Original Research Article

Inhibition of SENP5 by Cucurbitacin B Suppresses Cell Growth and Promotes Apoptosis in Osteosarcoma Cells

Jiang-Bo Zhong[1], Zhao-Rui Liu[2]*, Sen Liu[3] and Jian-Qiang Zhao[3]
[1]Department of Spine Surgery, [2]Department of Orthopedics, 3Department of Oncology, Jinan Central Hospital Affiliated to Shandong University, Jinian, 250013, China

*For correspondence: **Email:** liuzhaorui276@gmail.com; **Tel/Fax:** +86-531-85695114 or +86-531-86942457

Abstract

Purpose: To investigate the effect of cucurbitacin B on the expression of SUMO-specific proteases (SENP5).
Methods: Effect of cucurbitacin B (10-50 mg/mL) on viability of U2OS and Saos-2 cells was determined in a plate reader by recording absorbance at 570 nm. In Western blot analysis, bicinconinic acid (BCA) method was used to determine protein concentration. Flow cytometry was employed to measure DNA content.
Results: Ccucurbitacin B treatment inhibits the expression of SENP5 in U2OS and Saos-2 osteosarcoma cells in a dose- and time-dependent manner. Significant inhibition (p = 0.005) of SENP5 expression was observed at 50 mg/ml from day 10, reaching a maximum on day 20. It also induced a significant decrease (p = 0.005) in mRNA and protein levels of SENP5. The decrease in mRNA and protein levels of SENP5 led to decrease in proliferation of U2OS and Saos-2 cells. The 48 h cell cultures containing 50 mg of cucurbitacin B caused induction of apoptosis in 54.72 ± 5.42 % of the total cell population in U2OS cells. Similarly, in Saos-2 cells, exposure to 50 mg/mL cucurbitacin B increased apoptotic rate from 9.86 ± 8.89 % for 10 mg/mL to 48.54 ± 14.5 % with 50 mg/mL of cucurbitacin B.
Conclusion: Cucurbitacin B is a potential therapeutic strategy for the treatment of aggressive malignancy in osteosarcoma.

Keywords: Cucurbitacin B, Osteosarcoma, SUMO-specific proteases, Cell proliferation, Apoptosis, Malignancy

INTRODUCTION

Cucurbitacin B (Fig 1), an oxygenated triterpene is isolated from *Trichosanthes kirilowii* Maximowicz (Cucurbitaceae family). Cucurbitacins exhibit cytotoxicity and anti-cancer activity [1,2]. Cucurbitacin B exhibits anti-proliferative effects and acts as a dual inhibitor of the activation of both JAK2 and STAT3 in some malignancies [3]. Recent, reports demonstrate that cucurbitacin B has antiproliferative activity against human breast cancer, glioblastoma multiforme, and myeloid leukemia cells [4–6]. It inhibits growth by cell cycle arrest in G2/M phase and increases apoptosis by inhibition of the JAK/STAT pathway [7,8].

Osteosarcoma, a common primary bone sarcoma has five-year survival rate of ~70 % in children and adolescents. Patients with osteosarcoma have a poor prognosis, with overall survival rates of < 20 % [9]. 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 [10–15].

In SUMOylation a small ubiquitin-like modifier (SUMO) protein gets covalently attached to lysine in the substrate [16] and initiates a cascade of effects in the cell [17]. There are six SUMO-specific proteases (SENPs) for the removal of SUMO from specific substrates at specific subcellular localizations [18]. SENP1 in nucleus deSUMOylate HDAC1 to activate transcription of multiple genes [19,20], SENP2 in nuclear envelope [21-23] regulates transcription, and axin and/or –catenin pathways [24,25], a truncated form of SENP2 can alter the localization of promyelocytic leukemia (PML) body proteins in the nucleus [26,27]. The current clinical treatment strategies for osteosarcoma are inefficient. Therefore, the need for a novel molecule with role in the treatment of osteosarcoma is highly needed.
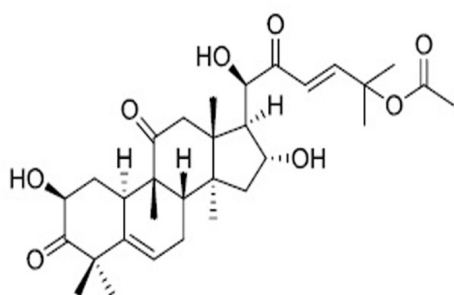

Fig 1: Structure of cucurbitacin B

EXPERIMENTAL

Cell culture

The osteosarcoma cell lines HOS, U2OS, Saos-2 and MG-63 cell lines were purchased from Sigma-Aldrich, USA. Cells were maintained in Minimum Essential Medium (MEM) supplemented with 10 % Fetal Bovine Serum (FBS) and incubated at 37 $^{\circ}$C in a humidified atmosphere containing 5 % CO_2/95 % air.

Tissue samples

We collected normal and osteosarcoma tissues from the Jinshan Hospital, Shanghai, China immediately after surgery. The samples were flash frozen in liquid nitrogen after surgical removal and stored at −80 $^{\circ}$C. The use of human tissues for study purposes was approved by

each patient and by the local ethics committee of the University Medical Centre.

Quantitative polymerase chain reaction (PCR)

Total RNA was isolated from the cells using Trizol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Total RNA was used for reverse transcription using DNA synthesis kit (Invitrogen). Primers for PCR were designed and PCR amplification of cDNA was performed at 35 cycles in a reaction mixture containing 10 μM Tris–HCl (pH 8.3), 1.5 μM $MgCl_2$, 50 μM KCl, 0.01 % (w/v) gelatin, 200 μM dNTP, SNEP5-specific primers (0.5 μM each), and 2.0 U of platinum Taq DNA polymerase (Invitrogen). For each reaction, two negative controls were performed consisting of omission of the RT step or omission of the target cDNA. The primers used were as follows: The primers for β-actin 5′-AGAGCTACGAGCTGCCTGAC-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′ and for SENP5 5′-GAGGAAAATTCTATGGAGGA-3′ and 5′-GAGGACAAAGTACTAACATT-3′.

Western blot analysis

The 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 proteins 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.

Cell proliferation assay

In each well of a 96-well plate, aliquots containing 3×10^5 cells were seeded. The cells were incubated overnight in a 5 % CO_2 incubator at 37 $^{\circ}$C and then cucurbitacin in DMSO diluted with RIMI 1640 (10 % FBS) was added to each well. After dilution with RIMI 1640 (10 % FBS), these were used to treat the tumor cells. RIMI 1640 (10 % FBS) with 0.1 % (v/v) DMSO was used as control. The incubation for 48 h was followed by addition of 25 μL of MTT (3 mg/mL in PBS) to each well and incubation was continued for 4 h more. To each well was added 100 μL of

SDS–HCl solution (SDS 10 % w/v, 0.01 M HCl) and incubated again for 12 h. An Infinite M200 pro reader (Tecan Austria GmbH, Salzburg, Austria) was used to measure the absorbance at 570 nm. The viable cells were expressed as percent of control and all the experiments were conducted in triplicate.

Cell cycle analysis

Cells were maintained in Minimum Essential Medium (MEM) supplemented with 10 % Fetal Bovine Serum (FBS) for 30 h. Then cells after fixing overnight in ethanol were stained with phosphate buffer 10 % Fetal Bovine Serum (FBS) containing 50 μg/mL propidium iodide and 100 μg/mL RNase A for 45 min at 37 °C. Accuri C6 flow cytometry system (BD Biosciences, Franklin Lakes, NJ, USA) was used to measure DNA content of the labelled cells.

Statistical analysis

The data represents the mean of three independent experiments. Student t test with the SPSS13.0 statistical program for windows was used for data analysis. $P < 0.05$ was considered to indicate statistically significant difference.

RESULTS

Cucurbitacin B inhibits SENP5 expression in osteosarcoma cell lines and tissues

The results from quantitative PCR and western blotting analysis are shown Fig. 2A and B. It is clear from the figure that SENP5 is significantly overexpressed in osteosarcoma cell lines (U2OS, Saos-2 and MG-63) compared with HOB cells (human osteoblasts isolated from normal human bone). There was also high level of SENP5 expression in clinical osteosarcoma specimens in comparison to normal adjacent bone tissues (Fig 2C and D).

Treatment of osteosarcoma cell lines (U2OS, Saos-2 and MG-63) with cucurbitacin B resulted in inhibition of SENP5 expression in a dose and time-dependent manner (Fig. 3A, B). We treated osteosarcoma cells with a range of cucurbitacin B amount from 10 to 50 mg. There was a significant decrease in SENP5 expression at of 30 mg.
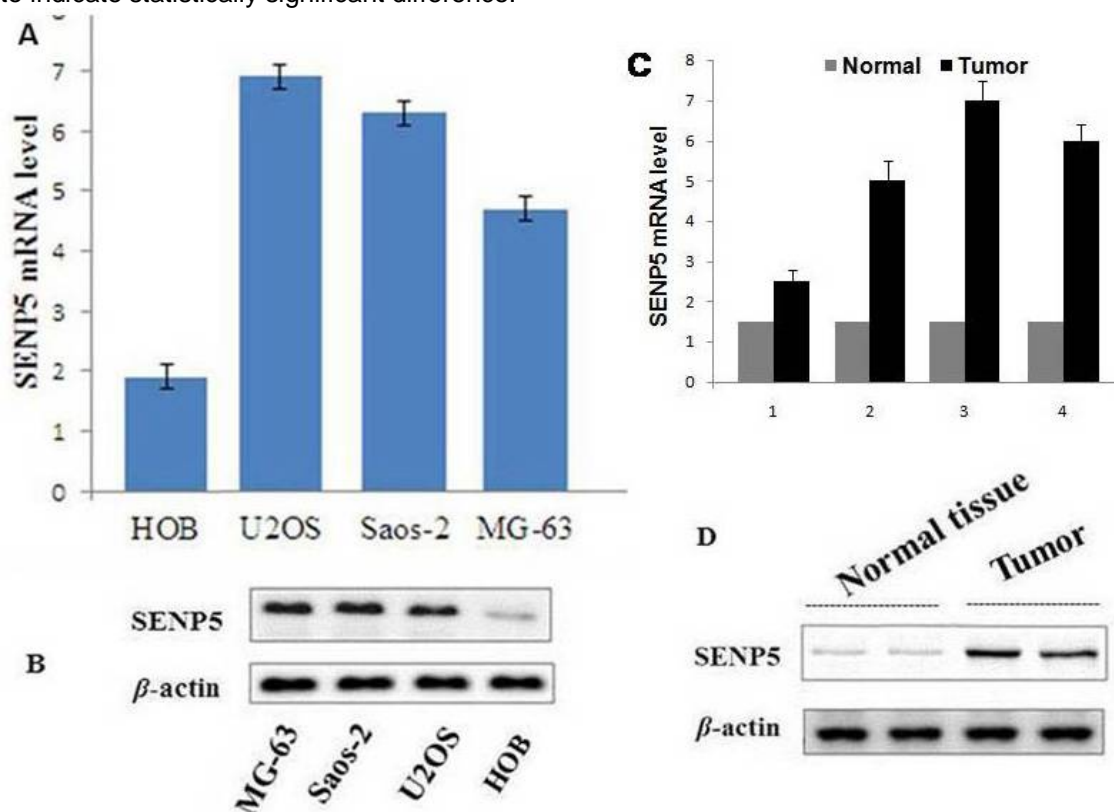
Fig 2: SENP5 is overexpressed in osteosarcoma cell lines and tissues. (A) mRNA expression and (B) protein levels of SENP5 in U2OS, Saos-2 and MG-63 osteosarcoma cell lines. (C) mRNA expression of SENP5 in four paired clinical specimens . (D) Protein levels of SENP5 in two paired clinical specimens

The results from RT-PCR analysis clearly demonstrated a significant inhibition of SENP5 expression from day 10 with a maximum effect on day 20.
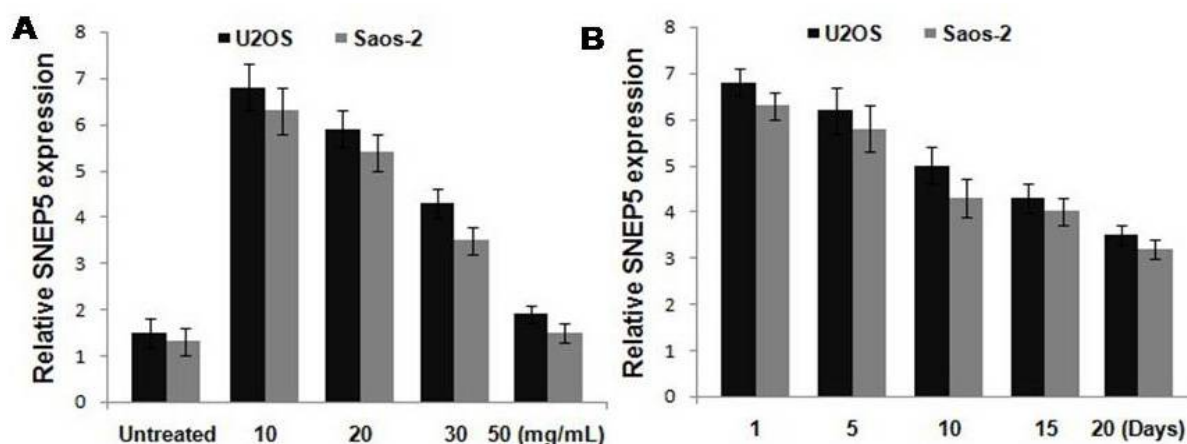
Suppression of SENP5 expression by cucurbitacin B significantly inhibits cell growth in osteosarcoma cells

The results from quantitative PCR and western blot analysis indicated a significant decrease in mRNA and protein levels of SENP5 on treatment with 50 mg of cucurbitacin B (Fig 4A, B). Although the decrease in mRNA and protein levels of SENP5 began from 30 mg/mL it was significant only at 50 mg amount. This decrease in mRNA and protein levels of SENP5 leads to

decrease in proliferation of U2OS and Saos-2 cells.

Cucurbitacin B treatment results in G2/M arrest and apoptosis in U2OS and Saos-2 osteosarcoma cells

Apoptotic cell death leading to reduction in U2OS and Saos-2 osteosarcoma cell growth was confirmed by flow-cytometric analysis and ssDNA detection assay. Exposure of the U2OS and Saos-2 osteosarcoma cells to cucurbitacin B resulted in apoptosis. In U2OS and Saos-2 osteosarcoma cells grown in control medium there was only 2.05 ± 1.01 % of the cells that underwent spontaneous apoptosis (Fig. 5A), after 24 h of incubation.

Fig 3: Inhibition of SENP5 expression by cucurbitacin B treatment. (A) Concentration dependent inhibition of SENP5 expression in U2OS and Saos-2 cells. (B). Time dependent inhibition of SENP5 expression in U2OS and Saos-2 cells
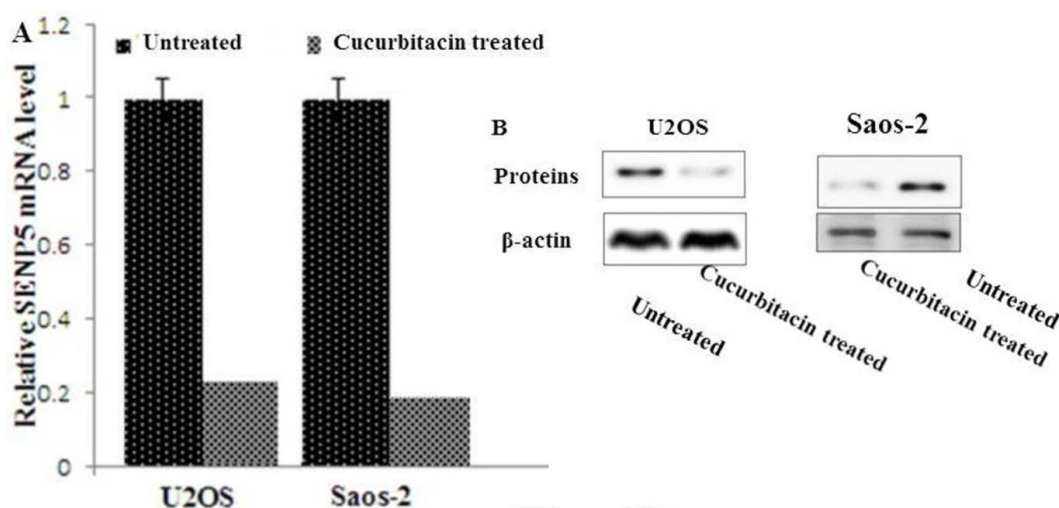
Fig 4: Inhibition of SENP5 expression by cucurbitacin B. (A) mRNA levels of SENP5 in U2OS and Saos-2 cells transfected with mock or Cucurbitacin B. (B) Protein levels of SENP5 in U2OS and Saos-2 cells treated with cucurbitacin B
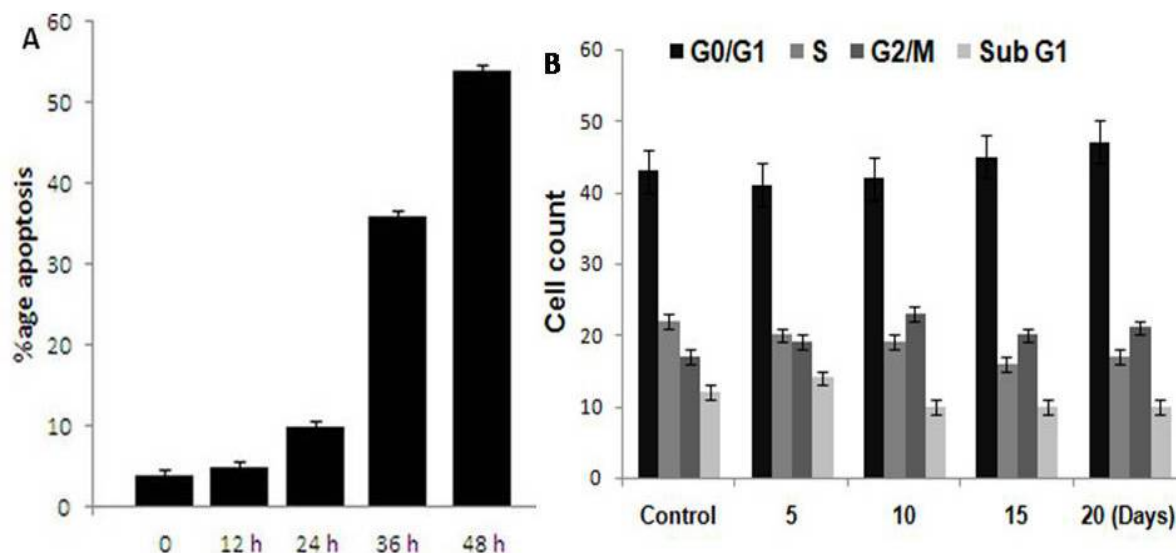
Fig 5: SENP5 inhibition results in G2/M arrest and apoptosis in U2OS and Saos-2 osteosarcoma cells. (A) Apoptosis in U2OS and Saos-2 cells. (B) Cell cycle distribution of U2OS and Saos-2 cells transfected with mock or cucurbitacin B. Cells were fixed with 70 % ethanol and stained with PI

The effective amount of cucurbitacin B (30 mg/ml) in the MTT assay caused an induction of apoptosis with results overlapping that obtained in control cell culture (5.89 ± 3.98 %). The 24 h cell cultures with IC_{50} concentration of cucurbitacin caused an induction of apoptosis in 54.72 ± 5.42 % of the total cell population (Fig. 5A). Similar results were observed in the Saos-2 cells, where in control group the percentage of apoptosis was 1.79 ± 0.23 %, and the exposure to cucurbitacin B was found to increase the apoptotic rate from 9.86 % ± 8.89 with 10 mg/ml to 48.54 ± 14.5 with 50 mg of cucurbitacin B (Fig. 5A).

The study of cell cycle phase distribution revealed that cucurbitacin B caused a significant accumulation of cell population in G2/M phase after treatment for 24 h. However, after 48 h, 20 % of cell population accumulated in sub-G1 phase, which is indicative of apoptosis (Fig 5B).

DISCUSSION

There is overexpression of SENP5 in osteosarcoma cell lines (U2OS, Saos-2 and MG-63) compared with HOB cells (human osteoblasts isolated from normal human bone) and clinical osteosarcoma specimens in comparison to normal adjacent bone tissues. In the present study, Cucurbitacin B was used to analyse its effect on SENP5 expression. The results indicated that Cucurbitacin B treatment causes inhibition of SENP5 expression, induces decrease in mRNA and protein levels, apoptosis

and cell cycle arrest in G2/M phase in U2OS and Saos-2 osteosarcoma cells.

The results from RT-PCR analysis demonstrated that cucurbitacin B inhibits the expression of SENP5 in U2OS and Saos-2 osteosarcoma cells at 50 mg. SENP5 inhibition was significant from day 10 with a maximum effect on day 20. Cucurbitacin B treatment at 50 mg also induced a significant decrease in mRNA and protein levels of SENP5. This decrease in mRNA and protein levels of SENP5 leads to decrease in proliferation of U2OS and Saos-2 cells. Exposure of the U2OS and Saos-2 osteosarcoma cells to cucurbitacin B resulted in apoptosis. In U2OS and Saos-2 osteosarcoma cells grown in control medium there was only 2.05 ± 1.01 % of the cells that underwent spontaneous apoptosis, after 24 h of incubation. The effective concentration of cucurbitacin (30 mg/mL) in the MTT assay caused an induction of apoptosis with results overlapping that obtained in control cell culture (5.89 ± 3.98 %). The 24 h cell cultures with IC_{50} concentration of cucurbitacin B caused an induction of apoptosis in 54.72 ± 5.42 % of the total cell population. Similar results were observed in the Saos-2 cells, where in the control group the percentage of apoptosis was 1.79 ± 0.23 %, and the exposure to cucurbitacin was found to increase the apoptotic rate from 9.86 % ± 8.89 with 10 mg to 48.54 ± 14.5 with 50 mg of cucurbitacin B. The cell cycle phase distribution revealed that cucurbitacin B caused a significant accumulation of cell population in G2/M phase after treatment for 24 h. However, after 48 h, 20 % of cell population accumulated in sub-G1 phase, which is indicative of apoptosis.

CONCLUSION

Thus, cucurbitacin B is a novel therapeutic agent with some potential for the treatment of aggressive malignancy in osteosarcoma.

CONFLICT OF INTEREST

No conflict of interest associated with this work.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Jiang-Bo Zhong and Zhao-Rui Liu contributed equally to this work.

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