Original Research Article

Synergetic effect of poly (ADP-ribosyl) polymerase (PARP) inhibitor and cisplatin on ovarian cancer

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Abstract

Purpose: To investigate the effect of the combination of poly (ADP-ribosyl) polymerase (PARP) inhibitor, talazoparib (BMN673), and cisplatin on the proliferation and apoptosis of ovarian cancer cells in vitro, and on xenograft tumors of ovarian cancer cells in vivo.
Methods: Cell viability was determined by CCK-8 assay, while cell proliferation and cell cycle were assessed using colony formation assay and flow cytometry, respectively. Cell apoptosis was evaluated by flow cytometry and TUNEL assays. Western blot assay was used to measure the expression levels of proliferation- and apoptosis-related proteins.
Results: The PARP inhibitor, BMN673, produced a dose-dependent synergistic effect with cisplatin (p < 0.05). Compared with when cisplatin or BMN673 was used alone, the combination of cisplatin and BMN673 significantly inhibited the growth of transplanted tumors (p < 0.05). Immunohistochemical analysis showed that cisplatin and BMN673 treatment increased the number of cells positive for TUNEL, but reduced the population of cells positive for Ki67.
Conclusion: Thus, BMN673 and cisplatin are synergistic against ovarian cancer cells, and therefore, should be subjected to further investigations, including clinical trials, to determine the potentials of the combination for the management of ovarian carcinoma.

Keywords: PARP inhibitor, Ovarian cancer, Xenografted tumor, Cisplatin

INTRODUCTION

Ovary carcinoma is the commonest gynecologic tumor and the topmost cause of mortality in patients with cancer of the reproductive organs [1]. Most cases of ovarian cancer have no early symptoms and are diagnosed in the late stages of metastasis (about 70%). The traditional treatment for advanced ovarian cancer is based on cell reduction surgery, followed by platinum-based chemotherapy [2]. Although some patients with epithelial ovarian cancer (EOC) respond reasonably well to clinical treatment, most patients remain uncured. Indeed, about seventy percent of patients relapse and eventually die from the disease [3].

Poly (ADP-ribose) polymerase is a rich nucleoprotein involved in DNA repair, cell death, regulation of telomere and maintenance of

genomic integrity [4,5]. In addition, a PARP inhibitor such as cisplatin has been known to strengthen cytotoxic effect in conventional chemotherapy in breast and ovarian cancers [6]. Cisplatin binds to DNA, causing cross-linking between and within strands, resulting in defects in DNA template, followed by disruption of the capacity of cancerous cells to synthesize and replicate DNA [7]. Some studies have reported the effect of combined application of PARP suppressors and cisplatin on several types of malignancies [8,9]. However, this has not been evaluated *in vitro* or *in vivo* in cells from ovarian cancer. Since recurrent ovarian cancer rapidly develops into chemo-resistant disease, there is no effective treatment available. Therefore, it is crucial to introduce new treatments to save the ovarian cancer patients.

It has been reported that BMN673 is a novel PARP1/2 inhibitor with an IC_{50} of 0.57 nM [5]. Although other drugs also suppress PARP, BMN673 produces 10 times or more the cytotoxic potency of other PARP inhibitors at nanomolar concentrations [10]. Thus, BMN673 produces the most potent inhibition of PARP, which may be attributed to the ability to sequester PARP bound to DNA [10]. The antitumor activity of BMN673 showed low potency in mammary and ovary cancer subjects with BRCA deficiency. Moreover, BMN673 has been evaluated in clinical trials, and it shows promise as an exciting new PARP1/2 inhibitor with optimal clinical benefits. In this study, it was found that BMN673, a PARP1 inhibitor, showed cooperative influence with cisplatin on ovarian cancer cells, and it enhanced the suppression of xenograft tumor. These results indicate that BMN673 has promising potential for use as therapy for ovarian carcinoma, in addition to conventional chemotherapy.

EXPERIMENTAL

Cells lines

Ovarian cancer cell lines (OVCAR-3 and SKOV-3) were cultured in RPMI1640 containing 10 % FBS. All cells were maintained in a 5 % CO_2 humidified atmosphere at 37 °C, and routinely passaged every 5 - 7 days. The test was carried out in a medium containing 1 % fetal bovine serum.

Drugs and irradiation preparation

The PARP inhibitor BMN673 purchased from Selleck Chem, was dissolved in DMSO for *in vitro* experiments. Radiation was generated by a 250 kv X-ray irradiator (RADSOURCE, USA).

Mouse xenograft model

Male BALB/C mice aged 6 weeks were kept under SPF state in an environment with equal light/dark periods, with unrestrained provision of standard chow and tap water. After the mice were anesthetized, the OVCAR-3 and SKOV-3 cells (5 × 10^6 cells/200 µL medium) were subcutaneously injected into the back skin of mice. Within two weeks, the tumor expanded to more than 7 millimeters in diameter. The cancer cell lines OVCAR-3 and SKOV-3 developed steadily into tumors. Therefore, the OVCAR-3 and SKOV-3 cell-derived xenograft tumors were employed for *in vivo* studies.

Ethical approval

All animal procedures conformed with the provisions of WHO guidelines for animal research [11]. The work was approved by the Ethical Committee of Shanghai Pudong Hospital (approval no. WZ-08).

Protocol for animal experiments

When tumor diameter reached up to 7 mm, 4 groups of mice were generated: control (0.02 mL physiological saline), Cisplatin (DDP) (2 mg/kg, solubilized in 200 µL sterile H_2O); (c) BMN673 (1 mg/kg, in 200 µL bactericidal medium), and (d) combination of DDP and BMN673. The drugs were injected into the abdomen five times every three days. BMN673 was injected intraperitoneally. Tumor size, tumor weight and tumor volume were measured [12].

Colony formation assay

The cells were treated according to specified conditions, cultured for 10 - 14 days, fixed in HCHO, washed with PBS and subjected to crystal violet staining. The colonies were photographed and counted with ImageJ software.

Cell viability studies

The CCK-8 assay kit was used to determine cell viability in line with the manufacturer's protocol. When the cell density reached 80 %, 0.01 mL of CCK-8 was added, followed by incubation for 2 - 3 h. The absorbance of CCK-8 was read at 450 nm using EPOCH plate reader (BioTek).

Cell cycle analysis

Cells (OVCAR-3 and SKOV-3) treated for 1 day with DDP or BMN673 or a combination of the two drugs, were fixed for 12 h in 70 % alcohol at 4

°C. Thereafter, they were suspended, stained with PI, and the cell cycle was measured using FACS (Beckman Coulter, USA).

Determination of apoptosis

Following treatment by addition of BMN673 at concentrations of 0.1, 1, 10 and 100 nM to the medium, the cells were incubated for 3 days, followed by rinsing in chilled phosphate buffer. According to each manufacturer's protocol, APC-linked Annexin V and PI (eBioscience, San Diego, CA) were used to stain the cells. Apoptosis analysis was performed on FACS-Caliber (BD Biosciences) using CellQuest software.

Immunohistochemistry

Paraffin-embedded tissue were sliced into sections (4 µm in thickness). The sections were dewaxed and endogenous peroxidase was blocked with 3 % H_2O_2. The tissue was heated in a microwave oven for 10 min to extract the antigen. Then, the tissue was subjected to incubation overnight with 1° immunoglobulins at 4 °C. This was followed by addition of biotinylated secondary antibody and incubation at room temperature for ¼ h. It was then treated with 3,3 '-diaminobenzidine (DAB) dye. The tissue was stained with hematoxylin.

Western blotting

Protein extraction was done with radioimmunoprecipitation assay buffer containing a mixture of protease blockers. The protein samples were resolved using 10% denatured SDS-PAGE and electro-transferred to PVDF membranes. The membranes were incubated with 5% defatted milk for 60 sec to block non-specific binding of the blot, followed by incubation for 12 h at 4 °C with 1° immunoglobulins for Ki67 (Abcam, 1:200), PCNA (Santa Cruz, 1:200), Bcl2 (Santa Cruz, 1:100), Bax (Abcam, 1:500), and caspase3 (Abcam, 1:1000). Thereafter, incubation was done with HR peroxidase-linked 2° immunoglobulin at room temperature for 1 h, after which the antigen-antibody complexes were detected using an ECL system (Amersham).

Statistics

Data were processed with SPSS 19.0 and are presented as mean ± SD. Two-group comparison was done with *t*-test to determine differences amongst groups. Values of $p < 0.05$ indicated significant differences.

RESULTS

Cytotoxic effects of BMN673 and DDP

Following 72-h treatment with BMN673 (0.1, 1, 10, or 100 nM) or DDP (2.5, 5, 10, 20 µg/mL), it was observed that BMN673 and DDP inhibited cancer cell proliferation in a concentration-based fashion, with 10 nM BMN673 or DDP (5 µg/mL) producing the most significant cytotoxicity (Figure 1 A and B).
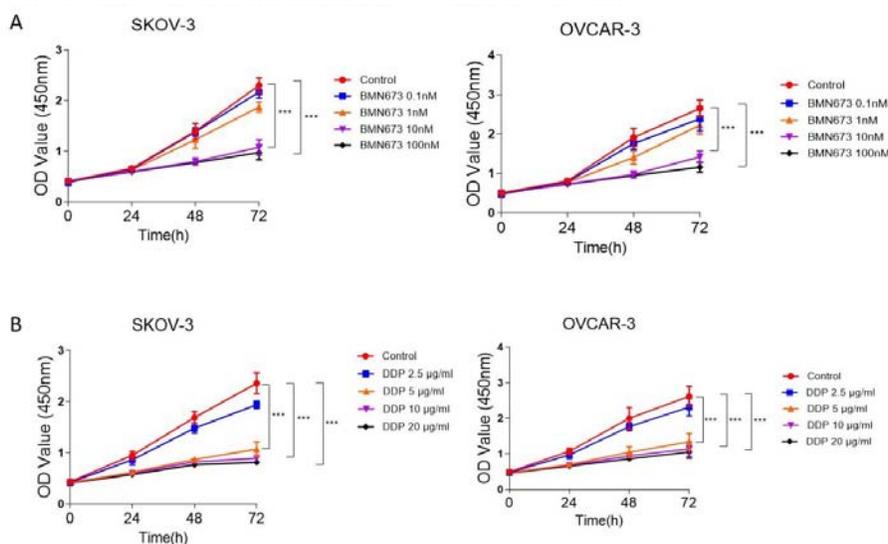
Figure 1: Cytotoxic effects of BMN673 and DDP on OVCAR-3 and SKOV-3 cells exposed to different doses of cisplatin or BMN673, followed by assay of cell viability. A & B: Cell viability; ***$p < 0.001$

Effect of combination of BMN673 with DDP on cell proliferation

The combined influence of BMN673 and DDP on the carcinoma cells was evaluated. The viability of the combined treatment group was significantly reduced, when compared with the group treated only with BMN673 or DDP (Figure 2 A). Clone formation assay also showed significant inhibition of cell proliferation in combined treatment group, relative to when BMN673 or DDP was used alone (Figure 2 B). Furthermore, Figure 2 C shows that protein expressions of PCNA and Ki67 were substantially reduced, particularly in cells given combined treatment. As shown in Figure 2 D, the combined application of BMN673 and DDP blocked cells in the G0/G1 phase. This may be responsible for the synergistic anticancer effect of BMN673 and DDP.

Effect of the combination of BMN673 and DDP on apoptosis

The apoptotic impact of combination of BMN673 and DDP was more effective than that of BMN673 or DDP alone. Moreover, TUNEL assay also indicated that combination treatment increased percentage apoptosis, relative to group given BMN673 or DDP-alone (Figure 3 B). Figure 3 C shows that, relative to cells that received single treatment, the combined treatment group had significantly increased protein expressions of Caspase-3 and Bax, and inhibited Bcl-2 protein (Figure 3 C).

Combination of BMN673 and DDP produced synergistic anticancer effect in xenograft tumor model

To determine the *in vivo* anticancer effects of BMN673 in combination with DDP, a nude mouse xenograft model was established. The combination of BMN673 and DDP inhibited tumor growth significantly, when compared to single treatment with BMN673 or DDP (Figure 4 A). Furthermore, compared to the BMN673 or DDP group, the tumor size and weight in the combined application group were significantly decreased (Figure 4 B and C). Immunohistochemical staining indicated synergistic effect of DDP and BMN673 on tumor growth and apoptosis in the BMN673 and DDP combination group, when compared to the BMN673 or DDP treatment group (Figures 4 D and E). In summary, results from xenograft model support the possibility that combination of DDP and PARP inhibitor could be a therapeutic strategy in ovarian cancer.

DISCUSSION

The results obtained in this research indicate *in vitro* synergism in the anticancer effects of BMN673 and cisplatin in ovarian carcinoma cells, and in a xenograft model *in vivo*.

The PARPIs are a new class of antitumor drugs which break DNA strands, leading to a build-up of single-stranded DNA [13]. Thus, PARPI has been used in the ongoing therapy for treatment of breast carcinoma susceptibility gene (BRCA) mutation ovarian cancer, with positive results and little side effects [14-17].
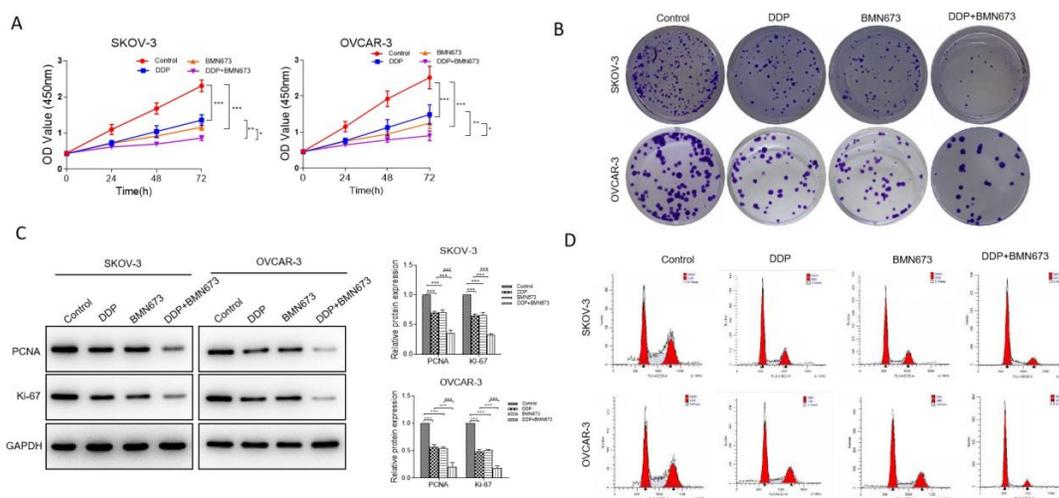
Figure 2: Effect of combination of BMN673 with DDP on cell proliferation. The cells were exposed to 10 nM BMN673 or DDP (5 µg/mL), alone, or in combination. (A) The CCK-8 assay results. (B) **C**olony formation assay results. (C) Results of western blotting. (D) Results of flow cytometry. *$P < 0.05$, **$p < 0.01$, ***$p < 0.001$
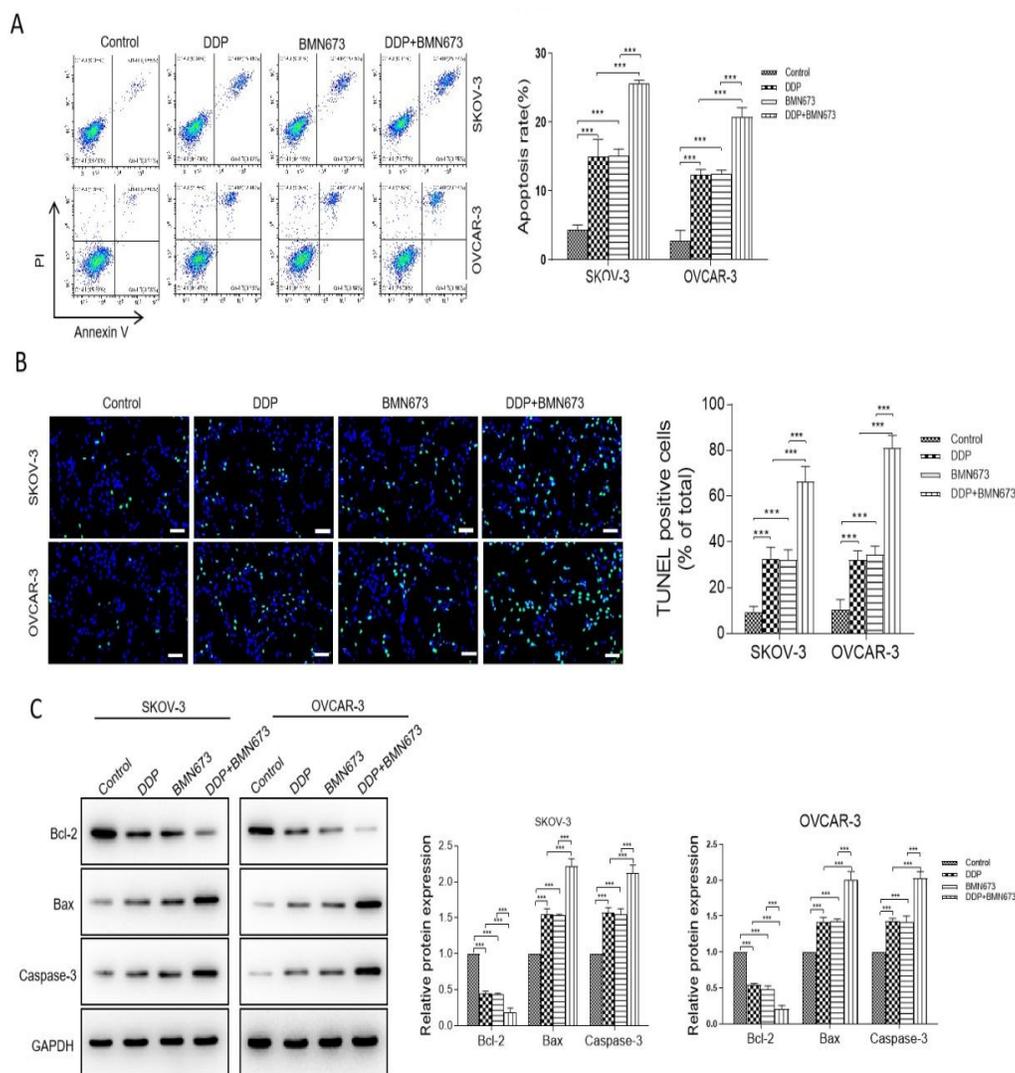
Figure 3: Effect of combination of BMN673 and DDP on apoptosis of ovarian carcinoma cells. (A) Results of flow cytometric analysis. (B) Results of TUNEL staining. (C) Results of Western blot analyses. *P < 0.05, **p < 0.01, ***p < 0.001
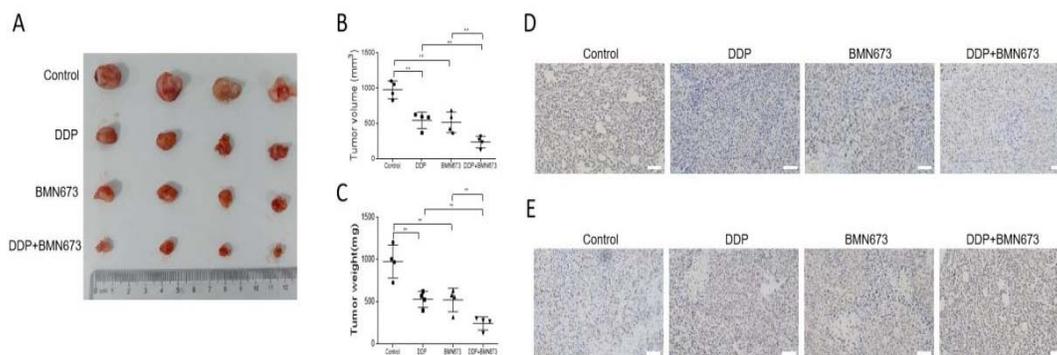
Figure 4: Combination of BMN673 and DDP produced synergistic anticancer effect in xenograft tumor model. A-C: Images showing tumor volume and weights in cells treated with cisplatin or BMN673 or DDP + BMN673. (D) Images of Ki67-positive cells in xenograft tumors. (E) Representative pictures of TUNEL staining of xenograft tumors

However, many BRCA patients do not respond well to this category of drugs [18]. Therefore, the application of PARPI in BRCA wild-type ovarian cancer has been the focus of research. In the past ten years, PARPI use has been extended to patients who do not have BRCA mutations. The Food and Drug administration (FDA) approved Olaparib and Niraparib as therapies for patients with total or incomplete platinum-responsive ovary carcinoma, regardless of BRCA profile [19,20]. In similar situations, Lynparza [21] and rucaparib [22] were used. Compared to other PARPIs, the evidence supporting the use of Talazoparib (BMN673) for EOC is limited since the drug is still in the early stages of clinical development.

In the present investigation, the impact of combination of BMN673 and DDP was determined in cell lines, and in a xenograft model. A novel PARP inhibitor approved by FDA, BMN673 inhibits tumor growth through sex hormone, ERK, Akt and other pathways [23]. The findings of this study showed that BMN673 or DDP alone inhibited cell viability, growth and colony formation, and increased cell apoptosis. Moreover, the combined treatment inhibited cell viability, cell growth and colony formation. It has been reported that PARPI enhanced antitumor impacts in the presence of cisplatin, especially in breast cancer [24-26]. The BMN673 combination therapy may reduce the occurrence of drug resistance, but the mechanism involved is not clear. Combined treatment with BMN673 and cisplatin weakened formation of new blood vessels, indicating that BMN673 is also implicated in suppression of angiogenesis.

CONCLUSION

This study has revealed a therapeutic mechanism whereby combined administration of BMN673 and cisplatin significantly improved the suppression of ovarian cancer. Therefore, PARP inhibitors may be used in combination with conventional chemotherapy for enhanced treatment of ovarian cancer.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yanyi Li, Ying Shan and Yuko Ohno conceived and designed the study, and drafted the manuscript. Yanyi Li, Ying Shan, Yingchun Duan, Cui Jiang and JISAIHAN A collected, analyzed and interpreted the experimental data. Ying Shan and Yuko Ohno revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

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