Inhibition of cell growth and induction of apoptosis in ovarian carcinoma cell lines CaOV3 and SKOV3 by natural withanolide Withaferin A

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ABSTRACT

Objective. Withaferin A, a natural withanolide, has shown anti-cancer properties in various cancers including breast cancer, but its effects in ovarian cancer remain unexplored. Notch 1 and Notch3 are critically involved in ovarian cancer progression. We decided to examine the effects of Withaferin A in ovarian carcinoma cell lines and its molecular mechanism of action including its regulation of Notch.

Methods. The effects of Withaferin A were examined in CaOV3 and SKOV3 ovarian carcinoma cell lines using MTS assay, clonogenic assay, annexin V/propidium iodide flow cytometry, and cell cycle analysis. Western analysis was conducted to examine the molecular mechanisms of action.

Results. Withaferin A inhibited the growth and colony formation of CaOV3 and SKOV3 cells by inducing apoptosis and cell cycle arrest. These changes correlated with down-regulation of Notch1, Notch3, cdc25C, total and phosphorylated Akt, and bcl-2 proteins.

Conclusions. Withaferin A inhibits CaOV3 and SKOV3 ovarian carcinoma cell growth, at least in part by targeting Notch1 and Notch3.

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Introduction

Ovarian cancer is the fifth most frequent cause of cancer death in women. Ovarian epithelial cancer accounts for 90% of all ovarian cancers and is the leading cause of death from gynecological cancers in North America and Europe [1]. Current chemotherapies for ovarian cancer often lead to resistance or relapse, making it necessary for identification of novel treatments and therapeutic targets.

Notch signaling is active in ovarian cancer, and is known to stimulate cell proliferation and survival in ovarian carcinoma cell lines [2–4]. So far, four Notch transmembrane receptors (Notch1–4) have been identified [5]. Upon specific ligand binding, Notch receptors undergo proteolytic cleavage, ultimately leading to liberation of the Notch intracellular domain (NICD), the activated form of Notch receptors, by γ-Secretase [5]. NICD then translocates to the nucleus to stimulate transcription of target genes such as Hes-1. Notch1 intracellular domain (N1ICD) is expressed in the majority of ovarian cancers and its depletion leads to growth retardation in ovarian cancer cells [2]. Notch3 is over-expressed in 55% of high-grade serous carcinoma, and is required

for proliferation and survival of Notch3-amplified tumors [3]. Therefore, Notch1 and Notch3 are potential therapeutic targets in ovarian cancers.

A number of natural withanolides including Withaferin A (WA) have been found to have anti-cancer effects in a variety of cancer cell lines and animal models [6–8], but their effects in ovarian cancer have not been explored. WA is steroidal lactone extracted from the medicinal plant *Withania somnifera* [9]. It has been found to down-regulate Notch1 in colon cancer cells [10]. Here we examined the effects of WA in CaOV3 and SKOV3 ovarian carcinoma cell lines and the molecular mechanism of its action. Marked down-regulation of Notch1 and Notch3 by WA was revealed in this study as one potential mechanism of its effects in ovarian cancer.

Materials and methods

Cell lines and reagents

Ovarian cancer cell lines

CaOV3 (adenocarcinoma), SKOV3 (adenocarcinoma), OVCAR3 (adenocarcinoma), TOV112D (endometrioid carcinoma), and TOV21G (clear cell carcinoma) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). CaOV3 and SKOV3 were cultured in DMEM containing 10% fetal bovine serum (FBS), 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. TOV112D and TOV21G were cultured in RPMI1640 containing 10% FBS, 100 units/mL

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penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. OVCAR3 was cultured in RPMI1640 containing 16% FBS, 10 μg/mL bovine insulin, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM glutamine. WA was purchased from ChromaDex (Irvine, CA). The antibodies against poly (ADP-ribose) polymerase (PARP), caspase-3, cdc25C, cyclin B1, total Akt, phospho-Akt ser473, Bcl-2, Bax, Notch1, and Notch3 were purchased from Cell Signaling Technology (Danvers, MA) and the anti-β-actin antibody was purchased from Millipore (Billerica, MA). The anti-annexin V-FITC-conjugated and propidium iodide (PI) were purchased from BD Bioscience (Rockville, MD).

MTS assay

Cells were seeded in 96-well microtiter plates (2.0×10^3 per well) in 90 μL of growth media. Cells were allowed to attach overnight and then treated with 10 μL of 10× of various concentrations of WA (0, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, 5, 10 μM) or cisplatin (as a control; 0, 0.313, 0.625, 1.25, 2.5, 5, 10, 20, 40 μM) for 72 h. The number of viable cells was determined using CellTiter 96 Aqueous non-radioactive Cell proliferation assay measuring A_{490} nm of the dissolved formazan product after addition of the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt, for 2 h as described by the manufacturer (Promega). All experiments were carried out in triplicates, and the viability was expressed as the ratio of the number of viable cells with WA or cisplatin treatment to vehicle group. The half-maximal inhibitory concentration (IC50) was obtained from the MTS viability curves using GraphPad Prism 5. Experiments were repeated twice, and data are representative of replicate experiments.

Clonogenic assay

CaOV3 (1000 cells/plate) or SKOV3 (300 cells/plate) cells were plated in 60 mm plates. After an overnight incubation, cells were treated with 0, 200, 400, 600, 800 or 1000 nM of Withaferin A for 24 h. Cells were then washed, and fresh media were added. After 14 days of incubation, surviving colonies were fixed and stained with Coomassie Blue stain. Experiments were repeated, and data are representative of replicate experiments.

Annexin V-FITC/PI flow cytometry

To study the effects of WA on cellular apoptosis, CaOV3 or SKOV3 cells were treated with increasing doses of WA for 24 or 48 h. Detached and adherent cells were then collected and labeled for 15 min at room temperature with annexin V-FITC-conjugated (1 μg/mL) and with prodidium iodide (PI; 40 μg/mL) and immediately analyzed on a flow cytometer. 10,000 cells were measured for each sample. All experiments were carried out in triplicate. Experiments were repeated, and data are representative of replicate experiments.

Cell cycle analysis

CaOV3 or SKOV3 cells were seeded in complete medium (2×10^5 per 100-mm plate). After an overnight incubation, cells were treated with increasing doses of WA for 24 or 48 h. Detached and adherent cells were collected and fixed in 70% ethanol in PBS at −20 °C overnight. Cells were then resuspended in PBS containing 40 μg/mL PI and 100 μg/mL RNAse and incubated for 30 min at 37 °C in the dark. Samples were then analyzed on a BD™LSRII flow cytometer (BD Biosciences) using BD FACSDiva6.0 software. 10,000 cells were measured for each sample. All experiments were carried out in triplicate. Experiments were repeated, and data are representative of replicate experiments.

Western analysis

Total proteins were extracted from CaOV3 or SKOV3 cells using radioimmuno-precipitation assay buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1% IGEPAL CA-630, 0.5% sodium deoxycholate, 1 mM

ethylenediaminetetra-acetic acid, and 0.1% SDS) containing a protease/phosphatase inhibitor cocktail (0.1 mg/mL PMSF, 30 μL/mL of aprotinin, 5 μg/mL of leupeptin, and 1 mM sodium orthovanadate; Sigma). Protein concentrations were determined using the BCA protein assay reagent kit (Pierce, Rockford, IL). Equal amount of proteins was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose membranes (Hybond; Amersham, Piscataway, NJ). After blocking with 3% nonfat dry milk in PBS for 1 h at room temperature, blots were incubated with appropriate primary antibodies overnight at 4 °C. Blots were then washed and incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h, and protein bands were detected using Pico or Femto chemiluminescence kit (Thermo Scientific, Rockford, IL). Next, blots were reprobed for β-actin to normalize protein expression. Experiments were repeated twice, and data are representative of replicate experiments.

Statistical analysis

Data were analyzed using SPSS Version 17.0 software (SPSS, Inc.). ANOVA was used for comparison across treatment regimes. When an F test indicated statistical significance, post hoc analysis was made using the Tukey's honestly significant difference procedure. Significance was set at $P < 0.05$ for all comparisons.
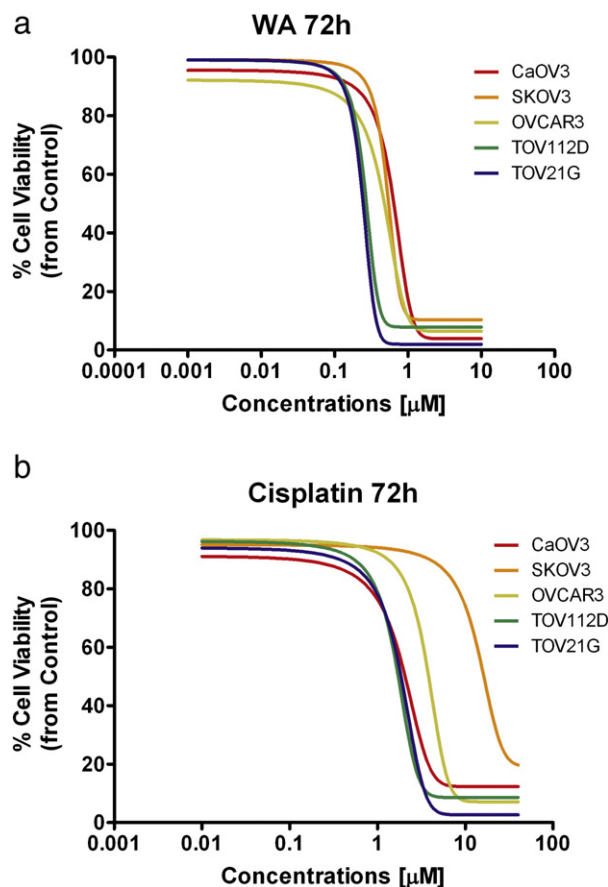

Fig. 1. Effect of WA versus cisplatin on the viability of ovarian cancer cell lines (CaOV3, SKOV3, OVCAR3, TOV21G, TOV112D) as measured by MTS assay. Cells (2×10^3 per well) were cultured in 96-well plates for 24 h, and treated with various concentrations of WA or cisplatin for 72 h. The viable cells were quantified by MTS assay in triplicates. a, Effects of WA; b, Effects of cisplatin.

Results

WA inhibits the viability of CaOV3 and SKOV3 cells

The effects of WA on ovarian cancer cell viability were analyzed using MTS assay after WA treatment for 72 h at various concentrations.

As shown in Fig. 1a, WA treatment caused marked decrease in cell viability in CaOV3, SKOV3, OVCAR3, TOV112D, and TOV21G ovarian cancer cells, with an IC50 concentrations at 520 nM, 627 nM, 452 nM, 262 nM, 243 nM, respectively. Cisplatin was used as a control, which decreased cell viability in CaOV3, SKOV3, OVCAR3, TOV112D, and TOV21G cells with IC50 concentrations at 1.8 μM, 13.8 μM, 3.7 μM,
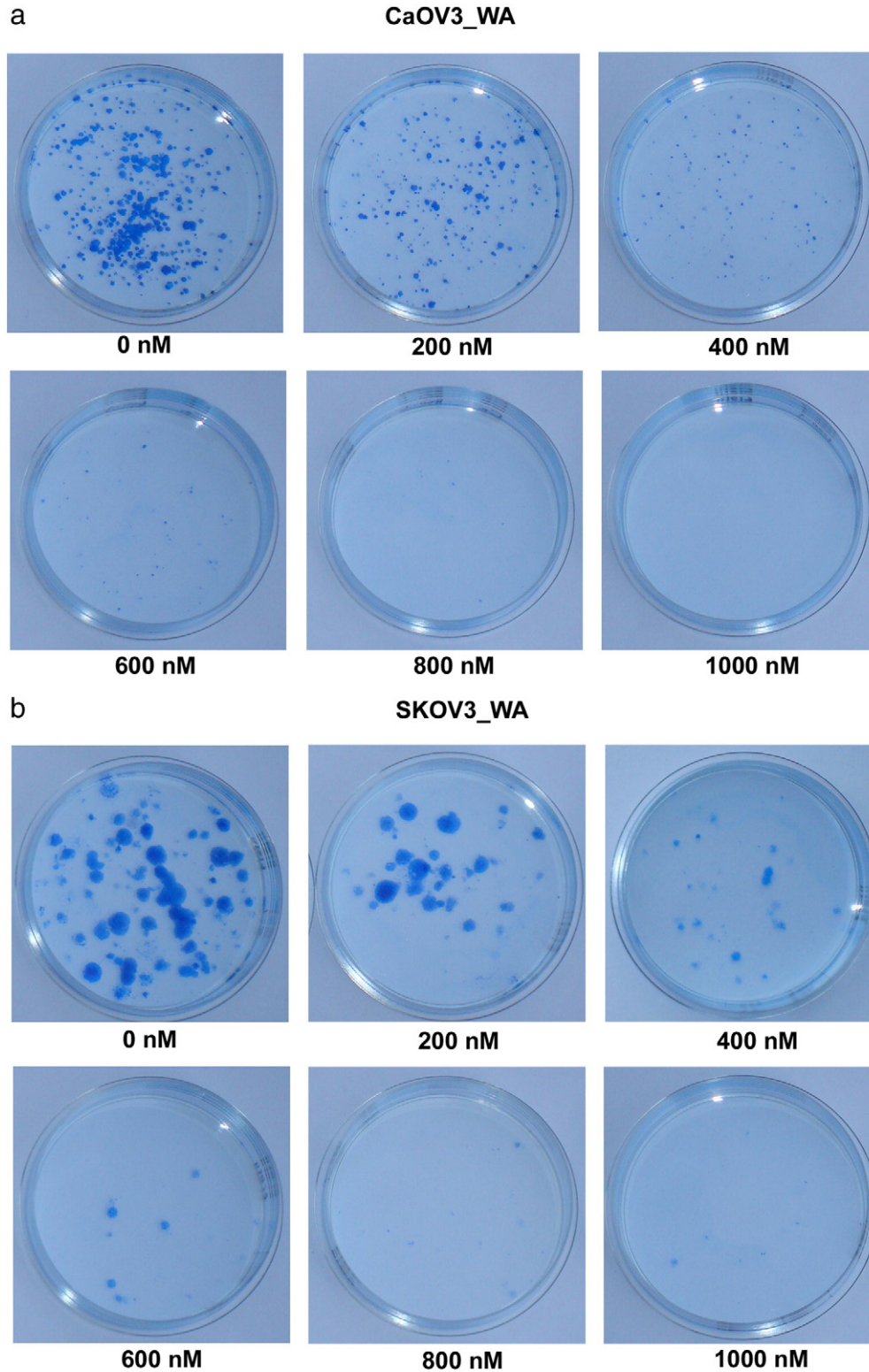
Fig. 2. Effects of WA on CaOV3 and SKOV3 colony formation as measured by clonogenic assay. Cells were treated with indicated concentrations of WA for 24 h and then cultured for 14 days. Survival colonies were stained with Coomassie Blue. a, Effects of WA on CaOV3 cell colony formation; B, Effects of WA on SKOV3 cell colony formation.

1.6 μM, 1.8 μM, respectively (Fig. 1b). These data demonstrated that WA inhibits the viability of various ovarian cancer cell lines, regardless of their sensitivity to cisplatin.

WA inhibits colony formation in CaOV3 and SKOV3 cells

We also determined the effects of WA on the colony formation efficiency of CaOV3 and SKOV3 cells. The results shown in Fig. 2 indicated that 24 h of WA treatment caused a dose-dependent inhibition of colony formation in both CaOV3 and SKOV3 cells. This inhibitory effect was observed when WA concentration was as low as 200 nM and increased with accelerating doses of WA. CaOV3 appears slightly more sensitive to WA as compared to SKOV3. After treatment with 1000 nM WA, no colonies could be seen in CaOV3 cells whereas in SKOV3 cells a couple of small colonies were observed.

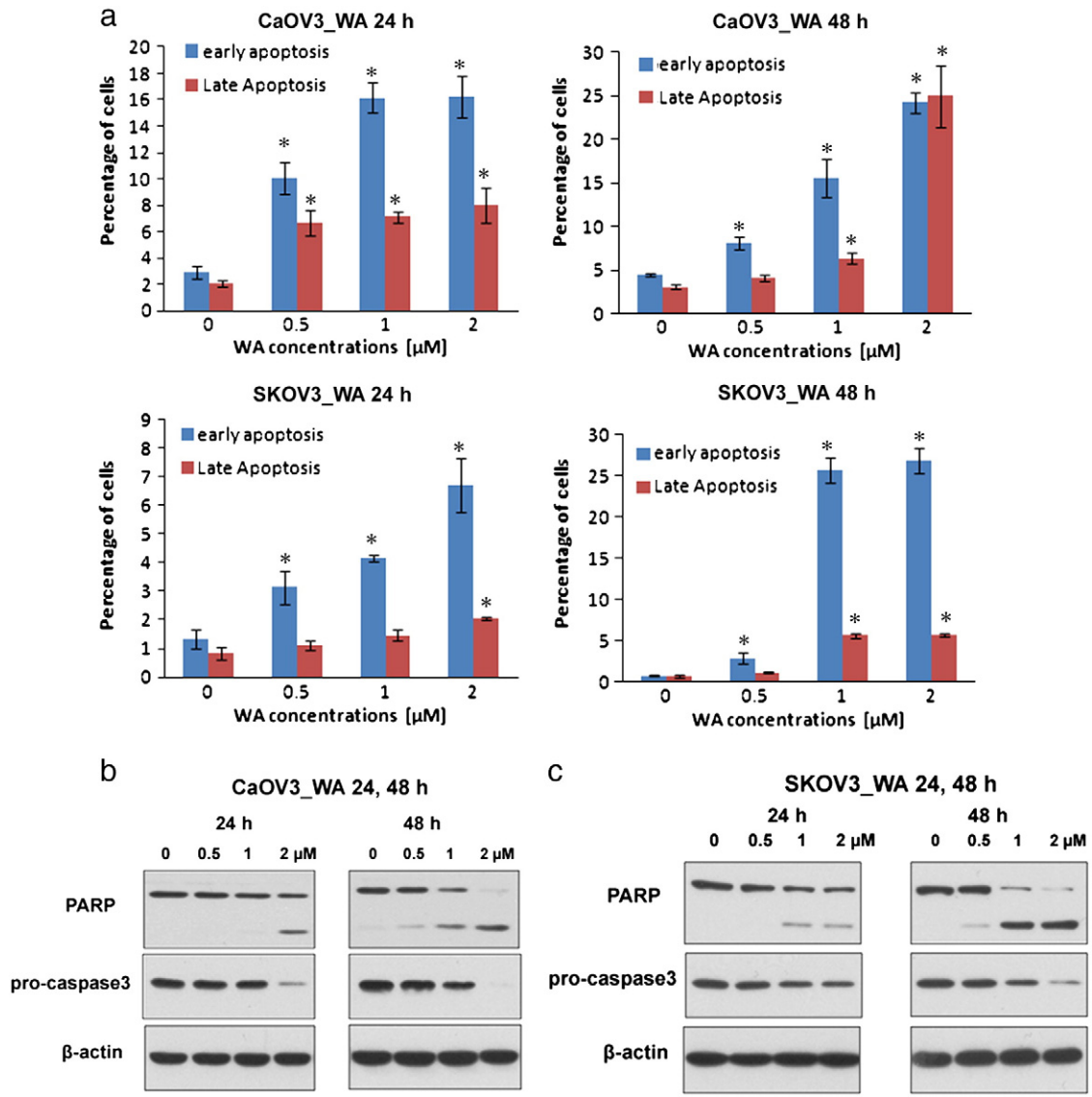*WA induces apoptosis in CaOV3 and SKOV3 cells*

To address whether WA causes cellular apoptosis, annexin V-FITC/PI flow cytometry was conducted. As shown in Fig. 3a, WA induced apoptosis in both CaOV3 and SKOV3 cells in a dose-dependent manner. A slight but significant increase in apoptosis was observed with 500 nM WA. At 1 or 2 μM, more cells underwent apoptosis. WA induced more early apoptosis than late apoptosis, except for 2 μM WA 48 h treatment, which caused approximately the same percentage of early and late apoptosis in CaOV3 cells.

Western analysis for PARP and caspase-3 revealed increased PARP cleavage and decreased pro-caspase-3 with accelerating doses of WA, confirming the pro-apoptotic effect of WA in CaOV3 and SKOV3 cells (Figs. 3b and c).

WA induces G2/M phase cell cycle arrest in CaOV3 and SKOV3 cells

To examine whether the inhibition of ovarian carcinoma cell growth by WA was also partly due to cell cycle arrest, we analyzed cell cycle distribution using PI staining. We found that WA differentially altered cell cycle distribution in CaOV3 and SKOV3 cells. Although both CaOV3 and SKOV3 cells showed an accumulation of cells in G2/M phase after WA treatment, this effect was more prominent in SKOV3 cells. The proportion of cells in G0/G1 phase was markedly decreased

Fig. 3. Effect of WA on CaOV3 and SKOV3 cell apoptosis. a, Annexin V-FITC-propidium iodide flow cytometry results. Cells were treated with DMSO or the indicated concentrations of WA for 24 or 48 h. Results were presented as mean (n = 3) ± SD. *, P<0.05, significantly different from control by one-way ANOVA. b and c, Western analysis of the effects of WA on PAPR cleavage and pro-caspase 3 activation. Cells were treated with DMSO or the indicated concentrations of WA for 24 or 48 h. Western for β-actin was conducted to confirm equal loading of proteins. B, CaOV3; C, SKOV3.
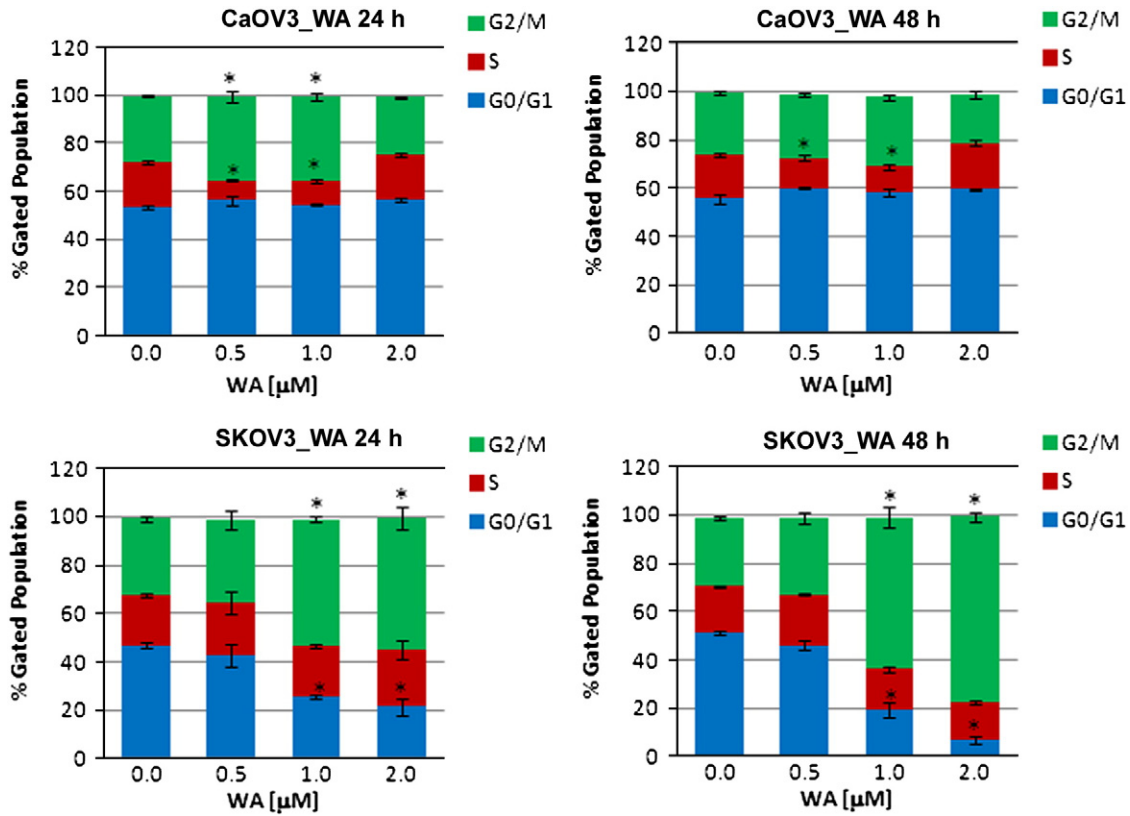
Fig. 4. Effect of WA on CaOV3 and SKOV3 cell cycle distribution as measured by propidium iodide flow cytometry. Cells were treated with DMSO or the indicated concentrations of WA for 24 or 48 h. Results were presented as mean $(n = 3) \pm SD$. *, $P < 0.05$, significantly different from control by one-way ANOVA.

in SKOV3 cells by WA, but remained unchanged in CaOV3 cells. The proportion of cells at S phase remained unchanged in SKOV3 cells, but was significantly reduced by WA in CaOV3 cells (Fig. 4).
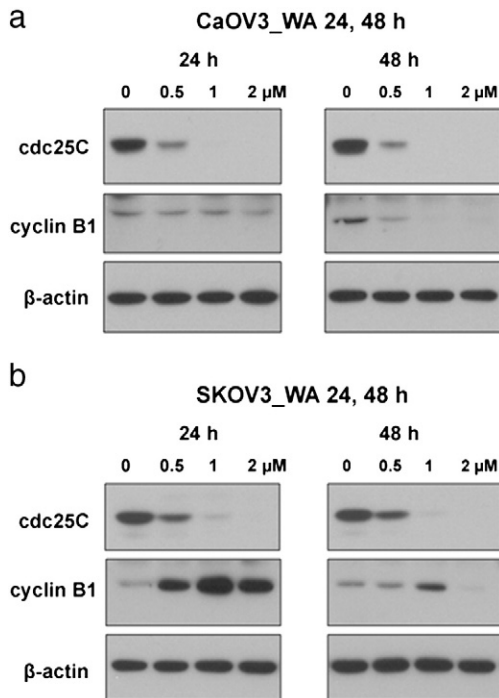

Fig. 5. Western analysis of the effects of WA on cdc25C and cyclin B1 protein levels. Ovarian cancer cells CaOV3 (a) and SKOV3 (b) were treated with DMSO or the indicated concentrations of WA for 24 or 48 h. Western for β-actin was conducted to confirm equal loading of proteins.

### WA depletes cdc25C in CaOV3 and SKOV3 cells

Western analysis was used to examine the expression of two important G2/M phase check point proteins, cdc25C and cyclin B1 (Fig. 5). In both CaOV3 and SKOV3 cells, cdc25C protein levels were markedly decreased by WA in a dose-dependent manner. At 24 h post treatment, its levels were significantly lower even with 0.5 μM of WA. The levels of cdc25C protein diminished with 1 μM of WA and became undetectable with 2 μM of WA by 24 h. No significant differences in cdc25C levels were observed between 24 h and 48 h.

Cyclin B1 levels were differentially altered by WA in CaOV3 and SKOV3 cells. In CaOV3 cells, cyclin B1 stayed unchanged at 24 h, but decreased by 48 h in a dose-dependent manner. In SKOV3 cells, however, cyclin B1 was transiently increased by WA at 24 h.

WA inhibits the Notch, Akt, and bcl-2 pro-survival pathways

We found that WA reduced the levels of phospho-Akt (ser 473) in both CaOV3 and SKOV3 cells. Total Akt levels were decreased too, but to a lesser extent. Bcl-2 protein levels were also decreased by WA in both cell types in a dose-dependent manner. The levels of Bax protein were decreased in parallel with bcl-2 in CaOV3 cells. In SKOV3 cells, the levels of Bax protein remained unchanged at 24 h, and decreased by 48 h after WA treatment (Fig. 6).

We then examined the effects of WA on Notch1 and Notch 3 expressions in CaOV3 and SKOV3 cells. We found that CaOV3 cells have abundant Notch3 (N3ICD) protein levels that can be easily detected using Pico luminescent kit, while their Notch 1 (N1ICD) protein levels are undetectable using Pico luminescent kit but could be detected using the more sensitive Femto luminescent kit. SKOV3 cells have abundant Notch1 (N1ICD) protein levels that can be easily detected using Pico luminescent kit, but their Notch3 (N3ICD) levels were undetectable even with Femto luminescent kit. Withaferin A treatment significantly
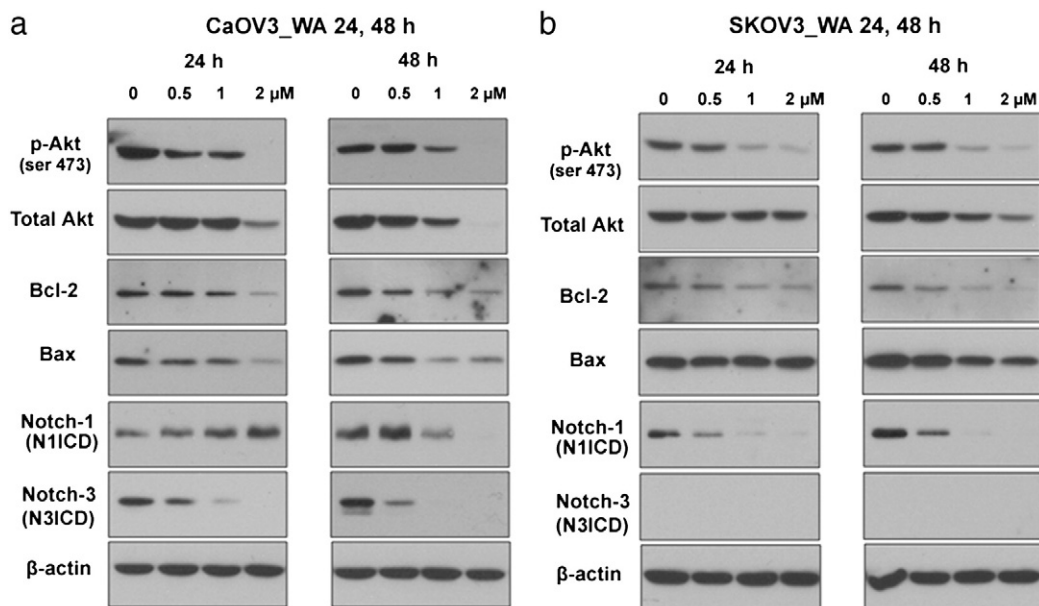
Fig. 6. Western analysis of the effects of WA on phospho-Akt (ser473), total Akt, bcl-2, bax, Notch1, and Notch3 protein levels. Ovarian cancer cells CaOV3 (a) and SKOV3 (b) were treated with DMSO or the indicated concentrations of WA for 24 or 48 h. Western for β-actin was conducted to confirm equal loading of proteins.

reduced Notch 3 protein levels in CaOV3 cells and Notch1 protein levels in SKOV3 cells in a similar dose-dependent manner. Down-regulation of Notch1 and Notch 3 occurred with as low as 0.5 μM WA, and became more effective with higher doses. In CaOV3 cells, Notch1 protein levels were transiently increased at 24 h after WA treatment, followed by a decline in its levels at 48 h. Expression of Notch 3 remained undetectable in SKOV3 cells after WA treatment (Fig. 6).

Discussion

The development of effective therapeutic agents for ovarian cancer is necessary to improve current chemotherapy. Our study demonstrates that WA has strong anti-cancer properties in various ovarian cancer cell lines, regardless of their subtypes or their sensitivities to cisplatin. Although WA has been shown to inhibit various cancers such as cancers of the breast [8,11], colon [10], and prostate [12], this is the first report on its effects in ovarian cancer.

We showed that WA-induced inhibition of cell viability in various ovarian cancer cell lines. The effects of WA in inhibiting ovarian cancer cell colony formation were demonstrated in CaOV3 and SKOV3 cells. We then asked the question whether the inhibition of ovarian cancer cell growth and viability is due to decreased cell proliferation rate, induction of cellular apoptosis, or both. We found that WA caused both G2/M phase cell cycle arrest and cellular apoptosis in CaOV3 and SKOV3 cells, indicating that inhibition of ovarian cancer cells is attributed to both induction of apoptosis and cell cycle arrest in these cell lines.

CaOV3 cells are slightly more sensitive to WA than SKOV3 cells regarding inhibition of clonogenic growth and induction of apoptosis, although the IC50 concentration of WA (defined as concentration of WA to induce 50% inhibition of cell viability as compared to the vehicle group) is slightly lower in SKOV3 cells than in CaOV3 cells. SKOV3 cells are a metastatic ovarian adenocarcinoma cell line, and they duplicate faster than CaOV3 cells in culture (so the number of SKOV3 cells in vehicle group is higher than that of CaOV3 cells in vehicle group by 72 h of treatment), which could explain the slightly lower IC50 of WA in SKOV3 cells as compared to CaOV3 cells.

We then asked if the G2/M phase cell cycle arrest is due to changes in the levels of cyclin B1 and cdc25C proteins, which play critical roles in G2/M phase [13]. We found a dose-dependent depletion of cdc25C by WA in both CaOV3 and SKOV3 cells. Cyclin B1 expression was unchanged in CaOV3 cells at 24 h and decreased at 48 h. In SKOV3 cells, however, its

levels were transiently increased at 24 h. Cyclin B1 and cdc25c kinase regulate the onset of the G2/M phase [14]. Just prior to mitosis, a large amount of cyclin B1 is present in the cells, but it is inactive due to phosphorylation of cyclin B1-bound Cdk1. The complex is activated by dephosphorylation of Cdk1 by the cdc25 family of phosphatase, which triggers entry into mitosis [14]. As such, the marked down-regulation of cdc25C by WA in CaOV3 and SKOV3 cells likely leads to prolonged G2/M phase. Down-regulation of cdc25C by WA treatment has also been reported in MCF-7 and MDA-MB-231 breast cancer cell lines [11]. The authors also found that cdc25C over-expression could partially reverse the WA-induced G2/M phase cell cycle arrest in breast cancer cells. Our data in ovarian carcinoma cells are in agreement with these findings. Cyclin B1 degradation is essential for completion of mitosis; whereas overexpression of stable cyclin B1 entailed metaphase arrest [15]. The differential response of CaOV3 and SKOV3 in cyclin B1 expression may explain why SKOV3 cells showed more conspicuous G2/M phase arrest following WA treatment.

We found that WA induced apoptosis in CaOV3 and SKOV3 cells at low micromolar concentrations, accompanied by a decrease in procaspase3 levels and an increased in PARP cleavage. These results are consistent with the annexin V-FITC/PI results. We then investigated the effects of WA on the expression of proteins critical to cell survival. Our data showed that WA drastically down-regulated Notch1 and Notch3 protein levels and suppressed Akt and bcl-2 as well. Notch signaling has been shown to inhibit apoptosis by activating Akt in breast epithelial cell lines [16]. In this study, we found that WA caused a marked decrease of Notch 3 in CaOV3 cells and a similar decrease of Notch 1 in SKOV3 cells at 500 nM by 24 h, which likely play a major role in mediating the pro-apoptotic and growth inhibitory effects of WA by suppressing pro-survival pathways such as Akt/bcl-2. The PI3K/Akt pathway is frequently highly activated in ovarian cancer, most commonly through mutational activation of PI3KCA or overexpression of AKT2 [17,18]. As such, inhibition of Akt activation by WA could have important value in this cancer type.

The Notch pathway is important in ovarian cancer progression. Most ovarian cancers over-express Notch 1 or Notch 3 [2–4]. Inhibition of Notch signaling led to decreased cell proliferation and survival [3,4]. Notch 3 is undetectable in SKOV3 cells, and this agrees with other reports in the literature [4]. Notch 1 levels were found to be low in CaOV3 cells, and could be detected only with the more sensitive Femto kit. WA transiently increased Notch 1 protein levels at 24 h, but decreased it at 48 h.

Notch 1 levels in SKOV3 cells and Notch 3 levels in CaOV3 cells were markedly decreased upon WA treatment at both 24 h and 48 h. To our knowledge, this is the first report of WA-mediated down-regulation of both Notch 1 and Notch 3. In pancreatic cancer cells, Withaferin A has recently been found to decrease Notch 1 expression [10]. The mechanisms of Notch inhibition by WA are currently under investigation in our laboratory.

In summary, we report here that WA down-regulates the Notch, Akt and bcl-2 pathways and causes growth inhibition via inducing G2/M phase cell cycle arrest and apoptosis in CaOV3 and SKOV3 ovarian carcinoma cells, suggesting that WA is a potential therapeutic agent for ovarian cancer. Further studies are needed to confirm these findings in animal models *in vivo* to demonstrate the efficacy of WA in treating ovarian cancer.

Conflicts of interest statement

The authors have no conflicts of interest to declare.

Acknowledgments

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