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RESEARCH ARTICLE

Astragaloside-IV Inhibits Metastasis by Suppressing the SDF-1/CXCR4 Axis and Activating Apoptosis in Cisplatin-Resistant Ovarian Cancer Cells

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ABSTRACT

Objective: Ovarian cancer has the highest mortality rate in women and it has a poor response rate to treatment due to its late diagnosis and is frequently resistant to currently used cisplatin-based treatment methods. Astragaloside IV (As-IV), a bioactive compound and natural tripeptide glycoside known as an antioxidant, has drawn attention in Chinese medicine for its healing properties. Many studies have shown that it has anti-inflammatory, antidiabetic, antitumoral, and anti-angiogenic properties.

Materials and Methods: In our study, we first rendered ovarian cancer cells (OVCAR-3) resistant to cisplatin and then applied determined doses of As-IV (40 µg/mL) and (70 µg/mL) to OVCAR-3 cells and cisplatin-resistant ovarian cancer cells (OVCAR-3-CisR). The cell viability capacity, variation of *BAX/BCL-2* gene expression, and regulation of the *SDF-1/CXCR4* chemokine axis protein and their gene expressions were investigated.

Results: According to the findings, As-IV administration suppressed metastasis by lowering the colony formation potential of cisplatin-resistant ovarian cancer and down-regulating the *SDF-1/CXCR4* axis, and increasing the ratio of *BAX/BCL-2* mRNA and protein levels due to *BAX* up-regulation and *BCL-2* down-regulation.

Conclusion: As a result, we showed that As-IV, used as an antioxidant, can be used as an effective anticancer agent to improve response to the currently used cisplatin-based treatment in cases of drug resistance in ovarian cancer.

Keywords: Ovarian cancer, Cisplatin, Astragaloside IV, Chemokine, Apoptosis, Antioxidant

INTRODUCTION

One of the most common cancers of the woman's reproductive system is ovarian cancer, with 313,959 new cases and 207,252 fatalities anticipated in 2020.[1] Ovarian cancer was the leading cause of death among all gynecologic cancer patients, outpacing only endometrial and cervical cancers in incidence rate.[2] The high death rate of ovarian cancer is attributed to delayed diagnosis and a lack of suitable treatments for resistant disease.[3] Despite advances in diagnostic strategies, surgical techniques, and novel therapeutic drugs, long-term survival in patients with ovarian cancer remains discouraging. Maximum tumor debulking followed by postoperative platinum-based chemotherapy is the current standard of care for ovarian cancer.[4] Cisplatin, a platinum-based antitumoral agent, is one of the most widely used chemotherapy drugs in the treatment of cancer patients suffering from lung, bladder, esophageal, testicular, ovarian, cervical, or breast cancer.[5] The cornerstone of current ovarian cancer chemotherapy, cisplatin-based chemotherapy showed favorable survival and response rates, and toxicity.[6] But as a result of drug resistance, cisplatin's effectiveness in ovarian cancer cells diminished or vanished.[7]

One of the main active components of Astragalus membranaceus is Astragaloside IV (As-IV), which is also referred to as astragalus saponin IV. Astragalus membranaceus is among the 50 most essential herbs used in traditional Chinese medicine, known as "Huang qi" in the Chinese Pharmacopoeia.[8] The chemical formula of AS-IV is 3-O-beta-D-xylopyranosyl-6-O-beta-D-glucopyranosyl-cycloastragenol and the molecular formula of As-IV is C14H68O14.[9] As-IV has a broad range of pharmacological effects, including cardioprotective, antioxidant, antidiabetes, anti-bacterial, anti-inflammatory, anti-fibrosis, antiviral, and immunoregulation effects. It also has protective effects against respiratory, cardiovascular, kidney, and immune system disease.[10] Furthermore, administration of the antioxidant As-IV was shown to increase the expression of the tight junction proteins occludin and zona occludens-1 and to protect the blood-brain barrier by contributing to the regulation of

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endothelial cells of mice subjected to ischemia/reperfusion.[11] In a study of rats with myocardial ischemia, As-IV was shown to increase myocardial antioxidant enzyme superoxide dismutase activity and to contribute to protection against ischemia through regulation of the nitric oxide inducing system.[12]

Numerous studies have shown that As-IV has anti-cancer effects against several tumor types by enhancing immune function, tumor growth inhibition, and tumor migration and invasion inhibition.We investigated the effect of treating cisplatin-resistant ovarian cancer cells with As-IV on chemokine receptors and apoptotic markers, as well as whether As-IV can be used as a potential treatment agent in ovarian cancer types that develop drug resistance.

MATERIALS AND METHODS

Ovarian Cancer Cells and Resistant to Cisplatin

Human ovarian cancer cells (OVCAR-3, ATCC-HTB-161) were used for MTT at 1-1000 µM cisplatin for 24, 48 and 72 h. The IC_{50} dose was 26.37 µM (Figure 1a). First, the living OVCAR-3 cells were passaged and incubated for 48 h with 10 µM cisplatin in the medium.[13] Second, the living OVCAR-3 cells were passaged and 5 µM cisplatin was added to the medium and incubated for 48 h. Finally, the OVCAR-3 living cells were passaged and incubated for 20 days with 2.5 µM cisplatin in the medium. In this way, there was a gradual reduction in the amount of cisplatin in the medium and the growth of OVCAR-3 cells with resistance. At this point the OVCAR-3 cisplatin resistance cells (OVCAR-3-CisR) were cultured in RPMI-1640 Medium (Gibco), including 2 mM glutamine, 10% FBS, 100 Units penicillin/streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cytotoxicity Assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) test was performed for the determination of cell viability. The OVCAR-3 and OVCAR-3-CisR cells were cultured in 96-well plates which designed to be 10,000 cells per well in order to obtain the desired results. Following overnight incubation at 37°C, OVCAR-3 and OVCAR-3-CisR cells were exposed to increasing (1-100 µg/mL) doses of As-IV (Sigma 74777) for 24 h.[14] As-IV substance was dissolved with dimethyl sulfoxide (DMSO) and an equal amount of DMSO was applied to the medium as a control batch in the cells. A 10 µL volume of MTT (final concentration: 5 µg/mL) was added to each well and incubated for 4 h at 37°C. After this, 100 µL of DMSO was added and the absorbance was measured at 570 nm.[15] The cell viability of the cells was calculated with the percentage of the treatment batch collate with the control.

Colony Formation Assay

Colony assay studies in OVCAR-3 and OVCAR-3-CisR cells were performed at concentrations closest to the IC_{50} of As-IV. For the colony formation experiment, 500 cells/well were cultured in a 24-well plate for 24 h. After 24 h of incubation, the OVCAR-3 cells were incubated 40 µg/mL As-IV and the OVCAR-3-CisR cells were incubated 70 µg/mL As-IV, both cells of which were incubated for 7 days. After removing the culture medium, the cells were fixed with a 1:1 solution of methanol and acetic acid, and then incubated for 20 min with crystal violet dye (0.5% in methanol).[15] The dye was then washed out with distilled water. The colonies formed were photographed in microscope (Axiovert, Zeiss), dissolved in ethanol and measured for absorbance at 595 nm. The percentage of formed colonies was calculated to collate with the control.

Measurement of CXCR4 and SDF-1 Protein Levels

OVCAR-3 and OVCAR-3-CisR cells ($1x10^6$ cells/well) were cultured in 6-well plates. As-IV (40 µg/mL or 70 µg/mL) was applied to the cells and incubated for 24 h. For the measurement of SDF-1 release, the cell medium was collected after incubation. For the measurement of CXCR4, the cell pellet was used. The plate was rinsed twice with PBS after collecting the cell medium. The cells were then harvested with trypsin-EDTA and centrifuged at 1000 x g for 10 min. The centrifuged OVCAR-3 and OVCAR-3-CisR cells were blasted with a cell lysis buffer containing 2% Triton X-100 and centrifugation was repeated at 4°C. After centrifugation, CXCR4 was measured using the supernatant. The measurements were carried out according to the protocol of the commercial ELISA kit used (CXCR4 ELISA kit CSB-E12825h-Cusabio Technology; SDF-1 ELISA kit CSB-E04722h-Cusabio Technology). After measuring the absorbance in an ELISA reader (Biotek, Epoch) at 450 nm, changes in protein levels were calculated according to the standard curve.

Measurement of *CXCR4, SDF1, BAX* and *BCL2* Gene Expression Levels

Apoptotic changes were analyzed by *BAX* and *BCL2* gene expression, and chemokine changes were analyzed by *CXCR4* and *SDF-1* gene expression using the qPCR technique. OVCAR-3 and OVCAR-3-CisR cells ($1x10^6$ cells/well) were cultured in 6-well plates and cells were incubated As-IV (40 µg/mL or 70 µg/mL) for 24 h. After incubation, cells were collected and lysed with cell lysis buffer in the total RNA isolation kit. The manufacturer's instructions for the RNA extraction kit (PureLink, Life Technologies) were followed to isolate total RNA. Using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystem) in accordance with the manufacturer's instructions, 1 µg of total RNA was reverse transcribed.

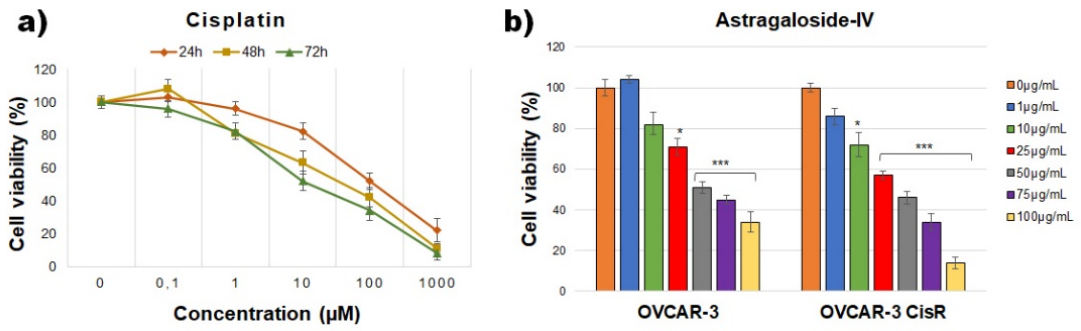

Figure 1. MTT cytotoxicity test results are depicted graphically. a) A percentage representation of the change in cell viability caused by the application of cisplatin doses ranging from 1-100 µM to OVCAR-3 cells for 24, 48, and 72 h. b) Percentage change in the viability of OVCAR-3 and OVCAR-3-CisR cells after 24 h incubation with As-IV doses ranging from 1-100 µg/mL

Statistical Analysis

Statistical analysis was accomplished with GraphPad Prism version 7.0 software. Data's significancy described as respectively *, p<0.05; ***, p<0.01; +++, p<0.001)

RESULTS

Effects of As-IV on Cell Viability in OVCAR-3 and OVCAR-3-CisR Cells

To determine the cytotoxic effect, As-IV was used in the concentration range of 1-100 µg/mL and incubated for 24 h, resulting in an IC_{50} dose of 43.11±4.36 µg/mL for OVCAR-3 cells, 71.02 ± 3.21 µg/mL for OVCAR-3-CisR cells found (Figure 1). In the experimental models, the dose of As-IV to be used in 24 h incubations was 40 µg/mL for OVCAR-3 cells and 70 µg/mL for OVCAR-3-CisR cells. As-IV appears to have a toxic effect on OVCAR-3 cells starting from 25 µg/mL (p<0.05) at concentrations above 50 µg/mL, and momentously reduce cell proliferation (p<0.001). It can be seen that As-IV momentously reduces cell proliferation in OVCAR-3-CisR cells starting from 10 µg/mL (p<0.05) at concentrations above 25 µg/mL (p<0.001).

Effect of As-IV on *BAX/BCL-2* Rate in OVCAR-3 and OVCAR-3-CisR Cells

Gene expression levels of *BAX* and *BCL2* markers were examined in order to understand whether the administration of As-IV treatment suppresses the apoptotic cascade in cells. According to the data obtained, it was observed that As-IV administration suppressed apoptosis in cells (Figure 2). *BAX* is an apoptotic gene and its protein forms localized to the mitochondria. *BAX* mRNA levels increased momentously with As-IV administration in OVCAR-3 cells versus the DMSO (Figure 2a, p<0.001). When the OVCAR-3-CisR cells were versus the OVCAR-3 cells control, there was no momentous change in *BAX* mRNA levels. In the OVCAR-3-CisR cells, As-IV administration momentously increased *BAX* mRNA levels versus the DMSO (p<0.001). At the same time, a momentous increase was found when this condition was against the OVCAR-3 cells control (p<0.001). *BCL2* is an apoptotic gene and its protein form is found in mitochondria and protects the cell from apoptosis. *BCL2* mRNA levels decreased momentously with As-IV administration in OVCAR-3 cells versus with the DMSO (Figure 2b, p<0.001). When the OVCAR-3 CisR cells were versus with the OVCAR-3 cells control, there was increased momentous change in *BCL2* mRNA levels (p<0.05). In the OVCAR-3-CisR cells, As-IV administration momentously decreased *BCL2* mRNA levels versus the DMSO (p<0.001). At the same time, a momentous decrease *BCL2* levels were found versus the OVCAR-3 control (p<0.001). The *BAX/BCL2* scale is more effective in assessing the severity of apoptosis. As-IV treatment momentously increased the *BAX/BCL2* scale in both OVCAR-3 and OVCAR-3-CisR cells (Figure 2c, p<0.001).

Effect of As-IV on Colony Formation in OVCAR-3 and OVCAR-3-CisR Cells

To assess the metastatic effect in colony formation, cells are assessed for their ability to proliferate alone in their region and for their behavior. Colonies formed as a result of incubation of OVCAR-3 and OVCAR-3 CisR cells with As-IV for 7 days were visualized by crystal violet staining (Figure 3a). It was shown that As-IV treatment momentously reduced colony formation (Figure 3b) in OVCAR-3 cells versus the DMSO (p<0.01). Colony formation in the OVCAR-3-CisR cells were found to be faster than in the OVCAR-3 cells and to increase momentously with cisplatin resistance (p<0.001). Colony formation was momentously reduced in OVCAR-3-CisR cells when the As-IV treatment was versus the DMSO (p<0.001). Compare with the OVCAR-3 cells DMSO, the OVCAR-3 CisR cells As-IV treatment had momentously reduced colony formation (p <0.05).

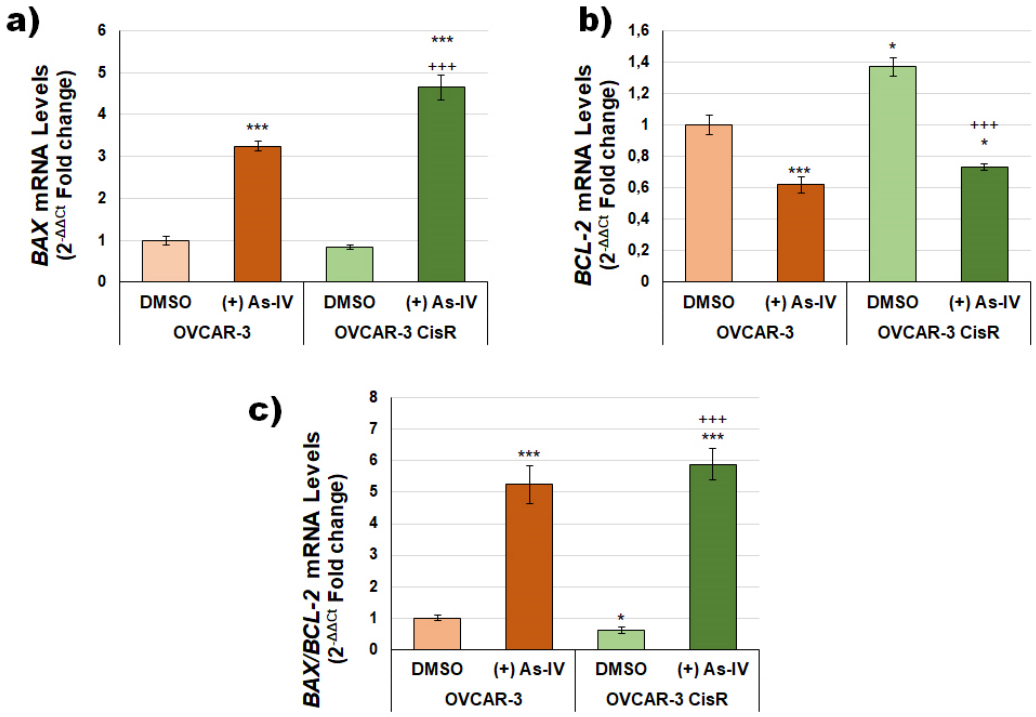

Figure 2. Apoptosis related gene expression levels of -/+As-IV on OVCAR-3 and OVCAR-3 CisR cells. (a) The change in *BAX* mRNA levels, (b) The change in *BCL2* mRNA levels, (c) The change mRNA levels ratio of *BAX/BCL2* (* p<0.05, ***p<0.001 compare to DMSO in OVCAR-3 cells, +++p<0.001 compare to DMSO in OVCAR-3-CisR cells).

Effect of As-IV on SDF-1 and CXCR4 in OVCAR-3 and OVCAR-3-CisR Cells

CXCR4 and SDF-1 are important chemokines for the evaluation of tumor microenvironment and drug resistance mechanisms. CXCR4 protein levels decreased momentously with As-IV administration in OVCAR-3 cells versus the DMSO (Figure 4a, p<0.001). When the OVCAR-3-CisR cells were versus the OVCAR-3 control batch, there was increased momentous change in CXCR4 protein levels (p<0.001). In the OVCAR-3-CisR cells, As-IV administration momentously decreased CXCR4 protein levels versus the DMSO (p<0.001). A momentous decrease in CXCR4 protein levels was found when As-IV administration was versus the OVCAR-3 cells control (p<0.01). SDF-1 protein levels decreased momentously with As-IV administration in OVCAR-3 cells versus the DMSO (Figure 4b, p<0.01). When the OVCAR-3-CisR cells were versus the OVCAR-3 control batch, there was increased momentous change in SDF-1 protein levels (p<0.001). In the OVCAR-3-CisR cells, As-IV administration momentously decreased SDF-1 protein levels versus the DMSO (p<0.001). There were no momentous changes in SDF-1 protein levels in As-IV treated OVCAR-3-CisR cells versus to OVCAR-3 control.

According to the data, *CXCR4* mRNA levels decreased momentously with As-IV administration in OVCAR-3 cells versus the DMSO (Figure 4c, p<0.001). When the OVCAR-3-CisR cells were versus the OVCAR-3 control, there was a momentous increase in *CXCR4* mRNA levels (p<0.001). In the OVCAR-3-CisR cells, As-IV administration momentously decreased *CXCR4* mRNA levels versus the DMSO (p<0.001). A momentous decrease *CXCR4* mRNA levels was found in As-IV administration OVCAR-3-CisR versus the OVCAR-3 control (p<0.001). *SDF-1* mRNA levels decreased momentously with As-IV administration in OVCAR-3 cells versus the DMSO (Figure 4d, p<0.001). When the OVCAR-3-CisR cells were versus the OVCAR-3 cells control, there was increased momentous change in *SDF-1* mRNA levels (p<0.001). In the OVCAR-3-CisR cells, As-IV administration momentously decreased *SDF-1* mRNA levels versus the DMSO (p<0.01). There were no momentous changes in *SDF-1* mRNA levels in As-IV treated OVCAR-3-CisR cells versus the OVCAR-3 cells control.

DISCUSSION

Ovarian cancer is the fifth deadliest cancer in the world and the most commonly diagnosed gynecological malignancy. Epidemiologic data show that over 22,000 new cases of ovarian cancer were diagnosed in 2018, with approximately 14,000 deaths occurring as a result of increased life expectancy worldwide, particularly among older women aged >65 years. Because of the inconspicuous location of the ovaries in the female reproductive tract, ovarian cancer is often referred to as a "silent

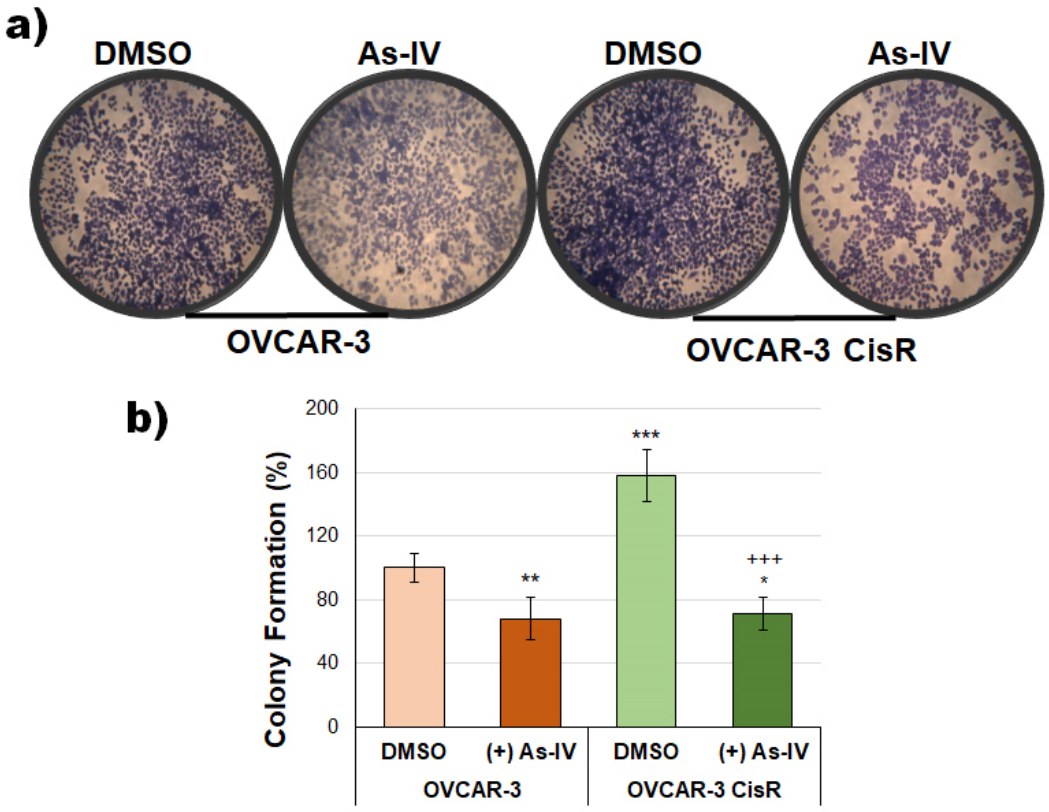

Figure 3. Colony formation of -/+As-IV on OVCAR-3 and OVCAR-3-CisR cells (a) Microscope images (4X) and (b) Colony formation graphics of percentage of cells (* p<0.05, ** p<0.01, ***p<0.001 compare to DMSO in OVCAR-3 cells, +++p<0.001 compare to DMSO in OVCAR-3-CisR cells).

killer," and nearly 70% of cases are prognosed with the progressive disease. Despite advances in detection and treatment, women with progressive ovarian cancer regularly have a five-year survival rate of only 30%.[16] Due to the increasing popularity of Chinese medicine, it has been widely used as an effective adjunct to standard cancer treatment. In Chinese medicine, the use of herbs included in the Chinese pharmacopoeia is important for their safety and includes herbs containing As-IV. As-IV is an important biomarker used for saponin screening in plants, and its importance in Chinese medicine is increasing day by day due to its antioxidant properties.[17] It can help reduce the side effects of chemotherapy and radiotherapy.[18,19] The main effects of the drug As-IV are its ability to induce cell cycle arrest and apoptosis[20,21] There are studies showing that As-IV has antioxidant effects.[22,23] As-IV acts as an antioxidant by increasing glutathione, superoxide dismutase, and catalase to reduce malondialdehyde release and increasing Nrf2 to increase NQO1 and HO-1 expression.[24] It has been proven that it reduces oxidative stress due to free oxygen radicals and has neuroprotective [25] and cardioprotective effects.[26] There are datas that the flavonoid components in its content can be used as an effective anticancer agent, by activating apoptotic mechanisms in the treatment of many cancers by showing anti-tumoral effect and leading to programmed death of cells.[10] As-IV also helps prevent cancer cells from growing and spreading.[27,28] In addition, the drug can improve the sensitivity of cancer cells to various chemotherapy drugs. It can also prevent tumor growth in models of cancer. [29] These effects were restricted to tumor cells and did not result in cytotoxicity in normal cells. Some of the other common cancers that can be affected by this drug include liver, lung, colorectal, ovarian, and breast cancer.[16,30–33] It also affects glioma[34], gastric cancer,[35] cervical cancer,[27] prostate cancer,[36] abdominal aortic aneurysm,[37] osteosarcoma,[38] and vulvar cancer.[39] As a result, As-IV is a promising anticancer agent with beneficial pharmacological effects. The diversity of pharmacological effects of As-IV is due to its structure. As-IV has high polarity due to its saponin derivative structure. Since As-IV is a tetracyclic triterpenoid saponin in the form of a lanolin alcohol, it has a high binding capacity to plasma proteins, but it has a low bioavailability due to its low lipophilicity.[40,41] The potential of an antioxidant molecule such as As-IV, which can bind to plasma proteins, to act on cancer cells is very important for studies. Antioxidants that are able to bind to plasma proteins will interact with cancer cells and cause them to undergo apoptosis while leaving healthy cells unharmed.

Apoptosis, also known as programmed cell death, is advan-

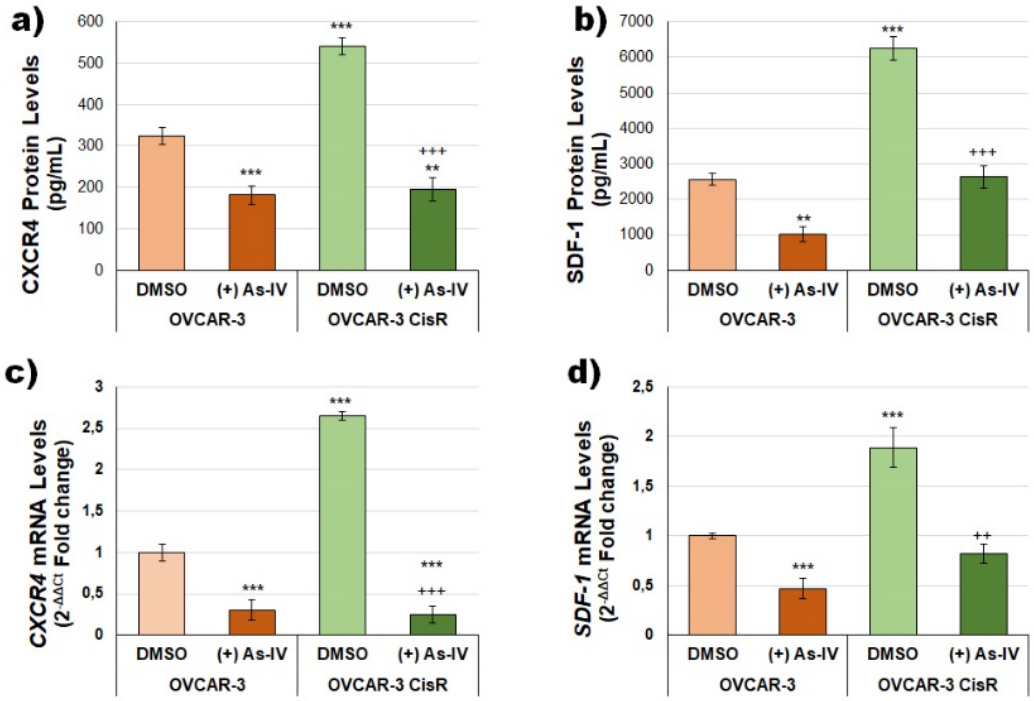

Figure 4. Demonstration of the effect of As-IV administration to OVCAR-3 and OVCAR-3 CisR cells on protein and gene expressions of CXCR4 and SDF-1 chemokines. (a) CXCR4 protein levels, (b) SDF-1 protein levels, (c) *CXCR4* mRNA levels, (d) *SDF1* mRNA levels. (** p<0.01, ***p<0.001 compare to DMSO in OVCAR-3 cells, ++p<0.001, +++p<0.001 compare to DMSO in OVCAR-3-CisR cells).

tageous for healthy cell division, organ development, and tissue homeostasis.[42] Apoptosis is a healthy physiological process that is crucial to the growth and homeostasis of living things. Most cancers have apoptosis defects.[43] The BCL-2 family has been found to play a vital role in stimulating or restraining the intrinsic apoptotic pathway caused by mitochondrial dysfunction.[44,45] As a result, the equity of pro- and anti-apoptotic members of this family can effect cellular fate. The main members of the BCL-2 family are BAX and BCL-2, whose potential roles in tumor advancement and diagnosis of various human malignancies have piqued the interest of several studies over the last decade. BAX causes the mitochondrial outer membrane to become permeable in reply to various cellular stresses, which encourages cell death. BCL-2, however, prevents apoptosis by inhibiting BAX activity.[46,47] Numerous studies have demonstrated that As-IV can affect the levels of *BCL2*, *BAX* expression, and the scale of *BAX/BCL2* in various cancer types, including lung, nonsmall-cell lung, liver, colorectal, breast, and vulvar cancers. It is shown in a study that by suppressing *BCL2* and *BCL-XL* levels and upregulating *BAX* and cleaved caspase 3 expression, As-IV treatment increased the mortality of vulvar squamous cancer cells.[39] In our study, we have showed that giving AS-IV to cisplatin-resistant ovarian cancer cells increases the *BAX/BCL2* scale by upregulating *BAX* expression while downregulating *BCL2* expression.

CXCR4 is usually overexpressed in cancerous cells of the breast, brain, lung, pancreas, colorectal, prostate, and ovarian, as well as melanoma and leukemia.[48–51] CXCR4 promotes epithelial cell migration by activating matrix metalloproteinases,[52] and elevates cancer cell motility by activating the NF-B and ERK-dependent pathways.[53] SDF-1 (also called CXCL-12), a chemokine, has been found in typical regions of tumor metastasis such as lungs and lymph nodes.[54] Tumor cells form metastatic tumors as a result of their interaction with SDF-1 and CXCR4.[55] Furthermore, SDF1 hypermethylation has been reported in a variety of cancers, including gastric, breast, colon, lung, and prostate cancer,[56] suggesting that SDF-1 may play a key role in carcinogenesis.[57] The SDF-1/CXCR4 axis promotes cancer progression and metastasis,[58,59] regulates angiogenesis,[60,61] induces epithelial-mesenchymal transition.[62,63] As-IV treatment may inhibit cancer cell migration, metastasis, and induce apoptosis by blocking the SDF-1/CXCR4 axis. The cisplatin resistance in ovarian cancer cells treated with As-IV showed a statistically momentous decrease trend in SDF-1 and CXCR4 protein and gene expression levels, and as a result, the chemoresistance developed against drug resistance was suppressed.

CONCLUSION

Ovarian cancer is one of the leading cancer types in the world in terms of mortality in women, and because it can progress

insidiously, its treatment is delayed. Chemotherapy based on cisplatin administration is one of the most effective treatment methods currently available. However, in most cases, resistance to cisplatin is observed, and the survival rate decreases due to poor response to treatment. As-IV is a natural triterpene glycoside that is very important in Chinese medicine due to its anti-tumoral and antioxidant effects. In our study, we looked at its anti-metastatic and apoptotic effects in ovarian cancer cells that had developed resistance to cisplatin. As a result, it has been demonstrated that As-IV administration can prevent metastasis to distant tissues caused by an epithelial-mesenchymal transition in drug-resistant ovarian cancer cells by inhibiting the SDF-1/CXCR4 axis, and can also induce apoptosis due to an increase in the *BAX/BCL2* scale. We believe As-IV has the potential to be used as an anticancer therapeutic agent in the treatment of patients with resistant ovarian cancer.

Ethics Committee Approval: Ethics committee approval is not required for the study.

Peer Review: Externally peer-reviewed.

Author Contributions: Concept: B.I.A, O.C; Design: B.I.A, O.C; Supervision: B.I.A, O.C; Materials: O.C; Data Collection and/or Processing: B.I.A, O.E, O.C; Analysis and/or Interpretation: B.I.A, O.E Literature Search: B.I.A, O.C; Writing: B.I.A, O.C; Critical Reviews: B.I.A, O.E, O.C.

Conflict of Interest: The authors declare that they have no conflicts of interest to disclose.

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