Berberine sensitizes mutliple human cancer cells to the anticancer effects of doxorubicin *in vitro*

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Abstract. The clinical use of doxorubicin (DOX), a potent antineoplastic agent, is limited by its serious side-effects, which include acute and chronic cumulative dose-related cardiotoxicity. Berberine (BER), a botanical alkaloid, has been reported to possess cardioprotective and antitumor effects. The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) assay was used to detect the cell viability of A549, HeLa and HepG2 cells after each cell line was treated with DOX, BER or a combination of DOX and BER for 24 h. Apoptosis was evaluated by acridine orange staining. The results showed that BER and DOX exhibited dose-dependent inhibitory effects on A549 and HeLa cells which were likely mediated by inducing apoptosis. The same result was found in the combination group. Isobologram illustration and combination index (CI) analyses revealed that the combination of DOX and BER generates synergistic effects in A549 (CI=0.61) and HeLa (CI=0.73) cells. These findings indicate that BER sensitizes cells to the anticancer effects of DOX.

Introduction

Doxorubicin (DOX), an anthracycline antibiotic and antineoplastic agent, was first isolated from *Streptomyces peucetius* (1). DOX is a potent chemotherapeutic agent that is used in the treatment of solid tumors and malignant hematological diseases (2). DOX exerts its antitumor activity by inserting into DNA, leading to double-stranded DNA breaks (DSB), and intercepting DNA topoisomerase II activity (3,4). However, the clinical use of DOX has been largely restricted due to its cardiotoxicity, which may lead to the development of cardiomyopathy and ultimately congestive heart failure (5). The molecular mechanisms underlying DOX-induced cardiotoxicity include the formation of free radicals, activation of transcription factor NF-κB, increased lipid peroxidation and Ca^{2+} overloading (6-8). The use of cardioprotective drugs is an alternative approach to reduce the cardiotoxicity of DOX. Pharmacological and clinical attempts to reduce the cardiotoxicity of DOX have had little success thus far. Consequently, it is important to develop a therapy to reduce DOX-induced cardiotoxicity and increase the antitumor effect of DOX.

Berberine (BER), a botanical alkaloid, is purified from the roots and bark of the *Berberis* species (9). BER reportedly possesses multiple biological and pharmacological properties, including anti-diarrheal, anti-fungal, anti-diabetic (10-12), hepatoprotective and cardioprotective effects. The possible mechanism of the hepatoprotective effect is that BER inhibits the activity of CYP 2E1 and CYP 1A2, reduces the production of nitric oxide and lowers the AST and ALT levels in serum (13,14). For the cardioprotective property, BER is known to modulate Cdk9 and cyclin T1 protein expression. BER possesses muscarinic agonist-like properties which may contribute to a reduction in myocardial damage (15-17). BER also suppresses tumor growth through the induction of apoptosis and cell cycle arrest in cancer cells (18-21). Notably, it has been reported that the acute toxicity of BER was not observed at normal dosage in mice (22).

Based on these findings, we hypothesized that combining DOX with BER as a novel strategy for tumor therapy would not only increase the effect of DOX, but also prevent the cardiotoxicity induced by DOX. The present study was therefore performed to test this hypothesis in A549, HepG2 and HeLa cells. Our observations revealed that BER enhances the antitumor effects of DOX in A549 and HeLa cells.

Materials and methods

Chemicals. BER was kindly provided by Professor Xue-Gang Li (Southwest University, Chongqing, China). Dimethyl sulfoxide (DMSO), trypsin, penicillin, streptomycin, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) and

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Abbreviations: AO, acridine orange; BER, berberine; CI, combination index; DOX, doxorubicin; DMSO, dimethyl sulfoxide; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide; IC_{50}, the 50% growth inhibition concentration

Key words: berberine, doxorubicin, synergistic effect, A549, HeLa

acridine orange (AO) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was obtained from Tianhang Biotechnology Company (Zhejiang, China). DOX was purchased from Shanxi Powerdone Pharmaceutics Company (Beijing, China).

Cell culture. The human lung carcinoma A549, human cervix carcinoma HeLa and human hepatoma HepG2 cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37˚C in 5% CO_2. The cells were subcultured at 90% confluence with 0.25% trypsin (w/v) every 2-3 days.

Cell viability assay. The cells were seeded in 96-well plates at different densities: A549, 7,000 cells/well; HeLa, 6,000 cells/well; and HepG2, 8,000 cells/well. The stock solutions of DOX and BER [both dissolved in phosphate-buffered saline (PBS)] were then diluted in culture medium to obtain the desired concentrations (BER: 0, 1, 10, 100, 200, 400 μM; DOX: 0, 0.1, 1, 10, 100, 200 μM; BER+DOX: 0+0, 1+0.2, 10+2, 50+10, 100+20, 200+60 μM). The MTT assay was used to detect cell viability. Briefly, 10 μl of MTT (at 5 mg/ml) was added to each well, at a final concentration of 500 μg/ml. Following 4 h of incubation under standard conditions, the cell supernatants were removed. DMSO (100 μl) was then added to dissolve the MTT crystals (formazan). The absorbance of the sample was read using a Bio-Rad microplate reader (model 630; Hercules, CA, USA) at 490 nm.

Analysis of drug synergism. The combination index (CI) was calculated for the analysis of the synergistic, antagonistic or additive effects of the two drugs (23). The CI is calculated using the formula: $CI=[(D)_1/(D_x)_1]+[(D)_2/(D_x)_2]$, in which $(D)_1$ is the concentration of the first drug required to achieve a particular effect in the combination; $(D_x)_1$ is the concentration of the first drug that causes an identical effect alone; $(D)_2$ is the concentration of the second drug which achieves a particular effect in the combination; $(D_x)_2$ is the concentration of the second drug that generates the same effect alone. CI>1 indicates antagonism, CI=1 indicates an additive effect and CI<1 indicates synergy.

Fluorescent microscopy measurements. To detect apoptosis, A549 cells were stained with AO. The cells were seeded in 6-well plates at a density of 800,000 cells/well. For the AO procedure, A549 cells were treated with different concentrations of BER and DOX (BER: 0, 75, 150, 300 μM; DOX: 0, 1.5, 3, 6 μM; BER+DOX: 0+0, 75+1.5, 150+3, 300+6 μM) for 24 h and then 10 μl of prepared AO working solution (100 μg/ml in PBS) was added. The cells were immediately examined with a fluorescence microscope (Olympus U-RFLT50, Tokyo, Japan). Morphologically apoptotic cells were counted from 10 visual fields of 5 different areas for each group.

Statistical analysis. Values are presented as the mean ± SEM. One-way ANOVA and the Student's t-test were performed. $P<0.05$ was considered to indicate a statistically significant result.

Results

BER enhances DOX-mediated cytotoxicity in solid tumor cells. To determine the cell viability following treatment with different concentrations of DOX and BER in the three cell lines, the MTT assay was performed. The results indicate that DOX and BER significantly inhibited cell viability in A549, HeLa and HepG2 cell lines in a dose-dependent manner (Fig. 1). As shown in Fig. 1, 100 μM BER caused 39.4% inhibition in A549 cell lines and 200 μM BER had an acute cytotoxic effect in A549 and HeLa cells. The 50% growth inhibition concentration (IC_{50}) of BER in A549, HepG2 and HeLa cells following 24 h of incubation was 139.4, 3,587.9 and 159.5 μM, respectively (Table IA). The IC_{50} of DOX and BER in the combination group are shown in Table IB. A549 and HeLa cells were found to be more sensitive to BER than HepG2 cells (Table I). A549 cells were the most sensitive to DOX. In the present study, we found that the IC_{50} of the combination of BER and DOX was lower than that of each drug used alone.

Synergistic action of BER and DOX. Isobolograms were used to evaluate whether combining BER and DOX generates a synergistic effect (24). As shown in Fig. 2, the Y-axis shows the IC_{50} of BER and the X-axis shows the IC_{50} of DOX. The straight line (additivity line) connects the IC_{50} values of DOX and BER when the drugs are used alone. In the present study, we found that the IC_{50} of combined DOX and BER was below the straight line, indicating that a combination of the two drugs may generate a synergistic antitumor effect in A549 and HeLa cells (Fig. 2). The CI was used to analyze the synergistic effect. The IC_{50} of DOX and BER was used to calculate CI. The CI was (1.7/3.1)+(8.6/139.4)=0.61 in the A549 cells, indicating that combined DOX and BER generates synergistic effect. In the HeLa cells, the CI was (1.9/16.7)+(98.9/159.5)=0.73. These results suggest the synergistic action of BER and DOX in cancer therapy.

Combined treatment with BER and DOX causes solid tumor cell apoptosis. To assess whether the decrease in viability was mediated by inducing apoptosis, cells that had been treated with the two drugs were stained with AO. Results showed that the single and combined treatment with DOX and BER induced apoptosis (Fig. 3). The number of apoptotic cells was increased in the combination group compared with the single treatment group (Fig. 3D), suggesting that the combination of DOX and BER synergistically induces the apoptosis of A549 cells.

Discussion

The findings of the present study indicate that BER, a botanical alkaloid, is able to enhance the anticancer effect of DOX in A549 and HeLa cells. Our results have shown that DOX and BER significantly reduced the viability of A549 and HeLa cells. From the IC_{50} of DOX and BER, we found that the IC_{50} of the combination of DOX and BER was lower than the IC_{50} of the drugs used singly. Notably, the results of this study demonstrate that combining DOX with BER generates a synergistic anticancer effect in A549 and HeLa cells.

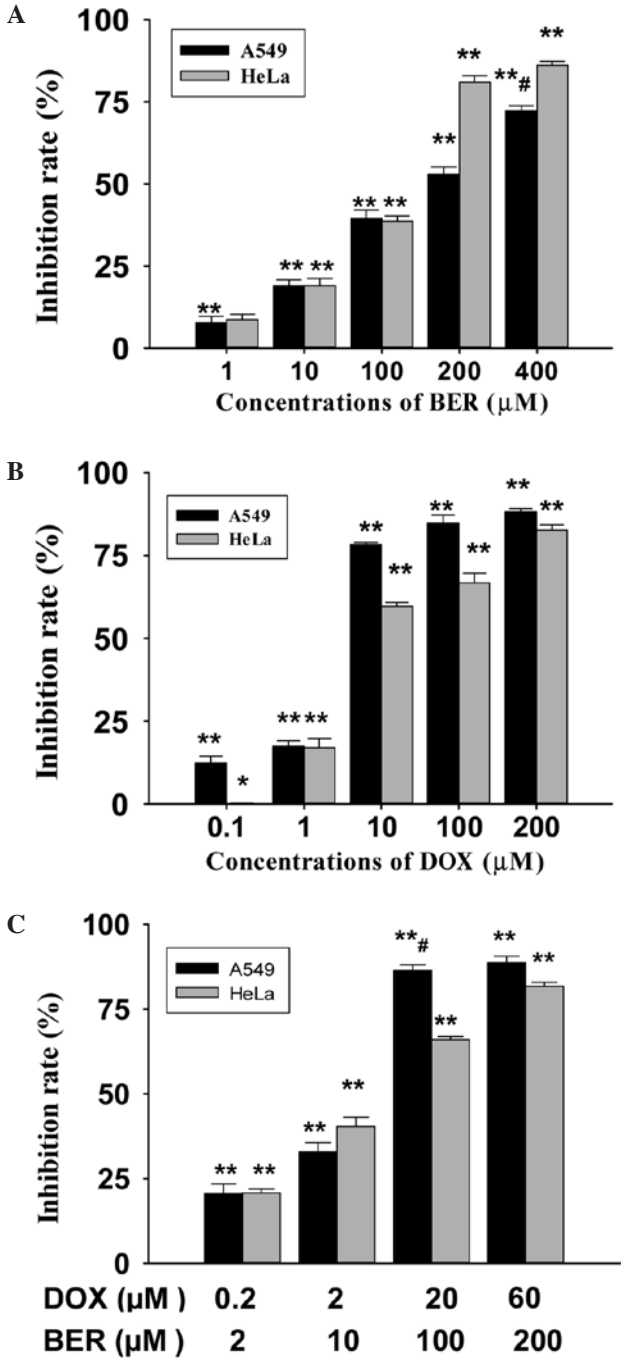

Figure 1. Inhibition rates of A549 and HeLa cells treated with (A) BER, (B) DOX and (C) a combination of DOX and BER for 24 h. The two drugs, administered singly and in combination for 24 h, significantly inhibited cell proliferation in A549 and HeLa cells in a dose-dependent manner. *P<0.05, **P<0.01, compared with the control group that was treated with no drugs. (A) #P<0.05 compared with A549 cells treated with BER (200 μM). (C) #P<0.05 compared with A549 cells treated with a combination of BER and DOX (200+60 μM, respectively). BER berberine; DOX, doxorubicin.

Table I. Sensitivity of A549, HepG2 and HeLa cells to the treatment with BER and DOX alone and in combination.

A, IC_{50} of BER and DOX alone (μM).

		A549 cells	HepG2 cells	HeLa cells
IC_{50}	BER	139.4	3,587.8	159.5
	DOX	3.1	9.2	16.7

B, IC_{50} of combined DOX and BER (μM).

		A549 cells	HepG2 cells	HeLa cells
IC_{50}	BER	8.6	-	98.9
	DOX	1.7	-	1.9

IC_{50}, 50% growth inhibition concentration; BER, berberine; DOX, doxorubicin.

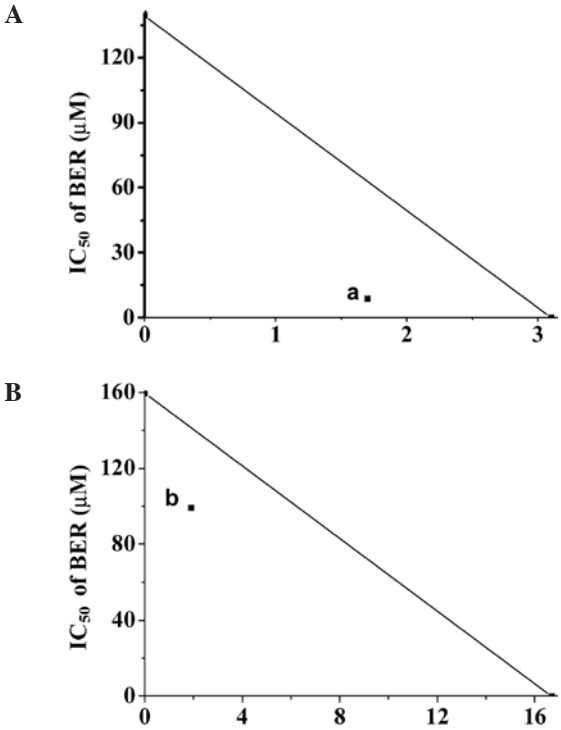

Figure 2. Isobologram for the combination of DOX with BER which generates a synergistic effect. (A) Isobologram illustration for BER+DOX-treated A549 cells; point 'a' is the IC_{50} of combined DOX and BER. (B) Isobologram illustration for BER- and DOX-treated HeLa cells; point 'b' is the IC_{50} of combined DOX and BER. Both points 'a' and 'b' are below the isobologram lines. BER berberine; DOX, doxorubicin; IC_{50}, 50% growth inhibition concentration.

DOX has been found to have anticancer activities against a range of solid tumors. However, the therapeutic use of DOX has been limited by its serious dose-related cardiotoxicity (25). BER has been reported to be safe and beneficial in the treatment of patients with chronic congestive heart failure (16). Therefore, combining DOX with BER is a novel strategy for the treatment of cancer and reduction of the cardiotoxicity induced by DOX.

BER is a naturally occurring botanical alkaloid that is found in the roots and bark of the *Berberis* species. In clinical use, BER possesses anti-inflammatory, anti-diarrheal and anti-fungal effects. BER has also been reported to possess anticancer properties and anti-metastatic effects in non-small cell lung cancer A549 cells (26). The mechanism of its antitumor effect is that BER induces apoptosis and cell cycle arrest in cancer cells (27,28). However, the anticancer effect of BER is

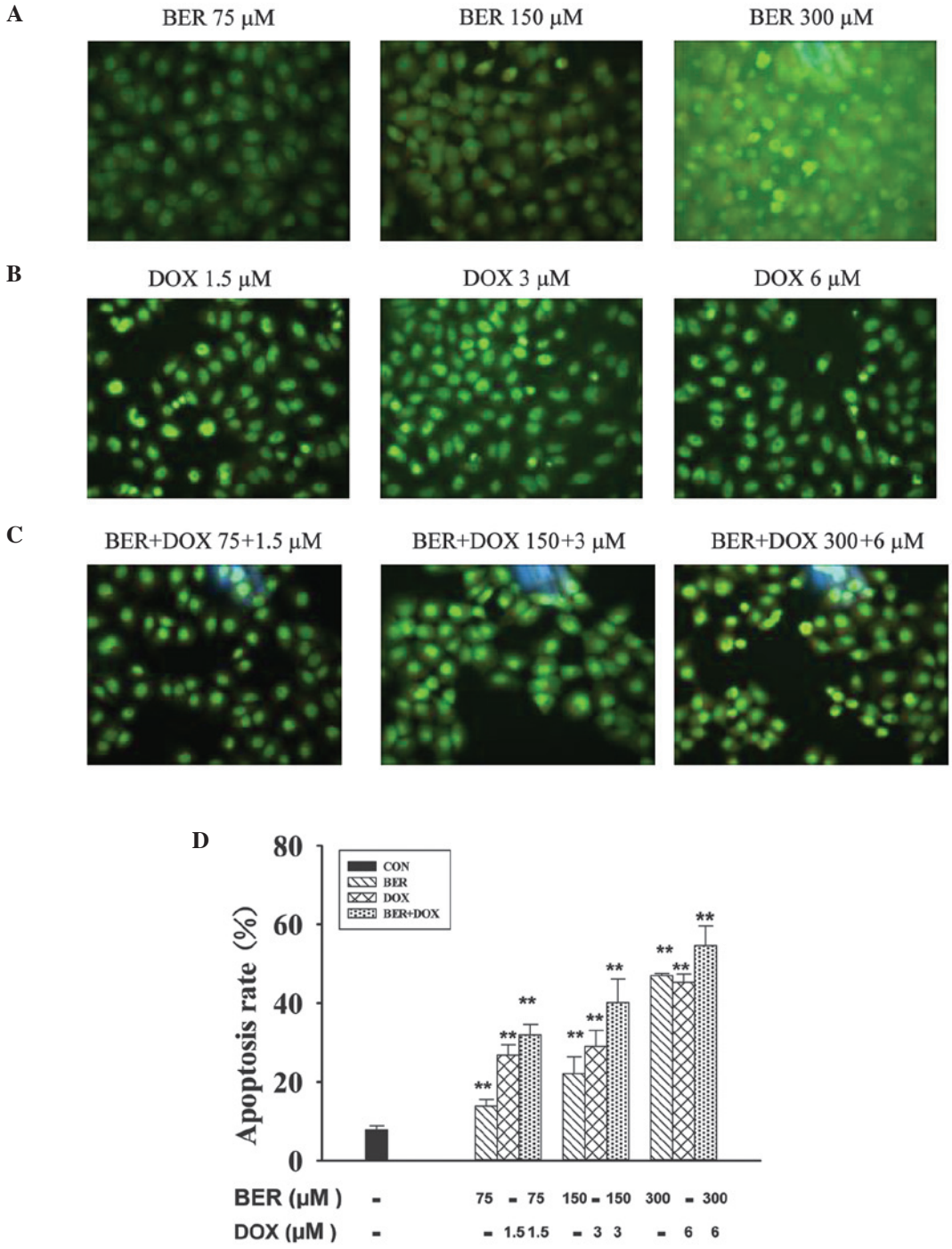

Figure 3. A549 cell apoptosis was assessed by AO staining. (A) A549 cells were preincubated with BER (75, 150, 300 μM) for 24 h. (B) A549 cells were preincubated with DOX (1.5, 3, 6 μM) for 24 h. (C) A549 cells were preincubated with DOX+BER (1.5+75, 3+150, 6+300 μM) for 24 h. (D) Apoptosis induced in BER-, DOX- or BER+DOX-treated cells. In the combination group, the apoptosis rates were increased compared with the single treatment groups. **P<0.01, compared with the control group that was treated with no drugs. N=10. BER berberine; DOX, doxorubicin; AO, acridine orange.

associated with the cell type; the IC_{50} of BER in the HepG2 cell line is 3,587.8 μM, which is extremely high for an antitumor drug, but the IC_{50} of BER is lower in A549 and HeLa cells. Our results have shown that BER induces apoptosis in A549 cells. Notably, the combination of DOX and BER also synergistically induced the apoptosis of A549 cells (Fig. 3). These data suggest that the induction of apoptosis is the mechanism by which the combination of DOX and BER inhibits the growth of A549 cells. However, more investigations are required to demonstrate the efficacy of the combination of DOX and BER in treating cancer patients.

The induction of apoptosis is one of the antitumor mechanisms of DOX and BER. This is in accordance with the theory of 'independent similar action' (29). Therefore, combining DOX with BER may achieve a synergistic antitumor effect. In the present study, we used isobologram illustrations to detect

the synergism. A combination of the two drugs generated synergistic antitumor effects in A549 (CI=0.61) and HeLa (CI=0.73) cells (Fig. 3). Thus, more studies should be conducted to detect the mechanism of the synergistic anticancer action of DOX and BER.

In conclusion, we confirmed that the combination of DOX and BER synergistically generates anticancer effects in A549 and HeLa cells *in vitro*, possibly mediated by inducing apoptosis. With regard to HepG2 cells, the IC_{50} of BER is extremely high for an antitumor agent. The combination of DOX with BER is a novel strategy that has potential in the treatment of cancer patients.

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