

Berberine enhances gemcitabine-induced cytotoxicity in bladder cancer by downregulating Rad51 expression through inactivating the PI3K/Akt pathway

XINGHUA GAO^{1,2}, JIKAI LIU¹, DAMING FAN³, XIAOFENG LI¹,
ZHIQING FANG¹, KEQIANG YAN¹ and YIDONG FAN¹

¹Department of Urology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, Jinan, Shandong 250012;
Departments of ²Urology and ³Pathology, Jinan Central Hospital, Jinan, Shandong 250013, P.R. China

Received July 11, 2021; Accepted October 19, 2021

DOI: 10.3892/or.2021.8244

Abstract. Although gemcitabine (GEM) has been used to treat bladder cancer (BC) for a number of years, severe adverse events or drug resistance frequently develops. A series of drugs have been proved to sensitize patients to GEM and reduce the side effects. The aim of the present study was to evaluate the potential effects of berberine (BER) on GEM-induced cytotoxicity in BC and to explore the possible underlying mechanisms. T24 and 5637 human BC cell lines were treated with GEM and/or BER before cell proliferation, apoptosis and migration were studied. Oncomine databases and Gene Expression Profiling Interactive Analysis (GEPIA) were used to retrieve RAD51 recombinase (Rad51) mRNA expression. Overexpression plasmid or specific Rad51 small interfering RNA were used to examine the role of Rad51 in drug-treated BC cells. BC model mice were administered with GEM and/or BER before changes in tumor volume, size and Ki67 expression were assessed. BER enhanced GEM-induced cytotoxicity, apoptosis and inhibition of migration, whilst attenuating the GEM-induced upregulation of phosphorylated Akt and Rad51 expression. According to Oncomine and GEPIA analyses, Rad51 was found to be significantly upregulated in BC tissues compared with that in normal tissues, where there was a weak positive correlation between Rad51 and Akt1 expression. Knockdown of Rad51 enhanced GEM-induced cytotoxicity, whilst overexpression of Rad51 reversed the suppressed cell viability induced by BER and GEM. Inactivation of the PI3K/Akt pathway by LY294002 or BER enhanced GEM-induced cytotoxicity and downregulated Rad51 expression, whilst overexpression of constitutively

active Akt restored Rad51 expression and cell viability that was previously decreased by BER and GEM. BER additively inhibited tumor growth and Ki67 expression when combined with GEM *in vivo*. These results suggest that BER can enhance GEM-induced cytotoxicity in BC by downregulating Rad51 expression through inactivating the PI3K/Akt pathway, which may represent a novel therapeutic target for BC treatment.

Introduction

Bladder cancer (BC) currently ranks as the 9th most common malignancy in the world and 13th in terms of cancer-associated mortality (1). Clinically, BC can be classified into non-muscle-invasive bladder cancer (NMIBC) and muscle-invasive bladder cancer (MIBC) (2,3). NMIBC tends to be confined only to the mucosa or submucosa, but makes up ~75% of all cases of BC (2). However, the 5-year probability of postoperative recurrence for NMIBC is at 31-78%, whereas the probability of postoperative disease progression after 5 years is 0.8-45% (2). Therefore, postoperative intravesical Bacillus Calmette-Guérin or intravesical chemotherapy, including mitomycin, epirubicin and gemcitabine (GEM), are essential for preventing recurrence and progression (2). In total, ~20-25% of all cases of BC are MIBC, where ~50% of these patients progress within 5 years after cystectomy (3). Although combined treatment with GEM and cisplatin has been applied as the standard chemotherapy regimen for patients with metastatic BC and MIBC, the disease response rate was reported to be only 49% (4). In addition, these treatment strategies may cause severe adverse reactions, including nausea, vomiting, neutropenia, thrombocytopenia, mucositis and febrile neutropenia (5), where >50% patients with BC remain unsuitable for cisplatin treatment due to the adverse reactions (6).

GEM belongs to a broad-spectrum class of antimetabolites (7). It is a cytosine analogue that can replace one of the building blocks of nucleic acids to induce 'masked chain termination', which in turn inhibits further DNA synthesis and leads to cell death (7). At present, GEM is widely applied for the treatment of various malignant tumors, such that it is considered to be an essential component of the first-line chemotherapy against BC, non-small-cell lung

Correspondence to: Professor Yidong Fan or Dr Keqiang Yan, Department of Urology, Qilu Hospital, Cheeloo College of Medicine, Shandong University, 107 Wenhua Road, Jinan, Shandong 250012, P.R. China

E-mail: fanyd@sdu.edu.cn

E-mail: yankeqiang1@126.com

Key words: berberine, gemcitabine, bladder cancer, RAD51 recombinase, PI3K/Akt signaling pathway, cytotoxicity tests

cancer (NSCLC) and pancreatic cancer (8,9). However, patients remain susceptible to developing recurrence and drug resistance (8,9). As a result, a series of drugs have been investigated with the aim of sensitizing the effects of GEM and reducing side effects. To this effect, everolimus, sunitinib, vitamin C and vitamin K3 have all been reported to enhance GEM-induced cytotoxicity in BC cells (10-12).

Traditional Chinese medicine (TCM) has been utilized for an extended period of time in China. A growing number of TCM-derived products and medicinal herbs have been previously shown to possess bioactive anticancer properties (13,14). As an isoquinoline alkaloid that can be isolated from *Berberis aquifolium* (Oregon grape) and *Berberis vulgaris* (barberry) (15), berberine (BER) has been recently revealed to exert several pharmacological properties, including anti-inflammatory, cardiovascular-protective, neuroprotective, anti-hyperglycemic, anti-hyperlipidemic, anti-hypertensive and antitumor effects (16-18). Additionally, BER can exert cytotoxicity on numerous types of tumor cells, including those of human esophageal (19), prostate (20) and ovarian cancers (21). Previous studies have demonstrated that BER can exert antitumor effects on BC cells by suppressing proliferation whilst promoting apoptosis and cell cycle arrest (22), in addition to sensitizing the response of BC cells to epirubicin (23).

DNA damage normally exerts a pivotal function in accelerating cell death, and DNA double-strand break (DSB) is one of the most cytotoxic types of DNA damage (24). Following DSB, the DNA repair system is swiftly initiated. RAD51 recombinase (Rad51) is a crucial element for the homologous recombination (HR) repair of DNA and is considered to mediate an error-free repair mechanism for maintaining genome integrity (25). High expression levels of Rad51 have been previously associated with invasiveness and therapeutic resistance in a number of tumors, including pancreatic adenocarcinoma (26), lung tumor (27), breast cancer (28) and ovarian cancer (29), whereas down-regulation of Rad51 has been shown to enhance the efficacy of chemotherapy or radiation sensitivity (30). Tsai *et al* (31) reported that GEM could induce the upregulation of Rad51 in NSCLC. In another study, Liu *et al* (32) previously reported that BER could sensitize esophageal cancer cells to radiotherapy by downregulating Rad51 expression.

Therefore, the present study hypothesized that BER could sensitize BC to GEM by regulating Rad51 expression. The aim of the present study was to evaluate the potential effects of BER on GEM-induced cytotoxicity in BC and to explore the possible underlying mechanisms, which may represent a novel therapeutic agent or target for BC treatment.

Materials and methods

Cell line and culture. Human BC cell lines 5637 and T24 (The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences) were cultured as a monolayer in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 mg/ml penicillin-streptomycin in an incubator at 37°C with a humidified atmosphere of 5% CO₂ and 95% air. BER (Beijing Solarbio Science & Technology Co., Ltd.) and GEM (MedChemExpress) were dissolved in DMSO. The maximum concentration (v/v) of DMSO in the final medium was 0.1%.

For *in vivo* experiments, BER was suspended in water supplemented with 0.5% carboxymethyl cellulose sodium (CMC-Na) and stored at 4°C. All experiments were conducted during the exponential growth phase of the cells.

Cell viability assay. Cell viability was examined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc.). In total, 2×10^3 cells in 100 μ l medium/well were seeded into 96-well plates and incubated overnight at 37°C. These cells were then treated with increasing concentrations of GEM (0, 5, 10, 20, 30, 40, 50 and 60 nM) or BER (0, 1, 5, 10, 20, 40, 80 and 160 μ M) for 48 or 72 h at 37°C. CCK-8 reagent was added to the culture medium with the ratio 1:10 and incubated for an additional 1 h. An ELISA plate reader (Bio-Rad Laboratories, Inc.) was used to measure the optical density in each well at 450 nm. The relative percentage of surviving cells was used to evaluate the influence of GEM or BER on cell viability. According to the results, the cells were then treated with BER (20 μ M for 5637 cells and 10 μ M for T24 cells) and GEM (40 nM for 5637 cells and 30 nM for T24 cells) alone or together for 48 h at 37°C. The cells in the control group were cultured in RPMI-1640 medium containing 10% FBS without drugs for 48 h at 37°C. The optical density was measured as aforementioned after CCK-8 was added. PI3K inhibitor (LY294002; 10 μ M; Beyotime Institute of Biotechnology) was used to block PI3K/Akt pathway activation for 48 h at 37°C.

Cell apoptosis assay. The FITC Annexin V Apoptosis Detection kit (BD Biosciences) was used to evaluate cell apoptosis. In brief, a total of 1×10^5 cells treated with GEM or/and BER were harvested, trypsinized and centrifuged at room temperature at 1,000 \times g for 5 min. After a washing step with PBS, cells were re-suspended in 0.1 ml binding buffer. Cells were incubated in the dark for 15 min at room temperature after 5 μ l Annexin V-FITC and 5 μ l PI were added. A BD FACSCalibur flow cytometer (BD Biosciences) was used to measure cell apoptosis at 1 h after 0.4 ml binding buffer was added. Flow cytometry data was analyzed using FlowJo Software V10 (FlowJo LLC). The apoptotic rate was calculated as the percentage of early plus late apoptotic cells.

ROS detection. BC cells treated with drugs were incubated with 10 μ M 2',7'-dichlorofluorescein diacetate (DCFH-DA) solution (Beyotime Institute of Biotechnology) for 30 min at 37°C in the dark. After incubation, the cells were harvested by trypsinization and washed with PBS. The fluorescence intensity was detected by flow cytometry (BD Biosciences).

Wound-healing assay. A total of 5×10^5 cells were seeded into a 6-well plate and incubated for 24 h at 37°C when the cells had reached a confluence of >90%, before a sterile 200- μ l pipette tip was used to create a scratch in each well. After washing three times with PBS, cells were cultured with GEM or/and BER in serum-free medium for another 24 h. Images were captured at 0 and 24 h using a fluorescence microscope (OLYMPUS IX73; Olympus Corporation). The wound width was measured and analyzed using ImageJ software (v1.53, National Institutes of Health). The cell migration rate was calculated as follows: (initial scratch width - scratch width at the time of experiment) / initial scratch width \times 100%.

Bioinformatics analysis. Rad51 mRNA expression data in BC were gathered from the Sanchez-Carbayo Bladder 2 datasets in the Oncomine database (<https://www.oncomine.org>) (33). Gene Expression Profiling Interactive Analysis (GEPIA) is an online application that can be used to analyze the differential expression of genes in cancer and normal tissues (<http://gepia.cancer-pku.cn/>) (34). GEPIA is a web-based tool for analyzing normal and tumor sample RNA sequencing data based on The Cancer Genome Atlas (TCGA) and Genotype-Tissue Expression (GTEx) data. In the present study, Rad51 mRNA expression in BC in the GEPIA database was also observed.

Western blot analysis. The cells were cultured in 6-well plates and then harvested after 48 h of treatment with GEM and/or BER. The cells were then washed twice with PBS, and RIPA lysis buffer (Beyotime Institute of Biotechnology) was used to lyse the cells. After centrifugation at 10,000 x g for 15 min at 4°C, a BCA Protein Assay kit (Beyotime Institute of Biotechnology) was used to determine the concentration of the protein supernatant. In total, 50 µg protein was fractionated by 8-12% SDS-PAGE and subsequently transferred onto PVDF membranes. After blocking with 5% non-fat milk in TBS with 0.05% Tween-20 at room temperature for 2 h, the membranes were incubated overnight with the respective primary antibodies at 4°C. At room temperature, the protein membranes were then exposed for an additional 2 h with secondary antibodies and detected using an ECL kit (Cytiva). Densitometry was performed by using ImageJ software (v1.53, National Institutes of Health). The specific antibodies used for western blot analysis were as follows: Anti-Akt (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.); anti-phosphorylated (p)-Akt (1:2,000; Ser473; cat. no. 4060; Cell Signaling Technology, Inc.); anti-Rad51 (1:1,000; cat. no. 8875; Cell Signaling Technology, Inc.); anti-GAPDH (1:1,000; cat. no. 5174; Cell Signaling Technology, Inc.); and the goat anti-rabbit IgG secondary antibody (1:5,000; cat. no. ab6721; Abcam).

Transfection of expression plasmids and small interfering RNA (siRNA). Exponentially growing 5637 and T24 cells were plated for 18 h in complete RPMI-1640 medium and the cells were transfected with constitutively active Akt expression plasmid (pcDNA3.1-myr-Akt; 1 µg plasmid/1x10⁵ cells; Shanghai Yaji Biotechnology Co., Ltd.), pcDNA3.1(+)-Rad51 plasmid (Rad51 OE; 1 µg plasmid/1x10⁵ cells; Wuhan Yipu Biological Technology Co., Ltd.) or siRNA targeting Rad51 (200 nM) at 37°C for 24 h using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The pcDNA3.1(+) empty vector plasmid or negative control scramble siRNA were used as negative controls. The sense-strand sequences of siRNA duplexes for Rad51 and scrambled (as a control) (Wuhan Yipu Biological Technology Co., Ltd.) were 5'-UGUAGCAUAGC UCGAGCG-3' and 5'-GCGCGCUUUGUAGGATTCG-3', respectively. At 48 h post-transfection, the cells were harvested and used for subsequent experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA of BC cells was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. RNA quality and concentration

were quantified using a NanoDrop™ 2000 Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc.). cDNA was synthesized from 1 µg total RNA at 42°C for 1 h and 70°C for 10 min using a PrimeScript™ RT reagent kit (Takara Bio, Inc.). qPCR was then performed using a SYBR Premix Ex Taq™ kit (Takara Bio, Inc.) in an ABI Prism 7500 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.). The thermocycling conditions for qPCR were as follows: 95°C for 5 min, followed by 40 cycles at 95°C for 10 sec and 60°C for 30 sec. β-actin expression was used as the internal control. The relative gene expression was calculated using the 2^{-ΔΔC_q} method (35). The primer sequences are as follows: β-actin forward, 5'-ATAGCACAGCCTGGATAG CAACGTAC-3' and reverse, 5'-CACCTTCTACAATGAGCT GCGTGTG-3'; and Rad51 forward, 5'-CTTTGGCCCCACA ACCCATTTTC-3' and reverse, 5'-ATGGCCTTTCCTTCA CCTCCAC-3'.

In vivo xenograft experiments. All animal experiments were approved by Jinan Central Hospital Experimental Animal Welfare Ethics Review Committee (Jinan, China; approval no. JNCH2021-19) and all experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (36). In total, 12 BALB/c female nude mice (age, 5 weeks old; body weight, 14-16 g; Beijing Vital River Laboratory Animal Technology Co., Ltd.) were kept in specific pathogen-free conditions and had access to sterilized food and filtered water freely. The vivarium was maintained at 23-25°C with a 12-h light/dark cycle and humidity at 40-70%. After 1 week of adjustable feeding, 5x10⁶ 5637 cells suspended in 200 µl PBS were subcutaneously injected into the flanks of mice. Once tumor masses became established and palpable, mice were randomized into the following four groups (three mice per group): i) Control group, which were orally gavaged with CMC-Na daily and intraperitoneally injected with PBS weekly; ii) BER group, which were orally gavaged with BER at 100 mg/kg/day and intraperitoneally injected with PBS weekly; iii) GEM group, which were orally gavaged with CMC-Na daily and intraperitoneally injected with GEM dissolved in PBS at 150 mg/kg/week; and iv) BER + GEM group, which were orally gavaged with BER at 100 mg/kg/day and intraperitoneally injected with GEM at 150 mg/kg/week. After initial detection, tumor volumes were evaluated every 4 days. Tumor sizes were measured with a digital caliper and tumor volume in mm³ was calculated using the formula volume: tumor volume = (width)² x length x 3.14/6 (37). The maximum tumor diameter observed in the present study was 12.41 mm. After 4 weeks, mice were euthanized by CO₂ asphyxiation using a 30% displacement rate of cage volume (30 l)/min before the xenografts were harvested and weighed.

Immunohistochemical (IHC) analysis. The xenografts were fixed with 4% paraformaldehyde for 12 h at room temperature, embedded with paraffin and sectioned at 4-µm each. Bovine serum albumin (5%; Beyotime Institute of Biotechnology) was used to block non-specific binding at 37°C for 30 min. IHC staining of Ki67 was performed using a Ventana Benchmark XT Staining system (Roche Diagnostics) on the sections with an anti-Ki67 antibody (ready-to-use without further dilution; cat. no. RMA-0542; Fuzhou Maixin Biotech Co., Ltd.)

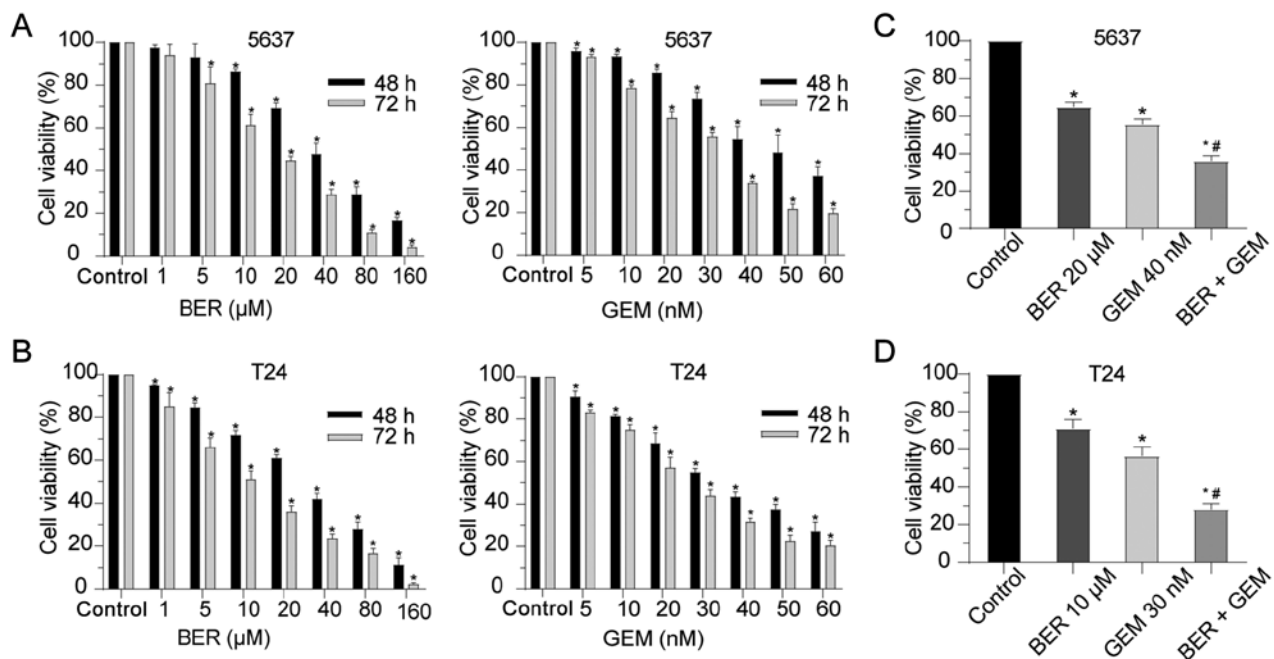


Figure 1. BER enhances the cytotoxic effects of GEM *in vitro*. Viability of (A) 5637 and (B) T24 cells after treatment with different concentrations of BER or GEM for 48 or 72 h. (C and D) The cells were then treated with the proper concentration of GEM and/or BER for 48 h. * $P < 0.05$ vs. control; # $P < 0.05$ vs. GEM group. GEM, gemcitabine; BER, berberine.

overnight at 4°C with gentle shaking. Then, the sections were incubated with the horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (1:50; cat. no. A0216; Beyotime Institute of Biotechnology) for 2 h at room temperature. Images were captured using a light microscope (LEICA DM4000; Leica Microsystems, Inc.). The nuclear expression of Ki67 was manually counted for each tumor in the area with the highest density of Ki67-positive nuclei ('hot spots').

Statistical analyses. Each experiment was repeated ≥ 3 times. Data are presented as the mean \pm SD. All data were graphed using GraphPad Prism 8.0 (GraphPad Software, Inc.) and analysis was performed using SPSS 19.0 software (IBM Corp.). Unpaired Student's *t*-test or one-way ANOVA followed by Tukey's post hoc test were performed to evaluate differences between or among groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

BER enhances the inhibitory effects of GEM on BC cell viability *in vitro*. 5637 and T24 BC cells were exposed to various concentrations of BER or GEM for 48 and 72 h. CCK-8 assay was then performed to assess cell viability. BER or GEM caused time- and dose-dependent reductions of BC cell viability (Fig. 1A and B). The IC_{50} of BER was calculated to be 33.29 μ M for 5637 cells and 28.52 μ M for T24 cells at 48 h, whilst the IC_{50} of GEM was calculated to be 43.69 nM for 5637 cells and 33.59 nM for T24 cells at 48 h.

To further optimize the response of BC cells to GEM and reduce potential side effects, GEM was combined with BER to investigate any possible effects on cell viability. According to results from the CCK-8 assay, the proper concentration of GEM was set at 40 nM for 5637 cells and 30 nM for T24 cells,

whilst that of BER was set at 20 μ M for 5637 cells and 10 μ M for T24 cells. The cells were then exposed to GEM with or without BER for 48 h. As shown in Fig. 1C and D, cell viability in the BER + GEM group was significantly lower compared with that in the GEM group in both cell lines. These data indicated that BER enhanced the cytotoxic effect of GEM *in vitro*.

BER enhances GEM-induced apoptosis, activation of ROS generation and inhibition of migration *in vitro*. To further explore the potential mechanism of action, the apoptotic rate and intracellular ROS were evaluated by flow cytometry. Although both single-drug treatments increased the apoptotic rate and the levels of intracellular ROS, BER + GEM treatment significantly increased apoptosis and ROS compared with that following GEM treatment alone in both cell lines (Fig. 2A and B). Wound-healing assay results indicated that single-drug treatment and BER + GEM treatment inhibited the migratory capacities of 5637 and T24 cells, where the migration rate in the BER + GEM group was lower compared with that in the GEM-only group (Fig. 2C). These results suggested that BER enhanced GEM-induced apoptosis and inhibition of migration.

BER reverses GEM-induced activation of PI3K/Akt signaling and upregulation of Rad51 expression in BC cells. The Sanchez-Carbayo Bladder 2 datasets in Oncomine indicated that Rad51 expression was upregulated in BC compared with that in normal bladder tissues (Fig. 3A). The same datasets also showed that Rad51 expression was upregulated in superficial BC (SBC) and infiltrating BC (IBC) tissues compared with that in normal bladder tissues, but there was no significant difference in Rad51 expression between SBC and IBC tissues (Fig. 3B). Consistently, GEPIA analysis also indicated that Rad51 expression was upregulated in BC compared with that

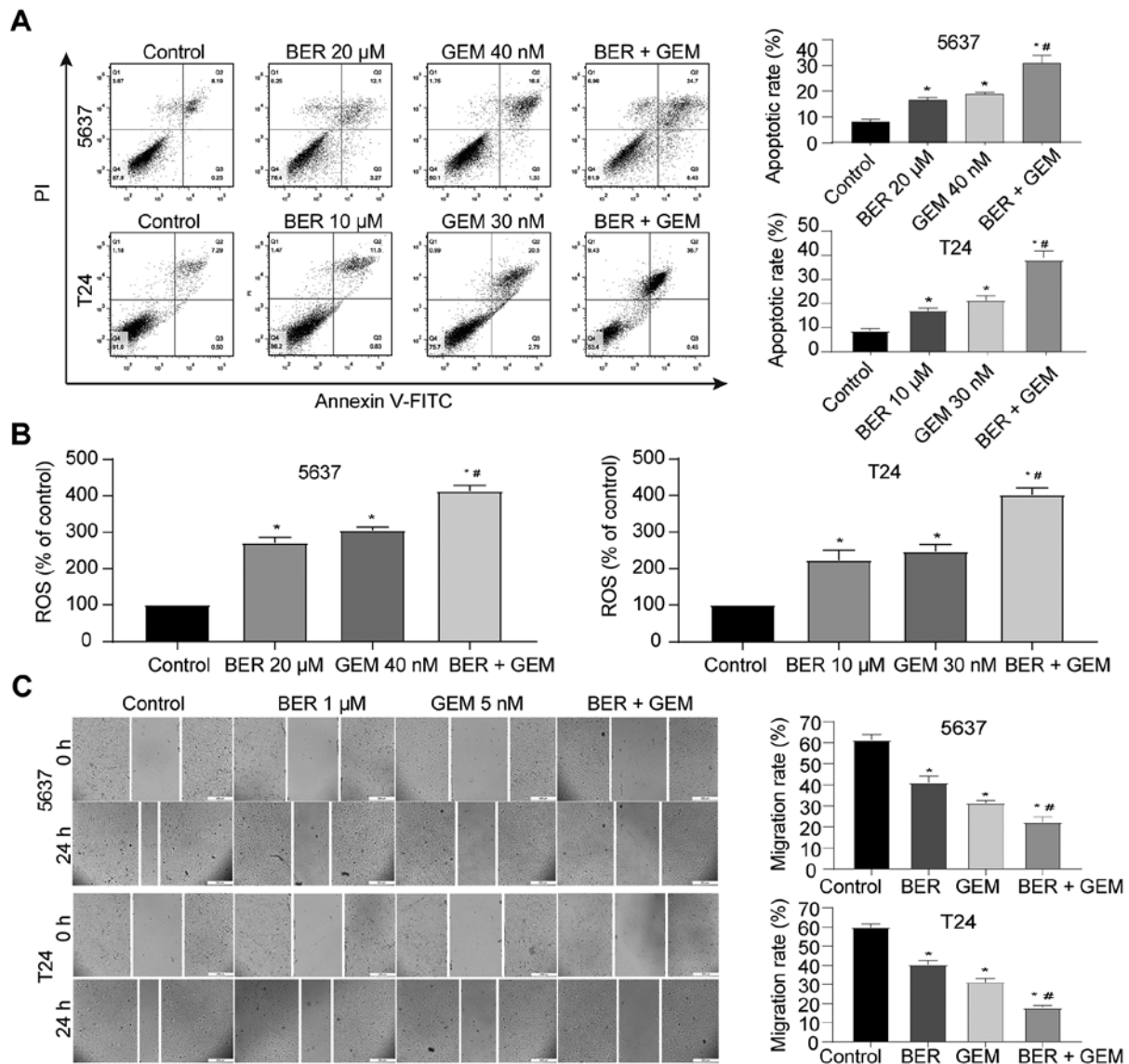


Figure 2. BER enhances GEM-induced apoptosis, activation of ROS generation and inhibition of migration *in vitro*. (A) The apoptotic rates of 5637 and T24 cells were observed after 48 h incubation with GEM and/or BER. (B) The intracellular ROS levels of 5637 and T24 cells were observed after 48 h incubation with GEM and/or BER. (C) Wound-healing assay was used to measure cell migration after treatment with GEM and/or BER (magnification, x40). * $P < 0.05$ vs. control; # $P < 0.05$ vs. GEM group. BER, berberine; GEM, gemcitabine; ROS, reactive oxygen species.

in normal bladder tissues (Fig. 3C), but there was no difference in the expression levels among stages II-IV (Fig. 3D).

PI3K/Akt signaling has been reported to regulate Rad51 expression in human NSCLC cells (31,38). GEPIA analysis also found that there was a weak, but significant positive correlation between Rad51 and Akt1 expression in BC ($R = 0.21$; Fig. 3E). Subsequently, it was found that the expression levels of Rad51 mRNA, p-Akt and Rad51 protein were all down- and upregulated in the BER and GEM groups, respectively (Fig. 3F and G). In addition, significant reductions were also observed in the BER + GEM group compared with those in the GEM-only group (Fig. 3F and G). These data suggested that BER attenuated GEM-induced activation of the PI3K/Akt pathway and upregulation of Rad51 expression.

Knockdown of Rad51 enhances GEM-induced cytotoxicity and inactivation of the PI3K/Akt pathway enhances GEM-induced cytotoxicity by downregulating Rad51 expression. To explore

the role of Rad51 and the PI3K/Akt pathway in GEM-induced cytotoxicity, Rad51 was knocked down using si-Rad51. As shown in Fig. 4A and B, si-Rad51 significantly increased the sensitivity of cells to GEM compared with the si-control group ($P < 0.05$) and decreased Rad51 protein expression induced by GEM, but did not interfere with the GEM-induced activation of the PI3K/Akt pathway. A PI3K inhibitor (LY294002) was used to block PI3K/Akt pathway activation in GEM-treated BC cells. LY294002 enhanced GEM-induced cytotoxicity (Fig. 4C) and decreased the activation of the PI3K/Akt pathway and Rad51 protein expression induced by GEM (Fig. 4D). These results indicated that inactivation of the PI3K/Akt pathway enhanced GEM-induced cytotoxicity by downregulating Rad51 expression.

BER enhances GEM-induced cytotoxicity by downregulating Rad51 expression. To explore the role of Rad51 in the BER-mediated enhancement of GEM-induced cytotoxicity,

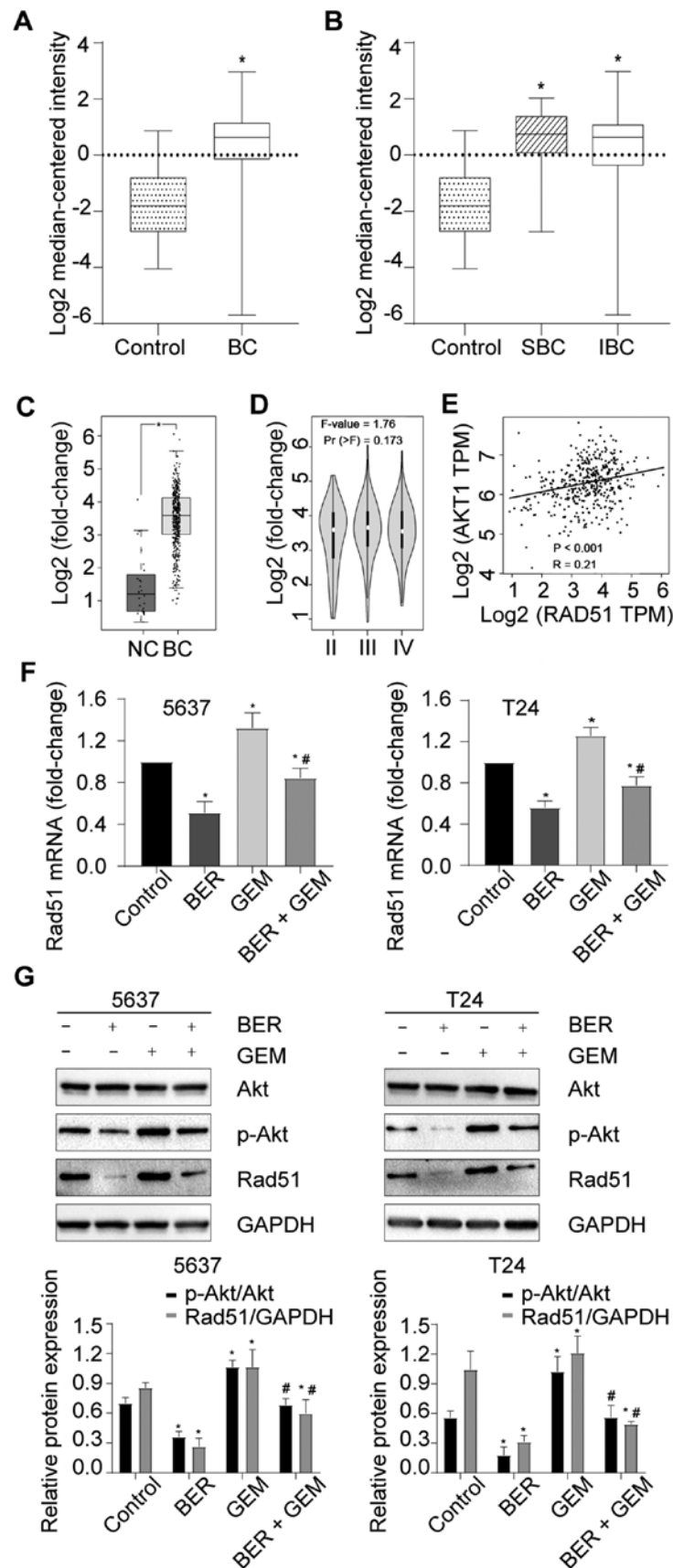


Figure 3. BER attenuates GEM-induced activation of PI3K/Akt signaling and upregulation of Rad51 expression in BC. (A) Rad51 mRNA expression data in BC and control tissues from the Oncomine database. (B) Rad51 mRNA expression data in SBC, IBC and control tissues from the Oncomine database. (C) Rad51 mRNA expression data in BC and normal control tissues as analyzed on GEPIA. (D) Rad51 mRNA expression data at different stages of BC, as analyzed using GEPIA. (E) Correlation analysis between Rad51 and Akt1 expression on GEPIA. (F) mRNA expression levels of Rad51 in BC cells after treatment with GEM and/or BER. (G) Protein levels of Rad51, Akt and p-Akt in BC cells after treatment with GEM and/or BER. *P<0.05 vs. control; #P<0.05 vs. GEM group. BER, berberine; GEM, gemcitabine; Rad51, RAD51 recombinase; BC, bladder cancer; SBC, superficial bladder cancer; IBC, infiltrating bladder cancer; GEPIA, Gene Expression Profiling Interactive Analysis; p-, phosphorylated; NC, negative control.

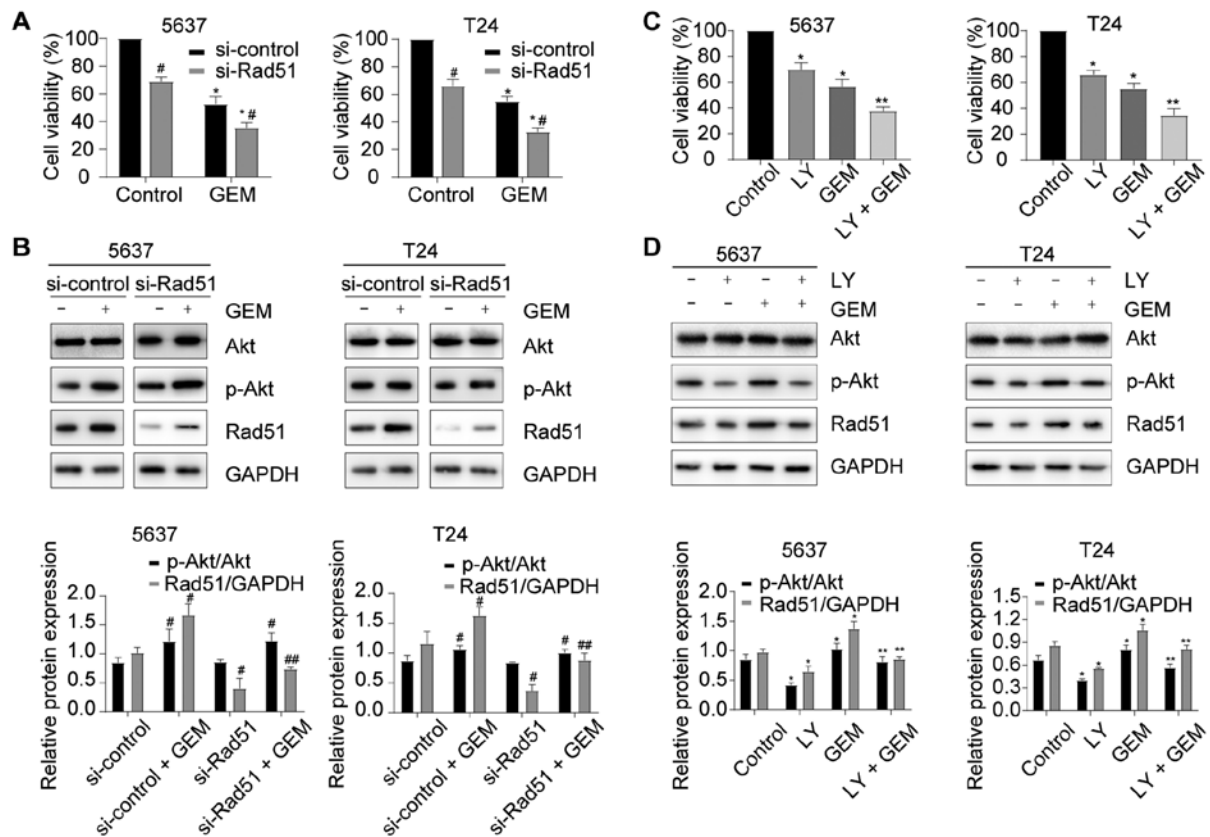


Figure 4. Knockdown of Rad51 or inactivation of the PI3K/Akt pathway enhances GEM-induced cytotoxicity. After transfection with si-Rad51 and/or treatment with GEM for 48 h, the (A) viability of BC cells and (B) protein levels of Rad51, Akt and p-Akt in BC cells were investigated. (C) Viability of BC cells and (D) protein levels of Rad51, Akt and p-Akt in BC cells after treatment with GEM and/or LY for 48 h. * $P < 0.05$ vs. control; # $P < 0.05$ vs. si-control; ** $P < 0.05$ vs. GEM group; ## $P < 0.05$ vs. si-control + GEM group. Rad51, RAD51 recombinase; GEM, gemcitabine; si-, small interfering; BC, bladder cancer; p-, phosphorylated; LY, LY294002.

5637 and T24 cells were transiently transfected with Rad51 OE plasmid, which upregulated Rad51 protein expression (Fig. 5A). The cells were then treated with GEM or BER either alone or in combination. It was observed that Rad51 OE reversed the BER-mediated reduction of Rad51 expression at both the mRNA and protein levels in addition to restoring the reduction in cell viability in the BER + GEM group (Fig. 5B-E). However, the ratio of p-Akt/Akt did not change (Fig. 5B and C). Therefore, these findings suggested that BER potentiated GEM-induced cytotoxicity by downregulating Rad51 expression, but PI3K/Akt signaling is unlikely to be regulated by Rad51.

BER enhances GEM-induced cytotoxicity and downregulates Rad51 expression by inactivating the PI3K/Akt pathway. To examine whether the enhancement induced by BER on GEM-mediated cytotoxicity and BER-induced Rad51 downregulation in BC cells were mediated by the PI3K/Akt pathway, BC cells were transiently transfected with myr-AKT plasmid, which upregulated the phosphorylation of Akt (Fig. 6A). It was observed that increased Akt phosphorylation upregulated Rad51 expression at both the protein and mRNA levels in all groups and reversed the BER-induced decrease in Rad51 expression in the BER + GEM group (Fig. 6B-D), suggesting that BER downregulated Rad51 expression by inactivating the PI3K/Akt pathway. Increased Akt phosphorylation also improved cell viability in all groups, whilst negating the

BER-induced reduction in cell viability in the BER + GEM group (Fig. 6E), suggesting that BER enhanced GEM-induced cytotoxicity by inactivating the PI3K/Akt pathway. These results indicated that BER enhanced GEM-induced cytotoxicity and downregulated Rad51 expression by inactivating the PI3K/Akt pathway.

BER enhances the anti-proliferative effects of GEM in vivo. Results from the xenograft mouse assays *in vivo* revealed that BER + GEM treatment significantly reduced the tumor weight and volume compared with those in mice treated with GEM (Fig. 7A and B). Cell proliferation in the tumor was assessed by measuring Ki67 staining in the xenograft tumor tissues. IHC analysis revealed that the expression levels of Ki67 in the xenografted tumors were significantly decreased in the BER + GEM group compared with those in tissues from mice treated with GEM alone (Fig. 7C), suggesting that BER enhanced the anti-proliferative effects of GEM *in vivo*.

Discussion

Despite the widespread use of GEM for BC, poor prognosis and severe adverse reactions underscore the need for identifying novel therapeutic agents and targets for this disease. BER has been shown to exhibit a broad spectrum of anticancer activities on numerous types of human cancers, including BC cells (22). BER has been previously demonstrated to sensitize

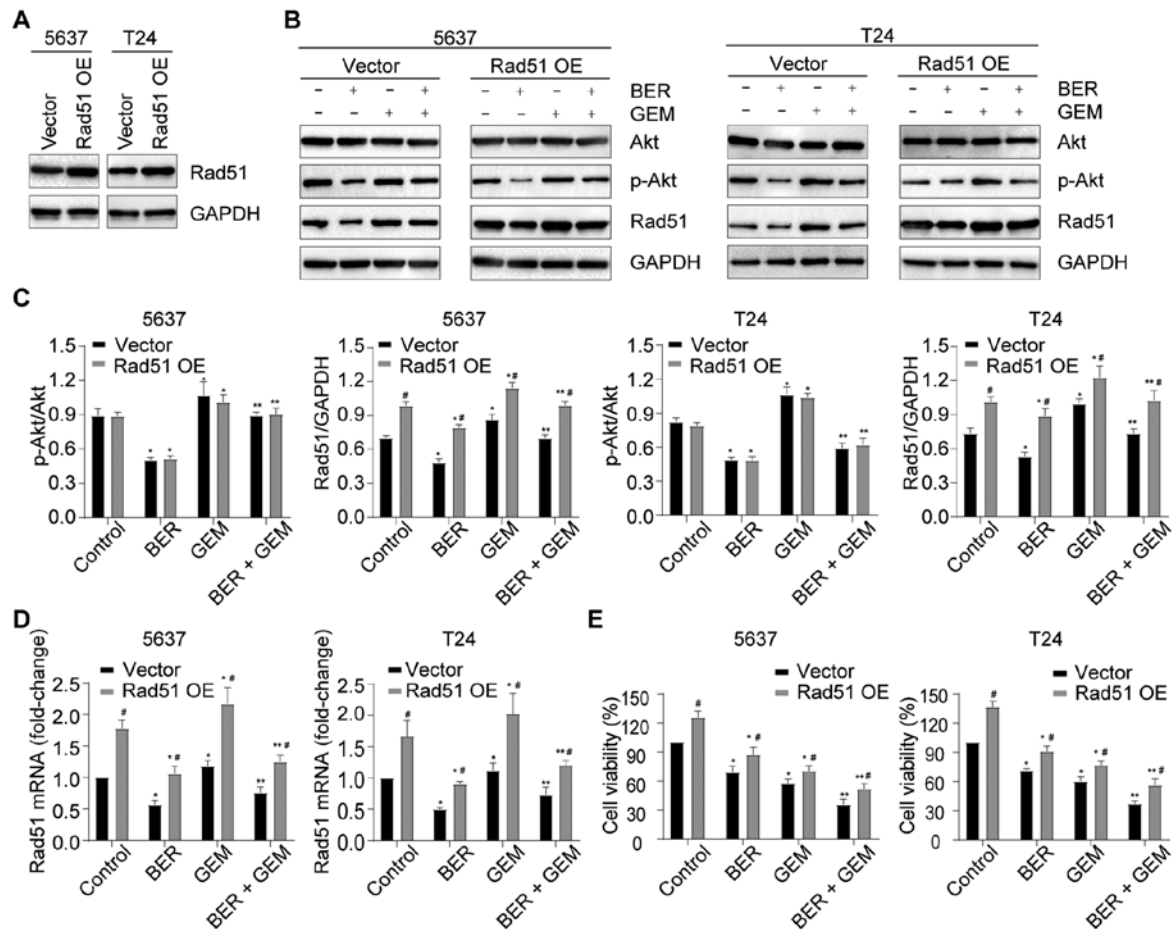


Figure 5. BER enhances GEM-induced cytotoxicity by downregulating Rad51 expression. BC cells were transfected with Rad51 OE plasmid. (A) Rad51 protein expression was measured. (B) The protein levels of Akt, p-Akt and Rad51 in BC cells after treatment with GEM and/or BER. (C) The ratio of p-Akt/Akt or Rad51/GAPDH relative protein expression. (D) The mRNA expression of Rad51 in BC cells after treatment with GEM and/or BER. (E) Cell viability of BC cells after treatment with GEM and/or BER for 48 h. * $P < 0.05$ vs. control; ** $P < 0.05$ vs. vector; *** $P < 0.05$ vs. GEM group. BER, berberine; GEM, gemcitabine; Rad51, RAD51 recombinase; BC, bladder cancer; OE, overexpression; p-, phosphorylated.

the response of BC cells to epirubicin (23), indicating that BER may serve an important role in modulating BC drug sensitivity. The main objectives of the present study were to determine the effects of BER on GEM-induced cytotoxicity in human BC cells and if any were found, to identify the possible molecular signaling pathways involved.

The present study showed that BER and GEM could inhibit the viability of 5637 and T24 BC cells in a time- and dose-dependent manner, such that BER potentiated the inhibitory effects of GEM on the viability and migration of BC cells. Suppression of growth is closely associated with apoptosis (39). Apoptosis is primarily modulated by specific relevant proteins and is characterized by a series of intracellular events, including the collapse of mitochondrial potential, caspase activation and DNA fragmentation (39). BER and GEM have both been previously reported to promote the apoptosis of BC cells (22,40,41). ROS is known to induce apoptosis (42). Takeuchi *et al* (43) reported that methyl 2-cyano-3, 11-dioxo-18 β -olean-1, 12-dien-30-oate induced apoptosis in BC cells through the induction of ROS. BER and GEM have been reported to induce ROS (23,44). Therefore, it was hypothesized in the current study that BER could enhance the pro-apoptotic effects of GEM by inducing ROS. Flow cytometry results reported that the apoptotic rates and

the levels of intracellular ROS of 5637 and T24 cells in the combination group were increased compared with those in the GEM-only group.

DSBs are critical cell fate defining events that can occur in the cell nucleus. As an intercalator, BER can induce DSBs in the DNA, which in turn activate p53 and ataxia telangiectasia mutated to activate apoptosis (45). Rad51 is a pivotal molecule in the process of DNA damage repair and a crucial element for HR (25). Rad51 expression was found to be upregulated in SBC and IBC tissues compared with that in normal bladder tissues, according to the data extracted from the Oncomine database (Sanchez-Carbayo Bladder 2) and analysis by GEPIA in the present study. In previous studies, Rad51 expression has been found to be upregulated in pancreatic adenocarcinoma, lung tumors, breast cancer and ovarian cancer, such that downregulation of Rad51 expression could enhance the sensitivity to radio- or chemotherapy (26-30). Consistent with previous reports (31,32), the present study found that GEM could upregulate the protein and mRNA expression levels of Rad51, but BER mediated opposite effects. Furthermore, BER attenuated the GEM-induced upregulation of Rad51 at both the mRNA and protein levels.

In the present study, it was also found that the GEM-induced upregulation of PI3K/Akt signaling, represented by Akt

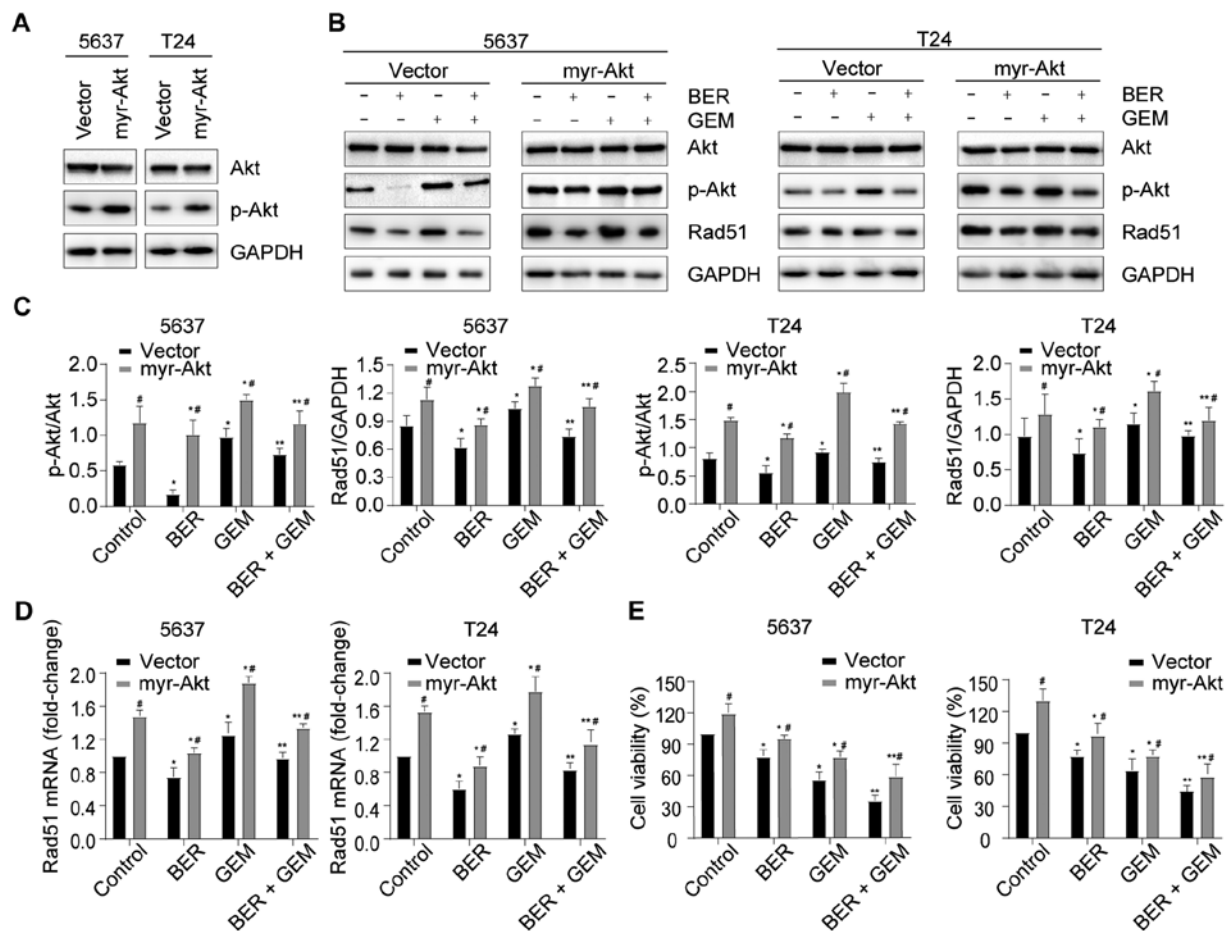


Figure 6. BER enhances GEM-induced cytotoxicity and downregulates Rad51 expression by inactivating the PI3K/Akt pathway. BC cells were transfected with the myr-AKT plasmid. (A) Akt phosphorylation was measured. (B) The protein levels of Akt, p-Akt and Rad51 in BC cells following treatment with GEM and/or BER. (C) The ratio of p-Akt/Akt or Rad51/GAPDH in relative protein expression. (D) mRNA expression of Rad51 in BC cells following treatment with GEM and/or BER. (E) Cell viability of BC cells following treatment with GEM and/or BER for 48 h. * $P < 0.05$ vs. control; # $P < 0.05$ vs. vector; ** $P < 0.05$ vs. GEM group. BER, berberine; GEM, gemcitabine; Rad51, RAD51 recombinase; BC, bladder cancer; p-, phosphorylated.

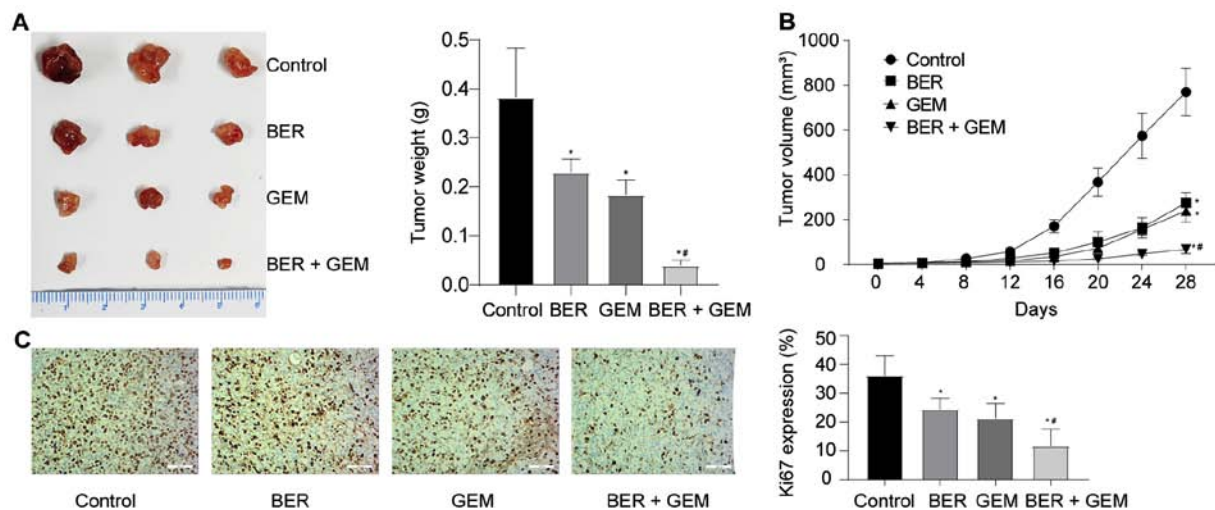


Figure 7. BER enhances the anti-proliferative effects of GEM *in vivo*. (A) Tumor weight in each treatment group was measured. (B) Tumor volume was measured on different days. (C) The expression of Ki67 was measured in the xenograft tissues (magnification, x100). * $P < 0.05$ vs. control; # $P < 0.05$ vs. GEM group. GEM, gemcitabine; BER, berberine.

phosphorylation, was attenuated by BER. GEPIA analysis also found a weak, but significant positive correlation between Rad51 and Akt1 expression in BC. It was therefore

hypothesized that the PI3K/Akt pathway and Rad51 may be involved in the additive physiological effects of BER on GEM in BC cells. Ko *et al* (38) previously reported that astaxanthin

downregulated Rad51 expression and Akt phosphorylation, whilst reducing cell viability in NSCLC, the effects of which were in turn enhanced by the PI3K inhibitor or Rad51 siRNA transfection. However, these aforementioned inhibitory effects of astaxanthin could be suppressed by Akt phosphorylation, suggesting that the PI3K/Akt pathway can upregulate the expression of Rad51. In another study, Tsai *et al* (31) found that GEM could upregulate the levels of Akt phosphorylation and Rad51 expression, but treatment with the PI3K inhibitor attenuated this GEM-induced upregulation of Rad51. By contrast, inactivation of the PI3K/Akt pathway or knocking down Rad51 expression could significantly increase GEM-induced cytotoxicity in NSCLC. A recent review documented that BER exerts promising anticancer effects, mainly by inhibiting the PI3K/Akt pathway (46). Consistently, the present study demonstrated that si-Rad51 enhanced GEM-induced cytotoxicity and inactivation of the PI3K/Akt pathway by LY294002 enhanced GEM-induced cytotoxicity by downregulating Rad51 expression. Akt phosphorylation and Rad51 expression were upregulated after GEM treatment in BC cells, in a manner that could be reversed by BER, which in turn led to the enhancement of GEM-induced cytotoxicity. In addition, overexpression of Rad51 attenuated this potentiation effect BER on GEM-induced cytotoxicity, suggesting that BER enhanced GEM-induced cytotoxicity by downregulating Rad51 expression. Activation of the PI3K/Akt pathway also upregulated the expression of Rad51 and increased cell viability in all groups, whilst reversing BER-induced reduction in Rad51 expression and cell viability in the BER + GEM group. These observations suggested that BER regulated Rad51 expression and cell viability via the PI3K/Akt pathway in GEM-treated BC cells.

Although the present study found that BER and GEM could inhibit BC cell viability, unlike BER, GEM can also upregulate the phosphorylation of Akt and Rad51 expression through other mechanisms that require further study. In addition, Takeuchi *et al* (41) reported that sequential GEM followed by tamoxifen treatment caused the largest increase in DNA fragmentation in BC cells, meaning that the timing of adjunct administration is critical for enhancing the effect of any co-treatment. Due to a lack of time, the present study did not determine the timing of adjunct administration or verify the expression of Rad51 in the xenograft tissues *in vivo*, which were limitations of this study. Despite these limitations, this study demonstrated the potentiating effects of BER on GEM in BC and its underlying mechanism. Nevertheless, further studies are warranted to determine the downstream mechanism of the PI3K/Akt pathway in regulating Rad51 expression in BC cells.

In conclusion, the findings in the present study suggested that BER functioned as a GEM sensitizer for BC chemotherapy, where Rad51, downstream of the PI3K/Akt signaling pathway, served a critical role. Therefore, a potential combinational adjuvant treatment strategy involving BER may restore the clinical efficacy of current treatment options for BC, such that Rad51 may represent a potential therapeutic target in patients with BC.

Acknowledgements

The authors would like to thank Dr Xianguang Meng at the Department of Dermatology of Jinan Central Hospital (Jinan,

China) and Ms. Xiaowen Xia at the Laboratory Animal Center of Jinan Central Hospital (Jinan, China) for their experimental assistance.

Funding

The present study was funded by the National Natural Science Foundation of China (grant no. 81672522).

Availability of data and materials

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

Authors' contributions

XG, KY and YF designed the study. XG, DF and XL carried out the experiments. XG, ZF and JL analyzed the data. XG, KY and YF confirm the authenticity of all raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal experiments were approved by the Jinan Central Hospital Experimental Animal Welfare Ethics Review Committee (Jinan, China; approval no. JNCH2021-19).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A and Bray F: Bladder Cancer Incidence and Mortality: A Global Overview and Recent Trends. *Eur Urol* 71: 96-108, 2017.
2. Babjuk M, Burger M, Comp  rat EM, Gontero P, Mostafid AH, Palou J, van Rhijn BWG, Roup  t M, Shariat SF, Sylvester R, *et al*: European Association of Urology Guidelines on Non-muscle-invasive Bladder Cancer (TaT1 and Carcinoma In Situ) - 2019 Update. *Eur Urol* 76: 639-657, 2019.
3. Gakis G: Management of Muscle-invasive Bladder Cancer in the 2020s: Challenges and Perspectives. *Eur Urol Focus* 6: 632-638, 2020.
4. von der Maase H, Hansen SW, Roberts JT, Dogliotti L, Oliver T, Moore MJ, Bodrogi I, Albers P, Knuth A, Lippert CM, *et al*: Gemcitabine and cisplatin versus methotrexate, vinblastine, doxorubicin, and cisplatin in advanced or metastatic bladder cancer: Results of a large, randomized, multinational, multicenter, phase III study. *J Clin Oncol* 18: 3068-3077, 2000.
5. Yu C, Hequn C, Jinbo C, Feng Z, Xiongbing Z and Jian D: Gemcitabine/cisplatin versus methotrexate/vinblastine/doxorubicin/cisplatin for muscle-invasive bladder cancer: A systematic review and meta-analysis. *J Cancer Res Ther* 14: 1260-1265, 2018.
6. Isono M, Hoffmann MJ, Pinkerneck M, Sato A, Michaelis M, Cinatl J Jr, Niegisch G and Schulz WA: Checkpoint kinase inhibitor AZD7762 strongly sensitises urothelial carcinoma cells to gemcitabine. *J Exp Clin Cancer Res* 36: 1, 2017.
7. Xie D, Zhang H and Shang C: Long non-coding RNA CDKN2B antisense RNA 1 gene inhibits Gemcitabine sensitivity in bladder urothelial carcinoma. *J Cancer* 9: 2160-2166, 2018.

8. Foschini F, Formisano L, Marciano R, Mozzillo E, Carratù A, Napolitano F, Santaniello A, De Placido P, Cascetta P, Servetto A, *et al*: FOLFIRINOX after first-line gemcitabine-based chemotherapy in metastatic pancreatic cancer: A mono-institutional experience. *Ann Oncol* 30 (Suppl 4): iv52-iv53, 2019.
9. Zhang X, Wang D, Li Z, Jiao D, Jin L, Cong J, Zheng X and Xu L: Low-Dose Gemcitabine Treatment Enhances Immunogenicity and Natural Killer Cell-Driven Tumor Immunity in Lung Cancer. *Front Immunol* 11: 331, 2020.
10. Kassouf W, Highshaw R, Nelkin GM, Dinney CP and Kamat AM: Vitamins C and K3 sensitize human urothelial tumors to gemcitabine. *J Urol* 176: 1642-1647, 2006.
11. Pinto-Leite R, Arantes-Rodrigues R, Palmeira C, Gaivão I, Cardoso ML, Colaço A, Santos L and Oliveira P: Everolimus enhances gemcitabine-induced cytotoxicity in bladder-cancer cell lines. *J Toxicol Environ Health A* 75: 788-799, 2012.
12. Yoon CY, Lee JS, Kim BS, Jeong SJ, Hong SK, Byun SS and Lee SE: Sunitinib malate synergistically potentiates anti-tumor effect of gemcitabine in human bladder cancer cells. *Korean J Urol* 52: 55-63, 2011.
13. Guo Z, Hu X, Xing Z, Xing R, Lv R, Cheng X, Su J, Zhou Z, Xu Z, Nilsson S, *et al*: Baicalein inhibits prostate cancer cell growth and metastasis via the caveolin-1/AKT/mTOR pathway. *Mol Cell Biochem* 406: 111-119, 2015.
14. Liu Z, Liu C, Yan K, Liu J, Fang Z and Fan Y: Huaier Extract Inhibits Prostate Cancer Growth via Targeting AR/AR-V7 Pathway. *Front Oncol* 11: 615568, 2021.
15. Tang QL, Lai ML, Zhong YF, Wang AM, Su JK and Zhang MQ: Antinociceptive effect of berberine on visceral hypersensitivity in rats. *World J Gastroenterol* 19: 4582-4589, 2013.
16. Lin X and Zhang N: Berberine: Pathways to protect neurons. *Phytother Res* 32: 1501-1510, 2018.
17. Imenshahidi M and Hosseinzadeh H: Berberine and barberry (*Berberis vulgaris*): A clinical review. *Phytother Res* 33: 504-523, 2019.
18. Mohammadinejad R, Ahmadi Z, Tavakol S and Ashrafzadeh M: Berberine as a potential autophagy modulator. *J Cell Physiol* 234: 14914-14926, 2019.
19. Jiang SX, Qi B, Yao WJ, Gu CW, Wei XF, Zhao Y, Liu YZ and Zhao BS: Berberine displays antitumor activity in esophageal cancer cells in vitro. *World J Gastroenterol* 23: 2511-2518, 2017.
20. Tian Y, Zhao L, Wang Y, Zhang H, Xu D, Zhao X, Li Y and Li J: Berberine inhibits androgen synthesis by interaction with aldo-keto reductase 1C3 in 22Rv1 prostate cancer cells. *Asian J Androl* 18: 607-612, 2016.
21. Hou D, Xu G, Zhang C, Li B, Qin J, Hao X, Liu Q, Zhang X, Liu J, Wei J, *et al*: Berberine induces oxidative DNA damage and impairs homologous recombination repair in ovarian cancer cells to confer increased sensitivity to PARP inhibition. *Cell Death Dis* 8: e3070, 2017.
22. Yan K, Zhang C, Feng J, Hou L, Yan L, Zhou Z, Liu Z, Liu C, Fan Y, Zheng B, *et al*: Induction of G1 cell cycle arrest and apoptosis by berberine in bladder cancer cells. *Eur J Pharmacol* 661: 1-7, 2011.
23. Zhuo Y, Chen Q, Chen B, Zhan X, Qin X, Huang J and Lv X: Berberine promotes antiproliferative effects of epirubicin in T24 bladder cancer cells by enhancing apoptosis and cell cycle arrest. *Int J Clin Pharmacol Ther* 55: 32-40, 2017.
24. Godin SK, Sullivan MR and Bernstein KA: Novel insights into RAD51 activity and regulation during homologous recombination and DNA replication. *Biochem Cell Biol* 94: 407-418, 2016.
25. Haber JE: DNA Repair: The Search for Homology. *BioEssays* 40: e1700229, 2018.
26. Nagathihalli NS and Nagaraju G: RAD51 as a potential biomarker and therapeutic target for pancreatic cancer. *Biochim Biophys Acta* 1816: 209-218, 2011.
27. Cortez MA, Valdecana D, Niknam S, Peltier HJ, Diao L, Giri U, Komaki R, Calin GA, Gomez DR, Chang JY, *et al*: In Vivo Delivery of miR-34a Sensitizes Lung Tumors to Radiation Through RAD51 Regulation. *Mol Ther Nucleic Acids* 4: e270, 2015.
28. Hong KJ, Hsu MC and Hung WC: RECK impedes DNA repair by inhibiting the erbB/JAB1/Rad51 signaling axis and enhances chemosensitivity of breast cancer cells. *Am J Cancer Res* 5: 2422-2430, 2015.
29. Wang B, Hou D, Liu Q, Wu T, Guo H, Zhang X, Zou Y, Liu Z, Liu J, Wei J, *et al*: Artesunate sensitizes ovarian cancer cells to cisplatin by downregulating RAD51. *Cancer Biol Ther* 16: 1548-1556, 2015.
30. Lee JO, Kang MJ, Byun WS, Kim SA, Seo IH, Han JA, Moon JW, Kim JH, Kim SJ, Lee EJ, *et al*: Metformin overcomes resistance to cisplatin in triple-negative breast cancer (TNBC) cells by targeting RAD51. *Breast Cancer Res* 21: 115, 2019.
31. Tsai MS, Kuo YH, Chiu YF, Su YC and Lin YW: Down-regulation of Rad51 expression overcomes drug resistance to gemcitabine in human non-small-cell lung cancer cells. *J Pharmacol Exp Ther* 335: 830-840, 2010.
32. Liu Q, Jiang H, Liu Z, Wang Y, Zhao M, Hao C, Feng S, Guo H, Xu B, Yang Q, *et al*: Berberine radiosensitizes human esophageal cancer cells by downregulating homologous recombination repair protein RAD51. *PLoS One* 6: e23427, 2011.
33. Sanchez-Carbayo M, Socci ND, Lozano J, Saint F and Cordon-Cardo C: Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J Clin Oncol* 24: 778-789, 2006.
34. Tang Z, Li C, Kang B, Gao G, Li C and Zhang Z: GEPIA: A web server for cancer and normal gene expression profiling and interactive analyses. *Nucleic Acids Res* 45 (W1): W98-W102, 2017.
35. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2⁻(Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
36. National Research Council (US) Committee for the Update of the Guide for the Care and Use of Laboratory Animals: Guide for the Care and Use of Laboratory Animals, 8th edition. The National Academies Press (US), Washington, DC, 2011.
37. Tomayko MM and Reynolds CP: Determination of subcutaneous tumor size in athymic (nude) mice. *Cancer Chemother Pharmacol* 24: 148-154, 1989.
38. Ko JC, Chen JC, Wang TJ, Zheng HY, Chen WC, Chang PY and Lin YW: Astaxanthin down-regulates Rad51 expression via inactivation of AKT kinase to enhance mitomycin C-induced cytotoxicity in human non-small cell lung cancer cells. *Biochem Pharmacol* 105: 91-100, 2016.
39. Jabbarzadeh Kaboli P, Rahmat A, Ismail P and Ling KH: Targets and mechanisms of berberine, a natural drug with potential to treat cancer with special focus on breast cancer. *Eur J Pharmacol* 740: 584-595, 2014.
40. Yin H, Yang X, Gu W, Liu Y, Li X, Huang X, Zhu X, Tao Y, Gou X and He W: HMGB1-mediated autophagy attenuates gemcitabine-induced apoptosis in bladder cancer cells involving JNK and ERK activation. *Oncotarget* 8: 71642-71656, 2017.
41. Takeuchi H, Mmeje CO, Jinesh GG, Taoka R and Kamat AM: Sequential gemcitabine and tamoxifen treatment enhances apoptosis and blocks transformation in bladder cancer cells. *Oncol Rep* 34: 2738-2744, 2015.
42. Miller WH Jr, Schipper HM, Lee JS, Singer J and Waxman S: Mechanisms of action of arsenic trioxide. *Cancer Res* 62: 3893-3903, 2002.
43. Takeuchi H, Taoka R, Mmeje CO, Jinesh GG, Safe S and Kamat AM: CDODA-Me decreases specificity protein transcription factors and induces apoptosis in bladder cancer cells through induction of reactive oxygen species. *Urol Oncol* 34: 337.e11-8, 2016.
44. Wang X, Bai Y, Zhang F, Yang Y, Feng D, Li A, Yang Z, Li D, Tang Y, Wei X, *et al*: Targeted Inhibition of P4HB Promotes Cell Sensitivity to Gemcitabine in Urothelial Carcinoma of the Bladder. *Oncotargets Ther* 13: 9543-9558, 2020.
45. Liu Z, Liu Q, Xu B, Wu J, Guo C, Zhu F, Yang Q, Gao G, Gong Y and Shao C: Berberine induces p53-dependent cell cycle arrest and apoptosis of human osteosarcoma cells by inflicting DNA damage. *Mutat Res* 662: 75-83, 2009.
46. Huang J, Feng W, Li S, Tang H, Qin S, Li W, Gong Y, Fang Y, Liu Y, Wang S, *et al*: Berberine Exerts Anti-cancer Activity by Modulating Adenosine Monophosphate-Activated Protein Kinase (AMPK) and the Phosphatidylinositol 3-Kinase/Protein Kinase B (PI3K/AKT) Signaling Pathways. *Curr Pharm Des* 27: 565-574, 2021.