Berberine in combination with cisplatin suppresses breast cancer cell growth through induction of DNA breaks and caspase-3-dependent apoptosis

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Abstract. Berberine (BBR) is an isoquinoline alkaloid extracted from medicinal plants such as Hydrastis canadensis, Berberis aristata and Coptis chinensis. BBR displays a number of beneficial roles in the treatment of various types of cancers, yet the precise mechanisms of its action remain unclear. Cisplatin is an effective cancer chemotherapeutic agent and functions by generating DNA damage, promoting DNA damage-induced cell cycle arrest and apoptosis; however, its efficacy is challenged by the resistance of tumor cells in clinical application. The aim of the present study was to investigate the effects of BBR in combination with cisplatin on human breast cancer cells. MTT assay showed that BBR inhibited breast cancer MCF-7 cell growth with a 50% inhibitory concentration (IC₅₀) value of 52.178±1.593 µM and the IC₅₀ value of cisplatin was 49.541±1.618 µM, while in combination with 26 µM BBR, the IC₅₀ value of cisplatin was 5.759±0.76 µM. BBR sensitized the MCF-7 cells to cisplatin in a time- and dose-dependent manner. After treatment of BBR and cisplatin, the cellular pro-apoptotic caspase-3 and cleaved caspase-3 and caspase-9 were upregulated and the anti-apoptotic Bcl-2 was downregulated. Importantly, BBR restrained the expression of cellular PCNA, and immunofluorescence analysis of γH2AX showed that BBR increased the DNA damages induced by cisplatin. Taken together, the results demonstrated that BBR sensitized MCF-7 cells to cisplatin through induction of DNA breaks and caspase-3-dependent apoptosis.

Introduction

Breast cancer is one of the most common malignancies among women, with 458,000 annual deaths worldwide (1,2). Treatment strategies for breast cancer include surgery, radiotherapy, hormone therapy, chemotherapy or a combination of these methods (3). A range of chemotherapeutic drugs are employed in the treatment of breast cancer, in which platinum agents represent a class of common chemotherapeutic drugs, such as cisplatin or carboplatin (4). Cisplatin is currently the most effective chemotherapeutic drug used to treat breast cancer. Cisplatin is a genotoxic agent and the mechanism of action includes induction of DNA damages; therefore it is considered to be dose-limiting (6). The efficacy of this chemotherapeutic agent is often low due to adverse side effects and drug resistance (7-10). High resistance to cisplatin is a major challenge in the successful treatment of breast cancer, and there is currently no effective cure for patients with advanced stage of the disease. Consequently, strategies designed to sensitize breast cancer cells to cisplatin are still under investigation.

Berberine (BBR) is an isoquinoline alkaloid extracted from the rhizomes of a variety of valuable medicinal plants, including Coptis chinensis and Coptis japonica (11). BBR has been reported to possess a wide variety of pharmacological activities as an anti-microbial and anti-inflammatory agent (12-15). Currently, the anticancer activities of BBR have been reported in a range of cancers including hepatoma, prostate cancer, glioblastoma, ovarian cancer, leukemia and breast cancer (16-24). BBR achieves its antitumor effect through inhibition of cell proliferation and induction of tumor cell apoptosis although the underlying molecular mechanisms of BBR involved in the inhibition of cancer cell growth have not been fully elucidated (25-29). BBR has been demonstrated to directly bind with DNA and interfere with DNA replication as a DNA topoisomerase I inhibitor, through which BBR eventually induces cellular apoptosis. Studies have also shown that BBR binds to DNA, and radiosensitized lung cancer and esophageal cancer cells by regulating...
the expression of DNA repair-associated proteins (30-33), and BBR was found to modulate the anticancer effects of doxorubicin and rapamycin in human cancer cells (34,35). Although the mechanisms through which BBR sensitizes cancer cells to radiation or chemotherapy agents remain unclear, it is likely that BBR increases DNA damage induced by various therapeutic drugs.

As resistance to cisplatin of breast cancer is still a major challenge for the successful treatment of this disease, in the present study, we focused on the effects of BBR on the sensitivity of breast cancer cells to cisplatin and the mechanisms through which BBR functions in breast cancer cells. In combination with cisplatin, a low dose of BBR suppressed the proliferation of MCF-7 cells, increased apoptotic-associated protein expression, and more importantly, BBR increased the DNA breaks induced by cisplatin. In conclusion, our findings demonstrated that BBR increased the genotoxic ability of cisplatin and sensitized breast cancer cells to cisplatin, which could be a potential strategy for the treatment of breast cancer patients with cisplatin resistance.

Materials and methods

Cell culture. The human breast cancer MCF-7 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (both from Hyclone, Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

Antibodies and reagents. Berberine (BBR), cisplatin and DMSO were purchased from Sigma (St. Louis, MO, USA). Antibodies to GAPDH were purchased from ProteinTech Group, Inc. (Chicago, IL, USA) and the antibody to γH2AX was obtained from CST (Boston, MA, USA).

Cell viability assay. Cell viability was determined by the MTT assay. Briefly, breast cancer cells were seeded at 4x10³ cells/well in 96-well plates overnight, cultured in fresh medium containing various concentrations of BBR and cisplatin was dissolved in DMSO. After incubation for 44 h, MTT (0.5 mg/ml; Sigma-Aldrich) was added and 4 h later the growth of the cells was measured at 492 nm using a microplate photometer (Thermo Fisher Scientific). The effect of the drugs on cell viability was assessed as the percentages of cell viability compared with the control cells which were arbitrarily assigned as having 100% viability.

Wound-healing assay. The cells were grown to full confluency in 6-well plates and incubated overnight. Cell monolayers were wounded with a sterile 10-μl pipette tip, washed with PBS, and treated with the indicated dose of BBR (13 μM) or cisplatin (3.3 μM) or the combination in complete medium. After a 48-h incubation, the medium was replaced with PBS, and the wound gap was observed and photographed using an Olympus microscope (Olympus, Tokyo, Japan).

Anchorage-independent colony formation assay. MCF-7 cells were treated with BBR (13 μM) and cisplatin (3.3 μM) for 48 h. The cells were washed with PBS and trypsinized with trypsin (0.25% trypsin, EDTA) and 400 cells were seeded into a well of the 6-well plates. The cultures were maintained in an incubator at 37°C with 5% CO₂ for 10 days. The cells were washed with PBS twice, fixed with methanol for 15 min, stained with Giemsa for 15 min, washed with water and air-dried. The colonies with more than 50 cells were counted under an ordinary optical microscope.

Western blot analysis. After incubation with 13 μM BBR and 3.3 μM cisplatin for 48 h, the cells were lysed in RIPA lysis buffer. Whole cell proteins were quantified using the BCA protein assay (KangChen Bio-tech, Shanghai, China), separated by electrophoresis using 10% SDS-PAGE and transferred to a PVDF membrane. Western blot analyses were probed with the specific antibodies at dilution conditions as follows: mouse anti-GAPDH (1:4,000), β-actin (1:4,000), caspase-9 (1:500), rabbit anti-caspase-3 (1:500), Bcl2 (1:500), anti-mouse and rabbit IgG (H+L) secondary antibodies (1:5,000); all the antibody were purchased from ProteinTech Group, Inc.

Immunofluorescence analysis. Cells grown on chamber slides were treated with BBR (13 μM) in combination with cisplatin (3.3 μM). After 48 h, the cells were washed with PBS and then fixed with 4% paraformaldehyde at room temperature for 30 min, and then washed with PBS for three times. After permeabilization in 0.2% Triton X-100 for 30 min, the cells were washed twice in PBS and blocked for 1 h in PBS containing 1% BSA (all from Solarbio, Beijing, China). The cell pellet was suspended in 100 μl of 1% BSA containing either 1:100 diluted anti-γH2AX polyclonal Ab (CST). The cells were then incubated overnight at 4°C. On the following day, the cells were washed twice with PBS and incubated in 100 μl of 1:100 diluted Alexa Fluor 488-conjugated anti-rabbit IgG (Thermo Fischer Scientific) for 2 h at room temperature in the dark. After washing with PBS three times, the cells were dyed with Hoechst 33342 (Sigma, St. Louis, MO, USA) for 3 min, and washed with PBS for three times, and then photographed under a microscope (Olympus).

Statistical analysis. Data analysis was carried out using SPSS 6.0 software. One-way ANOVA was used to determine the significance of the differences in multiple comparisons; p<0.05, p<0.01, p<0.001, p<0.0001 were considered statistically significant. All experiments were performed in triplicate. Data are expressed as the mean ± SD. We used Image J and IPP6.0 software to process and analysis the immunofluorescence image.

Results

Berberine in combination with cisplatin suppresses MCF-7 cell proliferation. We analyzed the effect of BBR in combination with cisplatin on human breast cancer MCF-7 cell proliferation by MTT assay. After a 48-h BBR treatment, the IC₅₀ value of BBR in the MCF-7 cells was 52.178±1.593 μM and the IC₅₀ value of cisplatin was 49.541±1.618 μM. In contrast, following combination with 26 μM BBR, the IC₅₀ value of cisplatin was 5.759±0.76 μM (Fig. 1A). BBR increased the sensitivity of MCF-7 cells to cisplatin in a dose and time-dependent manner (Fig. 1A and B).
Berberine modifies cell morphology and inhibits cell migration. Following treatment of the MCF-7 cells with BBR at the dose of 13 µM and with cisplatin at 3.3 µM, reduced cell-cell contact and the formation of filopodia were observed (Fig. 2A). The wound healing assay showed that BBR and cisplatin inhibited the migration of MCF-7 cells. BBR in combination with cisplatin further inhibited the migration of MCF-7 cells (Fig. 2B and C). Each drug administered
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alone suppressed cell colony formation. BBR in combination with cisplatin further suppressed MCF-7 cell colony formation (Fig. 3A and B).

**Berberine sensitizes MCF-7 cells to cisplatin through the caspase-3-dependent apoptotic pathway.** We next tested whether BBR and cisplatin induce apoptotic-associated proteins. The expression levels of pro-apoptotic proteins, caspase-3 and caspase-9 and anti-apoptotic protein Bcl-2 in MCF-7 cells were analyzed by western blot analysis. BBR increased the expression levels of caspase-3 and caspase-9 compared with these levels in the control group (Fig. 4A). A low dose of BBR (13 µM) in combination with cisplatin increased the expression of cleaved caspase-3 and caspase-9, but decreased expression of Bcl-2 compared with these levels in the cells treated with cisplatin alone (3.3 µM) (Fig. 4B). The results indicate that BBR sensitized MCF-7 breast cancer cells to cisplatin through a caspase-3-dependent apoptotic pathway.

Berberine increases DNA breaks and restrains the expression of PCNA. Immunofluorescence results showed that BBR increased the DNA breaks induced by cisplatin. Cells treated with DMSO were used as control group with mean γH2AX density set at 100%. (A) The percentage of the mean γH2AX density in each treatment group was calculated relative to cells treated with DMSO vehicle control. Data are presented as mean ± SD of three tests. *p<0.05, **p<0.01 indicate significant differences between the treatment and DMSO control group. (B) Effect of BBR on expression of cellular PCNA was detected through western blot assay.

**Berberine increases DNA breaks and restrains the expression of PCNA.** We used immunofluorescence analysis to test γH2AX foci in the cells. The cells were cultured with
BBR and cisplatin for 48 h, and γH2AX foci are shown in Fig. 5A. The result showed that cisplatin induced DNA breaks, and a low dose of BBR increased the DNA breaks induced by cisplatin. We also detected the effect of BBR on expression of PCNA, an important factor in DNA replication and DNA repair. BBR extensively reduced the expression of PCNA (Fig. 5B), suggesting that BBR may regulate the cellular DNA repair pathway to increase DNA breaks and sensitize cells to cisplatin.

Discussion

Currently, breast cancer treatment includes surgery, chemotherapy, hormone therapy, radiotherapy, and combinations of these methods. Conventional cisplatin is still the most effective chemotherapeutic agent in breast cancer treatment. However, the resistance of tumor cells to cisplatin is a considerable obstacle to effective breast cancer therapy. Due to the genotoxicity of cisplatin, the drug is often considered to be dose-limiting. Therefore, it would be beneficial for chemotherapeutic treatment if alternative reliable agents can sensitize cancer cells to cisplatin. Berberine is a traditional Chinese medicine and has been demonstrated to function in anticancer therapy with minor side effects. Thus, we evaluated the sensitization of MCF-7 cells to BBR in combination with cisplatin and the mechanisms of BBR action involved in the inhibition of breast cancer cells.

BBR inhibited breast cancer MCF-7 cell growth, and suppressed breast cancer cell colony formation and migration. We investigated the effect of a low level of BBR in combination with cisplatin on apoptosis and DNA breaks. A low level of BBR increased apoptotic caspase-3 and caspase-9 expression, reduced Bcl2 expression in combination with cisplatin. The results demonstrated that a low level of BBR greatly increased cisplatin-induced caspase-3 activation although this dose of BBR had a limited effect on the cell proliferation of the MCF-7 cells. To study the mechanism of BBR-induced apoptosis, we investigated the DNA breaks induced by BBR and cisplatin. A low level of BBR had a limited effect on cell growth, however, BBR greatly increased the sensitivity of the cells to genotoxic cisplatin. BBR in combination with cisplatin induced more γH2AX foci, suggesting that BBR increased the DNA damage induced by cisplatin. The increased cellular DNA damage may result in subsequent apoptosis and suppression of MCF-7 cell proliferation. BBR was reported to bind to DNA directly and to interfere with DNA replication (33), which would be a possible explanation for the ability of BBR to sensitize breast cancer cells to chemotherapeutic cisplatin.

To address the role of BBR in regulating cellular DNA repair, we detected the effect of BBR on expression of proliferating cell nuclear antigen (PCNA), a DNA sliding clamp required for DNA poly to replicate DNA and is crucial in DNA repair (36). BBR extensively restrained the expression level of PCNA, suggesting that BBR may decrease the cellular DNA repair ability to sensitize cells to genotoxic cisplatin.

In conclusion, our data demonstrated that BBR suppressed breast cancer MCF-7 cell proliferation, colony formation and migration. A low level of BBR sensitized breast cancer cells to cisplatin, regulated cleaved caspase-3, caspase-9, Bcl-2 protein expression, and more importantly, BBR increased the DNA damages induced by cisplatin and reduced the cellular PCNA level. These results suggest that a low level of BBR can regulate cellular DNA repair and promote the DNA breaks induced by cisplatin, further potentiating the breast cancer cells to cisplatin-induced apoptosis, which could be one of the mechanisms of BBR action in antitumor activity. Given the wide application of cisplatin and other platinum-based drugs in cancer treatment and the relatively limited side effects of a low dose of BBR, our studies suggest an alternative approach to circumvent the cancer resistance to cisplatin and to improve the efficacy of platinum-based chemotherapeutic treatment. Further studies are needed to determine the clinical relevance of BBR in combination with cisplatin.

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References


