

Astragaloside IV enhances Paclitaxel chemosensitivity in breast cancer cells via regulating Nrf-2 signaling

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ABSTRACT

Background: Chemotherapy resistance has become a problem for the treatment of breast cancer patients. This study aimed to investigate whether astragaloside IV (AS-IV) has synergistic effects to enhance Paclitaxel (PTX) chemosensitivity in breast cancer cells. **Materials and Methods:** CCK-8 assays were performed to evaluate the sensitivity of the cell to AS-IV and PTX. The flow cytometric analysis of Annexin-V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining was carried out to evaluate cell apoptosis. The western blotting assay was used to measure the apoptosis-related proteins and Nrf-2 levels. ROS levels were measured using Dichlorofluorescein (DCF) assay. **Results:** AS-IV enhances PTX chemosensitivity in MDA-MB-231 and MCF-7 cells by enhancing the inhibitory effect of cell viability and promotion effect on cell apoptosis of PTX. The synergistic effects of AS-IV to PTX could decrease the Nrf-2 expression and increase the ROS levels. Nrf-2 signaling was related to ROS generation. **Conclusion:** AS-IV may enhance PTX chemosensitivity in breast cancer via regulating Nrf-2 signaling. AS-IV might be used as a potential adjuvant drug to increase PTX sensitivity in breast cancer cells.

INTRODUCTION

Breast cancer has emerged as one of the most prevalent malignancies worldwide and is associated with high mortality rates, significantly impacting women's health globally ⁽¹⁾. In recent decades, the incidence of breast cancer in China has been increasing at an alarming rate ⁽²⁾. With advancements in diagnosis and treatment technology, as well as the in-depth understanding of breast cancer, the choice of treatment options have changed from single surgical treatment to comprehensive schedules including surgery, radiotherapy, chemotherapy, endocrine gene targeting, and other comprehensive programs ⁽³⁾. These comprehensive treatment regimens, particularly the use of chemotherapeutic agents, have contributed to a decline in breast cancer mortality. However, chemotherapy resistance has emerged as a critical challenge, leading to tumor metastasis and increased mortality among affected individuals ⁽⁴⁾.

Paclitaxel (PTX) is one of the most commonly used chemotherapeutic drugs in clinical practice, which can exert anti-tumor effects by increasing microtubule aggregation and inhibiting cell mitosis to induce cell apoptosis ^(5, 6). As one of the most active and effective chemotherapeutic drugs in breast cancer

chemotherapy, PTX has a high effective rate in the treatment of recurrent and metastatic breast cancer ^(7, 8). However, PTX is easy to induce drug resistance of tumor cells in clinical treatment ^(9, 10). So far, the mechanism of PTX resistance is still not fully understood. Astragaloside IV (AS-IV), a saponin derived from *Astragalus membranaceus*, has been shown in previous studies to inhibit tumor cell proliferation, metastasis, and enhance chemosensitivity ^(11, 12). Previous studies indicated that AS-IV could suppress tumor cell growth, metastasis, and enhance tumor cell chemosensitivity ⁽¹³⁻¹⁵⁾. For instance, AS-IV reduced the growth, metastasis, and angiogenesis of lung cancer through the AMPK signaling pathway ⁽¹⁴⁾. However, whether astragaloside IV can increase the chemosensitivity of paclitaxel in breast cancer and the mechanism are still unknown.

The activity of nuclear factor erythroid 2-related factor 2 (Nrf-2) is correlated with tumor progression and chemoresistance ⁽¹⁶⁾. Nrf-2 inhibits tumor cell apoptosis and promotes cell regeneration, and plays an anti-apoptotic role through the mitochondrial pathway ^(16, 17). As intermediate products of oxygen metabolism, reactive oxygen species (ROS) are mainly generated on complex I (NADH dehydrogenase) and complex II (cytC reductase) on

mitochondria^(18, 19). A previous study revealed that miR-153/Nrf-2/GPx1 pathway regulated radiosensitivity and stemness of glioma stem cells through ROS⁽²⁰⁾. A previous study indicated that PTX could alleviate the expression levels of the antioxidant molecules, including Nrf-2⁽²¹⁾. These studies implied that a potential mechanism of PTX in the treatment of breast cancer.

In the present study, we observed that AS-IV can promote the efficacy of PTX chemotherapy on breast cancer cells by increasing ROS level, and the Nrf-2 pathway is closely related to ROS level. This study aimed to investigate whether AS-IV enhances the efficacy of PTX in breast cancer cell chemotherapy through the Nrf-2 pathway.

MATERIALS AND METHODS

Cell lines and culture

Two breast cancer cell lines MDA-MB-231 and MCF-7 were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Both cell lines were cultured and grown in DMEM medium (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml penicillin, and 100 µg/ml streptomycin (15140122, Gibco, USA) at 37°C in a humidified atmosphere of 5% CO₂.

Cell transfection

Specific oligonucleotides for pcDNA3.1-Nrf-2 plasmid (Nrf-2) or control vector pcDNA3.1 plasmids (NC) were purchased from Sangon Biotech (Shanghai, China) and were transiently transfected in MDA-MB-231 and MCF-7 cells using the Lipofectamine 2000 (Invitrogen, Carlsbad, USA) as prescribed by the manufacturer. Untreated cells were used as control. After 24 h of transfection, the efficiency was confirmed by Reverse Transcription quantitative real-time PCR (RT-qPCR).

Cell counting kit-8 (CCK-8) cell viability assay

CCK-8 assays were performed to evaluate the sensitivity of the cell to AS-IV (84687-43-4, MCE, China) and PTX (33069-62-4, MCE, China). Briefly, the MDA-MB-231 and MCF-7 cells (3000 cells/well) were seeded to 96-well plates until cells attachment. 5 nM PTX or 30 µM AS-IV¹⁵ (purity above 98%; MUST BIO Technology Co.Ltd, Chengdu, China) were added to the wells. PTX was dissolved in DMEM medium directly, and AS-IV was dissolved by DMSO (D806645, Macklin, China) and then added in DMEM medium. Cell viability was measured using CCK-8 kit (C0038, Beyotime, China) and the absorbance was measured at 450 nm with a microplate reader (Thermo Fisher Scientific, Inc.).

RNA isolation and RT-qPCR assay

Total RNA was isolated from breast cancer cells

using Trizol reagent (Invitrogen, USA). After isolation, the RNA concentration was determined using NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Then, 2 µg total RNA was used to synthesize cDNA using SuperScript III first-strand cDNA synthesis kit (Invitrogen, USA). The RT-qPCR assay was performed using SuperScript III first-strand cDNA synthesis kit (Invitrogen) with 7500 Real-Time PCR System (Applied Biosystems, Singapore). The cycling condition included denaturation at 55°C for 2 min, 95°C for 10 min, followed by 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and then 72°C for 1 min. Normalization of the relative level was carried out using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels and calculating the relative Nrf-2 levels using the 2^{-ΔΔCt} method.

Cell apoptosis assay

The flow cytometric analysis of Annexin-V-fluoresceine isothiocyanate (FITC)/propidium iodide (PI) staining (Dojindo, Japan) was carried out to evaluate cell apoptosis. The breast cancer cells (2 × 10⁵ cells/ml) were added to 6-well plates. After incubation, the cells were harvested and washed with ice-cold PBS and mixed with 1 ml serum-free DMEM medium to make cell suspension. Then cell suspension was cultured with Annexin V-FITC and PI. The flow cytometry was used to separate the apoptotic cells.

Western blotting assay

Proteins were extracted from breast cancer cells by RIPA buffer (P0013B, Beyotime, China) and subjected to 10% SDS-PAGE, then transfected to PVDF membranes (Millipore, USA). Then, the membranes were blocked with 5% skim milk in TBST (ST675, Beyotime, China) for 3 h at room temperature and then incubated with primary antibodies: Bcl2 antibody (1:2000, Proteintech, 12789-1-AP, China), P53 antibody (1:5000, Proteintech, 10442-1-AP, China), Cleaved caspase 3 antibody (1:5000, Proteintech, 82707-13-RR, China), Caspase 9 antibody (1:1000, Proteintech, 28793-1-AP, China), Nrf-2 (1:1000, Proteintech, 80593-1-RR, China), at 4°C overnight. After washed in TBST, blots were incubated with corresponding horseradish peroxidase-conjugated secondary antibodies (GAPDH antibody, 1:5000, Proteintech, 10494-1-AP, China) for 1 h at room temperature. The western blots were visualized using enhanced chemiluminescence kit (P0018S, Beyotime, China) and quantified using ImageJ software (National Institute of Health, USA).

Dichlorofluorescein (DCF) assay

Both cell lines were incubated in 96-well plates with a density of 1.5 × 10⁴ cells/well for 24 h. Then cells were incubated in the medium supplemented with DCFH-DA (2',7'-Dichlorodihydrofluorescein diacetate, S0034S, Beyotime, China) for 30 min. After

incubation, the generation of ROS that was revealed by increased DCF was measured by fluorescence microscopy with excitation of 488 nm and emission of 525 nm at 37°C in no FBS-containing medium.

Statistical analysis

Statistical analyses were performed by using the SPSS 22.0 software applying a significance level of the data, which was represented with mean \pm SD. Differences between groups were determined using one-way ANOVA. All experiments were performed at least three times with three replicates.

RESULTS

AS-IV enhances PTX chemosensitivity in MDA-MB-231 and MCF-7 cells

MDA-MB-231 and MCF-7 cells were treated with 30 μ M AS-IV, 5 nM PTX, or a combination of 30 μ M AS-IV with 5 nM PTX. The results from the CCK-8 assay indicated that AS-IV alone did not have a significant effect on cell viability in either cell line. However, when AS-IV was combined with PTX, there was a significant enhancement in PTX cytotoxicity ($P < 0.001$, figure 1A). To explore whether this enhancement in cytotoxicity was associated with increased apoptosis, flow cytometric analysis was performed. The results in figure 1B show that cotreatment with AS-IV and PTX significantly increased cell apoptosis compared to treatment with PTX alone. These findings suggest that AS-IV may enhance chemosensitivity in breast cancer with a favorable safety profile.

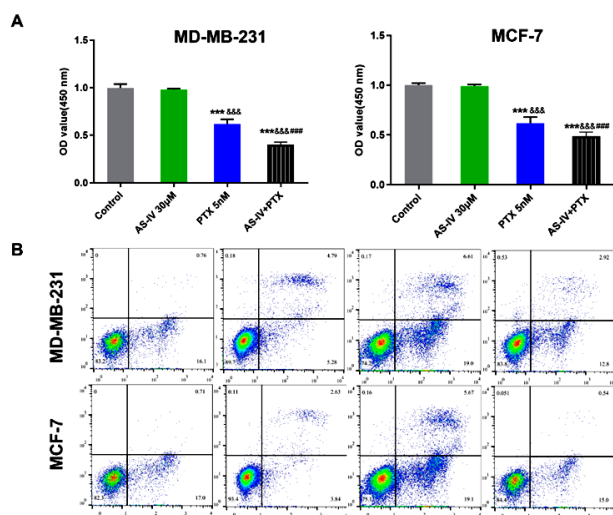


Figure 1. AS-IV enhances PTX chemosensitivity in MDA-MB-231 and MCF-7 cells. **A.** CCK-8 assays were used to measure cell viability. **B.** Cell apoptosis assay was carried out to detect the effect of AS-IV and PTX on cell apoptosis abilities. *** $P < 0.001$ vs. Control; &&& $P < 0.001$ vs. AS-IV alone; #### $P < 0.001$ vs. PTX alone.

Effect of AS-IV on apoptosis protein expression in PTX-treated MDA-MB-231 and MCF-7 cells

Moreover, on the basis of the effect of AS-IV with

PTX on cell apoptosis, a western blotting assay was carried out to measure apoptosis-related proteins. The data demonstrated after cotreatment of AS-IV and PTX, the expression of caspase-3, p53, and caspase-9 was increased accompanied by the downregulation of Bcl-2 (figure 2), suggesting that AS-IV may have synergistic effects to PTX in attributing to apoptosis induction.

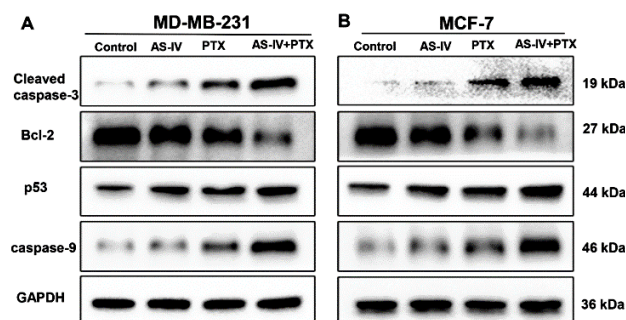


Figure 2. Effect of AS-IV on apoptosis-related proteins in PTX-treated MDA-MB-231 and MCF-7 cells. **A.** Western blot assay was used to measure the protein levels in MDA-MB-231 cells.

B. The protein levels in MCF-7 cells.

Effect of AS-IV on the expression of Nrf-2 in PTX-treated breast cancer cells

Aberrant Nrf-2 signaling plays an important role in the progression of cancer. Moreover, the balance of the Nrf-2 pathway is crucial for the response and sensitivity of chemotherapy. Thus, the Nrf-2 mRNA and protein expression levels were analyzed. The data revealed that PTX decreased the Nrf-2 mRNA and protein expression levels compared with control (figure 3A, B). Moreover, AS-IV cotreatment decreased the Nrf-2 mRNA and protein expression levels in both MDA-MB-231 and MCF-7 cells compared with cells treated PTX alone. These results suggest that AS-IV increased the PTX cytotoxicity, at least partly, through inhibition of Nrf-2.

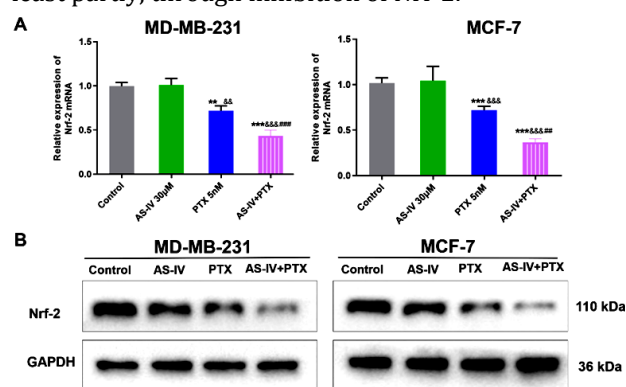


Figure 3. Effect of AS-IV on the expression of Nrf-2 in PTX-treated MDA-MB-231 and MCF-7 cells. **A.** The expression of Nrf-2 mRNA was measured using RT-qPCR assay. **B.** The Nrf-2 protein levels were measured using Western blot assay. *** $P < 0.001$ vs. Control; && $P < 0.01$, &&& $P < 0.001$ vs. AS-IV alone; #### $P < 0.001$ vs. PTX alone.

Effect of AS-IV on ROS generation in PTX-treated MDA-MB-231 and MCF-7 cells

ROS is associated with cell proliferation, which is a key mediator in several signaling ²². Moreover, ROS

was measured and the results indicated that AS-IV treatment alone has no significant effect on the ROS level, while PTX treatment alone increased ROS level in both MDA-MB-231 and MCF-7 cells compared with control ($P < 0.01$, figure 4). After cotreatment of AS-IV and PTX, ROS levels were further increased compared with control and PTX treatment alone (figure 4). These results suggested that AS-IV has synergistic effects on PTX on the ROS levels.

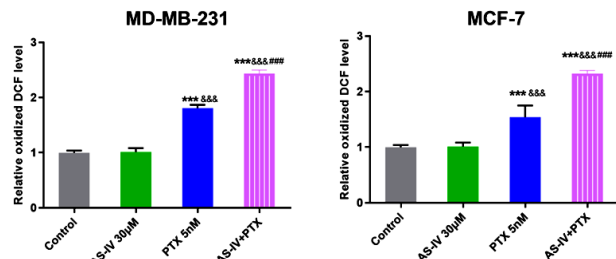


Figure 4. The effect of AS-IV and PTX co-treatment on ROS generation in MDA-MB-231 and MCF-7 cells based on relative oxidized DCF levels.

Effect of AS-IV on cell viability and apoptosis in PTX-treated Nrf-2 overexpression cells

Moreover, the effect of AS-IV on cell behaviors was measured in PTX-treated Nrf-2 overexpression cells. Firstly, pcDNA3.1-Nrf-2 was constructed to increase the expression of NRF-2 and the transfection efficiency was detected using the RT-qPCR assay. The data showed that Nrf-2 transfection significantly enhanced the mRNA expression of Nrf-2 in both MDA-MB-231 and MCF-7 cells ($P < 0.001$, figure 5A). Subsequently, the apoptosis-related protein caspase-9 was measured in Nrf-2 overexpression cells. The results in figure 5B exhibited that Nrf-2 overexpression significantly decreased the expression of caspase-9 compared with untreated control ($P < 0.01$).

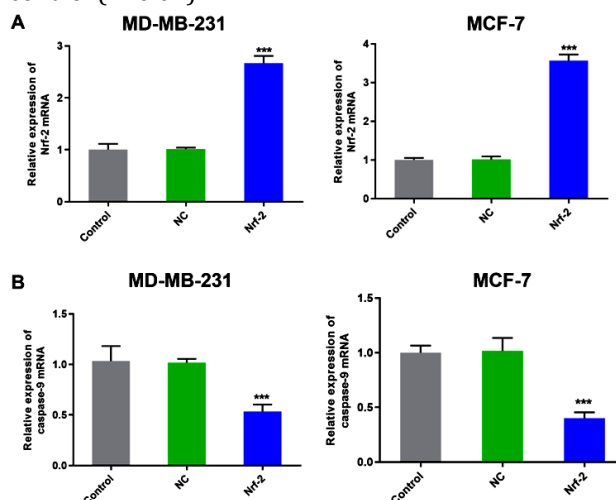


Figure 5. Effect of Nrf-2 overexpression on caspase-9 mRNA expression in MCF-MB-231 and MCF-7 cells. **A.** Transfection efficiency was confirmed using RT-qPCR assay. **B.** The relative expression of caspase-9 mRNA was measured in Nrf-2 overexpression cells. ** $P < 0.01$, *** $P < 0.001$ vs. Control.

Furthermore, the effect of AS-IV on cell viability

and apoptosis in PTX treated Nrf-2 overexpression cells was observed. As shown in figure 6A of the CCK-8 assay results, ectopic expression of Nrf-2 enhanced breast cancer cell growth compared with NC group ($P < 0.001$). Moreover, AS-IV cotreatment suppressed cell viability in Nrf-2 overexpression cells compared with PTX-treated Nrf-2 overexpression cells ($P < 0.001$, figure 6A). Then, flow cytometry analysis results exhibited AS-IV and PTX cotreatment facilitated cell apoptosis compared with the PTX treatment group alone. Ectopic expression of Nrf-2 inhibited breast cancer cell apoptosis compared with the NC group (figure 6B).

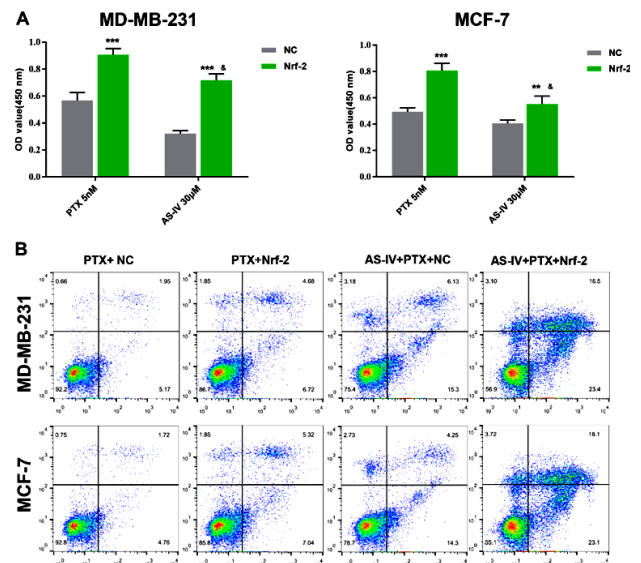


Figure 6. Effect of AS-IV on cell viability and apoptosis in PTX-treated Nrf-2 overexpression cells. **A.** Cell viability was measured using CCK-8 assays in MDA-MB-231 and MCF-7 cells. **B.** Cell apoptosis assay was carried out to detect the effect of AS-IV and PTX on cell apoptosis abilities in Nrf-2 overexpression cells. *** $P < 0.001$ vs. NC group; &&& $P < 0.001$ vs. PTX-treated Nrf-2 overexpression group.

Effect of AS-IV on ROS generation in PTX-treated Nrf-2 overexpression cells

Using the Nrf-2-transfected cells, the ROS levels were further explored after treatment PTX alone and cotreatment AS-IV and PTX. As shown in figure 7, upregulation of Nrf-2 could decrease the ROS levels compared with the NC group. After cotreatment of AS-IV and PTX, ROS levels were further increased compared with PTX treatment Nrf-2 overexpression group ($P < 0.001$).

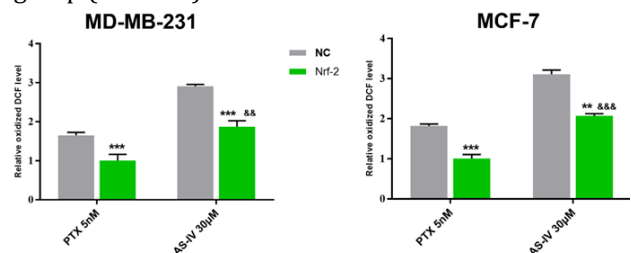


Figure 7. The effect of AS-IV and PTX cotreatment on ROS generation in Nrf-2 overexpression MDA-MB-231 and MCF-7 cells by using relative oxidized DCF levels.

DISCUSSION

Chemotherapy remains the primary treatment strategy for breast cancer patients. However, decreased sensitivity to chemotherapy, along with tumor recurrence and metastasis, significantly contributes to treatment failure in advanced stages of the disease ⁽²³⁾. In this study, we demonstrated that AS-IV may serve as a potential adjuvant drug to enhance PTX sensitivity in breast cancer cells.

AS-IV, an active component of *Astragalus membranaceus*, has shown protective effects against visceral damage caused by various conditions, including pulmonary diseases, liver fibrosis, and diabetic nephropathy. Additionally, AS-IV exhibits promising anti-tumor properties ⁽¹¹⁾. For instance, AS-IV improved insulin resistance (IR), dyslipidemia, oxidative stress, and inflammation, and accelerated suppressed autophagy in the liver of T2DM rats that may be associated with the promotion of AMPK/mTOR-mediated autophagy ⁽²⁴⁾. Previous studies have demonstrated that AS-IV has anti-tumor effects in several cancers, such as colorectal cancer ⁽²⁵⁾ and ovarian cancer ⁽²⁶⁾. Moreover, AS-IV has been shown to enhance chemosensitivity; in prostate cancer, it increased carboplatin sensitivity by inhibiting the AKT/NF- κ B signaling pathway ⁽²⁷⁾. In our findings, treatment with 30 μ M AS-IV reduced cell viability without significant differences compared to the control, indicating its safety at this concentration. Consistent with previous studies in other cancers, our data revealed that AS-IV enhanced the inhibitory effects of PTX on cell viability and increased apoptosis, indicating a synergistic effect between AS-IV and PTX in breast cancer cells. Notably, cotreatment with AS-IV and PTX elevated the expression of apoptosis-related proteins, including caspase-3, p53, and caspase-9, while downregulating Bcl-2. This supports the notion that AS-IV enhances PTX-induced apoptosis in breast cancer.

Interestingly, previous studies have shown that certain oncogenes can elevate Nrf-2 transcription, leading to a reduced intracellular ROS environment. AS-IV has been reported to alleviate tacrolimus-induced chronic nephrotoxicity by promoting p62 phosphorylation, enhancing Nrf-2 nuclear translocation, and reducing ROS accumulation ⁽²⁸⁾. ROS might have AS-IV could relieve tacrolimus-induced chronic nephrotoxicity by promoting p62 phosphorylation, thereby enhancing Nrf-2 nuclear translocation, and then alleviating ROS accumulation and renal fibrosis ⁽²⁹⁾. Furthermore, by using RT-qPCR and western blot assays, PTX decreased the Nrf-2 mRNA and protein expression levels compared with control. Interestingly, AS-IV and PTX cotreatment further decreased the Nrf-2 mRNA and protein expression levels in both MDA-MB-231 and MCF-7 cells compared with cells treated PTX alone. These results suggest that AS-IV increased the PTX

cytotoxicity, at least partly, through inhibition of Nrf-2. Moreover, after cotreatment of AS-IV and PTX, ROS levels were further increased compared with control and PTX treatment alone. These results suggest that AS-IV may promote the efficacy of PTX chemotherapy on breast cancer cells by increasing the ROS level, at least partly, through regulating the Nrf-2 expression.

AS-IV appears to enhance chemosensitivity across various cancer types through different mechanisms ^(15,30). For instance, in hepatocellular carcinoma, AS-IV increased cisplatin sensitivity by inhibiting MRP2 expression ⁽³⁰⁾. To explore whether Nrf-2 expression is a new mechanism for the activation of the Nrf-2 antioxidant program and has an effect on the ROS level, furthermore, experiments were carried out in Nrf-2 overexpression breast cancer cell lines. The data revealed that ectopic expression of Nrf-2 promoted cell viabilities while inhibited breast cancer cell apoptosis, as well as decreased ROS levels compared with the NC group. In Nrf-2 upregulation cells, cotreatment of AS-IV and PTX also exhibited synergistic effects. These results implied that AS-IV may enhance PTX chemosensitivity in breast cancer cells via Nrf-2 signaling. However, the detailed mechanism of the synergistic effect of AS-IV to PTX in breast cancer remains to need to be explored in future researches.

In summary, our study indicates that AS-IV can enhance PTX chemosensitivity in breast cancer cells by increasing ROS levels, which is associated with the Nrf-2 pathway. AS-IV holds promise as a potential adjuvant drug to improve PTX sensitivity in breast cancer treatment.

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Ethical Compliance: None.

Authors Contribution: T.R., L.C. and Z.Y. contributed equally to this work. All authors read and approved the final manuscript.

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