Enhanced antitumor activity of epirubicin combined with cerulenin in osteosarcoma

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Abstract. Osteosarcoma (OS) is the most common primary malignant neoplasm in children and adolescents. Epirubicin is one of the chemotherapeutic agents currently used for the treatment of OS; however, the efficacy of chemotherapy is hampered by the acquired drug resistance of OS. Cerulenin, an inhibitor of fatty acid synthase, has been defined as a candidate tumor suppressor. In this study, we explored the combined effect of cerulenin plus the chemotherapy drug, epirubicin, on human OS U2-OS cells *in vitro* and *in vivo*. We demonstrated that cerulenin plus epirubicin induced synergistic growth inhibition and enhanced apoptosis in U2-OS cells. We also demonstrated that cerulenin plus epirubicin synergistically suppressed tumor growth in subcutaneously xenografted U2-OS cells in athymic nude mice. Our results indicate that cerulenin enhances the anti-OS effects of epirubicin *in vivo* and *in vitro*.

Introduction

Osteosarcoma (OS) is the most common primary malignant neoplasm in children and adolescents. The estimated incidence rate worldwide is 4/million/year, with a peak incidence at the age of 15-19 years (1). By combining surgery with multiagent chemotherapy over the past three decades, the 5-year cumulative survival rate of primary OS patients has been significantly improved to 60-90% (2-8). Unfortunately, the efficacy of chemotherapy is hampered by acquired drug resistance (9). It is estimated that less than 30% of patients with recurrent disease are cured (10-12). Therefore, strategies on how to improve the key effect of chemotherapy is the focus for treating OS.

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Epirubicin is one of the chemotherapeutic agents used for the treatment of OS (13,14). Epirubicin exhibits growth inhibition of tumors by inducing apoptosis. Conversely, it reduces apoptosis in OS cells by activating NF-κB (15). At the same time, many other factors also result in the limitation of epirubicin in clinical application, including toxicity and adverse reactions. Fortunately, adjunctive therapies of drugs sometimes reduce adverse reactions and drug resistance. Therefore, we attempted to find a compound, which would enhance the effect of epirubicin against OS.

Cerulenin, a small-molecule fatty acid synthase (FASN) inhibitor, has been isolated from *Cephalosporium caerulens*. Cerulenin contains an epoxy group that reacts with the keto-acyl synthase domain of FASN. It is one of the first compounds to be found to inhibit FASN in breast cancer cell lines. Cerulenin has revealed significant antitumor activity in many malignant neoplasm cells by inducing apoptosis and growth inhibition (16-19).

In the present study, we investigated the enhanced antitumor activity of epirubicin combined with cerulenin *in vitro* and *in vivo*. We observed that there was increased apoptosis and growth inhibition in U2-OS OS cells when treated with cerulenin plus epirubicin *in vitro*. In addition, the size and weight of U2-OS OS subcutaneously xenografted tumors in athymic nude mice treated with cerulenin plus epirubicin were reduced compared to those treated with either drug alone. Further investigations are required to explore the mechanism of the enhanced effect of cerulenin plus epirubicin.

Materials and methods

Cell growth assay. A U2-OS cell line was cultured in 96-well tissue culture plates at a density of 5,000 cells/well in minimum essential medium (MEM) containing 10% fetal bovine serum and 2 mM L-glutamine. Following attachment overnight, the medium was replaced and cells were incubated with increasing concentrations (2.5, 5, 10 and 20 μg/ml) of cerulenin (Sigma, St. Louis, MO, USA), epirubicin (Zhejiang Hisun Pharmaceutical Co., China) or combined at different ratios (cerulenin:epirubicin = 1:1, 1:2 and 2:1). Following treatment for 48 h, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were carried out at 490 nm wavelength in triplicate. The inhibi-

tion ratio by cerulenin, epirubicin or the combination at each concentration was calculated, and the concentration-viability curves were fitted by Originpro7.5 program. The half maximal inhibitory concentration (IC_{50}) values of cerulenin, epirubicin or a combination were determined. All experiments were repeated three times. Interaction between epirubicin and cerulenin was assessed using the combination index (CI): CI = $C_{AC}/C_{AS} + C_{BC}/C_{BS}$, where C_{AC} and C_{BC} are the concentration of cerulenin and epirubicin, respectively, in a combined model when the inhibition ratio is 50%. C_{AS} and C_{BS} are the concentration of cerulenin and epirubicin, respectively, when used solely at 50% inhibition. When CI is <0.95, there is a synergistic effect between cerulenin and epirubicin. When CI is >1.05, there is an antagonizing effect between them. When CI is <1.05 but >0.95, there is an additive effect between them.

Fluorescence-activated cell sorting (FACS). U2-OS cells in exponential growth phase were treated with varying concentrations of cerulenin, epirubicin or the combination for 24 h. Cells were then fixed with 70% ethanol and stained with Propidium Iodide (PI). FACS analysis was performed to determine the percentage of apoptotic cells and cell cycle distribution by using the EPICS XL flow cytometer and System II software. Jin's Q value, which is also used to assess the adjunctive therapy, was determined by the following: Q = $E_{(A+B)}/[E_A + (1 - E_A) \times E_B]$; $E_{(A+B)}$ was the apoptotic percentage at various concentrations when combined, while E_A and E_B were those when cerulenin and epirubicin were used solely. When the Q-value is <0.85, there is an antagonizing effect between cerulenin and epirubicin. When the Q-value is >1.15, there is a synergistic effect between them. If the Q-value is <1.15 but >0.85, there is an additive effect between them.

Evaluation of cerulenin, epirubicin or combination in vivo. BALB/C nude mice (SPF grade, males) aged between 4 and 5 weeks, were obtained from the Shanghai Institute of Materia Medica (Shanghai, China), housed in sterile cages under laminar airflow hoods in a specific pathogen-free room with a 12-h light and 12-h dark schedule and fed autoclaved chow and water *ad libitum*. All animal procedures and maintenance were conducted in accordance with the institutional guidelines of the University of Nan Chang. Mice were injected with a 0.2-ml suspension containing 2×10^6 U2-OS cells. After 7 days, when tumors reached a volume of 0.1 cm³, 24 mice were randomly separated into four groups as follows: the control group which received a i.p. administration of 0.2 ml phosphate-buffered saline (PBS) containing 10% dimethyl sulfoxide (DMSO) (group A); the cerulenin group (group B) which received cerulenin treatment (40 mg/kg weight); the epirubicin group (group C) which received epirubicin treatment (2 mg/kg weight); the cerulenin combined with epirubicin group (group D) which received cerulenin (40 mg/kg weight) combined with epirubicin (2 mg/kg weight) treatment. All animals received i.p. administration once every 48 h which lasted for 12 days. A total of 18 days prior to the end of administration, the mice were sacrificed and the tumors were flaked off. The volume and weight of tumors were measured. The volume and weight inhibition ratios were calculated as follows: % volume inhibition = 100 x (1 - volume$_{treated}$/volume$_{control}$); % weight inhibition = 100 x (1 - weight$_{treated}$/weight$_{control}$).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assays. Tumor tissues were cut into slices, which were then paraffin-embedded, followed by TUNEL assays using In Situ Cell Death Detection kit (Roche Diagnostics Corp., Indianapolis, IL, USA). Briefly, following deparaffinization and rehydration, tissue sections were pre-treated with sodium chloride-sodium citrate buffer (pH 7.0) at 80°C for 20 min, followed by thorough washing in distilled water. Subsequently, sections were digested with Proteinase K for 1 h with gentle agitation at 37°C and digestion was discontinued by washing in running water. Tissue sections were then incubated with fluorescein-labeled deoxyuridine triphosphate (dUTP) reagent and TUNEL reagent in order, according to the manual instructions. Subsequently, the sections were stained with diaminobenzidine (DAB) via horseradish peroxidase (HRP)-conjugated fluorescein antibody and counterstained with 0.5% methyl green. The sections were mounted with coverslips and imaged; brown-stained cells were considered to be apoptotic cells, and the number of brown-stained cells was used to determine the apoptotic index (AI) by dividing this value by the total number of tumor cells.

Statistical analysis. Data were expressed as the means ± standard deviation (SD). A one-way ANOVA and least significant difference (LSD) tests were used for statistical analysis, and a p-value of <0.05 was considered to be statistically significant. All analyses were performed using SPSS version 13.0 (Statistical Software for Social Sciences, Inc., Chicago, IL, USA).

Results

Synergistic antitumor effect between cerulenin and epirubicin. Epirubicin is a type of antitumor agent that is widely used; at the same time, cerulenin inhibits cell growth in many types of cancer cell lines. We examined the effect of cerulenin and epirubicin used alone on the growth of the U2-OS cell line, as well as the adjunctive therapy between them, by MTT assay. The growth curves indicated that U2-OS cells were sensitive to both cerulenin and epirubicin, and that the growth inhibition occurred in a dose-dependent manner (Fig. 1A). The IC_{50} values of epirubicin and cerulenin for U2-OS cells were 9.85 and 22.63 µg/ml, respectively. When these two reagents were applied on U2-OS cells adjunctively in the proportions of 1:1, 1:2 and 2:1 (Fig. 1B), the IC_{50} values were 8.01, 11.54 and 11.85 µg/ml, respectively. All CIs of 0.51, 0.58 and 0.73 in proportions of 1:1, 1:2 and 2:1, respectively, were <0.95, indicated that a synergistic antitumor effect existed between epirubicin and cerulenin.

Epirubicin, cerulenin and combined treatment induces apoptosis in U2-OS cells. FACS analysis was used to examine the mechanism of cell growth inhibition by epirubicin, cerulenin and the combination treatment. Reagents of varying concentrations were added to U2-OS cell cultures in the exponential growth phase. Following treatment for 24 h, treated and untreated cell samples were taken and fixed for FACS analysis. FACS analysis revealed that apoptosis occurred in a dose-dependent manner, regardless of whether cells were treated with epirubicin, cerulenin or a combination (Fig. 2). The percentage of apoptotic cells found in the cells treated
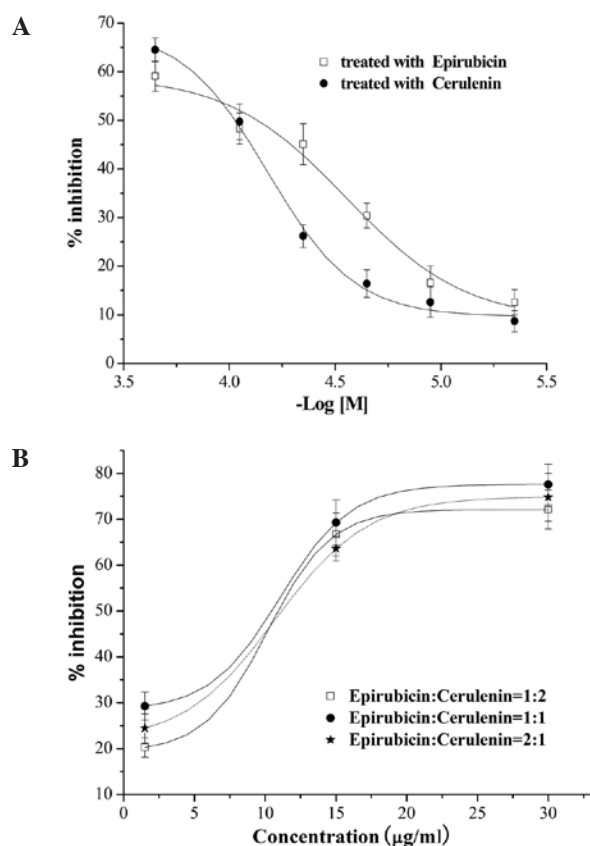
A

B

Figure 1. Inhibitory effect of epirubicin, cerulenin or combination treatment on the growth of U2-OS cells. (A) Epirubicin and cerulenin both inhibited U2-OS cell growth in a dose-dependent manner. (B) Epirubicin was applied to U2-OS cells adjunctively with cerulenin in a proportion of 1:1, 1:2 and 2:1. The adjunctive treatment also inhibited U2-OS cell growth in a dose-dependent manner.

Table I. Percentage of apoptotic cells in cells treated with epirubicin, cerulenin and their combination.

Groups	Percentage of apoptotic cells[a]	Q-value
Epirubicin treatment		
1 μg/ml	17.27±2.34	
5 μg/ml	26.07±3.17	
10 μg/ml	31.54±1.33	
Cerulenin treatment		
1 μg/ml	7.32±0.98	
5 μg/ml	11.17±1.22	
20 μg/ml	38.78±2.13	
Combination treatment with epirubicin plus cerulenin		
1 μg/ml epirubicin + 1 μg/ml cerulenin	26.94±1.65	1.17
10 μg/ml epirubicin + 5 μg/ml cerulenin	38.77±3.46	0.97
10 μg/ml epirubicin + 20 μg/ml cerulenin	66.19±3.97	1.14

[a]Values expressed as the mean ± standard deviation (SD).

with epirubicin was significantly higher than that in cells treated with cerulenin at the same concentration (Table I); 17.27, 26.07 and 31.54% in cells treated with 1, 5 and 10 μg/ml epirubicin, respectively. When these two reagents were applied to U2-OS cells in adjunct, the apoptotic percentage improved. In particular, when 1 μg/ml epirubicin was applied to cells in adjunct with 1 μg/ml cerulenin, the Q-value was 1.17, indicating a synergistic effect between them. Of note, the apoptotic percentage in cells treated by 10 μg/ml of epirubicin and 5 μg/ml of cerulenin in adjunct was only slightly higher than that in cells treated with 10 μg/ml epirubicin alone, with a Q-value of 0.97.

Epirubicin combined with cerulenin significantly inhibits the growth of xenografted tumors. A significant *in vivo* antitumor effect was also observed in mice treated with epirubicin combined with cerulenin (Fig. 3). The diameter of the tumor tissue was >2 cm in the control group, while in the groups treated with epirubicin and cerulenin the diameter was only ~1.5 cm, with volume inhibition ratios of 36.2 and 34.3%, respectively. However, when the mice were treated with epirubicin combined with cerulenin, the diameter of the tumor tissue was only ~1 cm, with a volume inhibition ratio of 75.1% compared to the control group. Accordingly, the tumor weights decreased with the treatment of epirubicin, cerulenin and combination treatment, with tumor weight inhibition rates of 35.3, 33.0 and

75.9%, respectively. There was no significant difference in volume and weight inhibition ratios between groups treated with epirubicin and cerulenin alone. However, when epirubicin was applied conjunctively with cerulenin in mice, both volume and weight inhibition ratios increased significantly. The Q_{weight} and Q_{volume} were 1.32 and 1.34, respectively, which indicated that a synergistic antitumor effect existed between epirubicin and cerulenin.

Epirubicin combined with cerulenin significantly induces apoptosis of tumor cells. The fact that epirubicin combined with cerulenin significantly inhibited the growth of xenografted tumors raised the possibility that adjunctive therapy may greatly induce apoptosis in tumor cells. TUNEL assays were carried out to test this theory. Epirubicin, cerulenin and the combination treatment all induced apoptosis in tumor cells (Fig. 4). As noted in Fig. 4B-D, brown-stained nuclei were evident throughout, whereas little staining was noted in Fig. 4A. The AI in groups A, B, C and D was 3.5±1.8, 12.1±6.1, 12.3±7.2 and 20.1±7.3%, respectively. Although there was no significant difference in AI between groups B and C, the AI in group D increased, which may partly explain the marked increase in volume and weight inhibition ratios when treated with epirubicin combined with cerulenin.

Discussion

OS is the most common malignant neoplasm of the bone. Owing to the rapid and aggressive nature of the disease, previously, the standard treatment for OS was amputation of the affected limb; however, the cure rate associated with
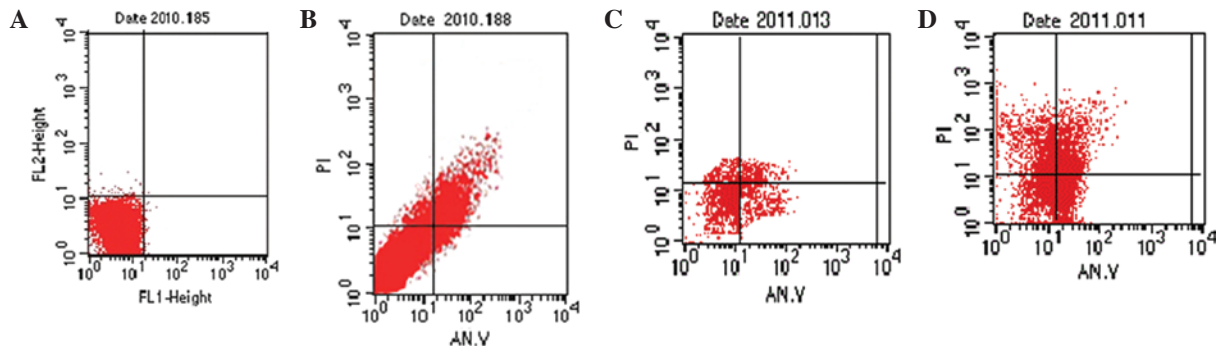
Figure 2. FACS analysis of U2-OS cells treated with epirubicin, cerulenin and the combination treatment for 24 h. (A) Few apoptotic cells were noted in the control group. Yet, the percentage of apoptotic cells was (B) 31.54% when treated with 10 μg/ml epirubicin and (C) 38.78% when treated with 20 μg/ml cerulenin. (D) The percentage of apoptotic cells rose to 66.19% when treated with 10 μg/ml epirubicin in conjunction with 20 μg/ml cerulenin. FACS, fluorescence-activated cell sorting; PI, propidium iodide.
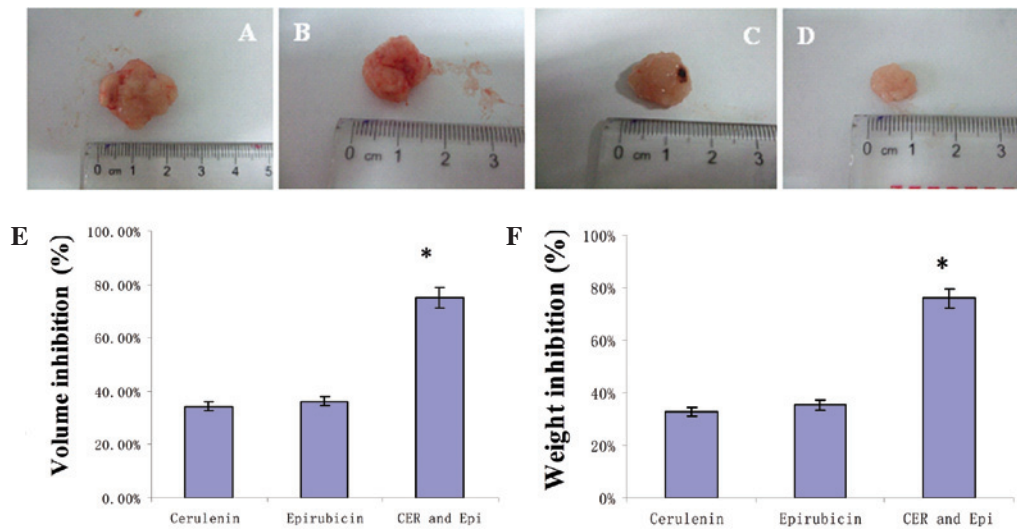

Figure 3. Tumor tissues peeled from xenografted nude mice following treatment with epirubicin, cerulenin and a combination treatment. Representative images are shown for each group. (A) In the control group, the diameter of the tumor tissue was ~2 cm. Following treatment with (B) epirubicin and (C) cerulenin, the diameter of the tumor tissue decreased to ~1.5 cm; concurrently, the weight of the tumor tissue was reduced. (D) When treated with epirubicin combined with cerulenin, the volume and weight of the tumor tissue decreased greatly. (E) The volume inhibition ratio of the cerulenin plus epirubicin group was significantly higher than that of the control, cerulenin and epirubicin alone groups. Columns, mean (n=6); bars, SD. *P<0.05 vs. control; cerulenin and epirubicin groups. (F) The inhibition ratio of group D was significantly higher compared to the cerulenin and epirubicin groups. Columns, mean (n=6); bars, SE. *P<0.05 vs. cerulenin and epirubicin groups. SD, standard deviation; Epi, epirubicin; SE, standard error; CER, cerulenin.
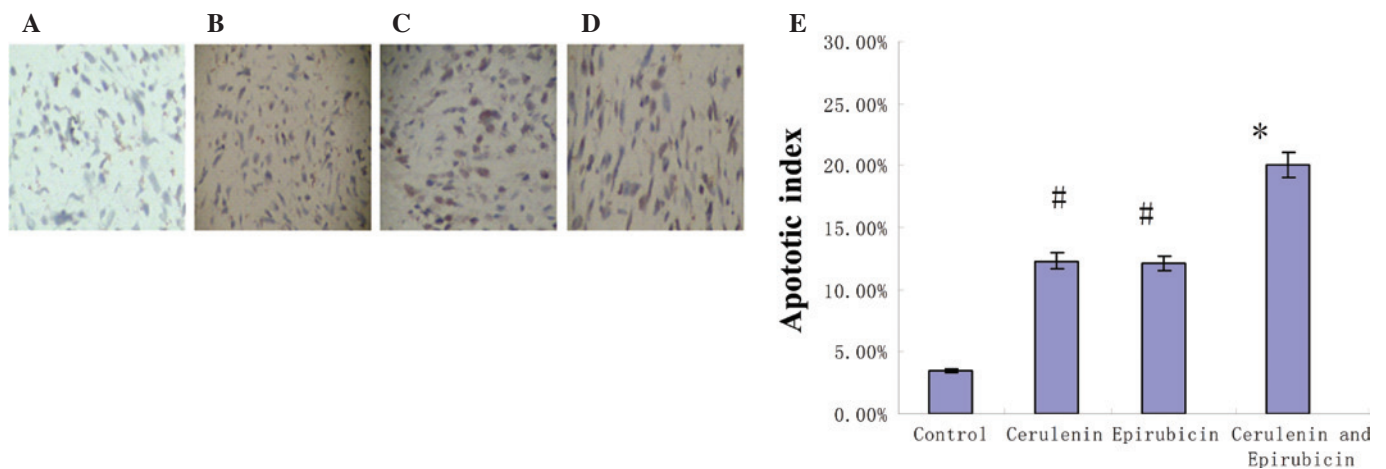

Figure 4. TUNEL assays of tumor tissue slides. (A-D) Representative images for each group. (A) Tumor-bearing mice received i.p. administration of 0.2 ml PBS and tumor tissue slides were prepared. TUNEL assays indicated that there were only a few fluorescent cells, with a ratio of <5%. Brown-stained cells were barely detected. When tumor-bearing mice were treated with (B) cerulenin and (C) epirubicin, the ratio of brown-stained cells increased significantly to ~12%. (D) When epirubicin combined with cerulenin was applied to tumor-bearing mice, there were widespread brown-stained cells, with a ratio of almost 20%. (E) The apoptotic index was significantly higher than that in the control, cerulenin and epirubicin alone groups. Columns, mean (n=6); bars, SD. *P<0.05 vs. control, cerulenin and epirubicin groups. PBS, phosphate-buffered saline; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; SD, standard deviation.

this method was less than 10% and almost all patients died within 1 year from diagnosis. Over the past three decades, the development of effective chemotherapy agents has reduced the incidence of metastasis and mortality (2,20). Chemotherapy is, thus, an important treatment for OS.

Epirubicin, a member of the anthracycline family, is widely applied for the treatment of OS patients, profiting from its weak side effects on the heart. However, the acquired resistance of cancer cells to epirubicin has become a major obstacle for successful cancer therapy (21). Various studies have reported that NF-κB plays an important role in drug resistance (22-24). Cerulenin, an inhibitor of FASN, inhibits the growth of tumor cells by inhibiting NF-κB and inducing apoptosis (25), which may reverse NF-κB activation by epirubicin. Therefore, we hypothesized that cerulenin enhances the anti-OS effects of epirubicin. Fortunately, this hypothesis was confirmed both *in vitro* and *in vivo* in our study. The *in vitro* study indicated that when epirubicin was applied in adjunct with cerulenin in proportions of 1:1 and 1:2, there was a synergistic antitumor effect between them, with Q-values greater than 1.15. However, when epirubicin was combined with cerulenin in a proportion of 2:1, there was only an additive effect between them, with a Q-value of 0.97. Our data suggest that cerulenin enhances the epirubicin anti-OS effect *in vitro*.

In the *in vivo* study, when epirubicin or cerulenin were applied alone, the volume inhibition ratios were 36.2 and 34.3%, respectively, and the weight inhibition ratios were 35.3 and 33%, respectively. However, when epirubicin was applied in adjunct with cerulenin, there were significant increases in volume and weight inhibition ratios, which were 75.1 and 75.9%, respectively, with Q_{weight} and Q_{volume} of 1.32 and 1.34, respectively. These data indicate that there were synergistic effects between cerulenin and epirubicin for anti-OS *in vivo*.

In conclusion, our results indicate that a synergistic or addictive effect exits between cerulenin and epirubicin for anti-OS *in vitro* and a synergistic effect *in vivo*. It has been suggested that cerulenin combined with epirubicin may be a potential treatment strategy for OS. However, the combination of cerulenin and epirubicin was administered using three ratios of the agents *in vitro*, and only one proportion *in vivo* was applied in this study. Whether another ratio of the agents, in particular increased doses of epirubicin, has enhancing effects between them requires further investigation. In addition, the mechanism involved in the synergistic effect between epirubin and cerulenin remains unknown. In this study, we did not verify that cerulenin reverses NF-κB activation by epirubicin. Perhaps some other factors contribute to the synergistic antitumor effect between them. Future experiments need to be carried out in order to try to elucidate this mechanism.

Acknowledgements

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