

Original Article

Sulforaphane enhances cisplatin sensitivity in human osteosarcoma cells through upregulation of p53-p21 pathway by enhancing G1 arrest

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Abstract: Background: Cisplatin (CisPt) resistance is one of the major problems for the treatment of osteosarcoma. The natural compound sulforaphane (SFN) are reported to have antitumor activity in many cancers. However, its effect to influence CisPt resistance in osteosarcoma cells has not examined. In this study, we intended to investigate the combined effects of SFN and CisPt in osteosarcoma cells and to investigate the related mechanism. Methods: Human osteosarcoma OS-732 and MG-63 cells were treated with SFN or cisplatin (cisPt) or combination of both for 72 h. The cell survival rate was measured by MTT assay. The cell cycle distribution and cell death were measured by flow cytometry. The expression of cell cycle and apoptosis related genes were analyzed by qRT-PCR and western blot. Results: The combination of SFN and CisPt had significantly greater cell growth inhibitory effects than either treatment alone. The combined treatment of SFN and CisPt increased the population of cells in the G1 phase and cell death than SFN or CisPt alone. The combination of SFN and CisPt treatment increased the expression of p53, p27, p21 and Bax and decreased the expression of cyclin D and E as compared to SFN or CisPt alone treatment. Conclusion: Taken together, we demonstrate that SFN enhanced CisPt sensitivity of osteosarcoma cells by inducing apoptosis through G1-phase arrest and by activating tumor suppressor p53-p21 pathway, suggesting that SFN may be used as a chemosensitizer for osteosarcoma treatment.

Keywords: Sulforaphane, cisplatin, osteosarcoma, apoptosis and cell cycle

Introduction

Osteosarcoma is the most common primary malignant bone cancer and is the eighth-most common form of malignancy that threatens the life of young people [1, 2]. In general, over 61% of patients are diagnosed at later stage when the disease has spread beyond the bone, which has a dismal 5-year survival rate with multidisciplinary chemotherapy treatment together with surgical techniques [3]. Cisplatin (CisPt) is a common chemotherapeutic agent used in the treatment of several cancers, including osteosarcoma and its application is restricted by significant variability in tumor response that could affect the clinical outcome [4]. CisPt-based chemotherapy is the most common treatment of advanced osteosarcoma, which yields high response rates and improved survival rates.

Conversely, most osteosarcoma patients will ultimately relapse and die of their cancer [5, 6]. CisPt-based chemotherapy is also associated with serious side effects that limit the doses and duration of the treatments. Furthermore, some common tumors are sensitive to CisPt treatment and others are inherently resistant to CisPt that cause failure in the curative therapy. Cytotoxicity of CisPt is mediated by cross-linking DNA, resulting in cell cycle arrest and eventually in the activation of apoptosis [7, 8]. A bioactive natural compound that increases the cisplatin sensitivity through the induction of apoptosis may potentially be useful for CisPt-resistant osteosarcoma therapeutic strategies.

Sulforaphane (SFN), an isothiocyanate, present in cruciferous vegetables (broccoli), has been reported to have majority of health-promoting

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and anticancer properties [9, 10]. SFN is one of the plant-based compounds and it was found to be induction of phase II enzymes [11], Nrf-2/Keap1/ARE-signaling pathway [12], epigenetic modifier [13] and now being referred to as nutrigenomic activity (Reviewed in [14]). The nutrigenomic potential of SFN is based on its ability to epigenetically modify the expression of critical cytoprotective genes, which involved in the regulation of cell cycle and apoptosis [13, 15-18]. A study reported that SFN affects gene expression is through inhibition of histone deacetylase (HDAC) activity that facilitate releasing DNA/chromatin interactions and permitting access to the promoters of transcription factors, including apoptosis mediating genes such as p21 and Bax [13].

The aim of this study was to investigate the effects of SFN on osteosarcoma cell growth when treated alone and in combination of SFN with CisPt and observed the molecular mechanisms underlying the alteration of CisPt sensitivity. We hypothesize that SFN increases CisPt sensitivity through cell cycle arrest and the induction of key regulators of apoptosis in osteosarcoma cells. Our findings demonstrate that SFN arrest cells at G1 phase and increases the apoptotic index of CisPt through activation p53-p21 signaling pathway.

Materials and methods

Cell lines and reagents

Sulforaphane was obtained from Calbiochem (Germany). Cisplatin was procured from Qilu Pharmaceuticals (Jinan, China). Human osteosarcoma cells OS-732 and MG-63 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS), 1X penicillin/streptomycin and incubated in a humidified incubator composed of 5% CO₂ at 37°C. All chemicals were purchased from Sigma-Aldrich (St Louis, MO), unless otherwise stated.

Measurement of cell growth by MTT assay

The cells were plated in 96-well plate at 4×10⁵ cells/ml and treated with different concentrations (0, 1, 5, 10, 15 and 20 M) of SFN or CisPt or DMSO (control) vehicle. After 72 h of treatment, 20 μl of methylthiazolotetrazolium (MTT) was added and incubated at 37°C for an addi-

tional 4 h. The medium was removed and dissolved the formazan crystals in 200 μl of DMSO. The absorbance was measured on an MRX microplate reader (DYNEX Technologies, Chantilly, VA) at 540 and 690 nm. Six samples were analyzed for each data point and the experiment was repeated three times.

Analysis of apoptosis

Apoptotic cells were determined using FITC Annexin V kit (BD Biosciences) according to the manufacturer's instruction as described previously [19]. Briefly, OS-732 and MG-63 cells were treated with SFN or CisPt or in combination of both at indicated concentrations for 72 h. After 72 h of treatment, cells were washed with cold PBS, trypsinized and pelleted by centrifugation at 1,000 rpm for 5 min. The pellets were then resuspended in 1× binding buffer, added 5 μl of Annexin V-FITC and 5 μl of propidium iodide (50 μg/ml) and incubated at room temperature for 5 min. FACS was performed using a FACScan flow cytometer (Beckman-Ultra, USA).

Cell cycle analysis

OS-732 and MG-63 cells were treated with SFN or CisPt and in combination of SFN and CisPt at indicated concentrations for 72 h and then fixed with 75% ethanol at -20°C. The ethanol fixed cells were resuspended in PBS with addition of RNase A (1 mg/ml) and incubated for 1 h at 37°C. Propidium iodide (50 μg/ml) was used for staining the fixed cells for 30 min at room temperature. The DNA contents of the stained cells were analyzed by using the CELL Quest Software with a FACS can flow cytometer (Beckman-Ultra, USA).

Analysis of mRNA expression by quantitative RT-PCR (qRT-PCR)

The total RNA was isolated by using AxyPre™ Multi-source RNA miniprepkit (Axygen, USA). Two μg of total RNA was converted into complementary DNA (cDNA) with PrimeScript RT reagent kit (Takara, Japan). TaqMan probes (Invitrogen) were used to measure p53, p27, p21, Bax, Cyclin D and Cyclin E mRNA expressions. The GAPDH probe was used as endogenous control. The PCR reactions were carried in a 20 μL mixture containing 150 ng of cDNA, 10 μL of TaqMan 2× universal PCR master mix and 1 μL of probes. ABI Prism 7900 Fast Real-time

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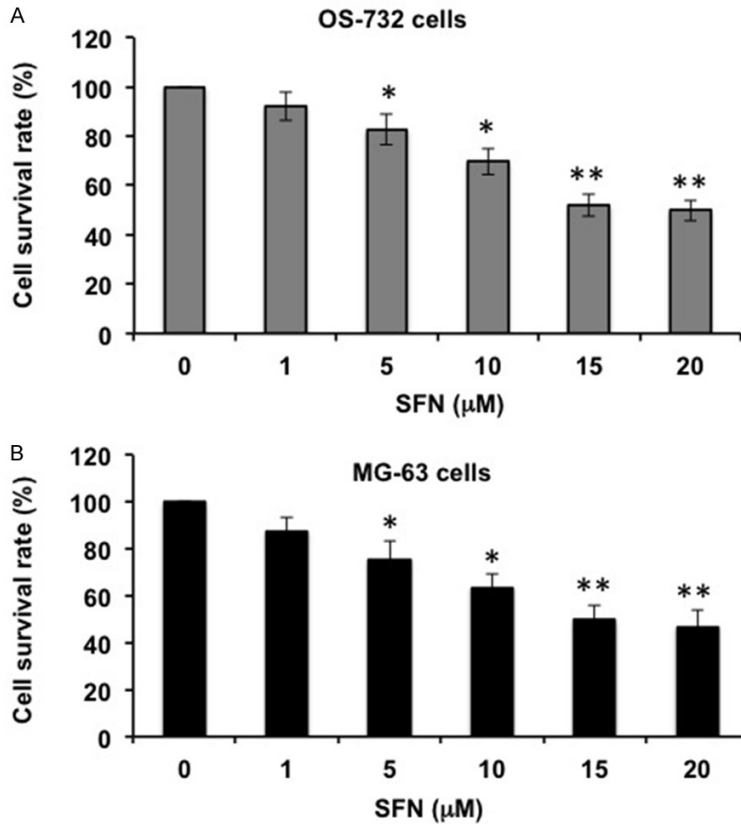


Figure 1. SFN inhibits osteosarcoma cell growth. Human osteosarcoma cells (A) OS-732 (B) MG63 cells were treated different concentrations (0, 1, 5, 10, 15 and 20 M) of SFN for 72 h. The cell survival rate was measured by MTT assay and expressed as percentage of cell survival rate. Data represent three independent experiments. *P < 0.001; **P < 0.0001 compared with DMSO (vehicle) treated cells (control).

PCR system were used for PCR reactions for each gene and each sample in triplicate as follows: 95°C for 10 min, 45 cycles of a 15-s denaturing at 95°C, and 1 min annealing at 60°C. The comparative 2- $\Delta\Delta$ CT method was used to calculate the relative gene expression level of each target gene.

Western blot analysis

At the end of treatment, cells were washed with PBS and dissolved in lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 1 mM PMSF with protease inhibitor cocktail. The protein concentrations of the supernatant were determined using the Bio-Rad assays. An equal amount proteins were separated in 12% SDS-PAGE, transferred to a nitrocellulose membrane. The membranes were incubated with primary antibodies

(1:1000 dilution for all antibodies), including mouse monoclonal anti-p53, anti-p21, anti-cyclin E and anti-actin, rabbit polyclonal anti-p27, and anti-cyclin D, overnight at 4°C. After 3 washes with TBST, the membranes were incubated with anti-mouse and anti-rabbit secondary IgG antibodies for 1 h at room temperature. The membranes were incubated with enhanced chemiluminescence reagent (ECL).

Statistical analysis

The data are expressed as mean \pm standard deviation (SD). One-way analysis of variance (ANOVA) and student's t test were used to analysis significant differences among the groups by using SPSS 17.0 software package. P < 0.05 was considered as statistically significant.

Results

Synergistic effect of SFN and CisPt on osteosarcoma cell growth inhibition

We compared the effects of SFN or CisPt alone and in combination of both SFN and CisPt on cell growth of well-established osteosarcoma cells, OS-732 and MG-63 cell lines *in vitro*. First, to examine the effect of SFN on osteosarcoma cell growth, OS-732 and MG-63 cells were treated with different concentrations of SFN (0, 1, 5, 10, 15 and 20 M) for 72 h and cell survival rate was determined by MTT assay. After 72 h of SFN treatment, the cell survival rate was decreased in a dose-dependent manner and 15 M of SFN significantly (P < 0.0001) inhibited 50% of cell growth in both OS-732 and MG-63 cells (**Figure 1A, 1B**).

We next find out the effect of CisPt on cell survival rate in these two cells lines. As shown in **Figure 2A, 2B**, CisPt significantly decreased the cell survival rate in both cells in a dose-

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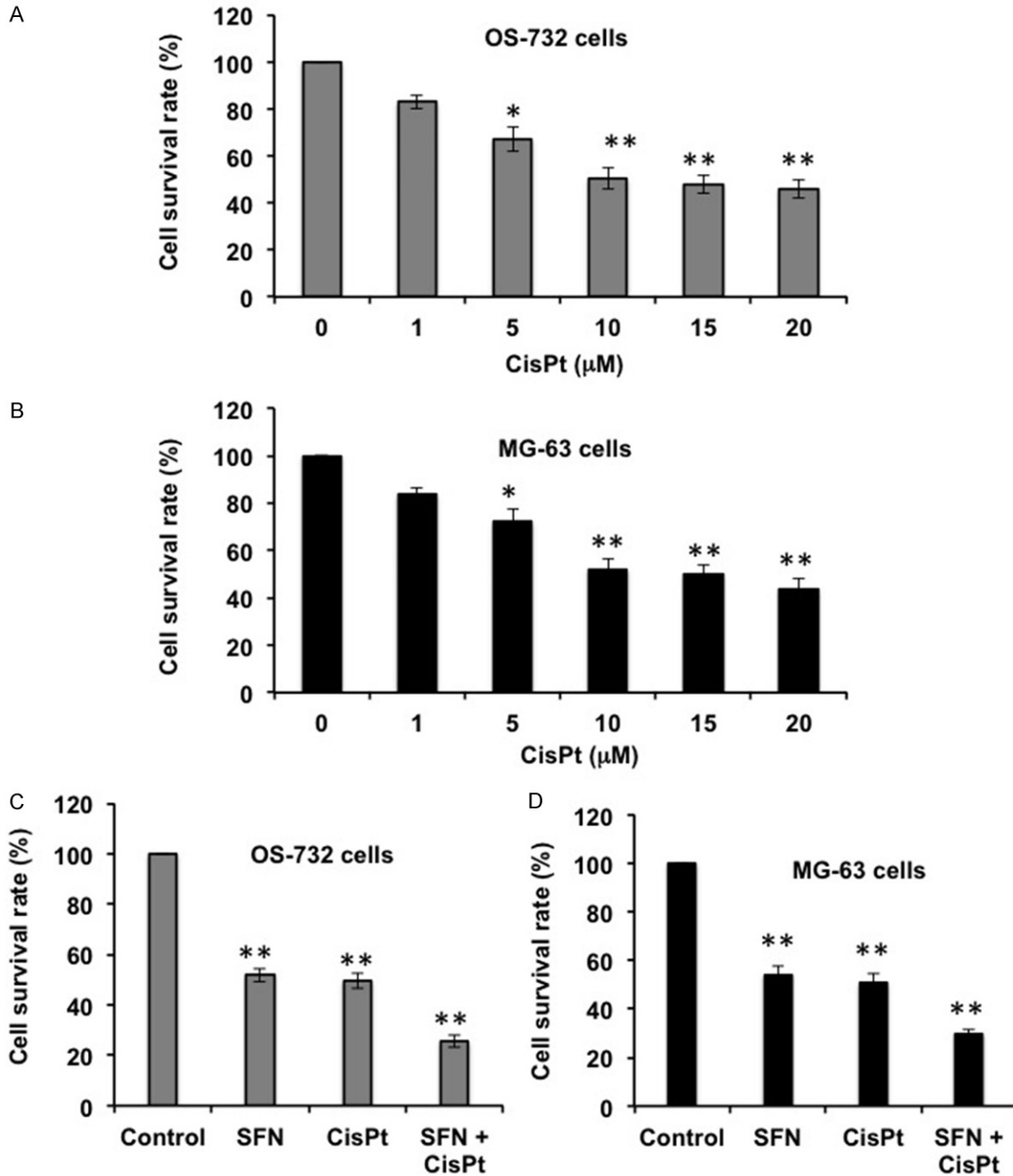


Figure 2. SFN and CisPt synergistically inhibit osteosarcoma cell growth. (A) OS-732 and (B) MG63 cells were treated with different concentrations (0, 1, 5, 10, 15 and 20 M) of CisPt for 72 h. (C) OS-732 and (D) MG-63 cells were treated with SFN (15 M) or CisPt (10 M) or combination of SFN (15 M) and CisPt (10 M). The cell survival rate was measured by MTT assay and expressed as percentage of cell survival rate. Data represent three independent experiments. *P < 0.001; **P < 0.0001 compared with DMSO (vehicle) treated cells (control).

dependent manner and 50% of inhibition was observed at the concentration of 10 M of CisPt. Based on these data, we have chosen 15 M of SFN and 10 M of CisPt for the combination experiments.

To assess the effects of SFN and CisPt combination, the cells were treated with 15 M of SFN and 10 M of CisPt for 72 h. A significant synergistic effect of SFN and CisPt was observed on OS-732 and MG-63 cells (Figure 2C, 2D), indi-

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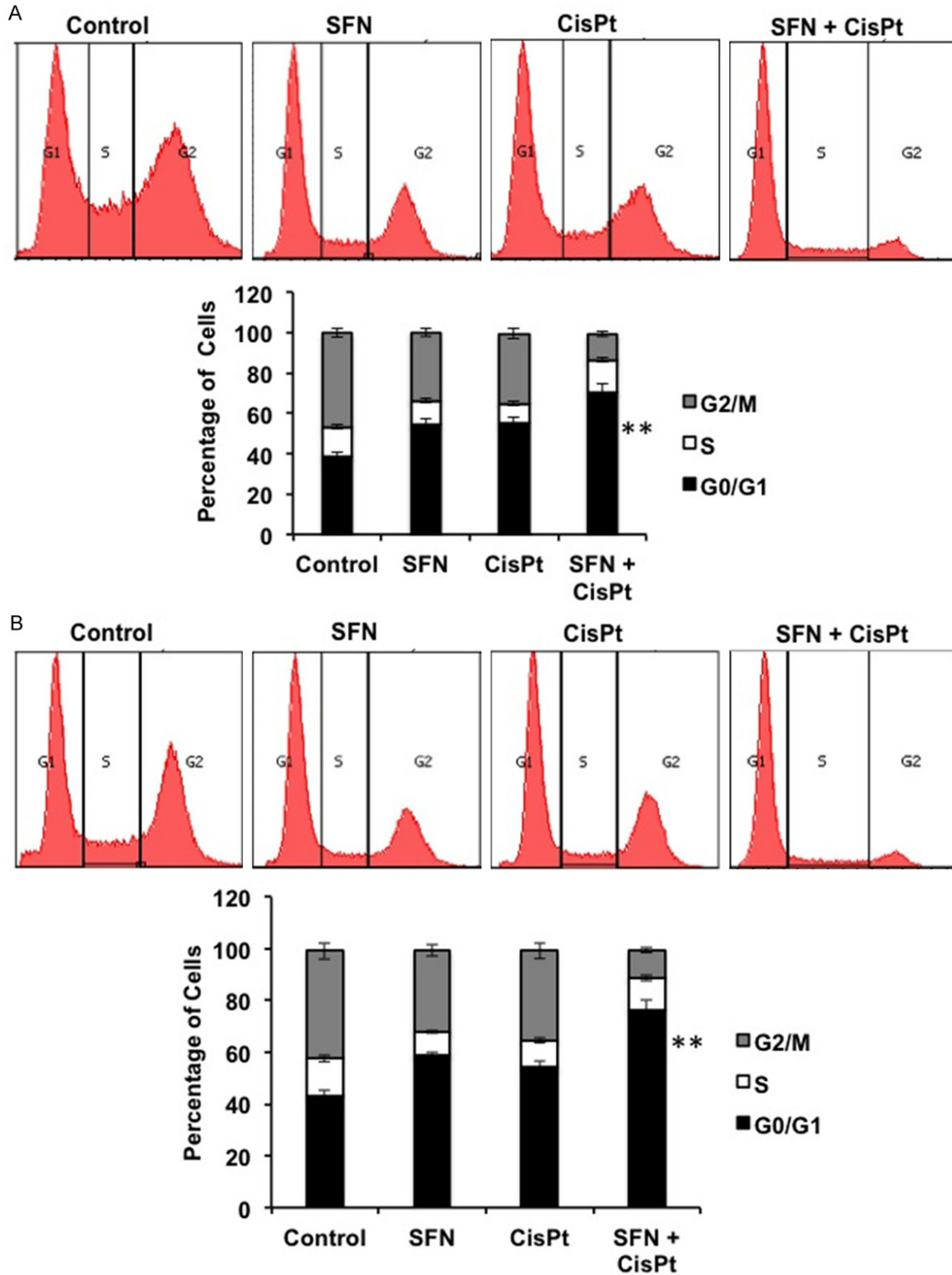


Figure 3. Combination of SFN and CisPt arrest cell cycle at G1 phase in osteosarcoma cells. (A) OS-732 and (B) MG-63 cells were treated with with SFN (15 M) or CisPt (10 M) or combination of SFN (15 M) and CisPt (10 M). After 72 h of treatment, cells were stained with propidium iodide (PI) and cell cycle distribution was assessed by flow cytometry. The representative cell cycle distributions images are shown in the top panel of each cells and percentage of cells at different phases are shown as bar graph. Data represent three independent experiments. **P < 0.0001 compared with DMSO (vehicle) treated cells (control).

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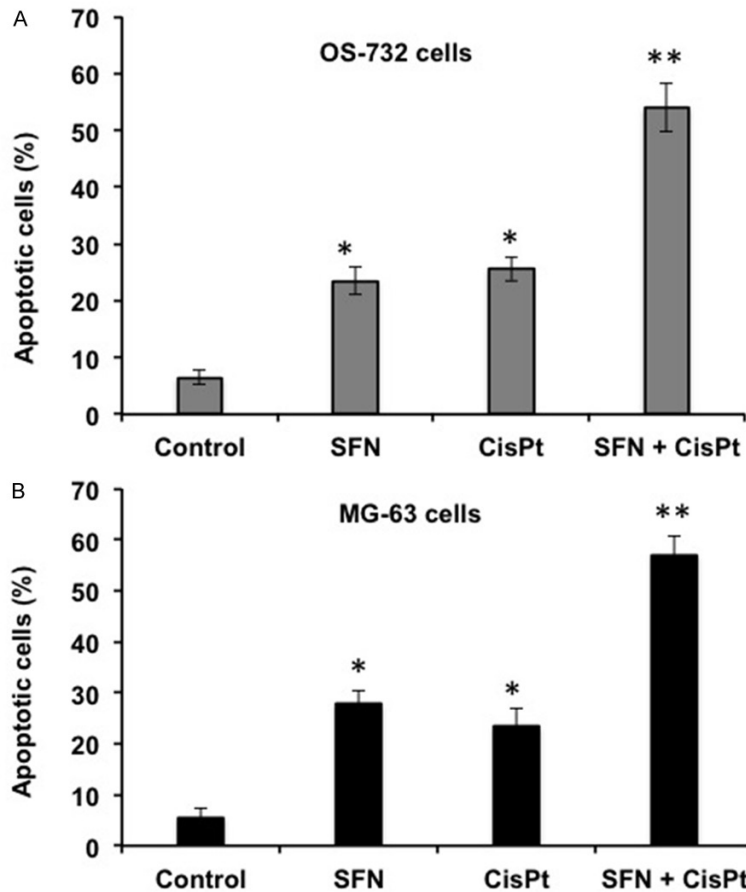


Figure 4. Combination of SFN and CisPt induces apoptosis in osteosarcoma cells. (A) OS-732 and (B) MG-63 cells were treated with SFN (15 M) or CisPt (10 M) or combination of SFN (15 M) and CisPt (10 M). Cells were harvested after 72 h of treatments and percentage of apoptotic cells were measured by FITC Annexin-V analysis. Data represent three independent experiments. * $P < 0.001$; ** $P < 0.0001$ compared with DMSO (vehicle) treated cells (control).

cating that SFN and CisPt synergistically inhibit cell growth than SFN or CisPt treatment alone.

Synergistic effect of SFN and CisPt on cell cycle progression

To examine the effects of SFN and CisPt on the status of cell cycle progression in osteosarcoma cells, OS-732 and MG-63 cells were treated with SFN or CisPt alone and in combination of both for 72 h and the nuclei DNA content was calculated by FACS. The cells treated with either SFN or CisPt moderately decreased the cell population in G2/M phase and a concomitant increased of G1 phase as compared to vehicle treated cells (Figure 3A, 3B). Interestingly, when cells treated with both SFN

and CisPt, significantly ($P < 0.0001$) decreased G2/M phase and arrest most of the cells at G1 phase.

Synergistic effect of SFN and CisPt on osteosarcoma cell death

To find out the combined effects of SFN and CisPt on the association of cell cycle arrest and cell death, the cells were treated with SFN or CisPt alone and in combination of both for 72 h and cell death was measured by apoptotic index using FITC Annexin V kit. There were significant increases of apoptotic cells when cells treated with combination of SFN and CisPt, comparing to cells treated with either alone agents or vehicle in OS-732 and MG-63 cells (Figure 4A, 4B), indicating that SFN and CisPt synergistically induces cell death to increases CisPt sensitivity.

Regulation of p53-p21 pathway and cell cycle markers by combined treatment of SFN and CisPt

We next investigated the functional relevance of p53-p21 pathway and cell cycle regulated genes for the promotion of CisPt sensitivity by SFN in osteosarcoma cells. The combined effects of SFN and CisPt on cell cycle and apoptosis-related genes, including p53, p27, p21, Bax, cyclin D and cyclin E mRNA (Figure 5A) and protein expressions (Figure 5B) were examined by qPCR and western blot analysis, respectively, in OS-732 cells. The SFN and CisPt alone increased p53, p27, p21 and Bax mRNA and protein contents and simultaneous decrease of cyclin D and cyclin E expression in OS-732 cells. However, significantly higher synergistic response in terms of these mRNA and protein expressions were observed when cells treated with both agents, suggesting that p53-p21

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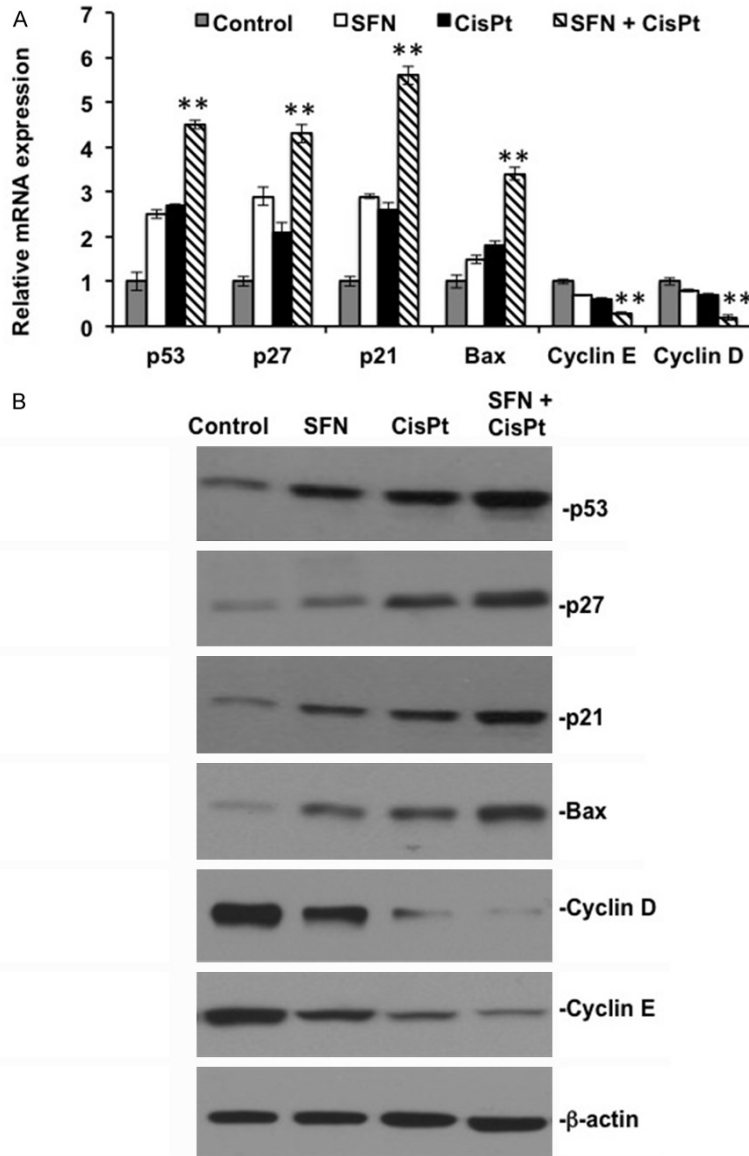


Figure 5. Combination of SFN and CisPt regulates apoptosis and cell cycle related genes in osteosarcoma cells. (A) RNA was extracted from OS-732 cells treated with SFN (15 M) or CisPt (10 M) or combination of SFN (15 M) and CisPt (10 M) for 24 h. The mRNA levels of p53, p27, p21, Bax, cyclin D and cyclin E were measured by qRT-PCR and normalized to GAPDH. (B) Cells were treated as in (A) and western blot were performed with indicated antibodies. β -actin was used as loading control. Data represent three independent experiments. **P < 0.0001 compared with vehicle treated cells (control).

pathway is a key role for SFN induced CisPt sensitivity in osteosarcoma cells.

Discussion

Osteosarcoma remains most common primary malignant tumor in children and adults. The use of multiagent and intensive chemotherapy are the major treatment of osteosarcoma

patients [20, 21] that increases patient survival but also has side effects that severely limit its clinical effectiveness such as acquisition of drug resistance. CisPt is one of the most common chemotherapy drugs for osteosarcoma but not all osteosarcoma patients are sensitive to CisPt treatment [22]. Therefore, novel therapeutic strategies that increase the chemo-sensitivity by diminishing the cumulative side effects of chemotherapy. Pro-apoptotic natural compounds that increase CisPt sensitivity and/or reduce its toxicity will have a great potential to improve osteosarcoma patient survival through synthetic agent lethality and can be used together with CisPt to better manage this deadly disease. Thus, the aim of this study was to examine the effects of SFN on CisPt sensitivity in osteosarcoma cells, with goal of reducing therapeutic CisPt concentrations that required for the induction of cancer cell death.

In the present study, we demonstrated for the first time that either SFN or CisPt alone inhibited osteosarcoma cell growth, whereas the combination of SFN and CisPt further significantly increased the growth inhibitory effects. Further, we investigated the molecular mechanisms in-

involved for the synergistic action of SFN and CisPt on the inhibition of osteosarcoma cell growth. Combined treatment of SFN and CisPt significantly increase the number of cells at G1 phase and subsequently induces apoptosis. Further our results show that increased G1 arrest and apoptotic responses to both SFN and CisPt are seemed to be the regulation of p53-p21 pathway.

SFN has been reported to increase cell cycle arrest and apoptosis, which is considered to be among most important mechanism of actions of SFN on cell growth inhibition. SFN also has been reported to increase p21 protein in a p53-independent manner in cancer cells [23]. Moreover, studies has shown that SFN inhibited the cell growth through increase of G2/M-phase arrest, as shown by the increase of cells with G1 DNA content and inducing apoptosis by increase cleaved caspase-3 [14, 24]. Other mechanism proposed that SFN regulates apoptosis and cell proliferation through the inhibition of both the PI3K/AKT and MAPK pathways [25]. We found that SFN alone increased the apoptotic cells (**Figure 4**) and number of cells at G1 phase (**Figure 3**), as consistent with previously described [26]. More interestingly, the combination of SFN and CisPt significantly increased the apoptotic cells and number of cells at G1 phase. Our findings suggest that combination of SFN and CisPt treatment may potentially induce cell death through apoptosis. We demonstrate that combined treatment of SFN and CisPt increased the number of cells at G1 phase and apoptotic bodies and activate the p53-p21 signaling cascades. Thus, the SFN-induced osteosarcoma cell death is considered to be apoptotic.

Many studies have revealed that p53-21 signaling pathways plays an important role in the induction of apoptosis in different types of tumor cells by CisPt [27, 28]. We also studied the combination effects of SFN and CisPt on the regulation p53-p21 pathway and cell cycle regulators such as cyclin E and D. CisPt-induced DNA damage activates p53 and the subsequent transcription of target genes including, p27 and p21 [29]. The present report shows that SFN and CisPt regulates the expression of p53, p27, p21, Bax, Cyclin E and D in osteosarcoma cells, suggesting the following sequence of actions leading to SFN-induced G1 arrest to increases CisPt sensitivity. Further, we found that combination treatment increased the apoptotic index and the expression of p53 activated p21 and Bax.

Our study showed that osteosarcoma cell survival rate significantly decreased with low-dose of SFN and CisPt when compared with SFN or CisPt alone (**Figures 1 and 2**). These data demonstrate that the combined use of SFN and

CisPt may have a stronger inhibitory effect with less toxicity. We also demonstrated that cell death measured by apoptotic index were significantly increased in the combined treatment of SFN and CisPt (**Figure 4**), suggesting that suppression of tumor growth by SFN and CisPt is due to the result of apoptosis induction.

In conclusion, our data demonstrated that SFN increases CisPt sensitivity in *in vitro* by increasing apoptosis and activating tumor suppressor p53-p21 signaling pathway. The combined treatment of SFN and CisPt would reduce the higher doses of CisPt for both chemoresistant and chemosensitive osteosarcoma cells. Our data indicate that SFN not only increase the chemopreventive effect of CisPt by overcoming the CisPt resistance but also decrease the side effects of CisPt by using low dose of CisPt. However, clinical trials of SFN with CisPt may provide an interesting therapeutic approach for osteosarcoma treatment.

Disclosure of conflict of interest

None.

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