

# Salidroside induces apoptosis in human ovarian cancer SKOV3 and A2780 cells through the p53 signaling pathway

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**Abstract.** Salidroside is one of the most potent compounds extracted from the plant *Rhodiola rosea*, and its cardiovascular protective effects have been studied extensively. However, the role of salidroside in human ovarian carcinoma remains unknown. The aim of the current study was to investigate the effects of salidroside on the proliferation and apoptosis of SKOV3 and A2780 cells using MTT assay and acridine orange/ethidium bromide staining. Salidroside activated caspase-3 and upregulated the levels of apoptosis-inducing factor, Bcl-2-associated X and Bcl-2-associated death promoter (Bad) proteins. Furthermore, salidroside downregulated the levels of Bcl-2, p-Bad and X-linked inhibitor of apoptosis proteins. Salidroside activated the caspase-dependent pathway in SKOV3 and A2780 cells, upregulating p53, p21<sup>Cip1/Waf1</sup> and p16<sup>INK4a</sup>. These results suggest that the p53/p21<sup>Cip1/Waf1</sup>/p16<sup>INK4a</sup> pathway may serve a key function in salidroside-mediated effects on SKOV3 and A2780 cells. The current findings indicate that salidroside may be a promising novel drug candidate for ovarian cancer therapy.

## Introduction

Ovarian cancer (OC) is the most lethal gynecological malignancy (1). For epithelial OC (EOC), the prognosis for premenopausal women with early-stage EOC is favorable (2). In the past few decades, patients with EOC were treated with the latest chemotherapeutic drugs and surgical techniques, but the 5-year survival rate was still  $\leq 40\%$  (3). A previous study reported that EOC demonstrates genomic instability (4). During treatment, numerous patients with EOC have recurrence and

become resistant to chemotherapy, indicating that new treatment strategies are required (5,6). Therefore, numerous studies have been performed to identify effective therapeutic agents and their associated mechanisms of action (6,7).

Salidroside, a p-hydroxyphenethyl- $\beta$ -D-glucoside (or phenylpropanoid glycoside), is one of the major active ingredients extracted from *Rhodiola rosea* and has a long history of use in Chinese medicine (8-11). Salidroside has primarily been used as a brain tonic, a roborant or headache relief agent (8,12). Recently, salidroside has been studied in experimental animals for its protective effects against hypoxia, cold, radiation and heavy physical exercise (11). It has been demonstrated that salidroside has various pharmacological properties (13), including antiaging (14), anticancer (15), anti-inflammation, hepatoprotective and antioxidative effects (16).

The aim of the current study was to investigate the effects of salidroside on OC, and to determine whether it may be a new therapeutic candidate in the treatment of OC.

## Materials and methods

**Cell culture.** Human ovarian cancer cell lines, SKOV3 and A2780 from the American Type Culture Collection (Manassas, VA, USA), were cultured in RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in an incubator containing humidified air with 5% (v/v) CO<sub>2</sub>.

**Cell viability assay.** SKOV3 and A2780 cells were seeded in 96-well plates at 5x10<sup>3</sup> cells/well and treated with salidroside at different concentrations (0, 50, 100, 500, 1,000 or 2,000  $\mu$ mol/l) for 48 h, at 37°C in an incubator containing humidified air with 5% (v/v) CO<sub>2</sub>. A total of 10  $\mu$ l MTT (5 mg/ml) was added to each well and incubated in the dark at 37°C for 4 h. The supernatant was removed and replaced with 150  $\mu$ l dimethyl sulfoxide. The plates were oscillated for 10 min and the absorbance was measured at 490 nm.

**Acridine orange/ethidium bromide (AO/EB) staining.** SKOV3 and A2780 cells in the logarithmic growth phase were deposited in 2x10<sup>3</sup> cells each well of a 96-well plate.

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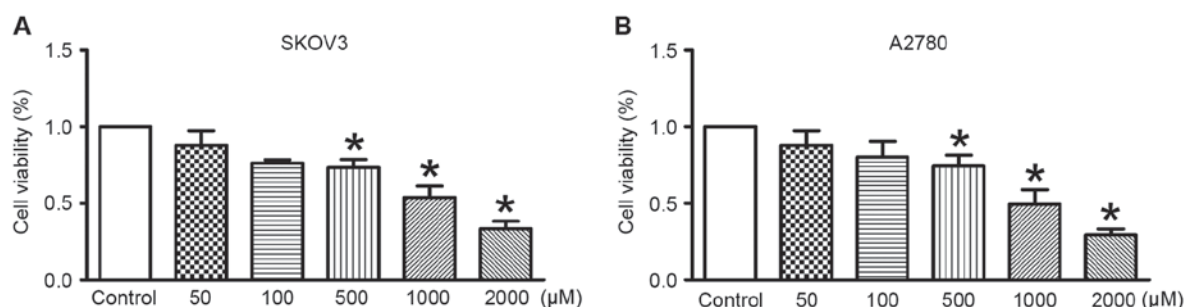


Figure 1. Salidroside inhibits the viability of SKOV3 and A2780 cells. (A) SKOV3 and (B) A2780 cells were treated with salidroside. Relative cell viability was determined by MTT assay. \* $P < 0.05$  vs. control;  $n = 6$  independent experiments for each condition.

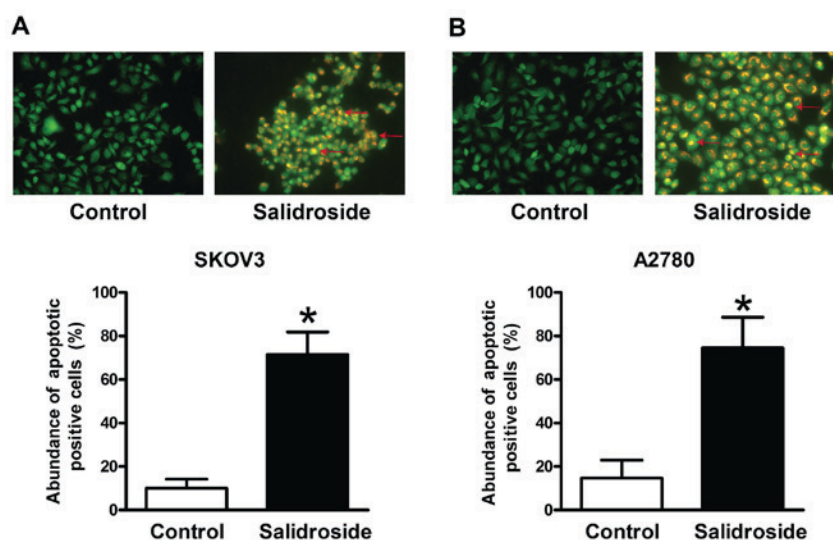


Figure 2. Salidroside induces apoptosis in SKOV3 and A2780 cells. Acridine orange/ethidium bromide staining was performed to detect changes in the nuclei of (A) SKOV3 and (B) A2780 cells. Red arrows indicate apoptotic cells. Magnification,  $\times 200$ . Data are expressed as the mean  $\pm$  standard error of the mean;  $n = 3$  for each group. \* $P < 0.05$  vs. control.

After 24 h, cells were subsequently treated with salidroside (0 or 1,000  $\mu\text{mol/l}$ ) for 48 h at  $37^\circ\text{C}$  in an incubator containing humidified air with 5% (v/v)  $\text{CO}_2$ , stained with AO (100  $\mu\text{g/ml}$  in PBS) and EB (100  $\mu\text{g/ml}$  in PBS) at room temperature and analyzed by fluorescence microscopy at  $\times 200$  magnification (17). Five fields were randomly selected for study. Cell apoptosis (%) was defined as the number of apoptotic cells divided by the total number of cells (18,19).

**Antibodies and western blotting.** SKOV3 and A2780 cells [treated with salidroside (1,000  $\mu\text{mol/l}$ ; salidroside group) or PBS (control group) for 48 h at  $37^\circ\text{C}$  in an incubator containing humidified air with 5% (v/v)  $\text{CO}_2$ ] were collected and lysed in ice-cold radioimmunoprecipitation assay buffer (Roche Diagnostics, Basel, Switzerland). The protein concentration of the lysates was measured using a bicinchoninic acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) according to the protocol of the manufacturer. Cell lysates were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (Pall Corporation, Port Washington, NY, USA). The membranes were blocked using 5% skim milk then incubated with primary antibodies against  $\beta$ -actin (cat. no. 3700; 1:2,000 dilution; Cell Signaling Technology, Inc.,

Danvers, MA, USA), p53 (cat. no. 2524; 1:500 dilution; Cell Signaling Technology, Inc.), p21<sup>Cip1/Waf1</sup> (cat. no. 610233; 1:500 dilution; BD Biosciences, Franklin Lakes, NJ, USA), p16<sup>INK4a</sup> (cat. no. sc-53392; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2-associated X protein (Bax) (cat. no. 5020; 1:1,000 dilution; Cell Signaling Technology, Inc.), Bcl-2-associated death promoter (Bad) (cat. no. 9268; 1:500 dilution; Cell Signaling Technology, Inc.), and p-Bad (cat. no. 9291; 1:500 dilution; Cell Signaling Technology, Inc.), Bcl-2 (cat. no. 15071; 1:1,000 dilution; Cell Signaling Technology, Inc.), apoptosis-inducing factor (AIF) (cat. no. 5318; 1:1,000 dilution; Cell Signaling Technology, Inc.) and X-linked inhibitor of apoptosis (XIAP) (cat. no. 14334; 1:1,000 dilution; Cell Signaling Technology, Inc.) at  $4^\circ\text{C}$  overnight. The membranes were incubated with rabbit (cat. no. 7054; 1:5,000 dilution; Cell Signaling Technology, Inc.) or mouse (cat. no. 7056; 1:5,000 dilution; Cell Signaling Technology, Inc.) secondary antibodies at room temperature for 1.5 h. Immunoreactive bands were visualized using an enhanced chemiluminescence reagent (GE Healthcare, Chicago, IL, USA). Intensities of immunoreactive bands were determined by densitometric analysis using ImageJ software (version 1.61; National Institutes of Health, Bethesda, MD, USA) and normalized against  $\beta$ -actin.

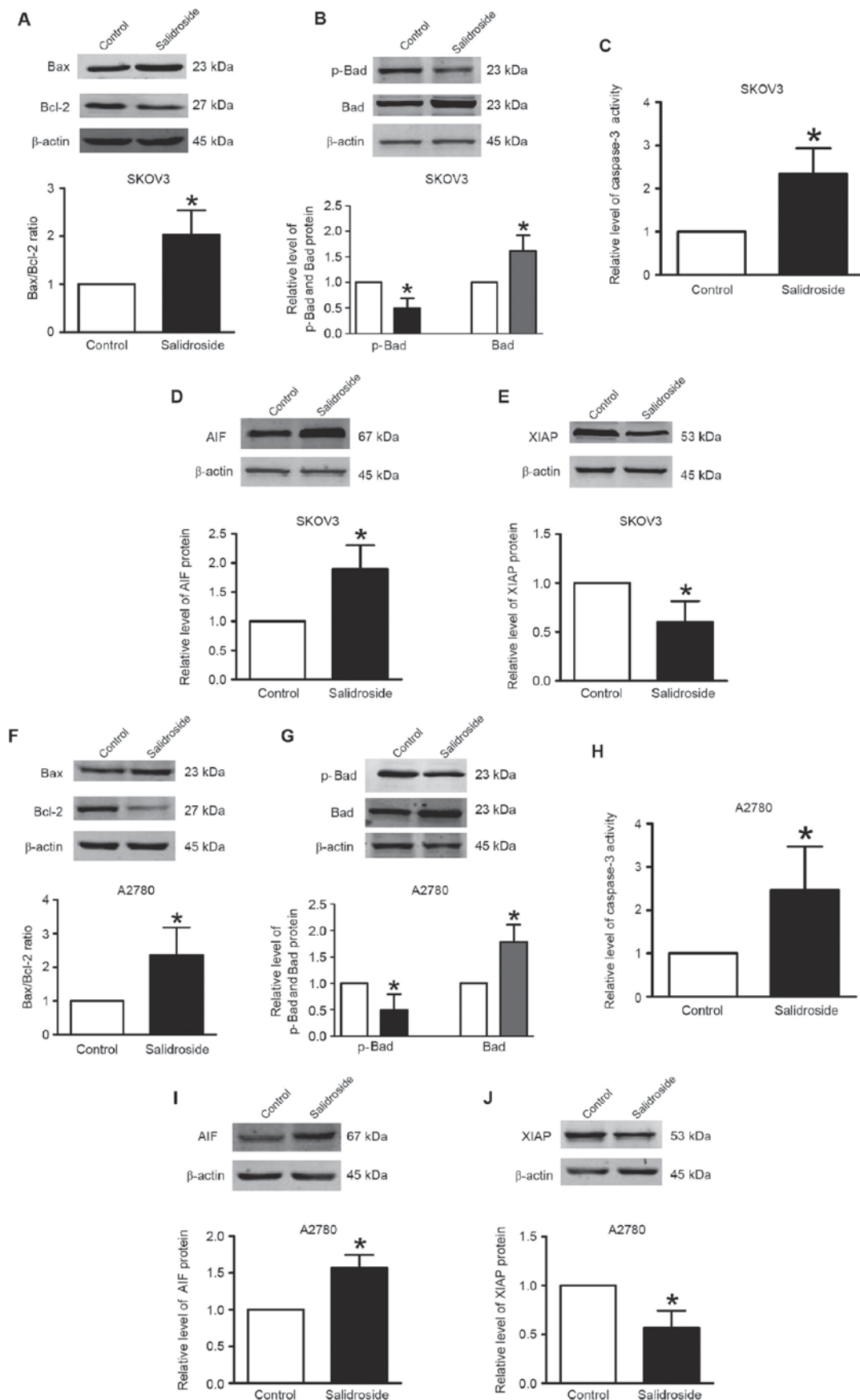


Figure 3. Salidroside alters p-Bad, Bad, Bax, Bcl-2, AIF and XIAP expression, and promotes caspase-3 activation. Western blotting was used to detect protein expression. Relative protein expression was normalized to  $\beta$ -actin. Caspase-3 activity was measured using a commercial assay kit. (A) Bax and Bcl-2 expression, (B) Bad and p-Bad expression, (C) caspase-3 activity, (D) AIF expression and (E) XIAP expression in SKOV3 cells treated with salidroside. (F) Bax and Bcl-2 expression, (G) Bad and p-Bad expression, (H) caspase-3 activity, (I) AIF expression and (J) XIAP expression in A2780 cells treated with salidroside. Data are expressed as the mean  $\pm$  standard error of the mean;  $n=3$  independent experiments for each group. \* $P<0.05$  vs. control. Bax, Bcl-2-associated X protein; Bad, Bcl-2-associated death promoter; AIF, apoptosis-inducing factor; XIAP, X-linked inhibitor of apoptosis.

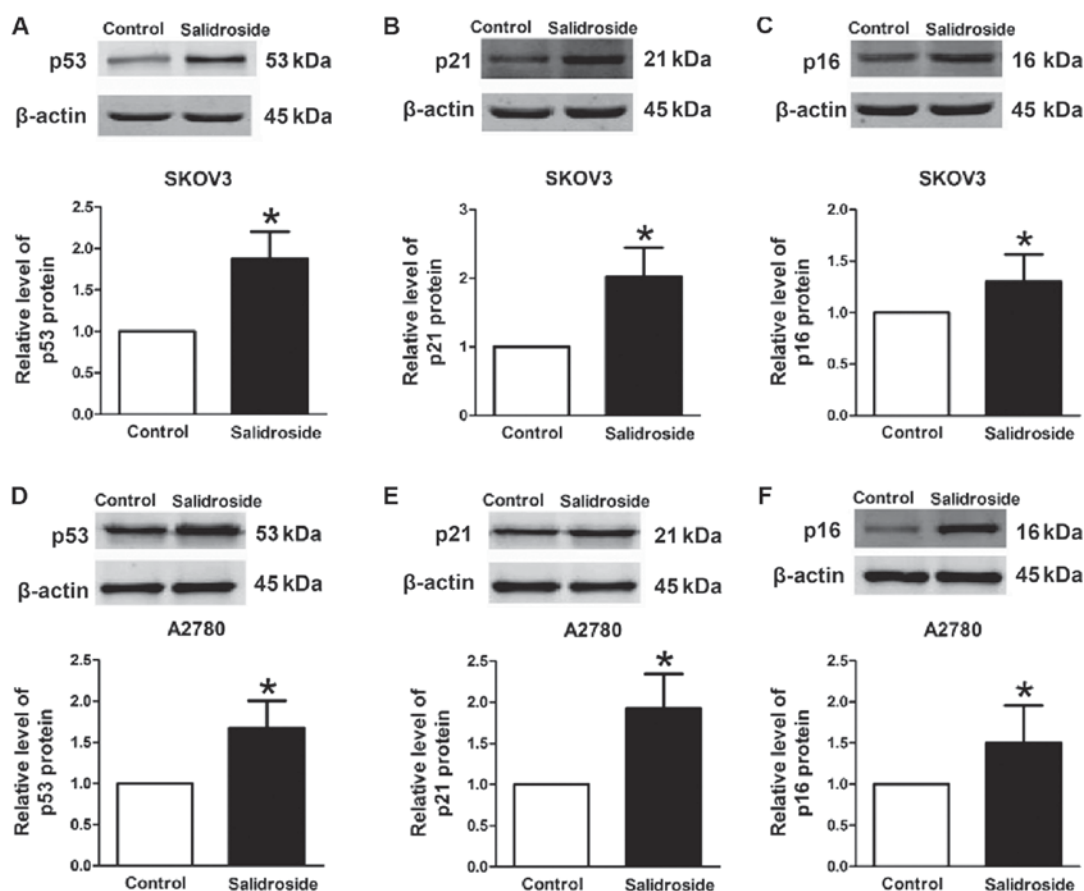


Figure 4. Salidroside induces the expression of p53, p21<sup>Cip1/Waf1</sup> and p16<sup>INK4a</sup> in SKOV3 and A2780 cells. Protein levels of (A) p53, (B) p21<sup>Cip1/Waf1</sup> and (C) p16<sup>INK4a</sup> in SKOV3 cells. Protein levels of (D) p53, (E) p21<sup>Cip1/Waf1</sup> and (F) p16<sup>INK4a</sup> in A2780 cells. Data are expressed as the mean  $\pm$  standard error of the mean; n=3 for each group. \*P<0.05 vs. control.

**Caspase-3 activity assay.** A caspase-3 activity assay kit (cat no. C1115; Beyotime Institute of Biotechnology, Haimen, China) was used to test the activity of caspase-3 following treatment with salidroside (0 or 1,000  $\mu$ mol/l) for 48 h in ovarian cancer (SKOV3 and A2780) cells. Caspase activity was expressed as a percentage of the control.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard error of the mean of three independent experiments. Statistical analysis was conducted using SPSS 19.0 software (IBM Corp., Armonk, NY, USA) and illustrated using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was determined using Student's t-test to compare two groups or analysis of variance with Tukey's post hoc test to compare multiple groups. P<0.05 was considered to indicate a statistically significant difference.

## Results

**Assessment of OC cell viability after treatment with salidroside.** Across different concentrations (500, 1,000 or 2,000  $\mu$ mol/l) of salidroside treatment, cell viability was significantly inhibited compared with the control (Fig. 1). The data revealed that salidroside treatment at 1,000  $\mu$ mol/l for 48 h inhibited viability of SKOV3 and A2780 cells by ~50%. For this reason, in subsequent experiments 1,000  $\mu$ mol/l was used as salidroside treatment.

**Salidroside induces apoptosis in SKOV3 and A2780 cells.** Cell viability is a balance between cell proliferation and apoptosis (20). AO/EB staining revealed that salidroside significantly increased the rate of apoptosis in SKOV3 (Fig. 2A) and A2780 cells (Fig. 2B).

**Salidroside activates pro-apoptotic signaling pathways.** To explore the functional mechanisms of salidroside, the expression of Bax, Bcl-2, Bad, p-Bad, AIF and XIAP proteins was investigated by western blotting in SKOV3 (Fig. 3A-E) and A2780 (Fig. 3F-J) cells. The results indicated that treatment with salidroside significantly upregulated the ratio of Bax/Bcl-2 and the expression of Bad and AIF, but significantly downregulated p-Bad and XIAP expression in SKOV3 and A2780 cells. Meanwhile, caspase-3 activity was significantly increased by salidroside in SKOV3 (2.1-fold increase) and A2780 (2.5-fold increase) cells.

**Salidroside activates p53 signaling pathways.** To define whether p53 signaling was involved in salidroside-induced apoptosis, the protein levels of p53, p21<sup>Cip1/Waf1</sup> and p16<sup>INK4a</sup> were evaluated. They were identified to be significantly upregulated in SKOV3 (Fig. 4A-C) and A2780 (Fig. 4D-F) cells after treatment with salidroside compared with the control. These results indicated that the p53/p21<sup>Cip1/Waf1</sup>/p16<sup>INK4a</sup> pathway serves a critical function in salidroside-induced apoptosis in SKOV3 and A2780 cells.



## Discussion

In the present study, *in vitro* experiments demonstrated that salidroside exerts potent anti-proliferative effects on SKOV3 and A2780 cells by inducing apoptosis. Furthermore, the mechanisms underlying the anticancer effects of salidroside on OC were investigated. The results indicated that activation of the caspase-3-dependent pathway and the p53 signaling pathway were involved in mediating these salidroside-induced effects. Therefore, the present results may provide an experimental basis for the potential role of salidroside in treating OC.

The current results are consistent with previous reports demonstrating that salidroside exerts anticancer effects in breast carcinoma (15), human fibrosarcoma (21,22) and neuroblastoma (23). The mechanism of action of salidroside has been reported to involve autophagy (24). However, the effect of salidroside in OC is not yet fully understood.

The present data suggested that salidroside has antiproliferative and pro-apoptotic effects on OC cells. It was revealed that salidroside inhibited the viability of SKOV3 and A2780 cells. Furthermore, AO/EB staining indicated that salidroside induced apoptosis in OC cells.

The effect of salidroside on the regulation of gene expression has been studied previously. Numerous studies have revealed that Bax, Bcl-2 and caspase-3 are involved in apoptosis in SKOV3 and A2780 cells (25). Liu *et al* (26) identified that caspase-mediated cleavage of Beclin1 inhibits autophagy and promotes apoptosis in SKOV3 cells. Furthermore, andrographolide radiosensitizes human SKOV3 cells (27), and miRNA-149 modulates the chemosensitivity of A2780 cells by modulating Bax protein expression (28). In the present study, it was demonstrated that salidroside could induce Bcl-2 and Bax expression, and upregulate caspase-3 in SKOV3 and A2780 cells. In addition, the ratio of Bcl-2/Bax was decreased, indicating that salidroside promotes apoptosis in OC. Bad is known to regulate apoptosis by forming heterodimers with Bax and Bcl-2 (29). XIAP inhibits activation of caspases by binding to them, preventing apoptosis of tumor cells (30). In the present study, Bad protein was significantly increased while p-Bad and XIAP levels were significantly decreased following treatment with salidroside. These results demonstrate that salidroside could induce apoptosis via caspase-3-dependent apoptosis signaling in OC (31,32). Furthermore, p53 can influence apoptosis by regulating Bcl-2 (33). Previous studies have revealed that >50% of tumors are associated with p53 gene mutation; wild-type p53 gene therapy has been suggested to strengthen sensitivity to cisplatin in SKOV3 cells (34-36). In the present study, it was demonstrated that salidroside has the ability to induce OC apoptosis. Salidroside also promoted the expression of p53, p21<sup>Cip1/Waf1</sup> and p16<sup>INK4a</sup> expression in SKOV3 and A2780 cells. It was identified that salidroside promotes the expression of caspase-3 and activation of the p53/p21<sup>Cip1/Waf1</sup>/p16<sup>INK4a</sup> pathway.

In summary, salidroside was demonstrated to reduce cell viability and promote apoptosis in OC. Furthermore, it was identified that salidroside activates caspase-3 and the p53/p21<sup>Cip1/Waf1</sup>/p16<sup>INK4a</sup> pathway. Therefore, salidroside is a promising new approach for the treatment of OC, but its underlying mechanism needs to be explored further.

## Competing interests

The authors declare that they have no competing interests.

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