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Berberine Abrogates Paclitaxel-Induced Tumor Repopulation and M2 Macrophage Polarization in Ovarian Cancer by Disrupting the COX-2 Signaling Axes

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Abstract

Background: Although Paclitaxel (PTX) is the frontline chemotherapy for ovarian cancer, PTX-induced apoptotic cells paradoxically alter the tumor microenvironment to stimulate the repopulation of residual cancer cells. This study aims to elucidate the precise molecular crosstalk between apoptotic cells, surviving cells, and macrophages, and to explore the therapeutic potential of Berberine, a highly safe natural alkaloid, in overcoming this treatment failure.

Methods: An in vitro Trans well co-culture system comprising PTX-treated SKOV3 cells, residual SKOV3 cells, and THP-1 macrophages was established. An in vivo xenograft mouse model using a mixture of apoptotic and ID8 cells was constructed to evaluate the therapeutic efficacy of Berberine.

Results: We demonstrated that PTX-induced apoptotic SKOV3 cells accelerated the proliferation of surviving cancer cells via the activation of the caspase 3/iPLA$_2$/AA/COX-2/PGE$_2$ pathway. Concurrently, these dying cells drove macrophage polarization toward the M2 phenotype. Crucially, Berberine acted as a potent dual-inhibitor. Both in vitro and in vivo results revealed that Berberine significantly abrogated tumor repopulation and suppressed the PGE$_2$-mediated M2 macrophage polarization.

Conclusion: Characterized by its pharmacological safety, Berberine emerges as an ideal adjuvant candidate for long-term maintenance therapy to synchronously block apoptosis-driven tumor repopulation and M2 macrophage polarization in ovarian cancer.

Keywords: Ovarian cancer; Tumor repopulation; M2 macrophage polarization; Berberine; PGE$_2$.

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Introduction

Ovarian cancer remains one of the most lethal gynecological malignancies worldwide, characterized by late-stage diagnosis and a high mortality rate [1]. Currently, Paclitaxel (PTX)-based chemotherapy is the frontline treatment for advanced ovarian cancer. Despite achieving significant initial tumor regression, a vast majority of patients eventually experience clinical treatment failure due to widespread metastasis and tumor recurrence. Accumulating evidence suggests that chemotherapy acts as a "double-edged sword". While it effectively induces tumor cell apoptosis, it paradoxically triggers a phenomenon known as chemotherapy-induced tumor repopulation [2-4]. Often referred to as the "Phoenix Rising" effect, this process occurs when massive numbers of dying cancer cells release potent paracrine signals that actively fuel the accelerated proliferation of a small fraction of surviving cells [4]. Consequently, deciphering the molecular mechanisms underlying this apoptosis-driven recurrence is critical for preventing ovarian cancer relapse.

Recent studies indicate that the executioner protein, active caspase 3, plays a crucial role not only in mediating cell death but also in orchestrating growth-promoting signals in the residual Tumor Microenvironment (TME) [5]. The activation of caspase 3 in dying cells can trigger cancer repopulation, notably through the calcium-Independent Phospholipase A2 ($iPLA_2$) pathway, leading to the accumulation of prostaglandin E2 (PGE_2) [6]. This PGE_2 surge subsequently activates downstream proliferation pathways in adjacent surviving cancer cells [7,8]. Beyond direct stimulation of residual tumor cells, chemotherapy-induced recurrence is profoundly dictated by the remodeling of the TME immune landscape [9]. Tumor-Associated Macrophages (TAMs) are highly plastic immune components within the TME [10]. Their polarization toward the pro-tumorigenic M2 phenotype is a hallmark of repopulation [11,12]. However, while the protumor genic role of M2 macrophages is well-established, the precise molecular crosstalk between chemotherapy-induced apoptotic cancer cells and TAM polarization remains largely elusive. Specifically, whether dying tumor cells actively recruit and polarize macrophages via specific chemokines requires urgent investigation.

To overcome this recurrence, there is a pressing need for novel adjuvant strategies capable of simultaneously targeting both tumor cell repopulation and the TAM [13]. Berberine, an important natural alkaloid extract, has garnered considerable attention for its broad-spectrum anti-tumor properties and high pharmacological safety [14]. Clinically, the evolution from initial chemotherapy to ultimate tumor recurrence is a protracted and insidious process. However, the dose-limiting toxicities of conventional chemotherapeutic agents or synthetic targeted drugs often preclude their continuous use. Due to its minimal systemic toxicity and excellent safety profile, Berberine is exceptionally well-suited for long-term maintenance administration, presenting a highly promising candidate for combination therapy [15].

In the present study, we hypothesized that PTX-induced apoptosis in SKOV3 ovarian cancer cells actively drives tumor recurrence through a dual-faceted mechanism: accelerating residual cell proliferation via AA pathway and simultaneously inducing macrophage M2 polarization via specific chemokines. Utilizing in vitro co-culture Tran swell systems and an in vivo mixture xenograft model, we aimed to elucidate the specific molecular dialogue, focusing on the caspase 3/$iPLA_2$/AA/COX-2/PGE_2 axis, between apoptotic cells, surviving cells, and macrophages. Furthermore, we comprehensively evaluated the therapeutic potential of Berberine in interrupting this detrimental cascade. Our findings provide novel mechanistic insights into chemotherapy failure and highlight Berberine as a potent dual-targeting adjuvant to re-educate the immune microenvironment and prevent ovarian cancer relapse.

Materials and methods

Chemicals and reagents

Berberine (Cat No. IB0120) were obtained from Solar bio-Science & Technology Co., Ltd. (Beijing, China) was dissolved with 0.2% DMSO culture medium to a final concentration of 200 μM as a stock solution for cell experiment. PTX (Cat No. 580555) was obtained from Sigma (St. Louis, MO, USA). Prostaglandin E2 ELISA Kit (Human: Catalog No. NBP3-00375, Mouse: Cat No. NBP3-00428) and Arachidonic acid ELISA Kit (Cat No. NBP2-59872) were purchased from R&D Systems (Minneapolis, MN, USA). $iPLA_2$ antibody (Cat No. 22030-1-AP) was form proteinates (Rochemont, Illinois, USA). Caspase-3(Cat No. ab184718), GLI1(Cat No. ab289368), COX-2 (Cat No. ab179800), Arg-1 (Cat No. ab212522), TGF-β (Cat No. ab315254), CD163 (Cat No. ab182422), Ki67 (Cat No. ab16667), CD206 (Cat No. ab64693), F4/80 (Cat No. ab300412) and β-actin (Cat No. Ab8227) were from Abcam (Cambridge, UK), diluted to a concentration of 1:1000 prior to use. Rabbit IgG Isotype Control Recombinant Antibody (Cat No. 98136-RR), CoraLite488-conjugated Goat Anti-Rabbit IgG (H+L) (Cat No. SA00013-2) and Rhodamine (TRITC)–conjugated Goat Anti-Rat IgG (H+L) (Cat No. SA00007-7) were acquired from proteinates (Rosemont, IL, USA). Dulbecco's Modified Eagle Medium (DMED) (Cat No. 1196092), RPMI medium 1640 (Cat No. 12633012) and fetal bovine serum (FBS) (Cat No. A5256701) were procured from Gibco (Thermo Fisher Scientific, Waltham, MA, USA). All chemical compounds utilized were of analytical grade.

Cell culture

SKOV3 cells were cultured in DMEM medium. All culture media were supplemented with 10% FBS and 1% penicillin-streptomycin mixture (100 U/mL penicillin and 100 μg/mL streptomycin). The human monocytic leukemia cell line THP-1 was maintained in suspension in RPMI-1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin and 0.05 mM β-mercaptothion (Cat No. 21985032, Thermo Fisher Scientific, Waltham, MA, USA). The murine ovarian cancer cell line ID8 was cultured in DMEM, supplemented with 4% fetal bovine serum FBS, 1% Insulin-Transferrin-Selenium (Cat No. 41400045, Thermo Fisher Scientific, Waltham, MA, USA), and 1% penicillin-streptomycin. Cells were incubated at 37℃ in 5% CO_2-95% air atmosphere. To ensure scientific rigor and reproducibility, all cell lines were recently authenticated using Short Tandem Repeat (STR) profiling prior to the experiments. Furthermore, the cells were routinely screened for mycoplasma contamination using a Mycoplasma Detection Kit (VA zyme, Nanjing, China) and were confirmed to be mycoplasma-free throughout the study.

Tran swell system model

The trans well system (Corning, NY, USA) with 0.4 μm pore size semipermeable membrane filter insert was used for preparing the co-culture system. SKOV3 and THP-1 cells were cultured in the co-culture system, with the cells in the lower chamber serving as feeder cells and those in the upper chamber as receptor cells.

Preparation of feeder cells

SKOV3 cells were seeded into the lower chambers of the 6-well plates at a density of 2×10^5 cells/well in 2.0 mL of complete culture medium. Then they were pre-treated with 20 nmol/L PTX for 24 h to induce robust apoptosis. Following treatment, these apoptotic feeder cells were thoroughly washed three times with PBS to remove any residual chemotherapeutic drug before being utilized in the co-culture system.

Co-culture and treatment protocol

Receptor cells, comprising either untreated SKOV3 cells or THP-1 macrophages, were seeded into the upper chambers of the 6-well plates at a density of 10000 cells/well in 1.5 mL of complete culture medium. After allowing the receptor cells to adhere overnight, the inserts were subsequently placed over the lower chambers. For the pharmacological intervention groups, Berberine (5 μmol/L) was added directly into the co-culture system (distributed in both upper and lower chambers to ensure uniform drug exposure) at the initiation of the co-culture period. The entire system was co-cultured for 48 h. Upon completion of the treatment period, the receptor cells, feeder cells and supernatants were collected. Receptor cells in the semipermeable filter inserts were fixed with methanol for Ki67 or CD206 immunofluorescence staining.

In vivo anti-tumor experiment

All animal care and experimental procedures were reviewed and strictly approved by the Institutional Animal Care and Use Committee (IACUC) of Dalian University of Technology (Approval Number: DUTFM260525-03). All in vivo experiments were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

1×10^5 ID8 cells were treated with 20 nmol/L PTX for 24 h, all cells, including apoptotic cells in the suspension and supernatant, were collected and combined with 2×10^6 fresh ID8 cells. Then the total cells were resuspended in 70 μL of PBS (pH 7.2) and mixed with 70 μmol/L of Matrigel Matrix, designated as the Apo group. 2×10^6 fresh ID8 cells were designated as Con group. This mixture was then injected subcutaneously into one side of the dorsal flank of 8-week-old female C57BL/6 mice. Once the tumor volume reached 60-100 mm^3. The control group received saline daily by oral gavage, while the berberine group was administered Berberine (100 mg/kg, dissolved in saline) daily by oral gavage. Body weights and tumor sizes were meticulously recorded every three days, with tumor volume calculated using the formula: length × $width^2$ × 0.52. The mice were sacrificed 25 days after the initiation of drug administration. At the conclusion of the study, immediately before sacrifice, blood samples were obtained from all groups by eyeball extirpation. The collected blood was allowed to stand at room temperature for 30 min for complete coagulation. Serum was separated by centrifugation at 3000 rpm for 15 min at 4°C and immediately frozen at -80°C for subsequent assays. Tumor tissues were isolated and immediately stored at −80°C for subsequent analysis.

Immunofluorescence (IF) assay

Tumor slices following deparaffinization, hydration, and antigen retrieval, the sections were blocked with 10% Bovine Serum Albumin (BSA) for 1 h. Subsequently, primary antibodies were applied to incubate the sections overnight at 4°C. After washing the sections three times with PBST, the secondary antibody was incubated for 1 h. Following another round of washing, DAPI (1 μg/mL) was utilized to stain the nuclei for 5 min. Images were captured using a fluorescence microscope (EVOSTMM5000, Invitrogen by Thermo Fisher Scientific). The fluorescence intensity was quantified using ImageJ software.

Immunohistochemistry (IHC) assay: Tumor tissues were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned at a thickness of 4 μm. The tissue sections were deparaffinized in xylene and rehydrated through a graded series of ethanol. For antigen retrieval, the slides were boiled in 10 mM sodium citrate buffer (pH 6.0) using a microwave oven for 15 min. After cooling to room temperature, endogenous peroxidase activity was quenched with 3% hydrogen peroxide (H_2O_2) for 10 min. To prevent non-specific binding, the sections were blocked with 5% BSA for 1 h at room temperature. Subsequently, the sections were incubated overnight at 4°C with primary antibodies. Following three washes with PBS, the slides were incubated with a Horseradish Peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The immunoreactive signals were visualized using a 3,3'-Diaminobenzidine (DAB) substrate kit (Cat No. DA1010; Solar bio, Beijing, China), and the nuclei were counterstained with hematoxylin. Finally, the slides were dehydrated, cleared in xylene, and mounted. The stained sections were observed, and representative images were captured using a microscope (EVOSTMM5000, Invitrogen by Thermo Fisher Scientific). For quantitative analysis, at least three randomly selected high-power fields per section were evaluated. The quantification of positive staining was performed using ImageJ software.

Western blot (WB) analysis

Total proteins were extracted from the treated cells using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Cat No. R0020; Solar bio, Beijing, China). Subsequently, the precise protein concentration of each sample was quantified using a Bicinchoninic Acid (BCA) protein assay kit (Cat No. PC0020; Solar bio, Beijing, China) according to the manufacturer's instructions. Subsequently, the proteins were mixed with loading buffer and denatured by heating. In brief, after performing Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with a gradient of 7.5%-12.5%, the samples were transferred to a Polyvinylidene Fluoride (PVDF) membrane (Cat No. IPVH00010; Solar bio, Beijing, China) and incubated with Bovine Serum Albumin (BSA) for 2 hours. The PVDF membrane was then incubated with primary antibodies overnight at 4°C, followed by secondary antibodies at room temperature 1 h. Bands were visualized using an Enhanced Chemiluminescence (ECL) detection system (Tenon 4600, Shanghai, China) for Western blotting. Subsequently, the densitometric quantification of the protein bands for statistical

analysis was performed using ImageJ software.

ELISA assay

Supernatants and serum samples were prepared for analysis using ELISA kits. The levels of PGE_2 and AA were measured according to the manufacturer's instructions. Absorbance readings were taken at a wavelength of 450 nm (Multiclan Sky-high, Thermo Scientific, Waltham, MA, USA).

Cell transfection

For overexpression experiments, the COX-2 overexpression plasmid and its empty vector control were obtained from Gene Pharma (Shanghai, China). Briefly, SKOV3 were seeded into 6-well plates and cultured until they reached approximately 60%–70% confluence. Cell transfections were performed using Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The plasmids (2 μg/well) and the transfection reagent were separately diluted in Opti-MEM reduced-serum medium (Gibco, Grand Island, NY, USA), mixed gently, and incubated for 15 min at room temperature to form complexes. The mixture was then added dropwise to the cells. After 6 hours of incubation, the medium was replaced with complete culture medium. Cells were prepared for Trans well system model as feeder cells.

Statistical analysis

Use Prism 8.0 statistical analysis software to statistic the data. The data was represented as the standard deviation of under 3 experiments. $P<0.05$ is deem to be significant.

Results

PTX chemotherapy accelerates the repopulation of SKOV3 cells

Researchers have found that apoptotic tumor cells can promote the repopulation of tumors from a limited number of surviving cells [3]. In our study, we established a Trans well system for the co-culture fresh SKOV3 cells with apoptotic SKOV3 cells. Treatment with PTX resulted in the apoptosis of feeder cells, and Ki67 staining of the receptor cells indicated that, compared to the control group, receptor cells in the model group exhibited significantly greater proliferation (Figure 1A). The activation of caspase 3 may stimulate the repopulation of cells. (Figure 1B) illustrated PTX activated caspase 3 in feeder cells. Subsequently, we focused on $iPLA_2$-COX-2-AA-PGE_2. We assessed the expression of $iPLA_2$ and COX-2 within the Trans well system. The levels of $iPLA_2$ and COX-2 in feeder cells treated with PTX were elevated compared to those in the control group (Figure 1B). Additionally, we analyzed the levels of AA and PGE_2 in the supernatants. Consequently, the amount of AA decreased, while the level of PGE_2 increased (Figure 1C). GLI1, which is activated by PGE_2, is associated with cell proliferation. Our findings demonstrated that apoptosis in SKOV3 cells could elevate the levels of GLI1 in receptor cells (Figure 1B). Collectively, these results support our hypothesis that PTX induced apoptosis may facilitate tumor cell repopulation by caspase 3-$iPLA_2$-COX-2-AA-PGE_2 pathway.

SKOV3 cells apoptosis promotes macrophage M2 polarization

M2 macrophages are pro-tumorigenic, contributing to tumor recurrence. However, it remains unclear whether apoptotic tumor cells can stimulate macrophage polarization. To investigate this, we employed a trans well system to co-culture THP-1 cells with PTX treated SKOV3 cells. THP-1 cells served as receptor cells. The surface marker CD206, which is predominantly expressed in M2 macrophages, was significantly elevated in the Model group (Figure 2A). Furthermore, Arg-1, TGF-β and CD163 levels also increased in the Model group (Figure 2B). PGE_2 plays a crucial role in promoting macrophage polarization toward the M2 phenotype. As shown in (Figure 2B), PTX-induced apoptosis resulted in increased COX-2 expression in feeder cells, which is the upstream of PGE_2. We also assessed the PGE_2 levels in the supernatants, finding that PGE_2 levels were elevated in the Model group (Figure 2C). These results confirm that chemotherapy drug-induced apoptosis may drive M2 polarization of macrophages via PGE_2.

Berberine reversed the PTX-induced repopulation of ovarian cancer cells: Previous studies have demonstrated that apoptotic tumor cells can stimulate the repopulation of cancer cells from a small number of surviving cells. In the SKOV3 co-culture Trans well system, treatment with PTX significantly increased the expression of Ki67 in SKOV3 cells located in the top chamber (receptor cells) compared to Con group (Figure 3A). What is more, we investigated the effects of Berberine on this specific type of repopulation. The addition of 5 μmol/L Berberine in both the upper and lower chambers markedly reduced the repopulation of receptor SKOV3 cells. In contrast, Berberine alone at the same concentration did not affect the receptor cells (Figure 3A). Receptor cells in the PTX group exhibited increased levels of COX-2 and GLI1 proteins, while Berberine partially reversed these elevations (Figure 3B). We subsequently assessed the levels of AA and PGE_2 in the supernatant of the trans well system. As illustrated in Figure 3C, treatment with 5 μmol/L Berberine did not result in significant alterations in the levels of AA and PGE_2 compared to the control group. In contrast, PTX treatment of feeder cells led to a decrease in AA levels (Figure 3C) and an increase in PGE_2 levels (Figure 3C). Notably, the addition of Berberine partially reversed these changes in AA and PGE_2 production compared to the PTX group (Figure 3C). These findings suggested that Berberine can counteract PTX-induced repopulation through the AA-COX-2-PGE_2-GLI1 pathway in SKOV3 cells.

Berberine reversed the PTX-induced macrophage M2 polarization

Our results demonstrated that PTX-induced apoptosis can promote macrophage M2 polarization. Consequently, we subsequently evaluated the impact of Berberine on M2 macrophage polarization. As shown in (Figure 4A), the PTX group exhibited high expression levels of CD206, which were reduced by Berberine (Figure 4A). Additionally, Berberine decreased the expression levels of COX-2 (feeder cells: SKOV3) and Arg-1 (receptor cells: THP-1) (Figure 4B). Simultaneously, under the influence of Berberine, the levels of PGE_2 were reduced (Figure 4C). These findings indicated that Berberine can inhibit PTX-induced apoptosis-mediated M2 polarization of macrophages.

COX-2, as the key regulator of "Phoenix Rising", is the main target of Berberine. COX-2 is the upstream of PGE_2 which promotes macrophages to M2 polarization. To further elucidated the role of COX-2 in promoting M2 polarization of macrophages following PTX-induced apoptosis, we utilized a COX-2-overexpressing plasmid. The results demonstrated that Berberine can suppress the expression of CD206 on macrophages, however, this inhibitory effect is partially restored following COX-2 overexpression (Figure 5A). Similarly, in comparison to the Berberine group, COX-2 reactivated the expression of Arg-1 (Figure 5B). Concurrently, under the influence of COX-2, PGE_2 was again increased (Figure 5C). These findings indicated that Berberine inhibits PTX-related apoptosis-induced M2 polarization of macrophages by suppressing COX-2.

Berberine inhibited the PTX induced repopulation of ovarian cancer in vivo

This study established a mouse model of PTX-induced recurrence mediated by apoptosis, achieved by mixing apoptotic ovarian cancer cells with normal ovarian cancer cells. Furthermore, it investigated the therapeutic effects of Berberine in vivo. As shown in (Figure 6A) and S1, the tumor volume in the group containing a mixture of apoptotic and normal cells was significantly larger compared to the CON group; however, tumors were significantly smaller following Berberine administration. Ki67 serves as a crucial marker for assessing tumor proliferation. The results indicated that Ki67 expression levels were significantly elevated in the Apo group, while they were reduced in the Berberine group (Figure 6B). These findings suggest that PTX-induced apoptotic cells promote the reproliferating of ovarian cancer in mice, whereas Berberine inhibits this reproliferating. Subsequently, we examined the role of Berberine in regulating the M2-polarized macrophages affected by PTX-induced apoptotic cells. The results demonstrated that the proportion of M2-polarized macrophages ($F4/80^+CD206^+$) increased in the Apo group, whereas this proportion decreased following Berberine administration (Figure 6C). These findings suggest that, consistent with in vitro results, Berberine inhibited M2 macrophage polarization induced by PTX-mediated apoptosis. Furthermore, we validated the mechanism by which Berberine suppressed the reproliferating of ovarian cancer cells in vivo. The results indicated that PTX-induced apoptotic cells activated the expression of $iPLA_2$, COX-2 and GLI1 in mouse tumor tissues, while Berberine administration decreased the expression of these proteins. Considering the role of the AA signaling pathway in PTX-induced apoptosis-mediated ovarian cancer reproliferating, we assessed the expression levels of key cytokines in the AA signaling pathway, specifically PGE_2 and AA, in mouse serum. The results demonstrated that the apoptotic cell microenvironment elevated PGE_2 levels and reduced AA levels, whereas Berberine treatment lowered PGE_2 levels and increased AA levels. In summary, Berberine inhibited the reproliferating of ovarian cancer cells induced by apoptotic ovarian cancer cells following PTX therapy, as well as the M2 polarization of macrophages triggered by these apoptotic cells. Moreover, this inhibitory effect of Berberine was mediated through the suppression of the AA metabolic pathway targeting COX-2.

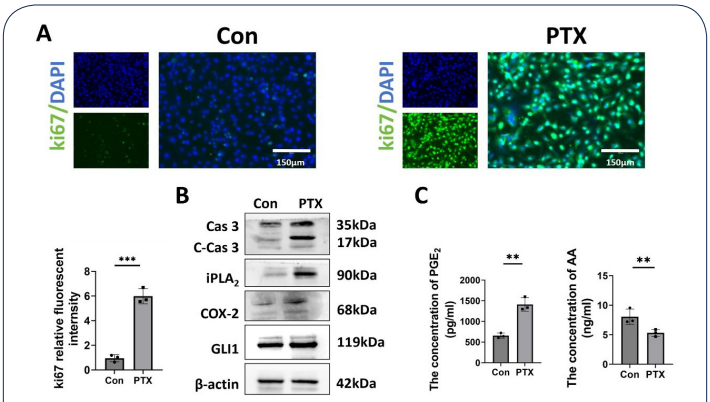


Figure 1: PTX accelerated the population of ovarian cell lines. (**A**) Ki67 expression in receptor cells. (**B**) protein expression of feeder cells (caspase 3, $iPLA_2$ and COX-2) and receptor cells (GLI1). (**C**) The AA and PGE_2 levels in supernatants. ** P<0.01, *** P<0.001 compared with the control group.

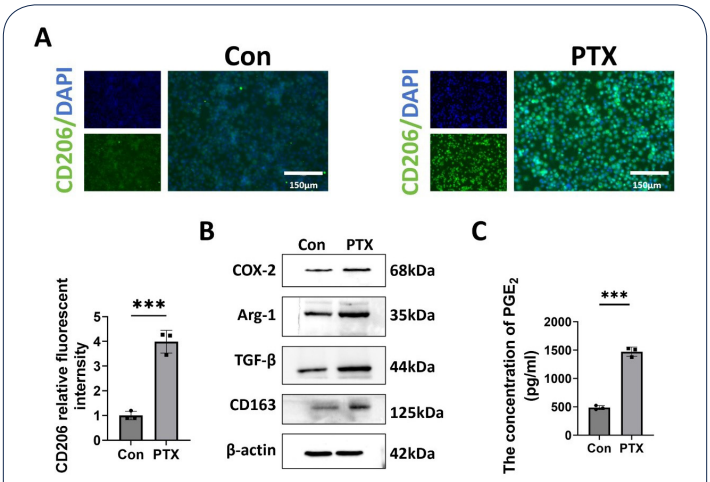


Figure 2: Berberine inhibits PTX-induced reproliferating of ovarian cell lines. (**A**) Ki67 expression of receptor cells. (**B**) protein expression of cells (feeder cells for COX-2 and receptor cells for GLI1). (**C**) The AA and PGE_2 levels in supernatants. * P<0.05, ** P<0.01, *** P<0.001.

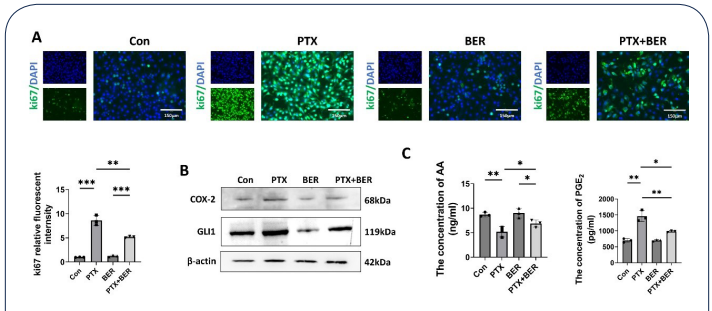


Figure 3: Berberine inhibits PTX-induced reproliferating of ovarian cell lines. (**A**) Ki67 expression of receptor cells. (**B**) protein expression of cells (feeder cells for COX-2 and receptor cells for GLI1). (**C**) The AA and PGE_2 levels in supernatants. * P<0.05, ** P<0.01, *** P<0.001.

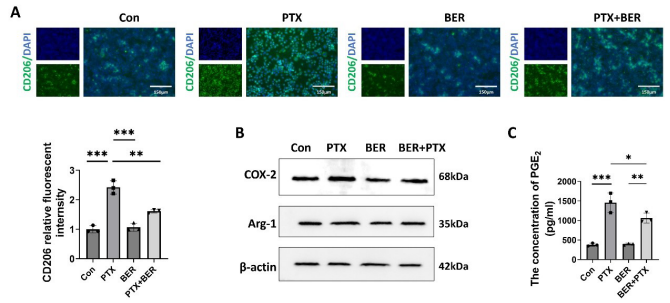


Figure 4: Berberine inhibits PTX-induced macrophage M2 polarization. (**A**) CD206 expression of receptor (THP-1) cells. (**B**) protein expression of feeder cells (COX-2) and receptor cells (Arg-1). (**C**) The PGE_2 levels in supernatants. ** P<0.01, *** P<0.001.

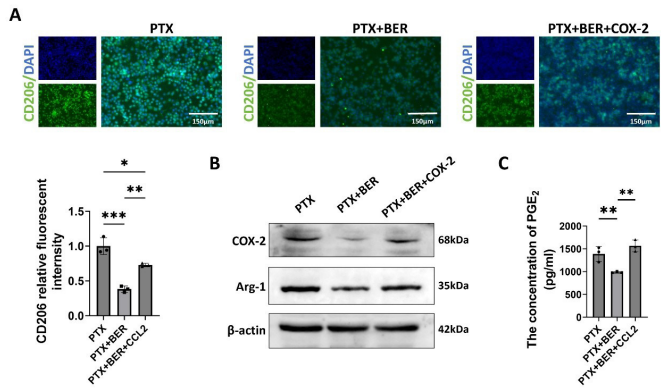


Figure 5: COX-2 reverses the inhibitory of berberine on M2 polarization of macrophages. (**A**) CD206 expression of receptor (THP-1) cells. (**B**) protein expression of feeder cells (COX-2) and receptor cells (Arg-1). (**C**) The PGE_2 levels in supernatants. * P<0.05, ** P<0.01, *** P<0.001.

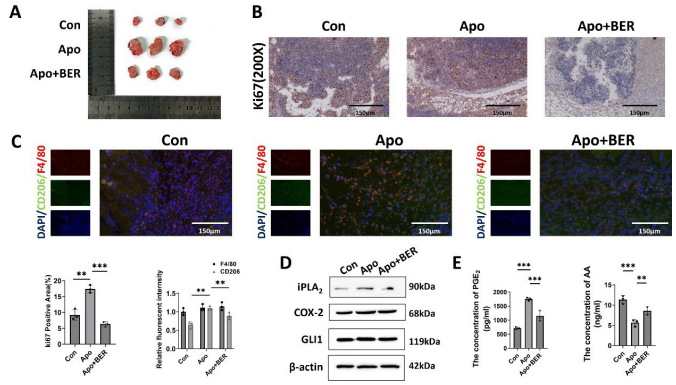


Figure 6: Berberine alleviates the repopulation of ovarian cancer in vivo. (**A**) Representative images of tumor tissues. (**B**) Ki67 expression in tumor tissue. (**C**) F4/80 and CD206 co-expression in tumor tissue. (**D**) protein expression in tumor tissue. (**E**) The AA and PGE_2 levels in serum. ** P<0.01, *** P<0.001.

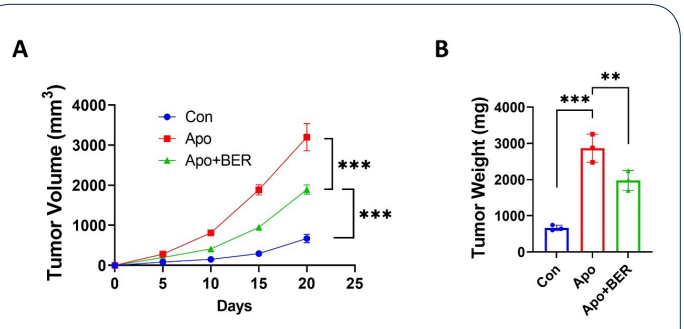


Figure S1: Berberine alleviates the repopulation of ovarian cancer in vivo. (**A**) Tumor growth curve. (**B**) Tumor weight. ** P<0.01, *** P<0.001.

Discussion

Despite the extensive application of PTX-based chemotherapy in ovarian cancer, tumor recurrence and metastasis remain the primary causes of clinical treatment failure [16]. Accumulating evidence suggests that although chemotherapy effectively induces tumor cell apoptosis, the dying cells can paradoxically stimulate the repopulation. However, the precise molecular crosstalk between PTX-induced residual tumor cells and immune cells remains incompletely understood. In the present study, we demonstrated that PTX-induced apoptosis of SKOV3 cells accelerates the proliferation of residual cancer cells via AA signaling pathway. Concurrently, we uncovered that these apoptotic tumor cells up-regulate PGE_2, driving the polarization of macrophages toward the pro-tumorigenic M2 phenotype. Most importantly, we identified Berberine as a potent dual-targeting therapeutic agent. Both our in vitro and in vivo results compellingly show that Berberine effectively abrogates PTX-induced tumor repopulation and reverses M2 macrophage polarization by concurrently suppressing the COX-2/PGE_2/GLI1 cascade. Collectively, our findings provide novel mechanistic insights into chemotherapy-induced tumor recurrence and highlight the promising translational potential of Berberine as an adjuvant therapy to overcome treatment failure in ovarian cancer.

The phenomenon of chemotherapy-induced tumor repopulation has been recognized as a major obstacle to long-term clinical efficacy, often referred to as the "Phoenix Rising" effect, where dying cells release paracrine signals to fuel the growth of neighboring surviving cells [17]. Consistent with previous studies, our data revealed that PTX-induced apoptosis in SKOV3 cells activates the downstream caspase 3-$iPLA_2$ cascade. This activation leads to a decrease in AA and a significant accumulation of PGE_2, which subsequently upregulates proliferation-related proteins (GLI1) in residual cells. Notably, the introduction of Berberine effectively blunted this PGE_2-driven repopulation, presenting a targeted pharmacological strategy to counteract the direct pro-proliferative signals released by apoptotic tumor cells. Beyond the direct stimulation of residual tumor cells, the remodeling of the TME plays a critical role in tumor recurrence. TAMs are highly plastic immune cells within the TME, and their polarization toward the immunosuppressive M2 phenotype (characterized by elevated CD206, CD163 and Arg-1) promotes tumor recurrence [18,19]. While M2 macrophages are known to be pro-tumorigenic, the specific interaction between chemotherapy-induced apoptotic cancer cells and TAM polarization has remained largely elusive [20].

Our findings demonstrated that PTX-treated apoptotic SKOV3 cells actively upregulate the PGE_2, which serves as a crucial molecular messenger orchestrating the M2 polarization of macrophages [21]. COX-2 is the upstream of PGE_2 in the PTX-induced repopulation. Strikingly, Berberine intervention profoundly reversed this apoptotic cell-mediated immune deterioration by downregulating COX-2 expression, thereby reducing M2 macrophage markers. To rigorously establish the mechanistic necessity of COX-2 in this cellular dialogue, we performed a rescue experiment using a COX-2-overexpression plasmid. The data unequivocally showed that ectopic overexpression of COX-2 effectively neutralized the inhibitory effects of Berberine, reactivating M2 polarization and restoring PGE_2 production. This robust line of evidence not only underscores COX-2 as the central mediator of apoptosis-induced TME remodeling but also highlights the unique capability of Berberine to re-educate the immune microenvironment by precisely disrupting the COX-2 signaling axis.

To substantiate our in vitro findings, we established an in vivo xenograft model using a mixture of apoptotic and normal ID8 cells, which elegantly mimics the complex pathological state of residual tumors post-chemotherapy. Consistent with the in vitro data, Berberine administration significantly hindered tumor repopulation in vivo, evidenced by diminished tumor volume, decreased Ki-67 expression, and a reversed immunosuppressive TME with reduced PGE_2 and M2 macrophage infiltration. Beyond its robust mechanistic efficacy, the translational value of Berberine is particularly noteworthy. As an important natural alkaloid extract, Berberine is widely recognized for its high pharmacological safety and minimal systemic toxicity [22,23]. Clinically, the evolution from initial chemotherapy to ultimate tumor recurrence is a protracted and insidious process that requires prolonged surveillance and intervention. Due to its excellent safety profile, Berberine is exceptionally well-suited for long-term maintenance administration during this extended window, overcoming the dose-limiting toxicities typically associated with synthetic targeted drugs [24,25]. Furthermore, Berberine intrinsically possesses broad-spectrum anti-tumor properties [26,27]. When implemented in combination with conventional chemotherapeutic agents like PTX, Berberine acts as a powerful synergist. It not only abolishes the chemotherapy-induced repopulation and M2 macrophage-mediated immune evasion but also enhances the overall chemosensitivity of ovarian cancer cells, thereby substantially reducing the risk of acquired chemoresistance [23,27]. Collectively, these compelling attributes highlight Berberine as an ideal, safe adjuvant candidate for long-term combination therapy to prevent ovarian cancer relapse.

Despite the promising dual-target therapeutic potential of Berberine demonstrated in our study, several limitations must be acknowledged to provide a balanced perspective. A major clinical hurdle for the translation of Berberine in cancer therapy is its remarkably poor oral bioavailability and rapid metabolism in the gastrointestinal tract, which severely restrict its effective systemic concentration and targeted accumulation at the tumor site [28-30]. To overcome this pharmacokinetic bottleneck, advanced nanotechnology-based drug delivery systems offer a highly viable solution. Previously, our research group successfully engineered a targeted nano-delivery system co-encapsulating Berberine and doxorubicin [22]. This nanocarrier not only achieved the synergistic therapeutic effect of the two drugs but also significantly enhanced the oral absorption, bioavailability, and tumor-specific targeting of Berberine. Given that PTX is the frontline chemotherapeutic agent for ovarian cancer-and the specific inducer of the apoptosis-driven tumor repopulation addressed in this study-the development of a novel co-delivery nanoplatform for PTX and Berberine is of great clinical significance. To date, there are no relevant studies on such a PTX/Berberine co-delivery system. Therefore, designing a customized nanocarrier to synchronize the spatial and temporal delivery of PTX (the apoptosis-inducer) and Berberine (the microenvironment-reeducator) represents a critical future direction for our laboratory, aiming to comprehensively tackle both the bioavailability limitation and chemotherapy-induced relapse in ovarian cancer.

Conclusion

In conclusion, the present study elucidates a dual-faceted mechanism underlying chemotherapy failure, wherein PTX-induced apoptosis inadvertently promotes ovarian cancer progression by accelerating residual cell repopulation via the caspase 3/$iPLA_2$/AA/COX-2/PGE_2 axis and concurrently driving M2 macrophage polarization. Crucially, Berberine emerges as a potent dual-inhibitor capable of abrogating both of these chemo-induced detrimental effects. Empowered by our advancing nanomedicine strategies, Berberine represents a highly promising, safe, and effective adjuvant candidate for long-term combination therapy to overcome tumor repopulation and prevent ovarian cancer relapse.

Declarations

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Use of artificial intelligence tools During the preparation of this work, AI tools were used to improve the readability and language of the manuscript, and subsequently, the authors revised and edited the content produced by the AI tools as necessary, taking full responsibility for the ultimate content of the present manuscript.

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