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Original Article

Enhanced antitumor activity of ursolic acid combined with cerulenin in osteosarcoma

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Abstract: The present study was devised to investigate the effect of ursolic acid and cerulenin combination on osteosarcoma with an aim to develop an effective treatment strategy. Inhibition of cell growth was observed by MTT assay while as SENP5 expression by quantitative PCR and western blotting analysis in U2OS osteosarcoma cell line and HOB cells. The percentage of apoptotic cells and cell cycle distribution was determined using EPICS XL flow cytometer and System II software. For TUNEL assays to determine apoptotic index (AI) in Situ Cell Death Detection kit (Roche Diagnostics Corp., Indianapolis, IL, USA) was used. The number of brown-stained cells was divided by total number of tumor cells to get AI. The results demonstrated that ursolic acid and cerulenin combination in the proportion of 1:1 1:2 and 2:1 exhibited synergistic effect on cell growth inhibition, SENP5 expression inhibition, and apoptosisinduction in U2OS cells. The IC_{50} values of cerulenin and ursolic acid combination in the proportion of 1:1, 1:2 and 2:1 were 2.0, 2.5 and 3.2 μg/ml, respectively. The combination index (CI) values of < 0.91 for inhibition of growth and < 0.90 for inhibition of SENP5 expression clearly indicated synergism between the two drugs. The Q-value for combination of 0.90 μg/ml cerulenin and 0.90 μg/ml ursolic acid was 1.09. AI calculated for tumor tissues treated with a combination of ursolic acid and cerulenin (23.5 ± 4.4%) was higher than that of the tissues treated separately with ursolic acid (12.4 ± 5.6%) or cerulenin (10.9 ± 6.1%). The AI for untreated tumor tissues was 3.0 ± 1.2%. Thus ursolic acid and cerulenin combination can be a promising treatment strategy for the osteosarcoma.

Keywords: Combination index, tumor tissues, apoptosis, inhibition, ursolic acid, Q-value

Introduction

Osteosarcoma is a commonly observed tumor of bones with a five-year survival rate of ~70% in children and adolescents. Patients with osteosarcoma have a poor prognosis, with overall survival rates of < 20% [1]. Osteosarcoma is a well-defined clinical entity with a characteristic radiographic appearance, histologic features, a relatively consistent spectrum of clinical presentations, and established standard treatments. These features have been the subject of many prior book chapters and reviews [2-7].

Ursolic acid (**Figure 1**), a pentacyclictriterpene present abundantly in the peels of Maluspumila Mill [8] exhibits a wide range of pharmacological properties, including antiinflammatory, antiallergic, antibacterial, antiviral, antitumor and cytotoxic activity being the most intriguing [9-13]. It was ranked as one of the most promising tumor preventive medication by Japanese researchers [14]. Ursolic acid arrests the cell cycle progression in the G1 phase and induces apoptosis [15]. It has many clinical applications in the development of chemotherapy for different types of cancers [16]. It has been reported that keeping polar substituents at the C-28 position of ursolic acid enhanced its antitumor potential against BGC-823 cell lines [17]. In the present study the effect of sequential treatment of UACT (**Figure 1**) followed by 5-FU on small cell lung cancer cells was investigated.

Cerulenin, has been isolated from the extract of Cephalosporiumcaerulens. The plant has a long traditional medicinal importance. Cerulenin is reported to act as inhibitor of fatty acid synthase (FASN) by reacting with the ketoacyl synthase domain of FASN. The role of Cerulenin as an antitumor agent has been reported in many malignant neoplasm [18-21]. In the present study the effect of ursolic acid combined with Cerulenin on cellular proliferation, inhibition of

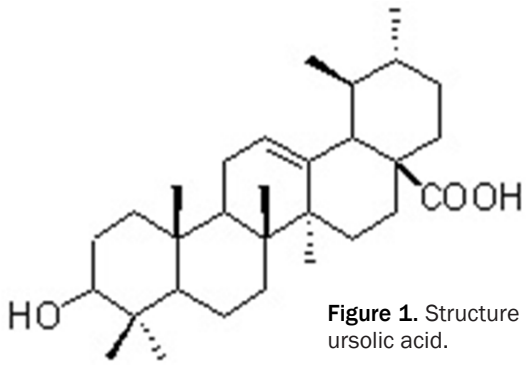

Figure 1. Structure ursolic acid.

SENP5 expression and apoptosis in human osteosarcoma cell line was investigated.

Materials and methods

Cell growth assay

The U2OS osteosarcoma cell line was purchased from the Sigma-aldrich, USA. Cells were maintained in Minimum Essential Medium (MEM) supplemented with 10% Fetal Bovine Serum (FBS) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 -95% air.

MTT assay

In 96-well tissue culture plates 5 × 103 U2OS cells were distributed per well and cultured in minimum essential medium (MEM) containing 10% fetal bovine serum and 2 mM L-glutamine. After 12 h attachment, the U2OS cells were exposed to different concentrations of cerulenin, ursolic acid (Sigma, St. Louis, MO, USA) or a range of sequential combination of cerulenin followed by ursolic acid for 36 h. Then 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to each well followed by addition of DMSO to dissolve farmazan crystals. The absorbance was recorded at 490 nm in a microplate reader (SpectraMax Plus; Molecular Devices) in triplicate. For each tested concentration of cerulenin, ursolic acid and their combination inhibition ratio was calculated and concentration-viability curves were plotted using Originpro7.5 program to get IC_{50} values. All experiments were repeated three times. The combination index (CI) for cerulenin and ursolic acid interaction was calculated. A value of CI < 0.95, CI > 1.05 and CI < 1.05 but > 0.95 indicated synergistic, antagonizing and additive effect respectively between cerulenin and ursolic acid.

Fluorescence-activated cell sorting (FACS)

In FACS analysis, U2OS cells were treated with different concentrations of cerulenin, ursolic acid and their combination for 24 h. EPICS XL flow cytometer and System II software was used to determine the percentage of apoptotic cells and cell cycle distribution. The Q = E (A + B)/[EA + (1-EA) × EB]; equation was used for calculation of Jin's Q value. Where, EA and EB are the apoptotic cell percentage at various concentrations of cerulenin and ursolic acid; E (A + B) is the apoptotic cell percentage at various concentrations of cerulenin and ursolic acid in combination. The Q-value is < 0.83, > 1.20 and < 1.20 but > 0.83 respectively indicates synergistic, antagonizing and an additive effect between cerulenin and ursolic acidB.

Western blot analysis

The transfected osteosarcoma cells were washed twice in PBS. Then, Lysis buffer (50 mM Tris-HCl pH 7.4, 137 mM NaCl, 10% glycerol, 100 mM sodium vanadate, 1 mM PMSF, 10 mg/ml aprotinin, 10 mg/ml leupeptin, 1% NP-40, and 5 mM cocktail) 2 ml was added to the cells. BCA method was used to determine protein concentration. The protein were loaded and resolved by electrophoresis on a 10% polyacrylamide gel. The semi-dry method was used to transfer proteins onto a PVDF membrane which was then blocked with 5% non-fat dry milk overnight. After TBST washing, membrane was incubated for 2 h with primary antibodies and then washed again with TBST before incubation with secondary antibodies for 2 h. Then X-ray autoradiography was performed and the gray scale images were analysed.

Terminal deoxynucleotidyl transferased UTP nick end labeling (TUNEL) assays

The frozen tumor tissues embedded in paraffin were cut into small slices and subjected to TUNEL assays. In Situ Cell Death Detection kit (Roche Diagnostics Corp., Indianapolis, IL, USA) was used for TUNEL assays. The sliced tumor tissues were deparaffinized and rehydrated. The tissue sections were then heated with sodium chloride-sodium citrate buffer (pH 7.0) at 80°C for 20 min. After washing thoroughly with distilled water the sections were treated with Proteinase K for 1 h with gentle agitation at 37°C. Incubation fluorescein-labeleddeoxyuridine triphosphate (dUTP) and TUNEL reagents

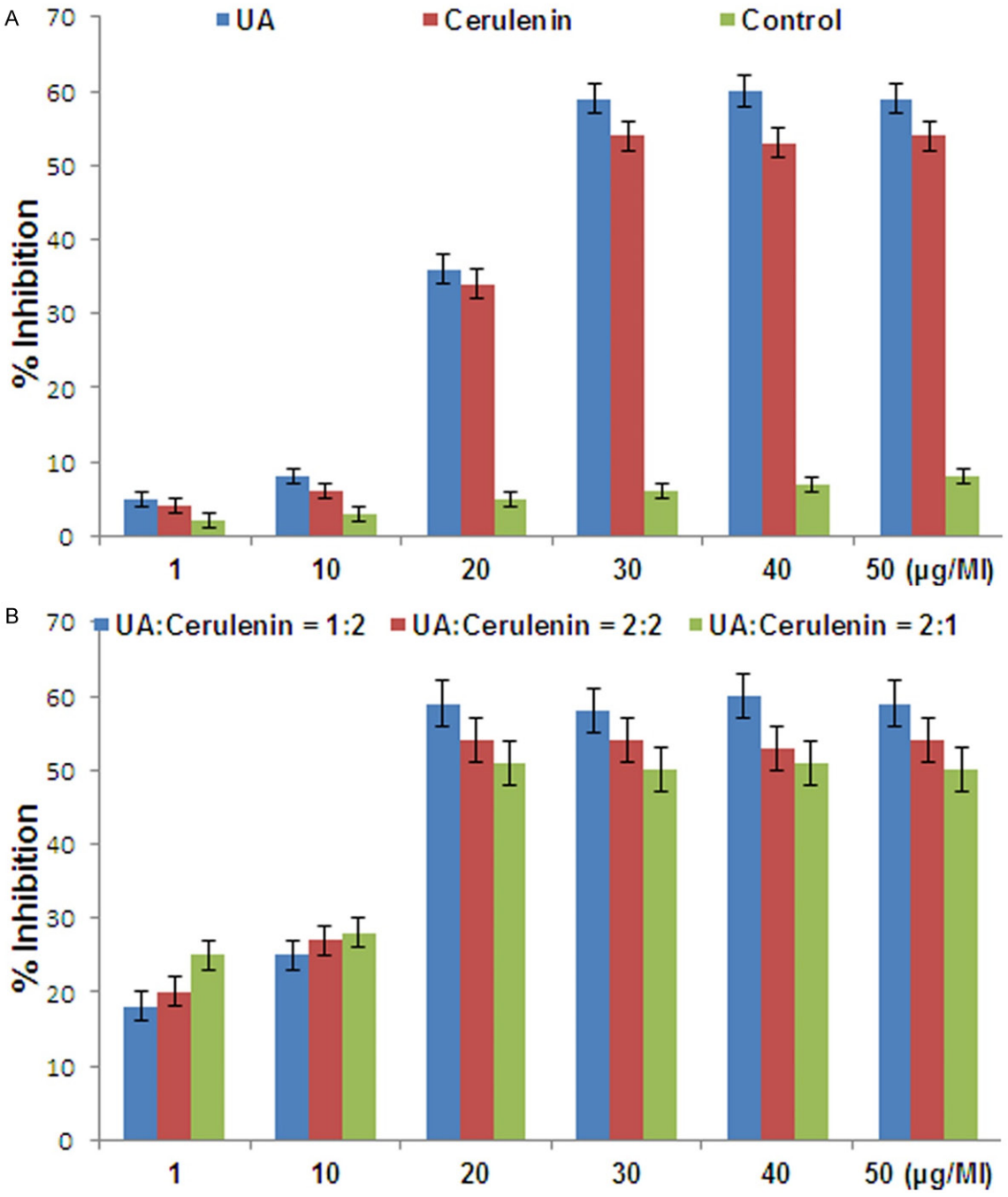

Figure 2. Inhibitory effect of ursolic acid, cerulenin or combination treatment on the growth of U2OS cells. A. Ursolic acid and cerulenin both inhibited U2OS cell growth in a dose-dependent manner. B. Ursolic acid was applied to U2OS cells adjunctively with cerulenin in a proportion of 1:1, 1:2 and 2:1. The adjunctive treatment also inhibited U2OS cell growth in a dose-dependent manner.

was performed as per the instructions of manufacturer. The sections stained with horseradish peroxidase (HRP)-conjugated fluorescein antibody were counterstained with 0.5% methyl green. To determine apoptotic index (AI), the number of brown-stained cells was divided by total number of tumor cells.

Statistical analysis

The data represents the mean of three independent experiments as standard error (SE). Student's t-test was used to analyse the significance. $P < 0.05$ was considered to indicate a statistically significant result.

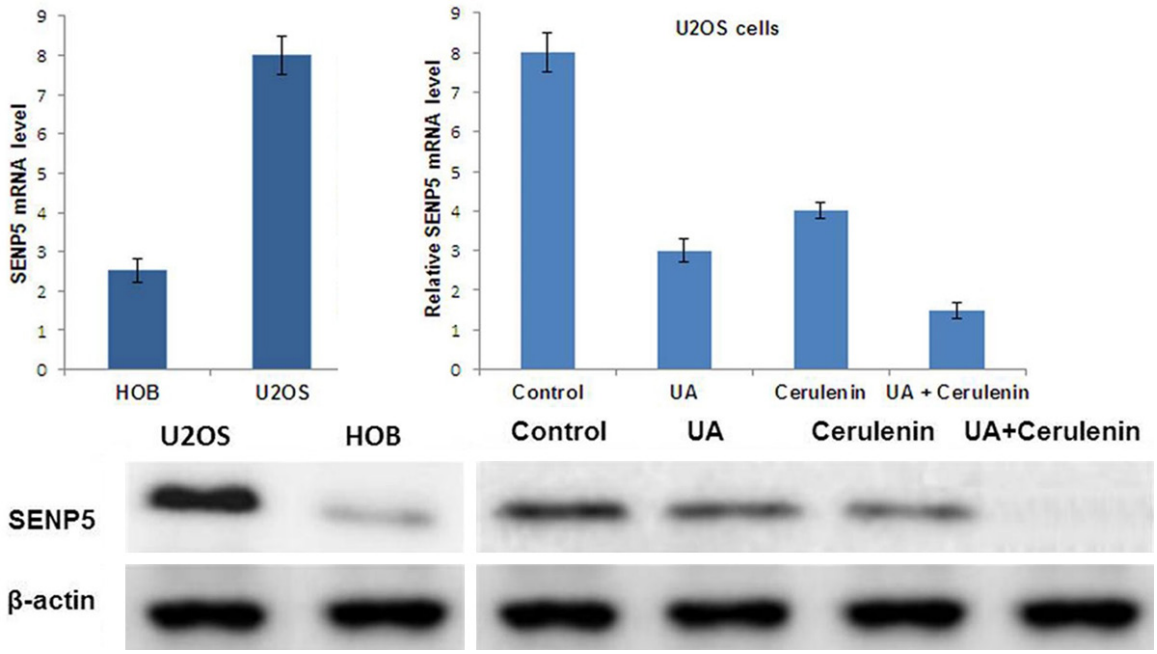

Figure 3. SENP5 is overexpressed in osteosarcoma cell lines. mRNA expression and protein levels of SENP5 in U2OS osteosarcoma cell lines. Inhibition of SENP5 expression by ursolic acid and cerulenin treatment. Concentration dependent inhibition of SENP5 expression in U2OS cells. Time dependent inhibition of SENP5 expression in U2OS cells.

Results

Synergistic effect of cerulenin and ursolic acid on antitumor activity in U2OS cells

The results from MTT assay indicated that U2OS cells were sensitive to both cerulenin and ursolic acid. Both the agents caused an inhibition in growth of U2OS cells in a concentration dependent manner (**Figure 2A**). The IC_{50} values of cerulenin and ursolic acid for antitumor activity against U2OS cells were 5.13 and 21.34 μg/ml, respectively. However, when U2OS cells were treated with a combination of cerulenin and ursolic acid in the proportion of 1:1, 1:2 and 2:1, the values for IC_{50} were 2.0, 2.5 and 3.2 μg/ml, respectively (**Figure 2B**). The calculated CI values for all the three combinations were < 0.91, indicating that cerulenin and ursolic acid exhibited synergistic effect on inhibition of growth in U2OS cells.

Synergistic effect of cerulenin and ursolic acid on inhibition of SENP5 expression in osteosarcoma cell lines

We used quantitative PCR and western blotting analysis to study the effect of ursolic acid and cerulenin combination on SENP5 expression. The results revealed that SENP5 is significantly overexpressed in U2OS osteosarcoma cells compared with human osteoblasts isolated from normal human bone (HOB) (**Figure 3**). These results were in correlation with the earlier reports [22].

Treatment of U2OS osteosarcoma cell line with ursolic acid or cerulenin resulted in inhibition of SENP5 expression in a dose and time-dependent manner (**Figure 3**). We treated osteosarcoma cells with 1:1, 1:2 and 2:1 proportion of ursolic acid and cerulenin. The results from RT-PCR analysis clearly demonstrated a significant inhibition of SENP5 expression on treatment with 1:1, 1:2 and 2:1 proportion of ursolic acid and cerulenin. The values for IC_{50} were 2.0, 2.5 and 3.2 μg/ml, respectively. The calculated CI values for all the three combinations were < 0.90, indicating that cerulenin and ursolic acid exhibited synergistic effect on inhibition of SENP5 expression in U2OS cells.

Synergistic effect of cerulenin and ursolic acid on induction of apoptosis in U2OS cells

The U2OS cell cultures were treated with a range of ursolic acid, cerulenin and their combination concentrations for 24 h. The results

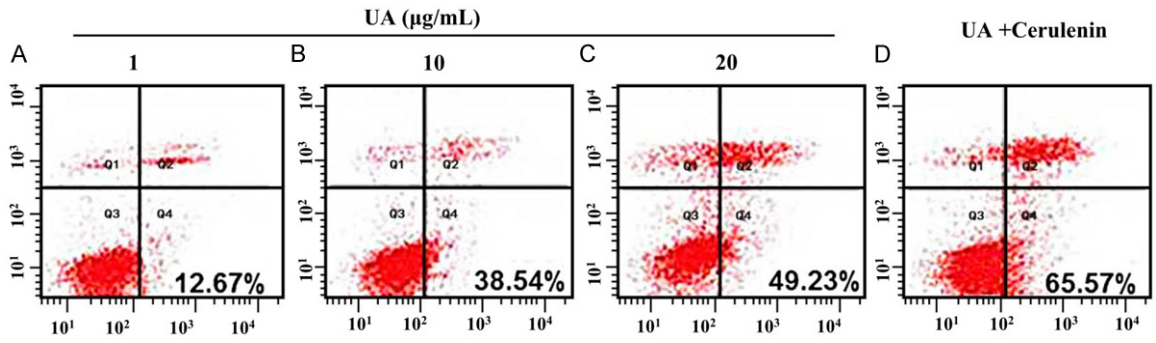

Figure 4. FACS analysis of U2OS cells treated with ursolic acid, cerulenin and the combination treatment for 24 h. (A) Few apoptotic cells were noted in the control group. Yet, the percentage of apoptotic cells was (B) 31.54% when treated with 10 μg/ml ursolic acid and (C) 38.78% when treated with 20 μg/ml cerulenin. (D) The percentage of apoptotic cells rose to 66.19% when treated with 10 μg/ml ursolic acid in conjunction with 20 μg/ml cerulenin. FACS, fluorescence-activated cell sorting; PI, propidium iodide.

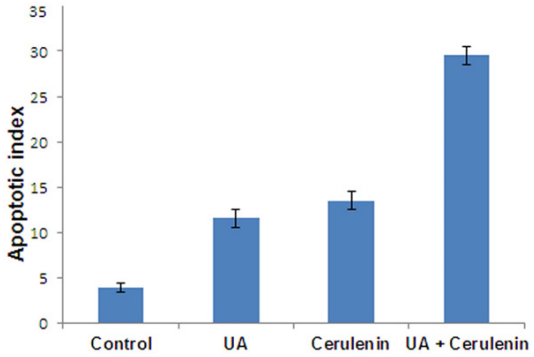

Figure 5. TUNEL assays of tumor tissue slides. The apoptotic index was significantly higher than that in the control, cerulenin and ursolic acid alone groups.

from FACS analysis indicated a concentration dependent induction of apoptosis in U2OS cell (**Figure 4**). There was apoptosis in 12.67, 38.54 and 49.23% cells treated with 1, 10 and 20 μg/ml of ursolic acid, respectively. The Q-value for combination of 0.9 μg/ml cerulenin and 0.9 μg/ml ursolic acid was 1.20 which indicated synergistic effect between the two drugs.

Synergistic effect of cerulenin and ursolic acid on induction of apoptosis in tumor cells

Treatment of tumor tissues with ursolic acid, cerulenin or their combination induced apoptosis in tumor cells (**Figure 5**). However the AI calculated for tumor tissues treated with a combination of ursolic acid and cerulenin (29.5 ± 6.5%) was higher than that of the tissues treated separately with ursolic acid (11.6 ± 5.5%) or cerulenin (13.5 ± 5.6%). The AI for untreated tumor tissues was 3.9 ± 0.89%.

Discussion

The incidence of metastasis and mortality in osteosarcoma is significantly decreased by effective chemotherapeutic agents developed over the past few decades [23, 24]. Cerulenin induced inhibition of tumor cell growth through inhibition of NF-κB and inducing apoptosis is well known [25]. Ursolic acid arrests the cell cycle progression in the G1 phase and induces apoptosis [15].

In the present study we investigated the effect of ursolic acid and cerulenin combination on the U2OS osteosarcoma cell line. The results revealed that the use of the two agents in combination exhibited synergistic effect on antitumor activity,inhibition of SENP5 expression and induction of apoptosis in osteosarcoma cell lines. The IC_{50} valuesfor cerulenin and ursolic acid combination in the proportion of 1:1, 1:2 and 2:1 were 2.0, 2.5 and 3.2 μg/ml, respectively. The calculated CI values for all the three combinations were < 0.93, indicating that cerulenin and ursolic acid exhibited synergistic effect on inhibition of growth in Saos-2 cells.

The combination of ursolic acid and cerulenin also inhibited SENP5 expression in Saos-2 cells through synergistic effect. Among three proportions tested the calculated CI values for all the three were < 0.90, indicating that cerulenin and ursolic acid exhibited synergistic effect on inhibition of SENP5 expression in Saos-2 cells. There was apoptosis in 12.67, 38.54 and 49.23% cells treated with 1, 10 and 20 μg/ml of ursolic acid, respectively. The Q-value for

combination of 0.9 μg/ml cerulenin and 0.9 μg/ml ursolic acid was 1.20 which indicated synergistic effect between the two drugs. AI calculated for tumor tissues treated with a combination of ursolic acid and cerulenin (29.5 ± 6.5%) was higher than that of the tissues treated separately with ursolic acid (11.6 ± 5.5%) or cerulenin (13.5 ± 5.6%). The AI for untreated tumor tissues was 3.9 ± 0.89%.

Conclusion

In conclusion, the results from our study demonstrate that cerulenin and ursolic acid combination exhibit synergistic effect against osteosarcoma *in vitro* and *in vivo*. Thus cerulenin combined with ursolic acid may be a potential treatment regimen for osteosarcoma treatment.

Disclosure of conflict of interest

None.

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