



Bruceine D Sensitizes Human Ovarian Cancer Cells to Paclitaxel Through JNK and STAT3 Signal Regulation

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Received: 29 September 2021 / Accepted: 28 February 2022 / Published online: 26 March 2022
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Abstract

Exploring novel regimens is essential to ensure the efficacy of paclitaxel-based chemotherapy in epithelial ovarian cancer. *Brucea javanica* (L.) Merr., Simaroubaceae, oil emulsion benefits the patients who received sequential chemotherapy, and bruceine D is identified as the major active tetracyclic quassinoid. On this premise, we explored the potential effects and related molecular mechanisms of bruceine D on epithelial ovarian cancer cells. Our study indicated an inhibitory effect of bruceine D treatment in epithelial ovarian cancer cells. Increased cell apoptosis and cancer stem cell elimination were observed with combined treatment of bruceine D and paclitaxel in ovarian cancer cells. Mechanism study indicated that JNK and STAT3 signaling regulation contributed to the synergistic effects of bruceine D in paclitaxel-based chemotherapy. Our study revealed a synergistic effect of bruceine D in paclitaxel-based chemotherapy. Our investigation provided a valuable strategy to enhance the efficiency of chemotherapy in ovarian cancer.

Keywords Ovarian cancer · Bruceine D · Paclitaxel resistance · Cancer stem cell · Sensitization

Introduction

Epithelial ovarian cancer is a common type of gynecological tumor, which shows high morbidity and mortality these years (Lheureux et al. 2019). Anticancer chemotherapy based on paclitaxel and platinum is recommended as the first-line treatment for epithelial ovarian cancer (Pignata et al. 2019). However, most ovarian cancer patients suffer disease progression within 2 years of the initial chemotherapy treatment, which is correlated with chemotherapy resistance (Pokhriyal et al. 2019). Further investigation is needed to ensure the effectiveness of chemotherapy agents by minimizing the occurrence of drug resistance.

Poor sensitivity is another major issue for the therapeutic efficiency of anticancer agents (Christie and Bowtell 2017). Apoptotic resistance accounts for the predominant contribution for paclitaxel-based chemotherapy failure (Stover et al. 2019). Further investigation indicated that the cancer stem cells subpopulation showed survival advantage in the

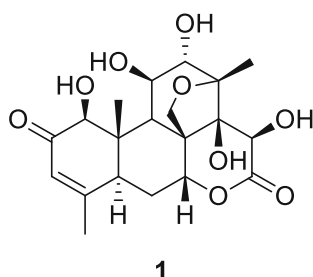
cytotoxic chemotherapy (Pieterse et al. 2019). Increased ovarian cancer stem cells are identified in the chemotherapy resistant ovarian cancer tissues, which was supported by increased CD133 positive cancer cell subpopulation (Burgos-Ojeda et al. 2012; Hatina et al. 2019). Therefore, it is essential to explore innovative strategies to eliminate ovarian cancer stem cells for improving paclitaxel-based chemotherapy efficiency.

Chinese medicinal therapy shows biological advantage in the management of malignancy (Wang et al. 2018b). Previous studies indicated that the use of traditional Chinese medicines improved the chemotherapy sensitivity in various solid tumors, such as the chemosensitivity effects of squellarin in prostate cancer (Gao et al. 2017) and corilagin in epithelial ovarian cancer (Jia et al. 2017). The clinical application of *Brucea javanica* (L.) Merr., Simaroubaceae, oil emulsion benefits the cancer patients who received sequential chemotherapy regimens, including epithelial ovarian cancer (Ye et al. 2016). However, the molecular mechanism of enhanced treatment efficiency is still elusive. Recent investigation identified bruceine D (**1**) as the major active ingredient in *B. javanica* oil emulsion (Dou et al. 2018). Bruceine D is an abundant natural active tetracyclic quassinoid from this plant species (Mao et al. 2019; Sin et al. 2020), which shows antitumor effects in several types of cancer, including pancreatic adenocarcinoma (Cheng et al. 2017), chronic myeloid leukemia (Fan et al.

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2020), hepatocellular carcinoma (Lau et al. 2009), and nonsmall cell lung cancer (Zhang et al. 2016; Xie et al. 2019). Further studies are needed to identify the role of bruceine D in epithelial ovarian cancer treatment, as well as the mechanism of its activity.



On these premises, we explored the potential inhibitory effects of bruceine D on epithelial ovarian cancer cells with paclitaxel co-treatment. We investigated the mechanism of apoptotic induction with bruceine D treatment, which demonstrated a reversal effect of apoptotic avoidance.

Material and Methods

Cell Lines and Reagents

Human epithelial ovarian cancer cell lines SKOV3 and A2780 were obtained from ATCC. The cells were cultured in DMEM which was supplemented with 10% fetal bovine serum (FBS). Cells were incubated at 37 °C in humidified 5% CO₂. Bruceine D (**1**) was provided by the Institute of Traditional Chinese Medicine and Natural Products, Jinan University (Guangzhou, China). The primary antibodies against CD133, cleaved caspase 3, Bcl-2, Bax, JNK, p-JNK, STAT3, and p-STAT3 were purchased from Abcam Inc. (Cambridge, MA).

Cell Proliferation Assay

Cell proliferation was examined with a CCK-8 assay kit (Beyotime, Shanghai, China). In brief, 3000 cells/well were cultured in a 96-well plate. The cells were treated with indicated regimens for the corresponding hours. Then, 10 µl CCK-8 medium was added in each well and incubated for 2 h. A Bio-Rad Model 680 microplate reader (Bio-Rad, Hercules, CA) was used to examine the cell proliferation with the absorbance of 490 nm following the manufacturer's directions. The IC₅₀ values (the regimen concentration of 50% of the cell viability inhibition) were determined with Graphpad software (GraphPad Instat Software, La Jolla, CA).

Cell Apoptosis and Necrosis Analysis

A total of 3000 ovarian cancer cells were planted in 96-well plates overnight. Then, cells were treated with paclitaxel and bruceine D for 12 h. RealTime Glo Annexin V Apoptosis and Necrosis Assay Kit (JA1011, Promega, Madison, WI) were used to evaluate the cell apoptosis and necrosis. In brief, the detection reagent (100 µl) was added to each well. GloMax microplate reader (Promega) was used to record the luminescence and fluorescence. Cell apoptosis and necrosis were analyzed following the kit instructions.

Tumor Sphere Formation Analysis

Serum-free DMEM/F-12 medium was prepared with 20 ng/ml recombinant human epidermal growth factor, 20 ng/ml basic fibroblast growth factor, and 2% B27. Ovarian cancer cells were cultured in a 6-well ultralow attachment plate (1000 cells/well). Oncospheres were counted manually after 7 days' culture with inverted phase contrast microscopy.

Immunofluorescence Analysis

Oncosphere cells were collected and fixed in 4% polyformaldehyde on coverslip. The primary antibody against CD133 (1:500, Abcam) was added and incubated at 4 °C overnight. FITC-labeled secondary antibody (Bioss, Beijing, China) was added to incubate for 2 h in room temperature. DAPI (Bioss) was used for cell counting. Then, cells were visualized with a confocal microscope.

Caspase 3 Activity Analysis

Caspase 3 Activity Assay Kit was purchased from Beyotime. Caspase 3 activity was analyzed following the kit instructions. In brief, bruceine D (**1**)-treated ovarian cancer cells were harvested and lysed with the lysis buffer. We incubated 30 µg collected cytosolic protein with 200 µM DEVD-pNA substrate at 37 °C for 1 h. The microplate reader (Bio-Rad) was used to examine the absorbance at 405 nm.

Western Blot Analysis

Protein preparation and quantification for western blot were performed according to our previous description (Wang et al. 2018a). The primary antibodies were used to examine the expression of cleaved caspase 3, Bcl-2, Bax, JNK, p-JNK, STAT3, and p-STAT3. GAPDH (AbCam) was used as an internal control for each analysis. The protein bands were analyzed with the Quantity One software (Bio-Rad) for comparison.

Flow Cytometry Analysis

Flow cytometry analysis was performed to assess the CD133⁺ percentage of ovarian cancer cells after different treatments. After removal of supernatant solution, the cells were stained with PE-conjugated CD133 antibody (ab253271, AbCam) at 4 °C for 20 min and subjected to fluorescence analysis on a FACS Calibur flow cytometer (BD Biosciences, Mansfield, MA).

Lentiviral Production and Transfection

The lentiviral vector carrying the STAT3-C gene or empty control was synthesized in Genepharm (Shanghai, China). The lentiviral transfection of SKOV-3 cells was performed with polybrene (Genepharm, Shanghai, China) and selected with puromycin (Sigma-Aldrich, St. Louis, MO) treatment.

Statistical Analysis

Experiments were repeated for at least three times. Data are presented as the mean $\pm$ standard deviation (SD). The SPSS 23.0 software (SPSS, Inc., Chicago, IL) and Prism GraphPad

were used for the statistical analysis. Differences between different groups were evaluated by the Student's *t*-test or one-way ANOVA. Statistical significances were accessed as $p < 0.05$.

Results and Discussion

Enhancement of Paclitaxel Cytotoxicity

The effects of bruceine D (**1**) treatment in SKOV-3 and A2780, human ovarian cancer cell lines, were examined. Bruceine D treatment was administrated for 24 h with a gradient of concentrations. CCK-8 assays showed that bruceine D has considerable cytotoxicity against ovarian cancer cells. Inhibited cell viability was observed with the treatment of bruceine D when the concentration was higher than 10 μ M (Fig. 1a). Further analysis identified the IC₅₀ of bruceine D for SKOV-3 cells as 33.54 μ M, and for A2780 cells as 18.04 μ M (Fig. 1b). More importantly, combined treatment with paclitaxel (5 nM) and bruceine D (≥ 5 μ M) induced higher percentage of cell proliferation inhibition, compared with those

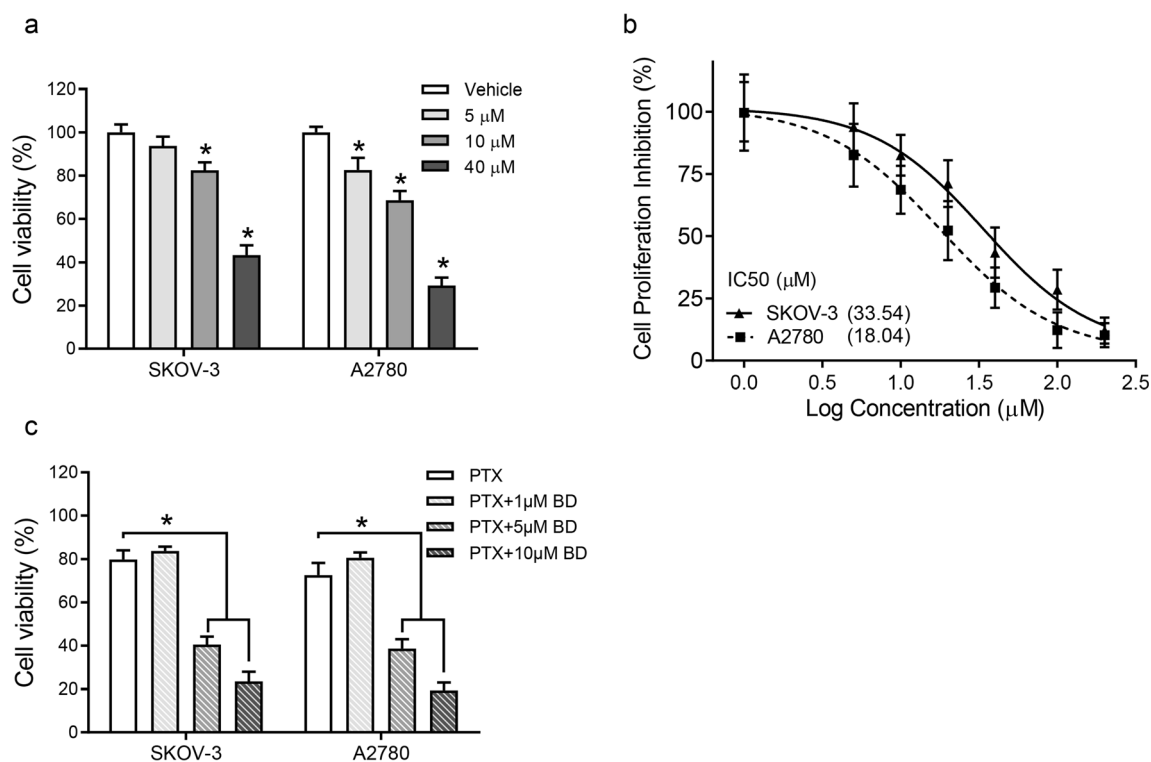


Fig. 1 Bruceine D enhances the cytotoxicity of paclitaxel in ovarian cancer cells. **a** CCK-8 assay was performed to evaluate the cell proliferation inhibitory efficiency of bruceine D. SKOV-3 and A2780 cells were treated with bruceine D for 24 h with the concentration of 5, 10, and 40 μ M. **b** Further analysis was performed for the IC₅₀ of cell proliferation inhibition. Ovarian cancer cells were treated with a serial concentration

from 1 to 100 μ M. **c** Combined treatment of bruceine D (1, 5, 10 μ M) and paclitaxel (PTX, 5 nM) was administrated in SKOV-3 or A2780 cells. Cell viability inhibition was compared with the vehicle control group. Each treatment group was prepared in triplicate. The results were presented with means $\pm$ SD. Significance was determined by the Student's *t*-test (* $p < 0.05$ vs. vehicle control)

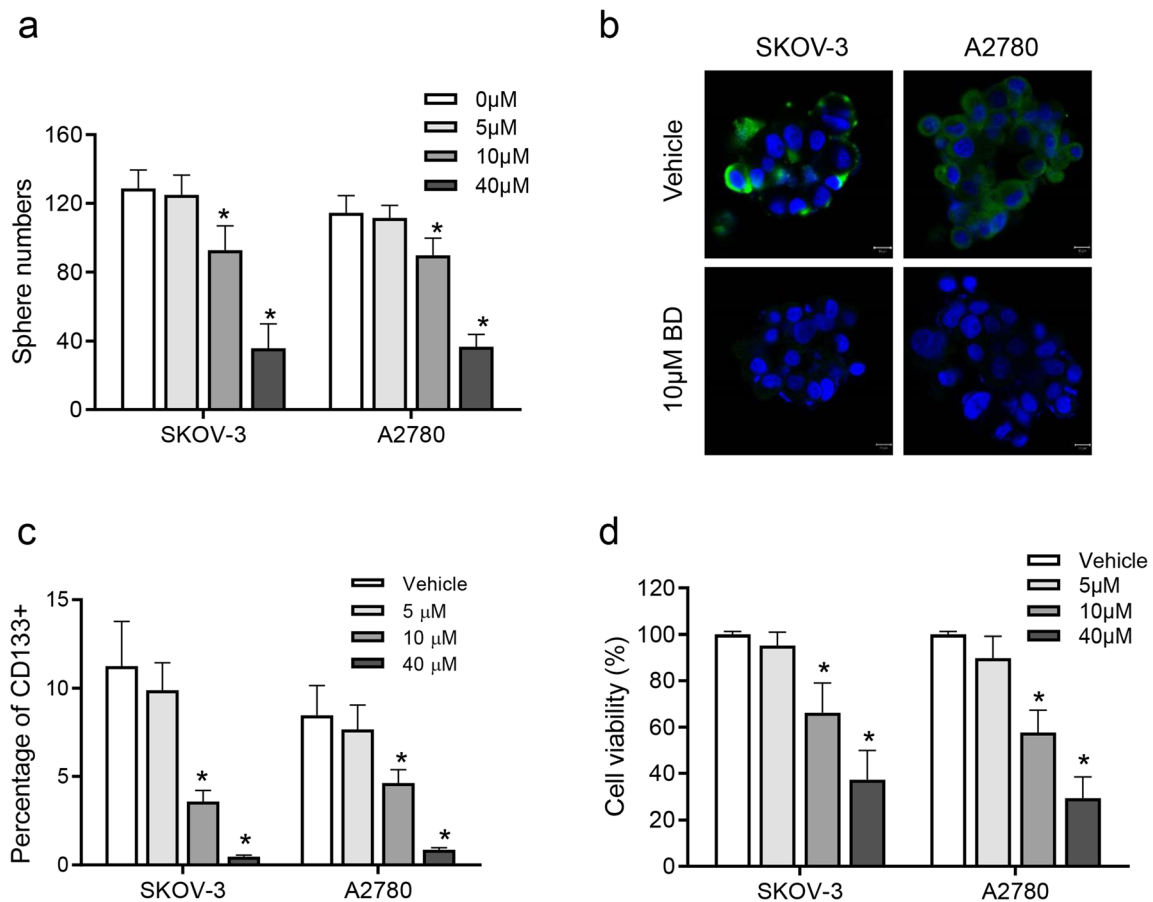


Fig. 2 Bruceine D eliminates cancer stem cell subpopulation in ovarian cancer cells. **a** Suspension culture of 1000 ovarian cancer cells (SKOV-3 and A2780) was treated with bruceine D for 7 days. The oncosphere number was compared among different groups. **b** Immunofluorescent staining of CD133 was performed with the ovarian cancer cells treated as in **a**. **c** Flow cytometry analysis of CD133⁺ was performed with the

cells treated with gradient concentration of bruceine D for 24 h. **d** Cell proliferation inhibition was analyzed with FACS sorted CD133⁺ ovarian cancer cells, which was treated with bruceine D for 24 h. Each treatment group was prepared in triplicate. Data represent the means $\pm$ SD. * $p < 0.05$

with only paclitaxel treatment (Fig. 1c). Therefore, bruceine D sensitizes human ovarian cancer cells to paclitaxel-based chemotherapy.

Elimination of Cancer Stem Cell Subpopulation

Further investigation was performed to study the stemness-related characteristics of bruceine D-treated ovarian cancer cells. Suspended tumor spheres were established with no-adhesive suspension culture system. SKOV-3 and A2780 cells formed floating spherical colonies in 7 days. Notably, 10 μ M bruceine D treatment significantly reduced the number of tumor spheres (Fig. 2a). Furthermore, confocal microscopy assays were performed with the collected sphere cells with bruceine D treatment, which indicated lower CD133 expression in bruceine D-treated group than the control ones (Fig. 2b). Moreover, after 48 h treatment in a gradient concentration of bruceine D, decreased percentages of CD133⁺ ovarian cancer cells were observed with flowcytometry analysis (Fig. 2c). Next, FACS sorted CD133⁺ cells were treated with bruceine

D for 48 h, which indicated bruceine D treatment inhibited the proliferation of CD133⁺ ovarian cancer cell subpopulation (Fig. 2d). These results indicate bruceine D as an effective agent in inhibiting self-renewal ability of ovarian cancer cells.

Increment of Cell Apoptosis

The efficacy of bruceine D on the apoptosis of ovarian cancer cells was analyzed. Combined treatment of bruceine D (10 μ M) and paclitaxel increased the SKOV-3 cell apoptosis, as compared with those with only paclitaxel treatment (Fig. 3a). Besides, no significant change was observed in cell necrosis with the combined treatment (Fig. 3b). Caspase 3 activity of the bruceine D- and/or paclitaxel-treated ovarian cancer cells was examined, which indicated that bruceine D treatment increased caspase 3 activity of the ovarian cancer cells (Fig. 3c). Western blot assays were performed to access the expression levels of cleaved caspase 3, Bcl-2, and Bax. Combined bruceine D treatment increased the cleaved caspase 3 expression, while decreased the levels of Bcl-2 and Bax (Fig. 3d).

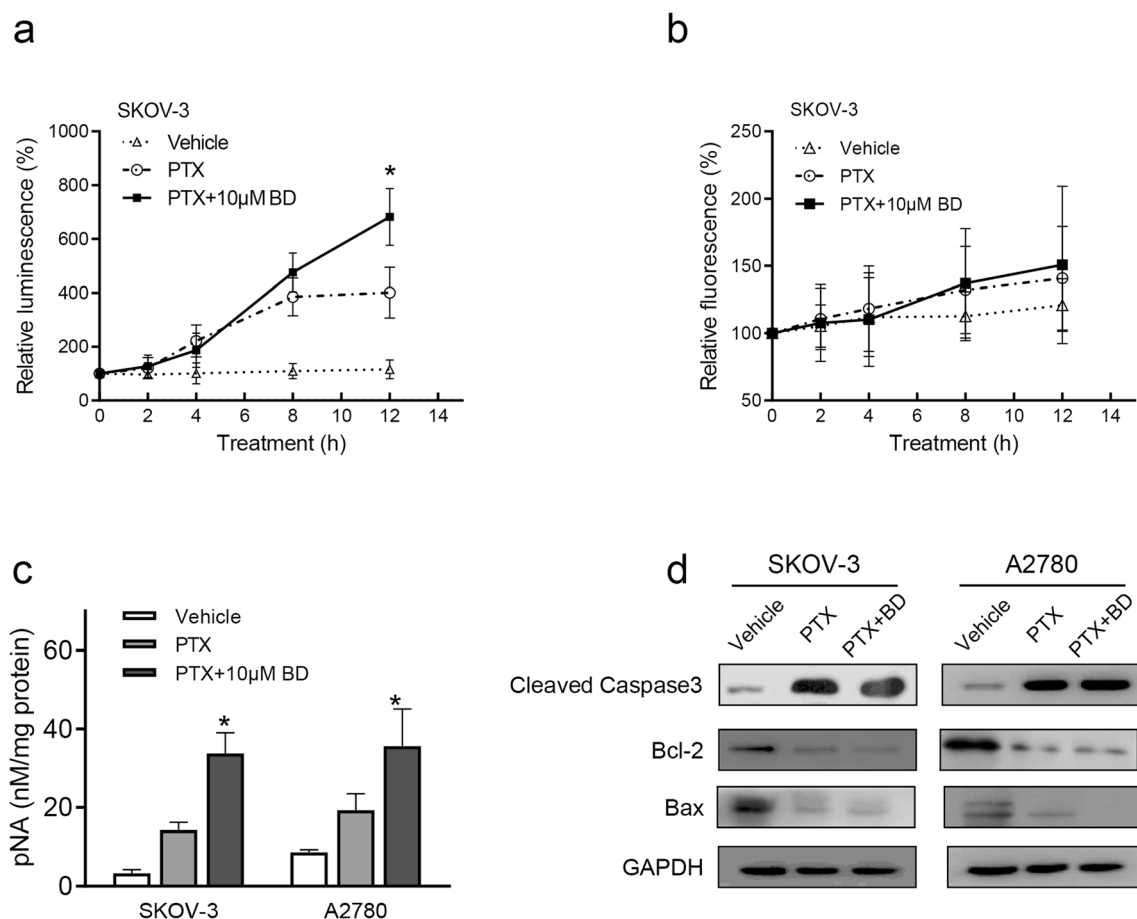


Fig. 3 Combined treatment of bruceine D and paclitaxel induces cell apoptosis of ovarian cancer cells. Cells were treated with paclitaxel (PTX) and bruceine D treatment as indicated for 12 h. The cell apoptosis (a) or necrosis (b) of ovarian cancer cells was analyzed with the RealTime Glo Annexin V cell apoptosis assays. c The pNA concentration analysis

was performed for the caspase 3 activity of the cells with combined treatment of PTX and bruceine D or only PTX. d Western blot assay was performed with the ovarian cancer cells with combined treatment of PTX and bruceine D or only PTX. Data represent the means $\pm$ SD. * $p < 0.05$ vs. control group

These results suggested that combined treatment of bruceine D enhances paclitaxel-induced cell apoptosis of ovarian cancer cells.

Paclitaxel Sensitization

Disrupt microtubule stability is the principal mechanism of paclitaxel-induced cytotoxic effects, in which JNK activation contributed predominantly to this activity (Tan et al. 2019). We further analyzed the regulation of JNK pathway in bruceine D (1) mediated paclitaxel sensitization. Combined treatment of bruceine D and paclitaxel or alone was administered for 24 h with SKOV-3 and A2780 cells. Western blot assays showed that combined bruceine D and paclitaxel treatment induced rapid phosphorylation of JNK (Fig. 4a). Higher JNK phosphorylation was observed in combined treatment group than with either bruceine D or paclitaxel treatment alone (Fig. 4b). We used JNK inhibitor (SP600125) to block JNK phosphorylation. Cell viability analysis indicated that JNK

inhibitor rescued cell proliferation in the cells with bruceine D and paclitaxel co-treatment (Fig. 4c), which indicated that p-JNK blockage attenuated the bruceine D-induced paclitaxel sensitization. Further analysis indicated that bruceine D down-regulated the phosphorylation of STAT3, and more apparent after co-treatment with bruceine D and paclitaxel. However, increased phosphorylation of STAT-3 was observed in the JNK inhibitor (SP600125) co-treated cells (Fig. 4d). Therefore, STAT3 signaling inhibition might be involved in the bruceine D-induced paclitaxel sensitization.

STAT3 Contribution to Cytotoxicity

The role of STAT3 phosphorylation in bruceine D-treated ovarian cancer cell was investigated. Exogenous constitutively activated STAT3 (STAT3-C) was expressed in SKOV-3 cells. Western blot assays indicated elevated CD133 expression in STAT3-C-expressing cells, whereas bruceine D treatment failed to decrease the protein levels of CD133 (Fig. 5a).

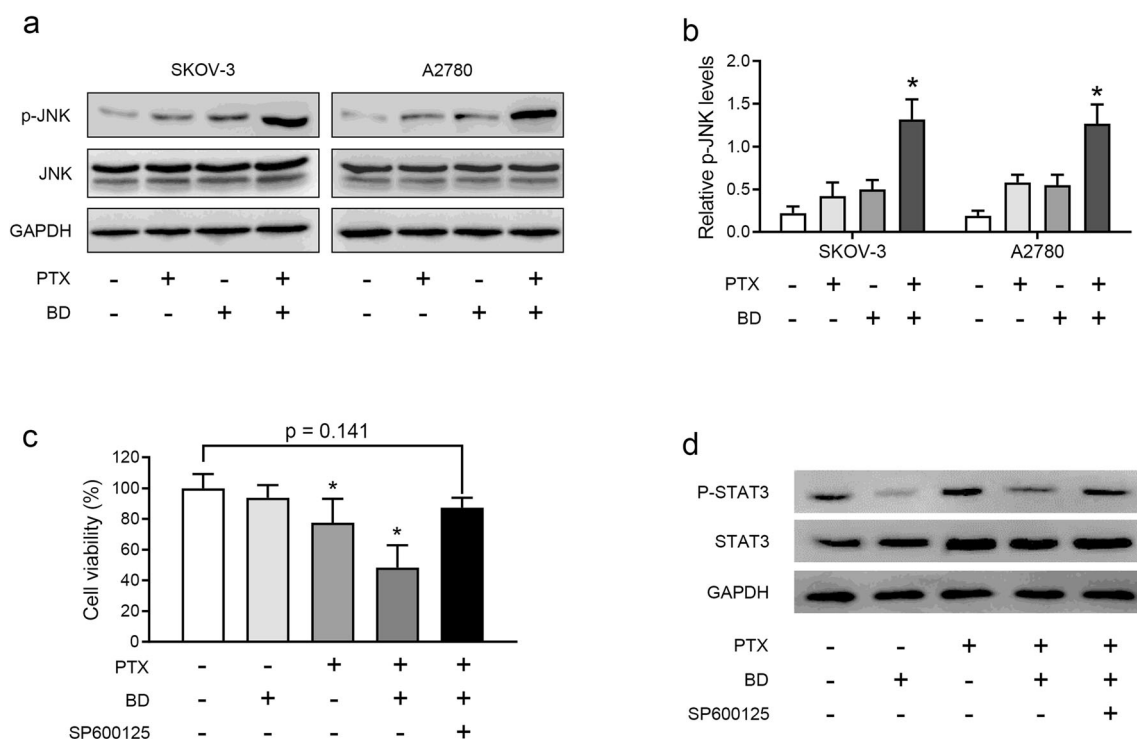


Fig. 4 JNK pathway is involved in bruceine D mediated paclitaxel sensitization. **a** Western blot assay indicated increased JNK phosphorylation in SKOV-3 and A2780 cells after bruceine D treatment for 48 h. **b** Quantitative analysis of JNK phosphorylation levels in the cells with different treatments as indicated. **c** Cell viability analysis of the

ovarian cancer cells with JNK inhibitor (SP600125), bruceine D, and PTX co-treatment, which was analyzed with CCK-8 assay. Data represent the means $\pm$ SD. * $p < 0.05$. **d** Western blot assay was performed for the levels of STAT3 and phosphorylated STAT3 after co-treatment with bruceine D, PTX, or JNK inhibitor (SP600125) as indicated

As expected, flowcytometry assay also showed that bruceine D treatment failed to eliminate the CD133⁺ subpopulation of STAT3-C-expressing cells (Fig. 5b). More importantly, STAT3-C expression rescued ovarian cancer cell proliferation with the combined treatment of bruceine D and paclitaxel (Fig. 5c). Furthermore, caspase 3 activity showed no significant change in the bruceine D and paclitaxel co-treated STAT3-C-expressing cells compared to the control group (Fig. 5d). These results suggested that the STAT3 signaling is involved in the induced bruceine D and paclitaxel chemotherapy sensitivity of ovarian cancer cells.

In this study, the therapeutic effects of bruceine D (**1**) in paclitaxel-treated ovarian cancer cells were explored, which was correlated with an increased ovarian cancer cell apoptosis and cancer stem cell elimination. Mechanism study indicated that JNK and STAT3 signaling regulation contributed to bruceine D-induced paclitaxel sensitization of ovarian cancer cells.

Paclitaxel-based chemotherapy is the recommended first-line regimen for epithelial ovarian cancer after surgical resection (Wright et al. 2016). Clinical analysis indicated a decreased sensitivity in patients treated with paclitaxel-based chemotherapy within 2 or 3 years, which was characterized as chemotherapy resistance (Shoji et al. 2018). Novel therapeutic regimens to sensitized paclitaxel-based chemotherapy

are urgently needed for improving the survival estimation (Lee et al. 2019). Paclitaxel cytotoxicity is a consequent from the microtubule stability disruption, which exhibits a dose- and time-dependent pharmacological action (Saari et al. 2015). Previous studies indicated that the mitogen-activated protein kinase/mitogen-activated protein kinase (MEK/MAPK) cascade participated in the antitumoral activity (Ren et al. 2018; Ribeiro et al. 2016). More importantly, JNK-apoptosis pathway also showed great influence in paclitaxel-induced cell apoptosis (Peng et al. 2016). Herein, we identified the synergistic effects of bruceine D and paclitaxel in ovarian cancer cell proliferation inhibition. Increased ovarian cancer cell apoptosis was observed in the combined treatment with bruceine D and paclitaxel. Our study provided valuable regimens for the management of ovarian cancer patients that develops chemotherapy resistance.

Furthermore, we provided experimental evidence that the synergistic effect of bruceine D in paclitaxel-based chemotherapy was correlated with increased ovarian cancer cell apoptosis, rather than necrosis. Previous mechanistic models indicated that bruceine D activated ROS/MAPK signaling and caspase 3 activation (Lau et al. 2009; Tan et al. 2019; Fan et al. 2020), which finally induced irreversible apoptotic cell death. Bruceine D also exhibited anti-proliferative effects against various tumors via inhibition of different signaling

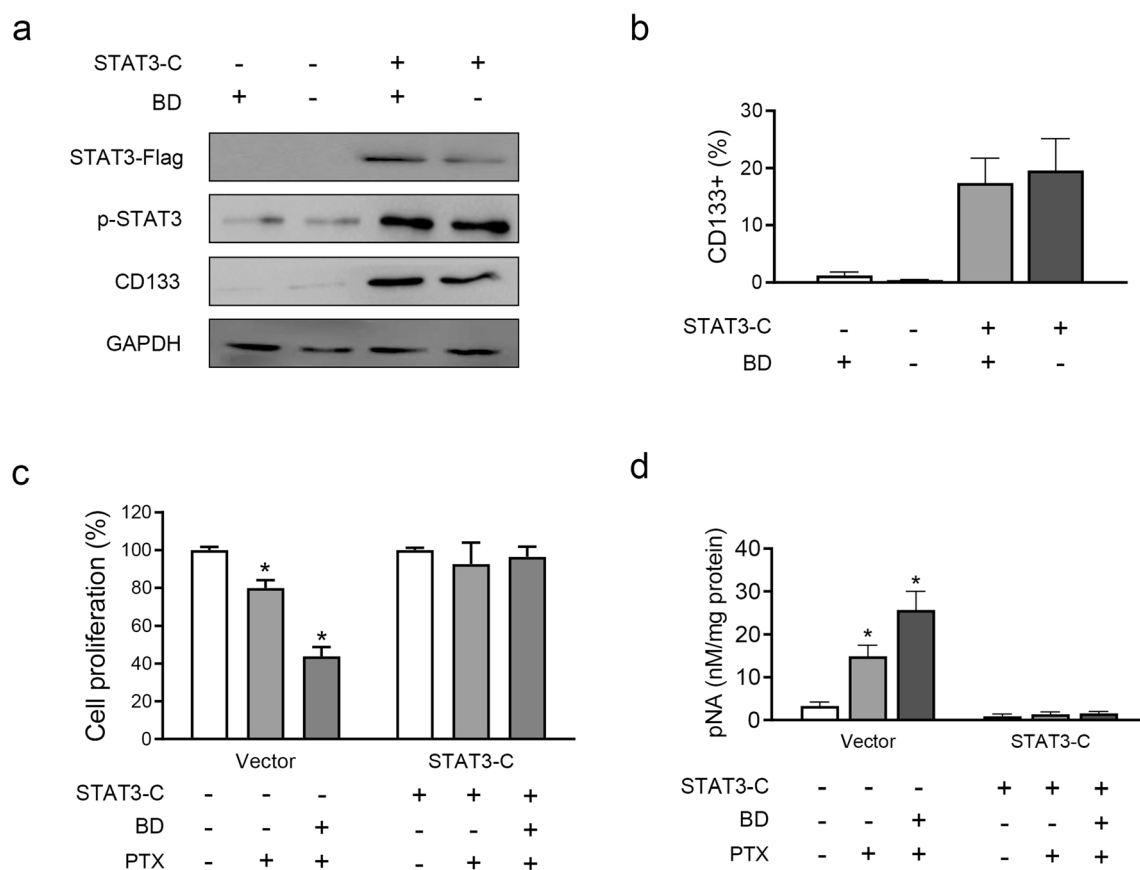


Fig. 5 STAT3 contributes to bruceine D-induced anti-ovarian cancer effects. **a** The expression levels of STAT3 and CD133 were examined with Western blot assays in the SKOV-3 cells, which was transfected with constitutively activated STAT3 (STAT3-C) or control plasmids. **b** Bruceine D failed to eliminate CD133⁺ cells of the STAT3-C-overexpressing cells, which was examined with flowcytometry analysis.

pathways including PI3K/AKT/mTOR (Zhang et al. 2016), STAT3 (Wang et al. 2019), and cyclin E1 (Li et al. 2020). To further elucidate the mechanisms underlying bruceine D-induced cell apoptosis and cancer stem cell elimination effects in ovarian cancer cells, the specific role of JNK signaling was focused on the present research. An increased JNK phosphorylation in bruceine D and paclitaxel co-treated ovarian cancer cells was observed. Previous studies indicated that activated JNK phosphorylated the *N*-terminus of the transcription factor c-Jun, which further resulted in the regulation of cell apoptosis activity. Further experimental data confirming that JNK inhibition rescued cell apoptosis with combined bruceine D and paclitaxel treatment were provided. Our results supported that bruceine D modulates JNK activation by regulation of the gene transcription associated with caspase 3-induced cell apoptosis.

Cancer stem cell is identified as the initiator for chemotherapy resistance via a series of modifications (Carnero et al. 2016). Enriched cancer stem cell population participate in cancer relapse, metastasis, and therapeutic resistance (Najafi et al. 2019). Current chemotherapy regimens

c Combined treatment of bruceine D and PTX failed to inhibit the proliferation of STAT3-C-overexpressing cells according to the CCK-8 assays. **d** The pNA concentration indicated that bruceine D failed to induce the caspase 3 activity of STAT3-C-overexpressing cells. Each treatment group was prepared in triplicate. Data represent the means $\pm$ SD. * $p < 0.05$

exhibit significant cytotoxicity for the bulk of cells to reduce tumor size, whereas little effect on cancer stem cells is observed (Carnero et al. 2016; Timaner et al. 2018). Similar phenomenon is also observed in ovarian cancer chemotherapy (Mihanfar et al. 2019). Therefore, exploring novel drugs to enhance the cytotoxicity to cancer stem cells are warranted for the current chemotherapy regimens. Meanwhile, cancer stem cell elimination also represented a promising way to overcome chemotherapy resistance (Mihanfar et al. 2019). In this study, we identified bruceine D as an effective therapeutic agent that could eliminate ovarian cancer stem cells. A decreased percentage of CD133 positive cells after bruceine D treatment was observed. Further clinical trials will guarantee the use of bruceine D as a chemotherapy sensitizer for paclitaxel-based chemotherapy in epithelial ovarian cancer.

Collective evidence supported that STAT3 signaling activation contributes to the maintains of cancer stem cells (Galoczova et al. 2018). The blockage of STAT3 activation decreases the cancer stem cell population (Wen et al. 2018). Consistent with these findings, the present study indicated that

STAT3 signaling activation contributed to cancer stem cell survival of epithelial ovarian cancer. Bruceine D increased the phosphorylation of JNK, which was alleviated by the JNK inhibitor SP600125. Further analysis observed an advantage for cancer stem cell survival in bruceine D-treated ovarian cancer cells with exogenous continuous activated STAT3 expression. Thus, these results demonstrated that JNK and STAT3 signaling is the predominant contributor for the bruceine D-induced cancer stem cell elimination effect.

Conclusions

Our study revealed the synergistic effect of bruceine D in paclitaxel-based chemotherapy of epithelial ovarian cancer. The inhibitory effects were correlated with increased cell apoptosis and cancer stem cell elimination. Mechanism study indicated that JNK and STAT3 signaling contributed to the synergistic effect of bruceine D in the paclitaxel-based chemotherapy of ovarian cancer, which supported bruceine D as an adjuvant agent in ovarian cancer chemotherapy.

Acknowledgements We appreciate the members of Clinical Laboratory of Hunan Cancer Hospital for their helpful assistance. Our research received no external funding.

Availability of Data and Materials The data supporting the study has been presented in the manuscript, and original data could be obtained from the corresponding author through suitable request.

Author Contribution CXS conceived and designed the study. ZPY, ZHJ, HYS, and KHP performed the cell experiments and molecular investigation. CXS supervised the study. ZPY and CXS interpreted the results and wrote the manuscript. All authors have read and approved the publication of the final manuscript.

Declarations

Ethics Approval and Consent to Participate There was no need for ethical approval or patient consent.

Conflict of Interest The authors declare no competing interests.

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