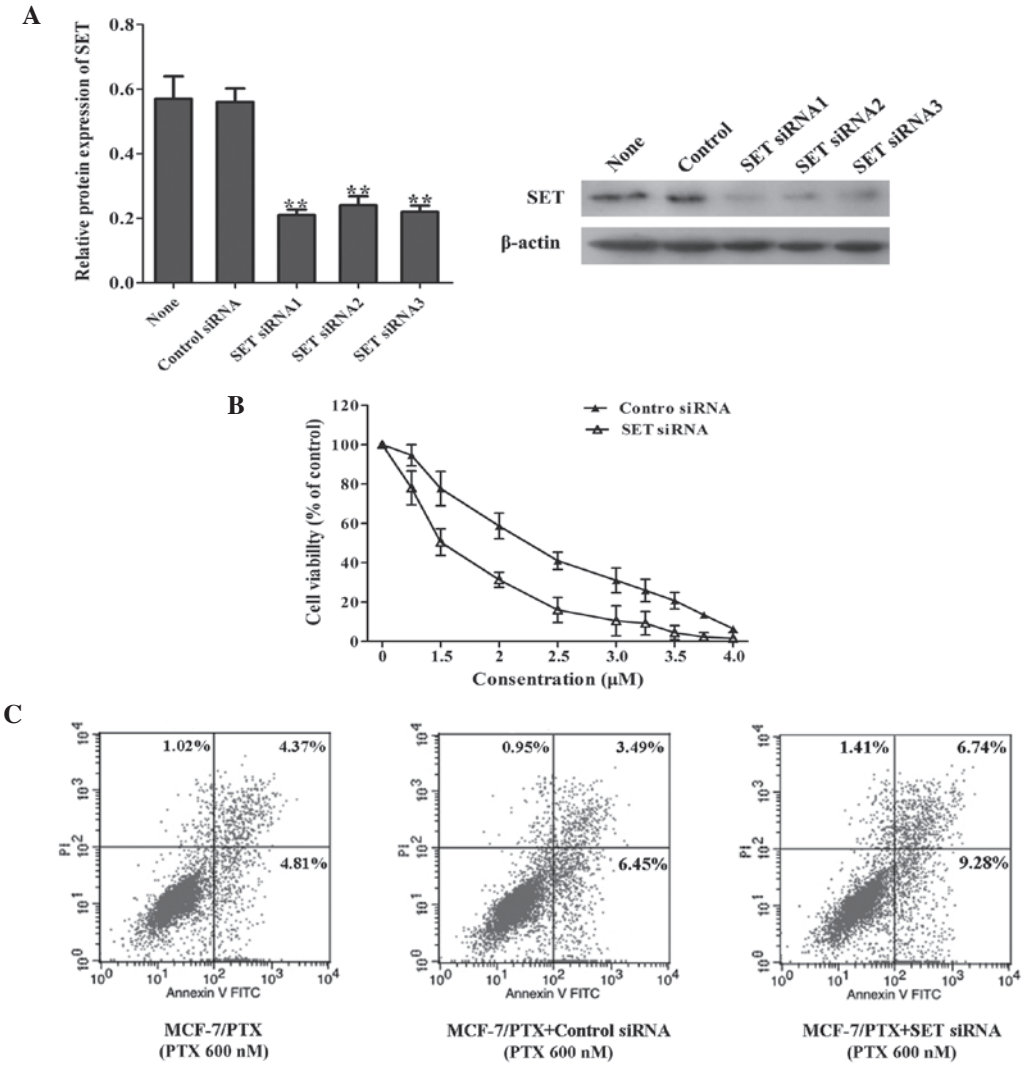

Figure 2. *SET* knockdown using siRNAs significantly attenuates paclitaxel resistance in MCF-7/PTX cells. (A) Reverse transcription-quantitative polymerase chain reaction and western blot analysis of the gene and protein expression of *SET*, respectively, in MCF-7/PTX cells transfected with either *SET* siRNA or control siRNA. β-actin was used as an internal control. (B) Cell viabilities of the MCF-7/PTX cells transfected with *SET* siRNA or control siRNA were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. (C) MCF-7/PTX cells transfected with *SET* siRNA or control siRNA were treated with paclitaxel for 48 h. The apoptotic rates of the cells were then detected using annexin V/PI-double staining and analyzed by flow cytometry. The values are expressed as the mean ± standard deviation. *P<0.05 and **P<0.01 vs. control. siRNA, small interference RNA; PI, propidium iodide; FITC, fluorescein isothiocyanate; MCF-7/PTX, paclitaxel-resistant MCF-7 human breast carcinoma cells.

PI3K/Akt pathway inhibits cell apoptosis by mediating the endogenous expression of Bcl-2 and Bax, which are mediators of apoptosis and are are the most frequently targeted genes regulating apoptosis in cells (36). In addition, activation of the PI3K/Akt pathway reduces the expression levels of P-gp, BCRP, MRP1 and other members of the ABC transporter superfamily (37-40). These two aspects of the PI3K/Akt pathway, at least in part, induce the development of drug resistance.

In the present study, siRNAs were used to knockdown the expression of *SET* in MCF-7/PTX cells. This resulted in a significant increase in the sensitivity of MCF-7/PTX cells to paclitaxel, including the promotion of apoptosis, decreased expression of ABC transporter proteins and Bcl-2, and increased expression of Bax to attenuation chemoresistance in breast cancer cells. In further mechanistic investigations, the knockdown of *SET* increased the expression of downstream PP2A and significantly reduced the phosphorylation of Akt. These results suggested that the dysregulation of SET mediated cell apoptosis and the expression of ABC transporter proteins, eventually leading to drug resistance by promoting the activity of the PP2A/PI3K/Akt pathway. In contrast to previous studies on SET, which were only performed in tumor cells, the present study revealed the expression patterns of SET in drug-resistant cells, using paclitaxel-resistant breast cancer cells as a model to elucidate the mechanism of SET-induced drug resistance. However, whether SET affects the activation of Akt signaling pathway and induces paclitaxel resistance in breast cancer primarily by inhibiting PP2A rather than other downstream factors, including tumor metastasis suppressor (nm-23-H1) or Ras-related C_3botulinum toxin substrate 1, requires further investigation (26).

Several previous studies have investigated the antitumor and drug-resistance-reversing effects of paeonol. A study demonstrated that paeonol induces apoptosis in ovarian

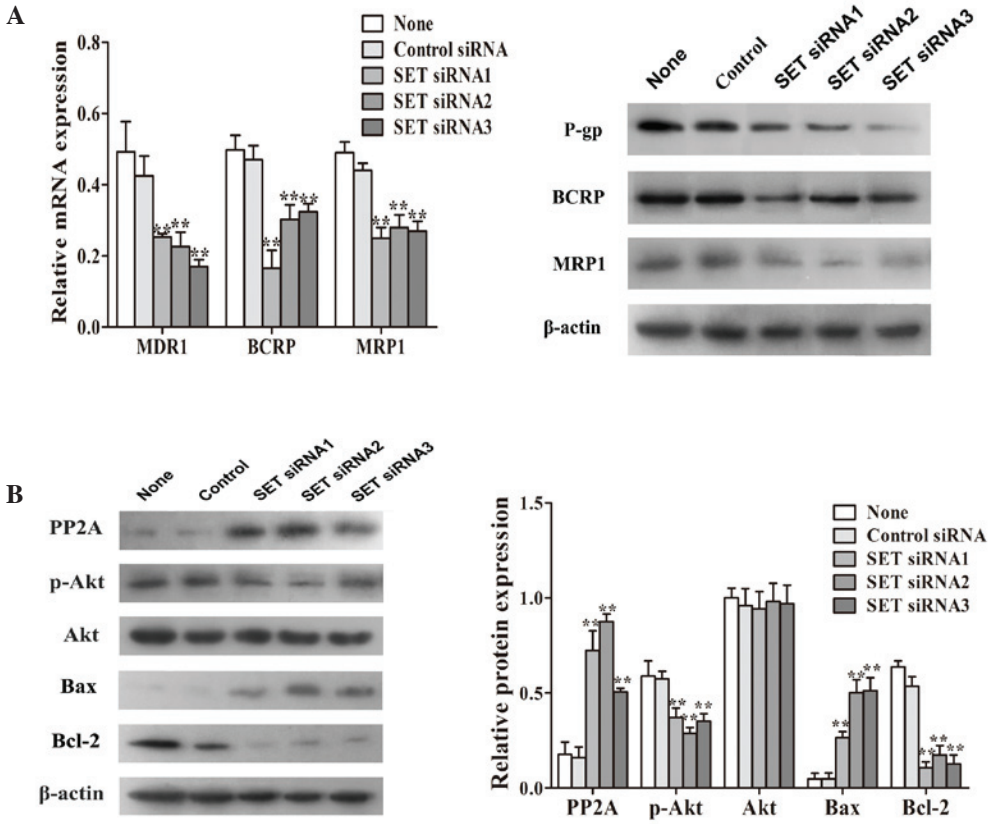

Figure 3. Adenosine triphosphate binding cassette transporter and PI3K/Akt pathway expression levels were evaluated using RT-qPCR and western blot analysis. (A) RT-qPCR and western blot analysis of the gene and protein expression levels of P-gp, BCRP and MRP1, repsectively in MCF-7/PTX cells transfected with either *SET* siRNA or control siRNA. (B) Protein expression levels of PP2A, p-Akt, Akt, Bax and Bcl-2 in MCF-7/PTX cells transfected with either *SET* siRNA or control siRNA were determined using western blot analysis. β-actin was used as an internal control. Results are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. untransfected cells. PI3K, phosphatidylinositol 3-kinase; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MRP1, multidrug resistance-associated protein 1; siRNA, small interference RNA; PP2A, protein phosphatase 2A; p-, phosphorylated; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; MCF-7/PTX, paclitaxel-resistant MCF-7 human breast carcinoma cells.

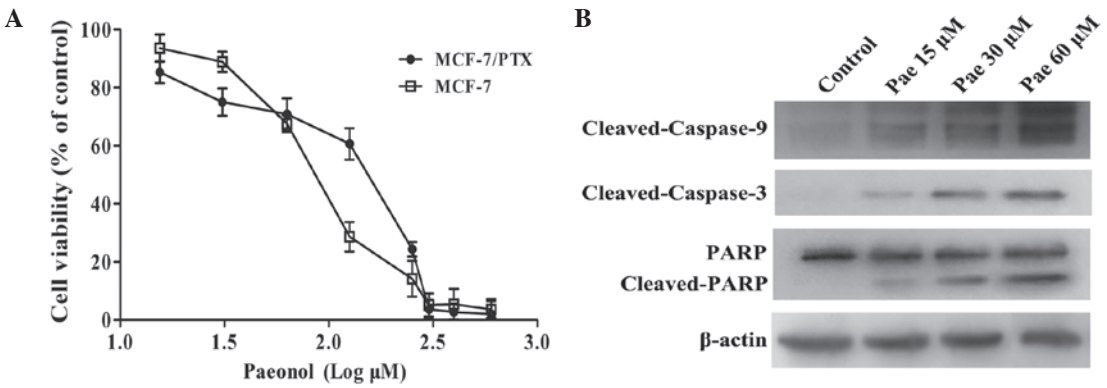

Figure 4. Effects of paeonol on cell proliferation of the MCF-7 and MCF-7/PTX cells and apoptosis-associated protein expression in MCF-7/PTX cells. (A) Effect of paeonol on cell viability. The MCF-7 and MCF-7/PTX cells were treated various concentrations of paeonol for 48 h and cell viability was determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay. (B) Protein levels of cleaved caspase-9, cleaved caspase-3 and PARP were enhanced by paeonol treatment in the MCF-7/PTX cells, as determined using western blot analysis. β-actin was used as an internal control. Values are presented as the mean ± standard deviation of three independent experiments. Pae, paeonol; PARP, poly adenosine diphosphate-ribose polymerase; MCF-7/PTX cells, paclitaxel-resistant MCF-7 human breast carcinoma cells.; MDRI, multidrug resistance gene 1; BCRP, breast cancer resistance protein.

cancer cells by promoting the activation of caspase-3 and inhibiting the protein expression of suvivin (41). Paeonol also inhibits tumor cell proliferation and migration through inhibition of the classic Akt and mitogen-activated protein kinase signaling transduction pathways (16). Furthermore, paeonol regulates expression of pro-apoptotic transcription factor CCAAT-enhancer-binding protein homologous protein in HepG2 cells (17), and paeonol significantly regulates

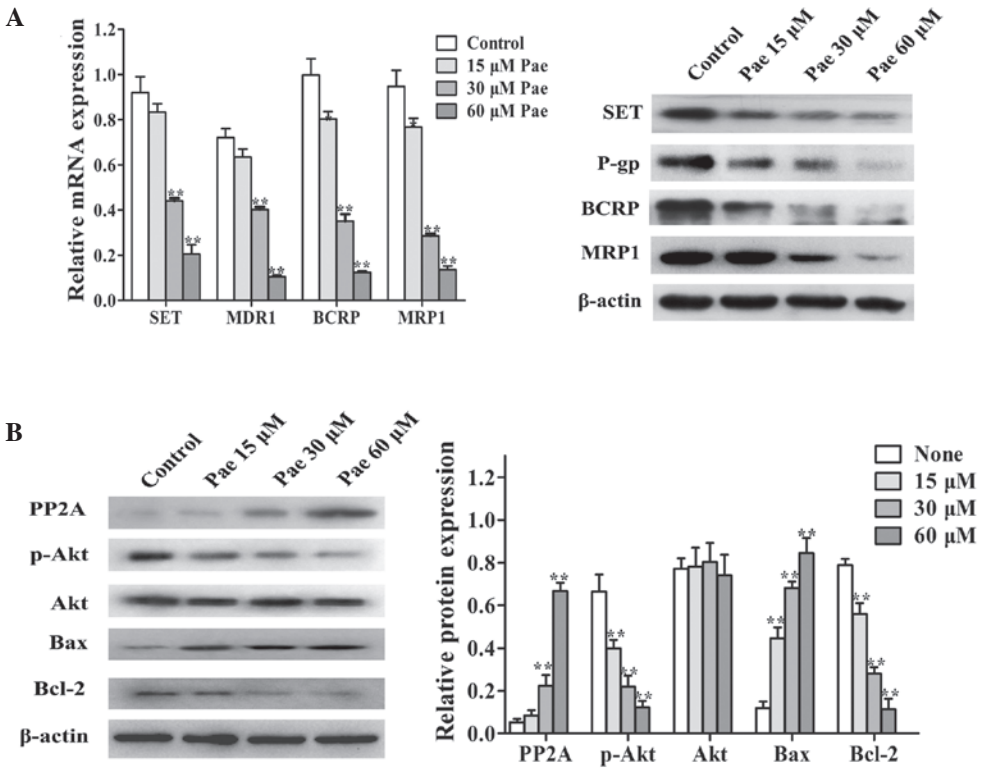

Figure 5. Effects of paeonol on the levels of SET, adenosine triphosphate binding cassette transporters and phosphatidylinositol 3-kinase/Akt pathway proteins in MCF-7/PTX cells. (A) Reverse transcription-quantitative polymerase chain reaction and western blot analysis of the gene and protein expression, respectively, of SET, P-gp, BCRP and MRP1 in the MCF-7/PTX cells treated with paeonol (15, 30 and 60 μM). (B) Protein expression of PP2A, p-Akt, Akt, Bax and Bcl-2 in MCF-7/PTX cells treated with various concentration of paeonol for 72 h were determined by western blot analysis. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control. Pae, paeonol, P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; MDRI, multidrug resistance gene 1; MRP1, multidrug resistance-associated protein 1; PP2A, protein phosphatase 2A; p-, phosphorylated; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; MCF-7/PTX, paclitaxel-resistant MCF-7 human breast carcinoma cells.

the expression of Bax and Bcl-2 in various types of cancer cells (42). However, no previous studies have investigated whether the antitumor effects of paeonol involve SET, or whether paeonol can be applied in the reversal of paclitaxel resistance in breast cancer.

The present study used RT-qPCR and western blot analysis to demonstrate that paeonol significantly upregulated the activated Akt downstream targets, cleaved-caspase 9, cleaved-caspase 3 and cleaved-PARP, promoting their function in inducing cell apoptosis. In addition, paeonol decreased the expression levels of SET and ABC transporters in a dose-dependent manner, promoting the expression of Bax and suppressing the expression of Bcl-2, which reversed paclitaxel resistance in breast cancer cells. In examining the potential reversal mechanism of paeonol, paeonol treatment led to increased expression of PP2A and attenuated the phosphorylation of Akt in MCF-7/PTX cells, in a dose-dependent manner. These results suggested that, by inhibiting the SET/PP2A/Akt signaling pathway, paeonol induced cell apoptosis and reduced the expression of ABC transporters, which eventually reversed paclitaxel resistance in the breast cancer cells. In contrast with previous antitumor studies, the present study introduced the potential application of paeonol in the reversal of paclitaxel resistance in breast cancer cells, and discussed the reversal mechanism underlying paclitaxel resistance. However, it is possible that other mechanisms are also involved in paeonol-regulated apoptosis in drug-resistant cells. There are numerous key downstream targets of the PI3K/Akt pathway, including mammalian target of rapamycin and p70S6 kinase, which are also important in regulating apoptosis (43). Therefore future studies are required to further investigate the reversal mechanisms of paeonol.

In conclusion, the present study provided the first evidence, to the best of our knowledge of SET protein as a potential molecular target in MCF-7/PTX cells, and confirmed that SET regulated the PP2A and Akt tumor-suppresor signaling pathways, including the expression of downstream apoptosis-associated proteins and ABC transporter proteins. In addition, paeonol reversed paclitaxel resistance in breast cancer cells by inhibiting the expression of the SET-mediated PI3K/Akt signaling pathway proteins in the paclitaxel-resistant cells. Therefore, paeonol may have potential as a novel reversal agent in the treatment of paclitaxel-resistant breast cancer. However, the present study was performed at the cellular level, and animal models and human clinical trials have yet to be performed.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (nos. 30973673 and 30973578).

References

1. Bhoo-Pathy N, Yip CH, Hartman M, *et al*: Breast cancer research in Asia: adopt or adapt Western knowledge? Eur J Cancer 49: 703-709, 2013.
2. Kim H, Park GS, Lee JE and Kim JH: A leukotriene B4 receptor-2 is associated with paclitaxel resistance in MCF-7/DOX breast cancer cells. Br J Cancer 109: 351-359, 2013.
3. Ajabnoor GM, Crook T and Coley HM: Paclitaxel resistance is associated with switch from apoptotic to autophagic cell death in MCF-7 breast cancer cells. Cell Death Dis 3: e260, 2012.
4. Carrara L, Guzzo F, Roque DM, *et al*: Differential in vitro sensitivity to patupilone versus paclitaxel in uterine and ovarian carcinosarcoma cell lines is linked to tubulin-beta-III expression. Gynecol Oncol 125: 231-236, 2012.
5. Yin S, Zeng C, Hari M and Cabral F: Random mutagenesis of beta-tubulin defines a set of dispersed mutations that confer paclitaxel resistance. Pharm Res 29: 2994-3006, 2012.
6. Zhang J, Zhao J, Zhang W, *et al*: Establishment of paclitaxel-resistant cell line and the underlying mechanism on drug resistance. Int J Gynecol Cancer 22: 1450-1456, 2012.
7. Bhattacharya R and Cabral F: Molecular basis for class V beta-tubulin effects on microtubule assembly and paclitaxel resistance. J Biol Chem 284: 13023-13032, 2009.
8. Miller AV, Hicks MA, Nakajima W, Richardson AC, Windle JJ and Harada H: Paclitaxel-induced apoptosis is BAK-dependent, but BAX and BIM-independent in breast tumor. PLoS One 8: e60685, 2013.
9. Youns M, Hoheisel JD and Efferth T: Traditional Chinese medicines (TCMs) for molecular targeted therapies of tumours. Curr Drug Discov Technol 7: 37-45, 2010.
10. Lee H, Lee G, Kim H and Bae H: Paeonol, a major compound of moutan cortex, attenuates Cisplatin-induced nephrotoxicity in mice. Evid Based Complement Alternat Med 2013: 310989, 2013.
11. Huang H, Chang EJ, Lee Y, Kim JS, Kang SS and Kim HH: A genome-wide microarray analysis reveals anti-inflammatory target genes of paeonol in macrophages. Inflamm Res 57: 189-198, 2008.
12. Lee B, Shin YW, Bae EA, *et al*: Antiallergic effect of the root of Paeonia lactiflora and its constituents paeoniflorin and paeonol. Arch Pharm Res 31: 445-450, 2008.
13. Ishiguro K, Ando T, Maeda O, *et al*: Paeonol attenuates TNBS-induced colitis by inhibiting NF-kappaB and STAT1 transactivation. Toxicol Appl Pharmacol 217: 35-42, 2006.
14. Zhou J, Zhou L, Hou D, Tang J, Sun J and Bondy SC: Paeonol increases levels of cortical cytochrome oxidase and vascular actin and improves behavior in a rat model of Alzheimer's disease. Brain Res 1388: 141-147, 2011.
15. Xu SP, Sun GP, Shen YX, Peng WR, Wang H and Wei W: Synergistic effect of combining paeonol and cisplatin on apoptotic induction of human hepatoma cell lines. Acta Pharmacol Sin 28: 869-878, 2007.
16. Kim SA, Lee HJ, Ahn KS, *et al*: Paeonol exerts anti-angiogenic and anti-metastatic activities through downmodulation of Akt activation and inactivation of matrix metalloproteinases. Biol Pharm Bull 32: 1142-1147, 2009.
17. Fan L, Song B, Sun G, Ma T, Zhong F and Wei W: Endoplasmic reticulum stress-induced resistance to Doxorubicin is reversed by paeonol treatment in human hepatocellular carcinoma cells. PLoS One 8: e62627, 2013.
18. von Lindern M, van Baal S, Wiegant J, Raap A, Hagemeijer A and Grosveld G: Can, a putative oncogene associated with myeloid leukemogenesis, may be activated by fusion of its 3' half to different genes: characterization of the set gene. Mol Cell Biol 12: 3346-3355, 1992.
19. Canela N, Rodriguez-Vilarrupla A, Estanyol JM, *et al*: The SET protein regulates G2/M transition by modulating cyclin B-cyclin-dependent kinase 1 activity. J Biol Chem 278: 1158-1164, 2003.
20. Zhang P, Compagnone NA, Fiore C, *et al*: Developmental gonadal expression of the transcription factor SET and its target gene, P450c17 (17alpha-hydroxylase/c17,20 lyase). DNA Cell Biol 20: 613-624, 2001.
21. Wagner S, Weber S, Kleinschmidt MA, Nagata K and Bauer UM: SET-mediated promoter hypoacetylation is a prerequisite for coactivation of the estrogen-responsive pS2 gene by PRMT1. J Biol Chem 281: 27242-27250, 2006.
22. Madeira A, Pommet JM, Prochiantz A and Allinquant B: SET protein (TAF1beta, I2PP2A) is involved in neuronal apoptosis induced by an amyloid precursor protein cytoplasmic subdomain. Faseb J 19: 1905-1907, 2005.
23. Almeida LO, Goto RN, Pestana CR, *et al*: SET overexpression decreases cell detoxification efficiency: ALDH2 and GSTP1 are downregulated, DDR is impaired and DNA damage accumulates. FEBS J 279: 4615-4628, 2012.
24. Boqun X, Xiaonan D, Yugui C, *et al*: Expression of SET protein in the ovaries of patients with polycystic ovary syndrome. Int J Endocrinol 2013: 367956, 2013.
25. Cervoni N, Detich N, Seo SB, Chakravarti D and Szyf M: The oncoprotein Set/TAF-1beta, an inhibitor of histone acetyltransferase, inhibits active demethylation of DNA, integrating DNA methylation and transcriptional silencing. J Biol Chem 277: 25026-25031, 2002.
26. Switzer CH, Cheng RY, Vitek TM, Christensen DJ, Wink DA and Vitek MP: Targeting SET/I (2) PP2A oncoprotein functions as a multi-pathway strategy for cancer therapy. Oncogene 30: 2504-2513, 2011.
27. Chen S, Dong Q, Hu S, *et al*: Proteomic analysis of the proteins that are associated with the resistance to paclitaxel in human breast cancer cells. Mol Biosyst 10: 294-303, 2014.
28. Chen LM, Liang YJ and Ruan JW, *et al*: Reversal of P-gp mediated multidrug resistance in-vitro and in-vivo by FG020318. J Pharm Pharmacol 56: 1061-1066, 2004.
29. Liu XD, Sun H and Liu GT: 5-Bromotetrandrine enhances the sensitivity of doxorubicin-induced apoptosis in intrinsic resistant human hepatic cancer Bel7402 cells. Cancer Lett 292: 24-31, 2010.
30. Fazly BB, Iranshahi M, Naderinasab M, Hajian S, Sabeti Z and Masumi E: Evaluation of the effects of galbanic acid from Ferula szowitsiana and conferol from F. badrakema, as modulators of multi-drug resistance in clinical isolates of *Escherichia coli* and *Staphylococcus aureus*. Res Pharm Sci 5: 21-28, 2010.
31. Cai J, Chen S and Zhang W, *et al*: Salvianolic acid A reverses paclitaxel resistance in human breast cancer MCF-7 cells via targeting the expression of transgelin 2 and attenuating PI3 K/Akt pathway. Phytomedicine 21: 1725-1732, 2014.
32. Chen SY, Hu SS, Dong Q, *et al*: Establishment of paclitaxel-resistant breast cancer cell line and nude mice models and underlying multidrug resistance mechanisms in vitro and in vivo. Asian Pac J Cancer Prev 14: 6135-6140, 2013.
33. Nakanishi T and Ross DD: Breast cancer resistance protein (BCRP/ABCG2): its role in multidrug resistance and regulation of its gene expression. Chin J Cancer 31: 73-99, 2012.
34. Li Z, Tian T, Hu X, *et al*: Six1 mediates resistance to paclitaxel in breast cancer cells. Biochem Biophys Res Commun 441: 538-543, 2013.
35. Leopoldino AM, Squarize CH, Garcia CB, *et al*: SET protein accumulates in HNSCC and contributes to cell survival: anti-oxidant defense, Akt phosphorylation and AVOs acidification. Oral Oncol 48: 1106-1113, 2012.
36. Rooswinkel RW, van de Kooij B, de Vries E, *et al*: Anti-apoptotic potency of Bcl-2 proteins primarily relies on their stability, not binding selectivity. Blood 123: 2806-2815, 2014.
37. Mao Z, Zhou J, Luan J, Sheng W, Shen X and Dong X: Tamoxifen reduces P-gp-mediated multidrug resistance via inhibiting the PI3K/Akt signaling pathway in ER-negative human gastric cancer cells. Biomed Pharmacother 68: 179-183, 2014.
38. Kazi AA, Gilani RA, Schech AJ, *et al*: Nonhypoxic regulation and role of hypoxia-inducible factor 1 in aromatase inhibitor resistant breast cancer. Breast Cancer Res 16: R15, 2014.
39. Cheng L, Luo S, Jin C, Ma H, Zhou H and Jia L: FUT family mediates the multidrug resistance of human hepatocellular carcinoma via the PI3K/Akt signaling pathway. Cell Death Dis 4: e923, 2013.
40. Pick A and Wiese M: Tyrosine kinase inhibitors influence ABCG2 expression in EGFR-positive MDCK BCRP cells via the PI3K/Akt signaling pathway. Chem Med Chem 7: 650-662, 2012.
41. Yin J, Wu N, Zeng F, Cheng C, Kang K and Yang H: Paeonol induces apoptosis in human ovarian cancer cells. Acta Histochem 115: 835-839, 2013.
42. Bao MH, Zhang YW and Zhou HH: Paeonol suppresses oxidized low-density lipoprotein induced endothelial cell apoptosis via activation of LOX-1/p38MAPK/NF-kappaB pathway. J Ethnopharmacol 146: 543-551, 2013.
43. Ponnurangam S, Standing D, Rangarajan P and Subramaniam D: Tandutinib inhibits the Akt/mTOR signaling pathway to inhibit colon cancer growth. Mol Cancer Ther 12: 598-609, 2013.