Progesterone Acts Synergistically with Niraparib in Ovarian Cancer by Promoting Ferroptosis via the PR/SCD1/ Fatty Acid Oxidation Pathway

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Article

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Abstract

PARP inhibitors (PARPi) play an increasing role in the treatment of ovarian cancer (OC). However, more than 40% BRCA1/2-deficient patients do not respond to PARPi, and wild-type BRCA cases have no significant benefit. Here, we demonstrated progesterone acted synergistically with niraparib in ovarian cancer cells with or without BRCA deficient. This synergism has been confirmed in the ovarian cancer organoid model and in vivo experiments. Furthermore, progesterone can enhance activity of niraparib in ovarian cancer via ferroptosis induction by upregulating progesterone receptor (PR)/SCD1-mediated fatty acid metabolism and enhancing mitochondrial damage. In clinical cases, PR and SCD1 were expressed at higher levels in PARPi sensitive cases compared with resistant cases, and high PR expression was associated with improved prognosis and higher sensitivity to PARPi in OC. This study provides new strategies for improving the efficacy of PARPi in ovarian cancer.

Introduction

Niraparib, one of PARP inhibitors approved as a maintenance therapeutic drug for recurrent ovarian cancer regardless of the presence of BRCA mutations, shows promising efficacy in patients with or without BRCA mutations [1, 2]. Although the PRIMA/PRIME and NOVA/NORA clinical studies found significant clinical efficacy of niraparib in patients with BRCA1/2 mutant ovarian cancer, the efficacy was reduced in wild-type BRCA patients, especially wild-type BRCA/HRD-negative[1-4]. Therefore, improving the efficacy of niraparib in patients without BRCA mutations is urgently required. Additionally, both the NOVA/NORA and PRIMA/PRIME studies found that niraparib causes adverse reactions in the digestive and vascular systems of patients with ovarian cancer. In the NOVA study, 14.7% of patients discontinued the treatment, 68.9% interrupted the treatment, and 66.5% reduced drug dosage due to side effects; after 4 months, only 25% of patients continued the treatment at the initial dose. Hence, de-escalating niraparib maintenance treatment while maintaining the clinical efficacy is important and feasible. The combination therapy of PARP inhibitors is a promising strategy to solve this problem[5, 6].

Progesterone is commonly used to reduce the toxic and side effects of primary cancer treatments, which protects the bone marrow and decreases digestive tract reactions in patients with malignant tumors [7-10]. Additionally, many studies have shown that progesterone can prevent the occurrence of ovarian cancer [10-15]. Furthermore, our previous study demonstrated progesterone could prevent ovarian cancer by inducing necroptosis of p53-defective fallopian tube epithelial cells through the TNF-a/RIPK1/RIPK3/MLKL pathway, accelerating ROS and DNA damage in ovarian cancer cells [16]. Intracellular ROS levels and the extent of DNA damage are closely associated with tumor cell sensitivity to PARP inhibitors [17, 18], suggesting that progesterone might improve the efficacy of niraparib in the treatment of ovarian cancer. In this study, we confirmed that P4 enhances the activity of PARP inhibitors in the treatment of ovarian cancer and provided new strategies for expanding their indications, reducing the related side effects, and overcoming tumor resistance to PARP inhibitors.

Materials And Methods
**Patient cohort**

The current study selected ovarian cancer patients from June 2018 to November 2021 based on the pre-defined inclusion and exclusion criteria. Inclusion criteria were: (1) recurrent ovarian cancer; (2) surgery and platinum-based chemotherapy as the initial treatment or platinum-based chemotherapy alone; (3) PARP inhibitors as maintenance therapy. Exclusion criteria were: (1) disease progression within 6 months of remission with platinum-based chemotherapy; (2) no platinum-based chemotherapy; (3) maintenance therapy with a combination of PARP inhibitors and other targeted agents. Progression-free survival (PFS) was the time starting from the date of ovarian cancer diagnosis to the date of disease recurrence or progression in patients with recurrent ovarian cancer or discontinuation of follow-up.

**Cell culture**

The human BRCA2 mutated ovarian cancer PEO1 and wild-type BRCA ovarian cancer OVCAR3, SKOV3, and A2780 cell lines, as well as the mouse-derived ovarian cancer ID8 cell line were obtained from the Cancer Institute, Central South University. PEO1 and ID8 cells were cultured in DMEM High-Glucose medium (Gibco, Life Technologies, Eugene, OR, USA) containing 10% FBS (Menlo Park, CA, USA), 100 μg/mL penicillin, and 100 U/mL streptomycin. OVCAR3, SKOV3, and A2780 cells were cultured in RPMI 1640 (Gibco, Life Technologies) containing 10% FBS, 100 μg/mL penicillin, and 100 U/mL streptomycin. All cell lines were cultured at 37°C with 5% CO2.

**CCK8 test**

Ovarian cancer cells were seeded into 96-well plates at 5×10^3/well and cultured for 24 hours. PEO1, OVCAR3, SKOV3, and A2780 cells were treated with vehicle control, niraparib, P4 plus niraparib (at concentration ratios of 1:10, 1:6, 1:4, 1:2, 1:1, and 1:0.5, respectively) or P4 for 48 hours. Then, 10 μl of the CCK8 reagent (K009-100; Zeta-Life) was added, and cells were incubated at 37°C with 5% CO2 in the dark for 2 h. Optical density was measured at 450 nm on a spectrophotometer. Based on the manufacturer’s instructions, combination indexes (CI) at indicated fraction affected (FA) levels were calculated with the CompuSyn software by the Chou-Talalay method with nonconstant-ratio combinations. IC50 values were calculated from optical density values. CI values were between 0.9 and 1.1 (additive effect), between 0.8 and 0.9 (low synergistic effect), between 0.6 and 0.8 (moderate synergistic effect), between 0.4 and 0.6 (high synergistic effect), and between 0.2 and 0.4 (strong synergistic effect), respectively.

**Colony formation assay**

PEO1 and OVCAR3 cells were seeded into a 6 cm-dish at 1×10^3/dish and cultured for 24 h. Then, the cells were administered niraparib (10 μM), P4 (10 μM), niraparib + P4 or vehicle control for 48 h. After formation, cell colonies were fixed with 95% alcohol for 30 min, stained with 1% crystal violet for 1.5 h and washed with water. The colony formation rate was determined after drying.

**Wound healing assay**
PEO1, OVCAR3, SKOV3, and A2780 cells were seeded into 6-well plates at 5×10^5/well. When the cells were ~90% confluent, using a sterile 10 µl pipette tip, the middle of the well was scratched. Next, the cells were administered niraparib (10 µM), P4 (10 µM), niraparib + P4 or vehicle control for 48 h, and cultured in DMEM High-Glucose medium or RPMI1640 containing 5% FBS. Images were acquired at 0, 24 h, and 48 h after drug administration, and the healing area on the scratch was calculated with the Image J software.

**Transwell migration assay**

PEO1, OVCAR3, SKOV3, and A2780 cells were seeded into a Transwell chamber at 5×10^5/well, in 160 µL of DMEM High-Glucose medium or RPMI 1640 without serum. The chambers were placed on a 24-well plate containing 600 µL/well of DMEM High-Glucose medium or RPMI 1640 with 10% serum. After 6 h of culture, the cells were administered niraparib (10 µM), P4 (10 µM), niraparib + P4 or vehicle control for 48 h. Then, the membranes with cells were fixed with 4% paraformaldehyde for 40 min and stained with 0.1% crystal violet solution for 20 min. Cells on the inner membrane were wiped with a cotton swab, and the chamber was washed with PBS to remove the excess staining solution. After drying, the chambers were imaged under a fluorescent inverted microscope, and the numbers of migrated cells were determined.

**Flow cytometry**

Cells apoptosis and cell cycle distribution in PEO1, OVCAR3, SKOV3, and A2780 cells were detected by flow cytometry. Cells seeded at 1×10^6/well into 6-well plates were administered niraparib (10 µM), P4 (10 µM), niraparib + P4 or vehicle control for 24 h. Cells were collected by trypsinization and centrifugation. The Annexin V/PI double-staining kit (cat. no. KGAV113; Nanjing KeyGen Biotech Co., Ltd., Jiangsu, China) was used to determine the proportion of apoptotic cells. For cell cycle analysis, cells were washed with pre-cold PBS and fixed in cold 90% ethanol at 4°C overnight. Then, the cells were washed with pre-chilled PBS, and the cell suspension was incubated with propidium iodide (PI) solution for 30 min and analyzed by flow cytometer.

**Immunofluorescence**

PEO1, OVCAR3, SKOV3, and A2780 cell slides were treated with niraparib (10 µM), P4 (10 µM), niraparib + P4 or vehicle control for 24 h. The cell slides were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% TritonX-100 in PBS. The samples were incubated with primary antibodies targeting γH2AX (ab81299; 1:200; Abcam, Cambridge, MA, USA), SCD1 (ab19862; 1:200; Abcam), and PR (ab2765; 1:200; Abcam), respectively, overnight at 4°C. This was followed by incubation with fluorescent-labeled secondary antibodies (A11034; 1:1000; Invitrogen, Eugene, OR, USA) in the dark for 1 h. Then, counterstaining was performed with DAPI, before analysis under a confocal laser scanning fluorescence microscope.

**Immunohistochemistry and immunofluorescence**
Paraffin sections from ovarian cancer tissue and mouse tumor tissue samples were deparaffinized with xylene and hydrated with different concentrations of alcohol. Antigen retrieval was performed after heating in citrate buffer (Beyotime Biotechnology, Shanghai, China). The tissue samples were incubated at room temperature with an endogenous peroxidase blocker (CWBio, Jiangsu, China). This was followed by overnight incubation at 4°C with primary antibodies against Ki67 (ab6667; 1:200; Abcam), Caspase-1 (ab138483; 1:200; Abcam), PR (ab2765; 1:200; Abcam), or SCD1 (ab19862; 1:200; Abcam). For immunohistochemistry, the tissue sections were incubated with enhanced enzyme-labeled goat anti-mouse/rabbit IgG (AWS0003a/AWS0002a; 1:1000; Abiowell) at room temperature for 20 min, with subsequent staining with the DAB kit (CWBio). For immunofluorescence, fluorescent-labeled secondary antibodies (A11034; diluted 1:1000 with BSA solution; Invitrogen) were added to the tissue samples, followed by incubation for 2 h at room temperature in the dark. After a PBS wash, 0.5 µg/mL DAPI 5% BSA solution was added (C1005; Beyotime Biotechnology) for staining and an appropriate amount of anti-fluorescence attenuation dose was added and mounted.

Animal experiments

Mouse ovarian in situ tumorigenesis model and intraperitoneal tumorigenesis experiment

Female C57BL/6 and BALB-nude mice at 6-7 weeks of age were purchased from SLA Laboratory. Female C57BL/6 mice were intraperitoneally injected with 1×10^5/mL ID8 cells, and female BALB-nude mice were injected 2×10^5/mL OVCAR3 cells into ovarian-tubal intrabursally or peritoneally. Then the mice were divided into 4 groups, including Vehicle, P4 (5 mg/kg P4 by intramuscular injection), Niraparib (50 mg/kg niraparib by gavage), and P4 plus Niraparib (5 mg/kg progesterone and 50 mg/kg niraparib) groups. Mice were continuously treated 3 times per week, and animal body weights and ascites were observed. Mice were sacrificed after 4 weeks of treatment, and tumor sizes, volumes, and tumor invasion and metastasis were recorded. Mouse tumors were fixed with 10% formalin, embedded in paraffin, and cut into 3 μm sections. Survival analysis of tumor-bearing mice after treatment with control, P4, niraparib, and P4 plus niraparib based on the methods mentioned above was also performed.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from OVCAR3, SKOV3, A2780, and PEO1 cells with TRIzol reagent (Ambion Inc., Austin, TX, USA). Total RNA was reverse transcribed into complementary DNA (cDNA) with PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa Biotechnology, Dalian, China), following the manufacturer's instructions. TB Green Premix Ex Taq II (TaKaRa Biotechnology) was used for qRT-PCR on a LightCycle 480 II instrument. All primers were synthesized by Sangon Biotechnology Company (Shanghai, China), and are listed in Supplementary Table 1. The relative mRNA expression was calculated by the 2^-ΔΔct method.

Western blot
Proteins from OVCAR3, SKOV3, A2780 and PEO1 cells were extracted with RIPA lysis buffer, and protein concentrations were determined with the BCA kit. Each sample was loaded at 30 µg, subjected to SDS-PAGE electrophoresis and transferred onto PVDF membranes (Millipore, Burlington, MA, USA). PVDF membranes were blocked with 5% skimmed milk (Biosharp, Hefei, Anhui, China) for 2 h and incubated overnight at 4°C with primary antibodies against PR, H2AX, and SCD1. Bound antibodies were then detected with HRP-conjugated secondary antibodies (AWS0003a; 1:1000; Abiowell). The ECL Western blotting substrate (Advansta, Munich, Germany) was used for visualization.

Statistical analysis

All data were obtained from three independent replicates, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed with SPSS version 18.0 or GraphPad. Two-sample t-test or non-parametric test was used to compare group pairs. The Chi-square test was used for categorical data. Kaplan-Meier analysis was performed to assess the prognosis of ovarian cancer patients, and the log-rank test was performed to compare survival. P<0.05 was considered statistically significant.

Results

1. P4 sensitizes ovarian cancer cells to niraparib by enhancing niraparib-mediated DNA damage and apoptosis.

The effects of combined P4 and niraparib (a PARP inhibitor) on cell growth were assessed in 4 ovarian cancer cell lines, including *BRCA* wild-type (*BRCA*WT) ovarian cancer OVCAR3, SKOV3, and A2780 cells and *BRCA2* mutated (*BRCA2*Mut) ovarian cancer PEO1 cells. Ovarian cancer cells were examined to determine whether P4 can sensitize to niraparib. PEO1 cells were treated with different concentrations of P4 plus niraparib (niraparib: P4=1:10, 1:6, 1:4, 1:2, 1:1 and 1:0.5) for 48 h. Synergetic effects (ED50 of <1) were observed for P4 plus niraparib at concentration ratios of niraparib:P4 between 1:4-1:0.5; the best combination index (CI) was found at niraparib: P4=1:1 in SKOV3, A2780, and PEO1 cells (Table 1, Figure 1A, and Supplementary Figure 1A). Niraparib-induced growth inhibition (green line) was significantly enhanced by the combined treatment (black line) in all 4 ovarian cancer cell lines tested regardless of *BRCA1* mutation status (Figure 1B and Supplementary Figure 1A). Moreover, P4 sensitized cancer cells to niraparib reflected by lower IC50 values for niraparib in OVCAR3, SKOV3, A2780, and PEO1 cells (Figure 1C & D). Further, compared with the *BRCA2* mutated cell line (PEO1), P4 significantly reduced IC50 in wild-type *BRCA* cell lines (OVCAR3, SKOV3, and A2780); the IC50 of OVCAR3 was reduced from 23.07 (20.79 to 25.60) µM to 5.903 (3.816 to 9.131) µM, the IC50 of SKVO3 was reduced from 23.39 (16.78 to 32.59) µM to 5.580 (2.671 to 11.65) µM, and the IC50 of A2780 was reduced from 33.22 (29.55 to 37.34) µM to 12.340 (7.978 to 19.08) µM, while the IC50 of PEO1 was reduced from 4.953 (4.414 to 5.558) µM to 2.410 (1.422 to 4.084) µM (Figure 1D). Importantly, the ovarian cancer organoid model confirmed the synergistic effects of P4 and niraparib: P4 combined with niraparib exerted satisfactory synergistic effects (ED50 of <1), and significantly inhibited cell viability both in niraparib-sensitive and resistant
cases (Figure 1E &F, Table 2). Both niraparib and P4 + niraparib inhibited organoid tissue formation in ovarian cancer (Figure 1F). Clone formation, cell scratching, and Transwell assays were performed in \( BRCA^{WT} \) and \( BRCA^{Mut} \) ovarian cancer cells. Combined treatment with P4 and niraparib significantly decreased the numbers and sizes of colonies (Supplementary Figure 1C) and inhibited cell invasion (Supplementary Figure 1D) and metastasis (Supplementary Figure 1E) in both \( BRCA^{WT} \) and \( BRCA^{Mut} \) cells compared with either niraparib or P4 alone.

DNA damage is related to sensitivity to the PARP inhibitor. To investigate whether P4 sensitizes niraparib in ovarian cancer cells by inducing DNA damage, the effect of P4 on niraparib-mediated induction of DNA DSBs was tested. P4 or niraparib both modestly increased the phosphorylation levels of H2AX (\( \gamma \)-H2AX), whereas combined treatment with P4 and niraparib in SKOV3, OVCAR8, A2780, and PEO1 cells increased the levels of \( \gamma \)-H2AX significantly (Figure 2A & B). Given that unrepaired DSB can trigger apoptosis and inhibit cell proliferation, the annexin V/PI kit was measured to determine whether the combination of P4 and niraparib induced apoptosis and cell cycle arrest. Flow cytometry analysis demonstrated cell cycle arrest in the S phase in the niraparib plus P4 group (Figure 2C). P4 combined with niraparib treatment significantly induced apoptosis compared with either single agent in OVCAR3, SKOV3, A2780, and PEO1 cells (Figure 2D), consistent with the critical prerequisite of DNA DSB repair for cancer cell survival. Taken together, these results suggested that P4 enhanced the sensitization of ovarian cancer cells to niraparib by preventing DNA DSB repair and inducing severe apoptosis.

2. P4 enhances the antitumor effect of niraparib \textit{in vivo} and prolongs ovarian cancer survival.

While treatment with P4 induced niraparib-mediated inhibition in ovarian cancer cells, the peritoneal tumorigenesis mouse model and ovarian in situ tumorigenesis mouse model were established to confirm the synergistic effects of P4 and niraparib in vivo. Mice were divided into 4 groups and treated with vehicle, P4, niraparib, or the P4 and niraparib combination. In the ovarian \textit{in situ} tumorigenesis model, after 15 days of administration of the drug, CT images showed that ovarian tumor volumes were smaller in the P4+niraparib combination group compared with the niraparib alone group. Representative images were shown in Figure 3A&B. The animals were sacrificed after 28 days of dosing and niraparib, P4+niraparib, and P4 inhibited tumor growth (Figure 3C & D); ovarian tumor volumes were smaller in the combination group compared with the niraparib alone group both in the ovarian \textit{in situ} (Figure 3E) and peritoneal (Figure 3F) tumorigenesis mouse models. IHF showed obvious \( \gamma \)-H2AX expression in the P4+niraparib group (Figure 3G). IHC and the TUNEL assay demonstrated that the cell proliferation-associated antigen Ki67 was suppressed and apoptosis-related markers were induced in the P4+niraparib group, compared with the niraparib alone cohort in the mouse tumor tissue (Figure 3H). Survival experiments showed that the P4+niraparib group significantly prolonged the survival time compared with niraparib alone in C57BL/6j mice (Figure 3I). In vivo experiments confirmed the above in vitro data, with P4 enhancing niraparib antitumor activity by inducing niraparib-mediated DNA damage and apoptosis in ovarian cancer cells.
3. P4 acts synergistically with niraparib by promoting SCD1 expression activated by progesterone receptor (PR) in ovarian cancer cells.

To further explore the mechanisms by which P4 promotes sensitivity to niraparib in ovarian cancer cells, transcriptomic sequencing of ovarian cancer cells administered vehicle control, niraparib, niraparib plus P4, or P4 was performed. Transcriptomic sequencing revealed that multiple lipid metabolism-related genes were significantly upregulated in the combined treatment group compared with niraparib (Figure 4A). In transcriptomic sequencing analysis of differential gene expression, 69 genes were upregulated and 7 were downregulated compared with niraparib+P4 and niraparib alone (Figure 4B). To confirm the RNA-Seq results, 10 significantly upregulated genes involved in fatty acid metabolism (SCD, FASN, DHCR7, MVD, EVT4, Per1, ACSS2, INSIG1, PSCK9, and FDFT1) were selected and analyzed by quantitative reverse-transcription PCR (RT-qPCR) in OVCAR3, SKOV3, A2780, and PEO1 cells (Supplementary Figure 2A). Treatment with P4 combined with niraparib significantly increased the expression of stearoyl-CoA desaturase (SCD) compared with niraparib alone in both cell lines tested (Figure 4C). WB and IHF confirmed that the SCD1 protein was upregulated in the combination group compared with niraparib or P4 treatment group both in ovarian cancer cells (Figure 4D & E) and mouse ovarian tumor tissues (Supplementary Figure 2B). Flow cytometry analysis demonstrated that a SCD1 inhibitor rescued the apoptosis induced by P4 plus niraparib (Figure 4F); the CCK8 test showed that the SCD1 inhibitor rescued the inhibitory effect of P4 plus niraparib on ovarian cancer cells (Supplementary Figure 2C). The overexpression of SCD1 in ovarian cancer cells (Supplementary Figure 2D) significantly inhibited cell proliferation (Supplementary Figure 2E) and enhanced the inhibitory effect of P4 plus niraparib on ovarian cancer cells (Supplementary Figure 2F). Suggested that P4 enhanced the antitumor activity of niraparib by upregulated SCD1 expression in ovarian cancer cells.

How P4 and niraparib combination upregulates SCD1 was further explored. We predicted the transcription factors of the SCD promoter via http://genome.ucsc.edu/, it’s shown that SCD promoter could be bound by progesterone receptor-A(PR-A) and PR-B, SREBP1 and SP1 etc. (Supplementary Figure 2G). It’s well known that progesterone action is mediated by intracellular progesterone receptors (PR-A, and PR-B) that regulate target gene transcription[19-21]. However, whether progesterone receptors can regulate the expression of SCD1 has not been reported. Chromatin immunoprecipitation assay (ChIP) provides direct evidence for PR binding to SCD gene promoter in this study (Figure 4G-H); the SREBP1, which was identified as positive effectors of SCD transcription [22, 23], was selected as positive control. WB and IHF found that PR was expressed in both cytoplasm and nucleus of ovarian cancer cells (Figure 4I-J). According to the above work, suggested that progesterone receptors can directly bind to the promoter of SCD gene, and act as a positive effector of SCD transcription.

4. P4 enhances the activity of niraparib in ovarian cancer by promoting ferroptosis through SCD1-mediated fatty acid metabolism.

SCD1 was the rate-limiting enzyme required for the production of monounsaturated fatty acids from saturated fatty acids, e.g., converting palmitic acid (16) to palmitoleic acid (16, 1) [24, 25]. To assess
whether P4 enhances the antitumor activity of niraparib in ovarian cancer cells by regulating fatty acid metabolism, the metabolomics of ovarian cancer cells treated with vehicle control, niraparib, niraparib plus P4, or P4 were investigated. Lipid oxidation was significantly enhanced in the niraparib plus P4 group compared with niraparib treatment alone (Figure 5A). Further, quantitative metabolomics demonstrated that fatty acid metabolism was significantly enhanced in the combination treatment group (Figure 5B), which was consistent with the above RNA-Seq analysis (Figure 4A). Interestingly, quantitative metabolomics also demonstrated that production of palmitoleic acid (POA) and myristoleic acid (MA) mediated by SCD1 was increased in the P4 plus niraparib group compared with the niraparib alone group (Figure 5C). P4 may upregulate the oxidative metabolism of fatty acids by upregulating SCD1. The CCK8 assay showed that POA, not MA, enhanced the inhibitory effect of niraparib in ovarian cancer cells (Figure 5D). Similar to P4, POA enhanced niraparib’s antitumor activity by inducing niraparib-mediated DNA damage, whereas combined treatment with POA and niraparib significantly increased the levels of γ-H2AX (Figure 5E&F).

The oxidative metabolism of fatty acids, especially lipid peroxidation, is closely related to ferroptosis [26]. In this study, BODIPY™ 581/591C11 staining was used to quantify lipid peroxidation. Niraparib plus POA markedly induced lipid peroxidation in ovarian cancer cells (Figure 5G) and inhibited the expression of GPX4, which detoxified phospholipid peroxidation and protected the cells from ferroptosis (Figure 5H). Similar results were observed in cells treated with P4 in combination with niraparib; treatment with P4 combined with niraparib significantly induced lipid peroxidation in the ovarian cancer cell lines, and the potent ferroptosis inhibitor liproxstatin-1 abolished the accumulation of lipid peroxides induced by niraparib plus P4 (Figure 6A). Consistently, niraparib plus P4 significantly inhibited the expression of GPX4 both in vivo (Figure 6B & C) and in vitro (Supplementary Figure 3A), and liproxstatin-1 rescued the inhibitory effect of P4 plus niraparib on GPX4 expression (Figure 6B & D). Meanwhile, IHF and flow cytometry revealed Fe²⁺ amounts were significantly increased in the combination group compared with niraparib or P4 alone (Figure 6A and Supplementary Figure 3B). P4 increased the inhibitory effect of niraparib in ovarian cancer cells by promoting ferroptosis.

Several metabolic processes in mitochondria play important roles in triggering ferroptosis [27], and the generation of mitochondrial ROS is critical for lipid peroxidation and ferroptosis onset. In this study, P4 combined with niraparib induced severe mitochondrial damage (Figure 6F) and increased ROS levels in OVCAR3 cells compared with niraparib alone (Supplementary Figure 3C). In addition, liproxstatin-1 and the ROS scavenger N-acetyl-L-cysteine (NAC) rescued the inhibitory effect of niraparib plus P4 on ovarian cancer cells (Supplementary Figure 3D). Hence, P4 may enhance the efficacy of niraparib in ovarian cancer by promoting mitochondrial damage and ROS production.

5. Elevated expression of progesterone receptor (PR) is linked to better prognosis and higher sensitivity to PARP inhibition in ovarian cancer patients.

To evaluate the clinical relevance of the study, patients with ovarian cancer treated with PARP inhibitors as first-line maintenance therapy according to the inclusion and exclusion criteria were selected. IHC and
IHF showed that PR was expressed at higher levels in PARPi sensitive cases compared with PARPi resistant cases (Figure 7A & B). Kaplan-Meier survival analysis found that high expression of PR was associated with improved prognosis (Figure 7C); the chi-square test revealed that high expression of PR was associated with greater sensitivity to PARP inhibitors in ovarian cancer patients (Supplementary Table 1). Meanwhile, low GPX4 and high SCD1 expression levels were found in PARPi sensitive cases, versus high GPX4 and low SCD1 amounts in PARPi resistant cases (Figure 7D, E & G, H), consistent with our previous results. In addition, low GPX4 expression was associated with improved prognosis (Figure 7F) and higher sensitivity to PARP inhibitors in ovarian cancer patients (Supplementary Table 2). TCGA data analysis showed that high SCD1 expression was associated with improved prognosis in ovarian cancer patients (Figure 7I).

**Discussion**

Based on PRIMA and NOVA trial, niraparib was approved by the Food and Drug Administration (FDA) for maintenance treatment of patients with newly diagnosed advanced or recurrent epithelial ovarian, fallopian tube, or primary peritoneal cancer who have had a response to platinum-based chemotherapy, regardless of homologous recombination (HR) or BRCA status. Although niraparib can achieve all comer benefits, it is not difficult to find that BRCA1/2-mutant ovarian cancer patients benefit the most from niraparib maintenance therapy, followed by wild-type BRCA. [1, 2, 28-30]. In this study, progesterone and niraparib exhibited synergistic effects in ovarian cancer cells regardless of BRCA mutation status, but these synergistic effects were more significant in wild-type BRCA ovarian cancer cells. The mass ratios of progesterone to niraparib in the combination were 1:1-4:1, and the best concentration ratio was 1:1. Niraparib plus P4 significantly inhibited cell viability and reduced IC50 compared with niraparib alone in ovarian cancer cell lines with or without BRCA mutation. Importantly, the ovarian cancer organoid model confirmed the synergistic effects of P4 and niraparib; P4 combined with niraparib had satisfactory synergistic effects (ED50<1) and significantly inhibited cell viability both in niraparib-sensitive and resistant cases (Figure 1, Table 2). Combined treatment with P4 and niraparib significantly decreased colony formation and metastasis in both BRCAWT and BRCA2Mut cells compared with either niraparib or P4 alone.

Progesterone has been used for over 80 years [31-33]. Progesterone, a low-toxicity therapeutic that targets various key pathways, is crucial for cancer treatment and prevention [16, 34]. In this study, WB and IHF revealed obvious γ-H2AX expression in ovarian cancer cells and tumor tissues in the niraparib plus P4 group. Meanwhile, flow cytometry analysis demonstrated combined treatment with P4 and niraparib significantly induced apoptosis and cell cycle S phase arrest in the niraparib plus P4 group. It should be noted that DNA damage may be an important determinant of PARP inhibitor sensitivity. Niraparib is a highly selective inhibitor of PARP-1 and PARP-2, which are sensors of DNA damage that are most active during the S-phase of the cell cycle [35]. These findings suggested that P4 may enhance the effects of PARP inhibitors by causing DNA damage and inducing severe apoptosis.
Progesterone, which is traditionally known to function via nuclear receptors for transcriptional regulation, increases oxidative cellular respiration and beta-oxidation [36, 37]. This study demonstrated that progesterone promotes lipid oxidation and palmitoleic acid (POA) production through upregulation of SCD1, resulting in the promotion of ferroptosis. Further, we demonstrated for the first time that that progesterone upregulated SCD1 by PR, which is a transcription factor that binds to the SCD promoter.

PARP inhibition promotes ferroptosis and synergizes with ferroptosis inducers in BRCA-proficient ovarian cancer, as shown in our previous research [38]. Ferroptosis is driven by the accumulation of lipid peroxides generated on phospholipid membranes, damages the cell membrane, and is mainly associated with the production of divalent iron or ester oxygenase-mediated lipid peroxidation from unsaturated fatty acids, with significant increases in lipid peroxidation and ROS due to reduced expression/activity of glutathione peroxidase 4 (GPX4), which is a core regulatory enzyme of the antioxidant system (glutathione system) [39-41]. In this study, transcriptomic sequencing and metabolomics identified that lipid oxidation was significantly enhanced in the niraparib plus P4 group compared with the niraparib alone group, and niraparib plus P4 markedly induced lipid peroxidation in ovarian cancer cell lines and significantly inhibited GPX4 expression, while liproxstatin-1 rescued the above changes. The sources of ROS responsible for lipid peroxidation were also increased in the niraparib plus P4 group.

Both the NOVA/NORA and PRIMA/PRIME studies found that niraparib causes adverse reactions in the digestive and vascular systems in patients with ovarian cancer. In the PRIMA study, 12% of drug withdrawal, 79.5% of discontinued treatment, and 70.9% of reduced drug dosage occurred due to side effects. Inspirationally, after adopting an individualized starting dose of niraparib, a significant improvement in safety was observed in PRIME study. The proportion of patients with treatment suspension decreased to 62.7%, dose reductions related to TEAEs only occurred in 40.4% of patients in the niraparib groups, TEAEs resulting in discontinuation of treatment was only 6.7% in the niraparib treatment. What's more, the hazard ratio for PFS of wild-type BRCA/HRD-negative subgroup in the PRIME study showed a comparable reduction in risk of disease progression or death for niraparib versus placebo (PRIME: HR = 0.41; PRIMA: HR = 0.68), and the median PFS value of wild-type BRCA/HRD-negative patients reported in niraparib group of the PRIME trial was also numerically higher than those in the PRIMA trial, which may reflect the enhanced tolerability of the individualized starting dose, and may also be due to variability in the patient populations of the two studies. However, the median PFS value of wild-type BRCA/HRD-negative patients in niraparib group of PRIME study was still only 8.5 months higher than that of the placebo group, which means to improve the efficacy of niraparib in patients with wild-type BRCA/HRD-negative is still extremely urgent. Progesterone is often used to reduce the toxic and side effects associated with tumor treatment, to protect the bone marrow, and to treat anorexia and cachexia in patients with malignant tumors [7-10]. The combination of progesterone and niraparib may not only enhance the efficacy of niraparib in ovarian cancer but also reduce its toxic side effects and improve the quality of life of patients.

Taken together, Progesterone enhances niraparib activity synergistically in ovarian cancer by promoting ferroptosis via PR/SCD1-mediated fatty acid oxidation (Figure 8). This study provides new strategies for
expanding clinical indications, reducing toxic side effects and overcoming drug resistance to PARP inhibitors in ovarian cancer.

**Declarations**

**Acknowledgments**

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**Author Contributions**

NYW contributed to molecular experiments and manuscript writing. CF, XZ and MCZ contributed to sample collection and animal experiments. CF, LJ, LWT and XMX contributed to mouse CT photography and analysis. ZBW, YW and HL contributed to technical support for data analysis and study conception. QJL, YJZ and JW contributed to study conception and design, and the final approval of the submitted version.

**Declaration of Interests**

No potential conflict of interest was reported by the authors.

**Ethics approval and consent to participate**

The study protocol was approved by the Institutional Review Board of Hunan Cancer Hospital and the Animal Ethics Committee of Hunan Cancer Hospital.

**References**


Tables

Table 1 Synergistic effect of P4 plus niraparib at different ratios.

<table>
<thead>
<tr>
<th>Ratio(Nira:P4)</th>
<th>1:10</th>
<th>1:6</th>
<th>1:4</th>
<th>1:2</th>
<th>1:1</th>
<th>1:0.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>PEO1 CI(ED50)</td>
<td>1.02</td>
<td>0.92</td>
<td>0.91</td>
<td>0.45</td>
<td>0.38</td>
<td>0.45</td>
</tr>
<tr>
<td>OVCAR3 CI(ED50)</td>
<td>1.26</td>
<td>1.34</td>
<td>0.56</td>
<td>0.33</td>
<td>0.45</td>
<td>0.01</td>
</tr>
<tr>
<td>SKOV3 CI(ED50)</td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>0.21</td>
<td>0.19</td>
<td>0.34</td>
</tr>
<tr>
<td>A2780 CI(ED50)</td>
<td>\</td>
<td>\</td>
<td>\</td>
<td>0.53</td>
<td>0.25</td>
<td>0.41</td>
</tr>
</tbody>
</table>

ED50: median effective dose

CI value:

0.9≤CI≤1.1, the combination of the two drugs has superposition effect;

0.8≤CI<0.9, the combination of the two drugs has slight synergistic effect;

0.6≤CI<0.8, the combination of the two drugs has moderate synergistic effect;

0.4≤CI<0.6, the combination of the two drugs has satisfied synergistic effect;

0.2≤CI<0.4, the combination of the two drugs has great synergistic effect.

Table 2 Synergistic effect of niraparib plus P4 in organoid tissue.
<table>
<thead>
<tr>
<th>Case</th>
<th>CI</th>
<th>Niraparib Sensitivity (ED50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case2</td>
<td>0.55</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Case3</td>
<td>0.73</td>
<td>Resistance</td>
</tr>
<tr>
<td>Case9</td>
<td>1.02</td>
<td>Sensitive</td>
</tr>
<tr>
<td>Case10</td>
<td>0.61</td>
<td>Resistance</td>
</tr>
<tr>
<td>Case11</td>
<td>0.65</td>
<td>Resistance</td>
</tr>
<tr>
<td>Case12</td>
<td>0.54</td>
<td>Sensitive</td>
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<tr>
<td>Case13</td>
<td>0.78</td>
<td>Resistance</td>
</tr>
<tr>
<td>Case14</td>
<td>0.72</td>
<td>Resistance</td>
</tr>
<tr>
<td>Case15</td>
<td>0.68</td>
<td>Resistance</td>
</tr>
<tr>
<td>Case16</td>
<td>0.47</td>
<td>Sensitive</td>
</tr>
</tbody>
</table>

**ED50** median effective dose

**CI value**

0.9 ≤ CI ≤ 1.1, the combination of the two drugs has superposition effect;

0.8 ≤ CI < 0.9, the combination of the two drugs has slight synergistic effect;

0.6 ≤ CI < 0.8, the combination of the two drugs has moderate synergistic effect;

0.4 ≤ CI < 0.6, the combination of the two drugs has satisfied synergistic effect;

**Figures**
Figure 1

Progesterone enhances the inhibitory effect of niraparib in ovarian cancer.

(A) P4 plus niraparib combination treatment at a ratio of 1:1 in human BRCA2 mutated ovarian cancer PEO1 and wild-type BRCA ovarian cancer OVCAR3, SKOV3 and A2780 cells; CI of <1 (under the line) suggested a synergy of the two drugs. (B) The CCK8 assay was performed to assess cell viability in
PEO1, OVCAR3, SKOV3 and A2780 cells treated with vehicle, P4 (blue line), niraparib (green line), and P4 plus niraparib (black line) for 48 h. (C, D) The half maximal inhibitory concentrations (IC50s) of PEO1, OVCAR3, SKOV3, and A2780 cells were calculated with GraphPad Prism. (E) Cell viability in 10 ovarian cancer organoid tissue cases was tested after treatment with vehicle (NC), niraparib or P4 plus niraparib for 5 days. (F) Representative images of ovarian cancer organoid tissues, acquired by light microscopy. *P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 2

**P4 enhances the sensitivity of ovarian cancer cells to niraparib by inducing DNA damage and apoptosis.**

Ovarian cancer cells were treated with vehicle, niraparib (10 µM), P4 (10 µM) plus niraparib (10 µM), or P4 (10 µM) for 24 h, and immunofluorescence (A) and Western blot (B) were performed to assess γ-H2AX expression. Flow cytometry was carried out to examine the cell cycle (C) and apoptosis (D). *P < 0.05; **P < 0.01; ***P < 0.001.
Figure 3

P4 enhances the efficacy of niraparib in ovarian cancer in vivo.

Representative 3D CT images (A) and CT scans (B) of ovarian in situ tumorigenesis mice before treatment (0 day) and 15 days after drug administration; (C,E) ovarian in situ tumorigenesis mice and (D,F) intraperitoneal tumorigenesis mice treated with vehicle, niraparib (50 mg/kg), P4 plus niraparib (5 mg/kg) and P4.
mg/kg progesterone and 50 mg/kg Niraparib), or P4 (5 mg/kg); mice were sacrificed after 28 days of drug administration, the ovaries and fallopian tubes as well as tumors were removed, and tumor sizes and weights were obtained. (G) Immunofluorescence and γ-H2AX expression in mouse tumor tissue samples; (H) IHC of the cell proliferation-associated antigen Ki67 and the TUNEL test for apoptosis-related markers in the mouse tumor tissue. (I) Kaplan-Meier survival analysis of the survival of C57BL/6 mice in 4 different treatment groups.
Figure 4

**P4 enhances the activity of niraparib by upregulating SCD1 in ovarian cancer cells.**

(A) GO analysis of differentially expressed genes shared by P4 plus niraparib and niraparib treatments. (B) The numbers of transcripts upregulated and downregulated at 2-fold or more after treatment with P4 plus niraparib or niraparib were presented by volcano maps. (C)qPCR, (D)Western blot and (E)IHF were performed to assess ovarian cancer cell expression of SCD1 after treatment with vehicle, niraparib (10 µM), P4 (10 µM) plus niraparib (10 µM), or P4 (10 µM) for 24 h. (F) In ovarian cancer cells treated with vehicle or P4 (10 µM) plus niraparib (10 µM) with or without SCD1 inhibitor (1 µM), flow cytometry analysis of cell apoptosis was performed. (G) ChIP assays were performed in SKOV3 and A2780 cells using PR antibody or SREBP1 antibody as positive control. Q-PCR and agarose gel electrophoresis analyses of ChIP samples were performed. (I) Western blot and (J)IHF were performed to assess ovarian cancer cell expression of PR after treatment with vehicle, niraparib (10 µM), P4 (10 µM) plus niraparib (10 µM), or P4 (10 µM) for 24 h.*P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 5

P4 promotes ferroptosis by upregulating fatty acid oxidation and POA production.

(A) Enrichment analysis results of metabolomics. Each pathway corresponds to a horizontal bar, the darker the color, the smaller the p-value of the differential metabolites enriched in the pathway; the length of the horizontal bar represents the fold enrichment. (B) The Z score heatmap of the overall profile of
metabolites. (C) Detection of POA and MA in OVCAR3 cells after 48 h of treatment with vehicle, niraparib (20 µM), P4 (20 µM) + Niraparib (20 µM), or P4 (20 µM). (D) CCK8 was used to assess the viability of OVCAR3 cells administered different treatments. IHF (E) and Western blot (F) were performed to assess the expression of γH2AX in OVCAR3 and SKOV3 cells. (G) BODIPY™ 581/591C11 staining was performed to determine the percentages of lipid peroxidation in OVCAR3 cells administered vehicle, niraparib, niraparib plus POA, and POA. (H) IHF and WB were performed to assess GPX4 expression. *P < 0.05; ** P < 0.01; *** P < 0.001.
Figure 6

P4 enhances the activity of niraparib in ovarian cancer by promoting ferroptosis and mitochondrial damage.

(A) BODIPY™ 581/591C11 staining was performed to quantify lipid peroxidation in OVCAR3 and SKOV3 cells administered vehicle control, niraparib (10 µM), P4 (10 µM) + Niraparib (10 µM), P4 (10 µM), and P4 (10 µM) + Niraparib (10 µM) + Liproxstatin-1 (1 µM). (B, C, D) IHF and Western blot were performed to test GPX4 expression in ovarian cancer cells administered different treatments. (E) IHF was carried out to determine Fe^{2+} levels in OVCAR3, A2780, SKOV3 and PEO1 cells administered vehicle control, niraparib (10 µM), P4 (10 µM) + Niraparib (10 µM), and P4 (10 µM) for 24h. (F) Transmission electron microscopy analysis of mitochondrial damage in OVCAR3 cells administered vehicle control, niraparib (10 µM), P4 (10 µM) + Niraparib (10 µM), and P4 (10 µM).
Figure 7

Expression of PR, GPX4, and SCD1 in PARP inhibitor-sensitive and resistant ovarian cancer tissues and their relationships with prognosis in ovarian cancer.

(A, B) PR, (D, E) GPX4 and (G, H) SCD1 levels in PARP inhibitor-sensitive and resistant ovarian cancer tissues. (C, F) Kaplan-Meier survival analysis for assessing progression free survival (PFS) in ovarian cancer.
cancer patients administered a PARP inhibitor as the first-line maintenance therapy. (I) Kaplan-Meier Plotter Website analysis of SCD1 expression and prognosis of ovarian cancer patients in TCGA.

Figure 8
Legend not included with this version.

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