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Original Research Article

Florofangchinoline inhibits proliferation of osteosarcoma cells via targeting of histone H3 lysine 27 trimethylation and AMPK activation

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Abstract

Purpose: *To investigate the effect of florofangchinoline on osteosarcoma cell growth in vitro, and the underlying mechanism of action.*
Methods: *Changes in the viability of KHOS and Saos-2 cells were measured using water soluble tetrazolium salt (WST) assay, while apoptosis was determined using Annexin V/PI staining and flow cytometry. Increases in mtDNA, and expressions of PGC-1α and TFAM were assayed with immunoblot analysis and quantitative real-time polymerase chain reaction (qPCR), respectively.*
Results: *Microscopic examination of florofangchinoline-treated cells showed significant decrease in cell density, relative to control cells ($p < 0.05$). Treatment with 10 μM florofangchinoline increased apoptosis in KHOS and Saos-2 cells to 56.32 and 63.75 %, respectively ($p < 0.05$). Florofangchinoline treatment markedly enhanced cleavage of caspase-3, caspase-8, caspase-9 and PARP. It elevated Bax level and reduced Bcl-2 in KHOS and Saos-2 cells. Moreover, florofangchinoline increased p21 and p-AMPKα levels, and mtDNA counts in KHOS and Saos-2 cells ($p < 0.05$). Moreover, in florofangchinoline-treated KHOS cells, the expressions of EED, EZH2 and SUZ12 were significantly suppressed ($p < 0.05$).*
Conclusion: *Florofangchinoline inhibits osteosarcoma cell viability by activation of apoptosis. Moreover, it activates AMPK and down-regulates histone H3 lysine 27 trimethylation in osteosarcoma cells. Therefore, florofangchinoline has potentials for development as a therapeutic drug for osteosarcoma.*

Keywords: *Osteosarcoma, Histone H3, Florofangchinoline, Apoptosis, Chemotherapeutic*

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INTRODUCTION

Osteosarcoma, the most common bone tumor in children and adults, is presently treated with surgical resection [1]. There have been advances in adjuvant chemotherapeutic strategies for osteosarcoma treatment [1]. Some of the therapeutic molecules discovered during the last two decades have shown toxicity during phases I and II clinical trials [2]. Thus, there is need for

new and effective compounds for osteosarcoma treatment. Mitochondria are vital cellular organelles that control energy metabolism and also regulate programmed cell death [3].

Anticancer therapies have identified mitochondrial dysfunction and its downstream cellular biogenetics as important targets for cancer treatment [4]. Studies have revealed quantitative reductions in mitochondrial count in liver, renal, gastric and breast cancer cells [5]. On the other hand, mitochondrial count was markedly increased in head, neck, ovary and esophageal cancer cells [6]. The reduction in mitochondrial count in liver and breast cancer cells has been found to be associated with tumor progression and poor prognosis of patients [5].

Studies have shown lower mitochondrial count in osteosarcoma cells than in normal muscular or musculoskeletal cells [7]. Peroxisome proliferator activated receptor-gamma coactivator-1α (PGC-1α) plays a vital role in regulation of gene transcription by encoding transcription factor A (TFAM) in mitochondria [8]. It has been reported that enhancement of mitochondrial count by PGC-1α up-regulation leads to mitochondrial apoptosis in sarcoma cells [7].

Fangchinoline has an alkaloid structure, and is a member of bisbenzylisoquinoline family obtained from the herb *Stephania tetrandra* S. Moore [9]. Initially, the herb was used in traditional medicine and later-on, fangchinoline was found to possess anti-cancer, anti-hypertensive and anti-inflammatory properties [10]. Studies have reported that fangchinoline induced apoptosis in carcinoma cells through phosphorylation of Akt and ERK [11]. Fangchinoline enhanced efficiency of radiotherapy and chemotherapy for pulmonary carcinoma cells and activated pro-apoptotic factors in bladder carcinoma cells [12]. It has also been reported that fangchinoline upregulated the expressions of anti-apoptotic factors such as Bcl-xl, Bcl-2 and survivin [13]. The present study was carried out to determine the apoptotic potential of triflorofangchinoline (Figure 1) in osteosarcoma cells, and the associated pathways.

EXPERIMENTAL

Cell and culture

The KHOS and Saos-2 cell lines were obtained from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cell culture was carried out in RPMI-1640 medium containing 10 % fetal bovine serum (FBS) and mixture of 1% penicillin and streptomycin. Incubation of cells was performed for 24 h in an incubator at 37˚C and 5 % CO_2 under a humid atmosphere.

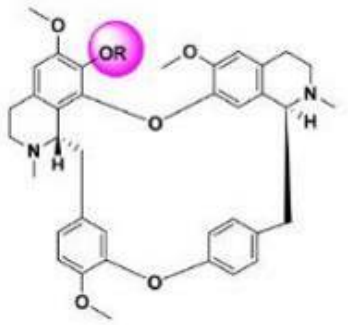

Figure 1: Chemical structure of triflorofangchinoline (FFC)

Cell proliferation assay

The effect of various doses of florofangchinoline (1, 2, 4, 6, 8 and 10 μM) on the proliferation of KHOS and Saos-2 cells was determined with WST-8 assay. The cell lines were seeded in 96-well plates at a density of 6,000 cells per well and cultured for 24 h. Then, fresh medium mixed with florofangchinoline was added to the plates and incubated with the cells for 48 h. Thereafter, 10 μL of CCK-8 solution was added to each well and incubation was continued for 90 min. The optical density of each plate was read at 455 nm using a Microplate Reader (Model 680; Bio-Rad).

Apoptosis analysis

Flow cytometry was used to determine the effect of florofangchinoline on apoptosis of KHOS and Saos-2 cells. The cell lines were seeded in 96-well plates at a density of 6,000 cells per well, cultured with florofangchinoline at doses of 2, 4 and 10 μM for 48 h, and washed with PBS. Then, the cells were put in 1% paraformaldehyde plus PBS, followed by re-suspending in ice-cold ethyl alcohol (70 %). Staining of the cells was performed with Annexin V/PI (BD Biosciences) as per the manufacturer's protocol. To detect apoptosis, flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) was used to examine the cells after 20 min of incubation with Annexin V/PI in the dark.

Immunoblot analysis

Protein expressions in cells treated with10 μM florofangchinoline for 48 h were assayed with western blotting. The cells were lysed with lysis buffer [Tris-hydrochloric acid (40 mM at pH 7.4) consisting of NaCl (160 mM) and Triton X-100 (1.2 %; v/v)] mixed with protease inhibitors. The

protein content of the lysate was determined using BCA reagent kit (Bio-Rad, Hercules, CA, USA). Equal amounts of protein samples were loaded onto 8 – 15 % SDS polyacrylamide gel for resolution with electrophoresis, and then transferred onto PVDF membranes. The membranes were blocked by treatment with skimmed milk (5 %) in TBS containing Tween-20 (0.1 %), followed by incubation with primary antibodies at 4 ^{O}C for overnight. The primary antibodies used for incubation of membranes were: anti-p-AMPKα, anti-AMPKα, anti-PGC-1α, anti-TFAM, anti-cleaved PARP, anti-cleaved caspase-3, anti-cleaved caspase-9, and anti-α-tubulin (Cell Signaling Technology, Danvers, MA, USA). The membrane was then washed with PBS, and incubated for 2 h with secondary antibody conjugated to horseradish peroxidase at room temperature. The immunoblots were visualized using ECL detection system reagents (GE Healthcare Biosciences, Piscataway, NJ, USA). Semi-quantification of bands was carried out using ImageJ software (version 1.47) with a densitometer.

Quantitative real-time polymerase chain reaction (qPCR)

Cells treated with florofangchinoline were assessed for mitochondrial count at 48 h by measuring the amount of mtDNA, relative to total nuclear DNA. GenElutedNA Miniprep kit (Sigma-Aldrich) was used for isolation of genomic DNA from KHOS and Saos-2 cells. The primer sequences designed for amplification of genes were as follows:

Forward: 5'-GCAGATTTGGGTACCACCCAAG TAT TGACTCACCC-3' and reverse: 5'-GCATGGAGAGCTCCCGTGAGTGGTTAATAG GGTGATAG-3'.

The PCR reaction sequence used was: 94 ˚C for 18 min, 38 cycles at 94 ˚C for 28 s, 56 ˚C for 28 s, and 70 ˚C for 85 s. The quantity of mtDNA, relative to total nuclear DNA was determined using the 2-ΔΔCt method.

Statistical analysis

The results are presented as mean ± standard error of the mean (SEM) of three independently performed experiments. Data comparisons were made using one-way analysis of variance, followed by Tukey's post-hoc test. Differences were considered statistically significant at $p < 0.05$. Data analysis was carried out using SPSS version 17.0 software (SPSS, Inc, Chicago, IL, USA).

RESULTS

Florofangchinoline suppressed growth of KHOS and Saos-2 cells

The anti-proliferative effect of florofangchinoline (1, 2, 4, 6, 8 and 10 μM) on KHOS and Saos-2 cells was measured with MTT assay (Figure 2). Florofangchinoline treatment suppressed proliferative ability of both cell lines in a dose-based manner (Figure 2). The proliferation suppression by florofangchinoline became significant at a dose of 1 μM, and was maximum at a dose of 10 μM in KHOS and Saos-2 cells (Figure 2 A). Microscopic examination of florofangchinoline-treated cells showed significant decreases in cell density, relative to control cells (Figure 2 B).

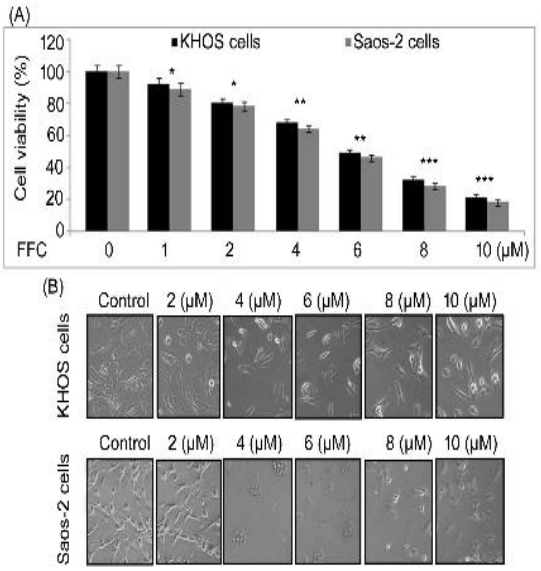

Figure 2: Effect of florofangchinoline on KHOS and Saos-2 cells. (A) Florofangchinoline-induced changes in cellular viability, as determined with WST-8 assay. (B) Microscopy of cells treated with florofangchinoline; *$p < 0.05$, **$p < 0.02$; ***$p < 0.01$, vs. control cells

Florofangchinoline induced apoptosis in KHOS and Saos-2 cells

Florofangchinoline induced apoptosis in KHOS and Saos-2 cells in a dose-based manner (Figure 3). Treatment with 10 μM florofangchinoline at raised apoptosis in KHOS and Saos-2 cells to 56.32 and 63.75 %, respectively. The proportions of apoptotic cells in KHOS and Saos-2 cells were 10.32 and 14.54 %, respectively on treatment with 2 μM florofangchinoline.

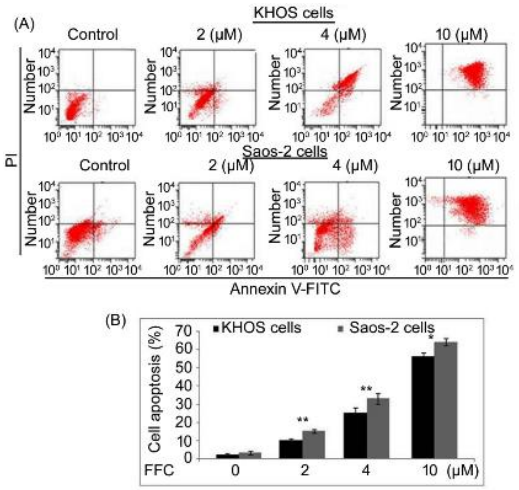

Figure 3: Apoptotic effect of florofangchinoline in KHOS and Saos-2 cells. (A) Florofangchinoline-induced apoptosis in cells, as determined with flow cytometry. (B) Quantified immunofluorescence data; *p < 0.05, **p < 0.02, vs. control cells

Florofangchinoline upregulated apoptotic proteins in KHOS and Saos-2 cells

In KHOS and Saos-2 cells, florofangchinoline treatment markedly promoted cleavage of caspase-3, caspase-8 and caspase-9 (Figure 4). The level of Bax in florofangchinoline-treated cells was also increased, whereas Bcl-2 level was suppressed in both cells. The florofangchinoline treatment enhanced p21 level in both KHOS and Saos-2 cells, and increased the cleavage of PARP.

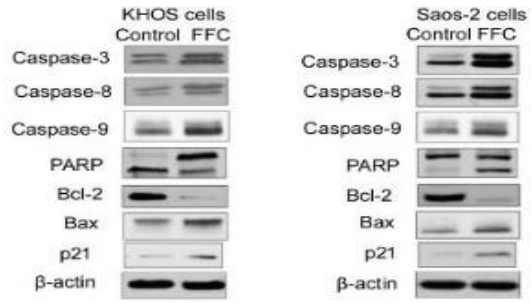

Figure 4: Effect of florofangchinoline on apoptotic factors. (A) The expressions of caspases-3, caspase-8 and caspase-8, caspase-9, p21 and Bcl-2 in florofangchinoline-treated cells were assayed with immunoblot assay. Semi-quantitative densitometrical analysis was carried out using ImageJ software

Florofangchinoline upregulated p-AMPK expression in osteosarcoma cells

As shown in Figure 5, treatment with 10 μM florofangchinoline resulted in marked up-regulation of p-AMPKα expression. Moreover, western blot assay showed marked elevations in p-AMPKα expression levels in KHOS and Saos-2 cells treated with 10 μM florofangchinoline.

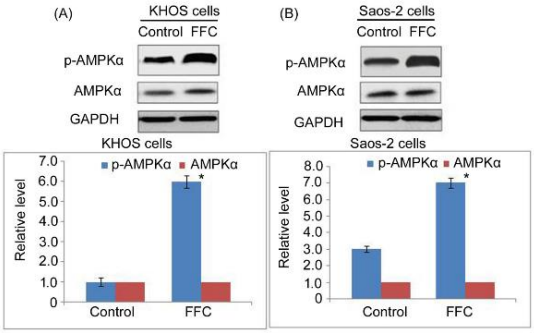

Figure 5: Effect of florofangchinoline on p-AMPKα expressions in KHOS and Saos-2 cells. A: Up-regulation of p-AMPKα by florofangchinoline, as determined using immunoblot assay. (B) Densitometric analysis, using ImageJ software, with α-tubulin as control; *p < 0.05, **p < 0.02, vs. control cells

Florofangchinoline enhanced levels of mtDNA and expressions of PGC-1α and TFAM

Florofangchinoline promoted mtDNA levels in KHOS and Saos-2 cells (Figure 6). The count of mtDNA was enhanced significantly (p < 0.05) by 10 μM florofangchinoline in both cell lines (Figure 6 A). The florofangchinoline treatment markedly elevated the expressions of PGC-1α and TFAM in KHOS and Saos-2 cells (Figure 6 B and C). The enhancement of PGC-1α and TFAM by florofangchinoline was more prominent at a higher concentration of 10 μM.

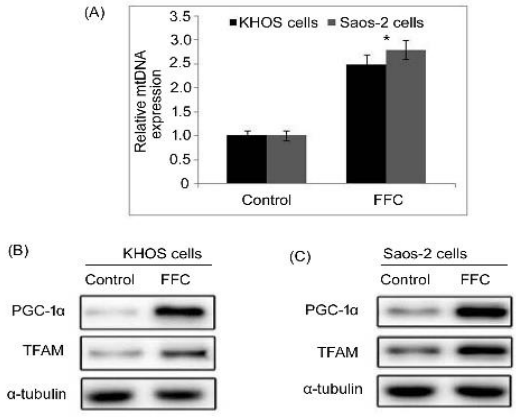

Figure 6: Effect of florofangchinoline on mtDNA. A: KHOS and Saos-2 cells treated with florofangchinoline were assayed for mtDNA using qRT-PCR. B & C: In florofangchinoline-treated cells, PGC-1α and TFAM expressions were assayed with immunoblot assay, with α-tubulin as control; *p < 0.02, vs. control cells

Florofangchinoline downregulated H3K27me3 in osteosarcoma cells

As shown in Figure 7, treatment of KHOS and Saos-2 cells with 10 μM florofangchinoline markedly suppressed the level of H3K27me3. Moreover, the expressions of EED, EZH2 and SUZ12 were markedly suppressed in florofangchinoline-treated KHOS and Saos-2 cells.

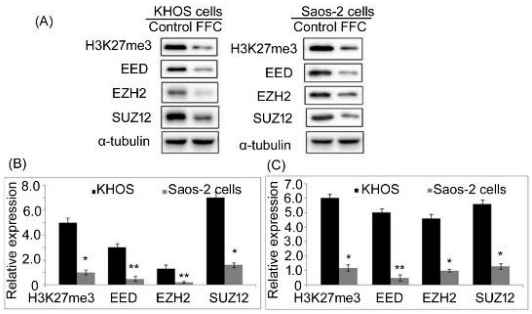

Figure 7: Effect of florofangchinoline on H3K27me3 expression. Florofangchinoline treatment was followed by assay of expressions of H3K27me3, EED, EZH2 and SUZ12 in KHOS and Saos-2 cells. *$P < 0.05$; **$p < 0.02$, vs. control cells

DISCUSSION

Osteosarcoma, a clinically aggressive carcinoma in children and adolescents, is a common primary tumor of bones. In approximately 65 % of patients, the average survival is improved by application of latest therapeutic strategies consisting of surgery and combination therapies [14,15]. However, the present chemotherapeutic strategies are not effective for metastatic or recurrent stage of osteosarcoma, and prognosis of such patients is very poor [14,15]. The biogenesis of mitochondria and maintenance of mtDNA are regulated by the PGC-1α/TFAM pathway [7]. Studies have revealed alterations in the count of mitochondria in various malignancies in humans [5]. The decrease in mitochondrial count is crucial in the progression of tumor and prognosis of carcinoma patients [16].

Many anticancer therapies target mitochondrial function, with promising results [5]. Studies have revealed that mitochondrial count is reduced in osteosarcoma patients, and its up-regulation through PGC-1α activation has a therapeutic role [8]. The present study showed that florofangchinoline increased levels of mtDNA in a concentration-dependent manner in KHOS and Saos-2 cells. The florofangchinoline treatment markedly elevated the expressions of PGC-1α and TFAM in KHOS and Saos-2 cells. These findings indicate that florofangchinoline increases mtDNA levels in KHOS and Saos-2 cells via activation of the PGC-1α/TFAM pathway.

It has been reported that the growth of cancer cells is related to AMPK, an enzyme for regulation of energy metabolism, through its influence on checkpoints in cell cycle [17]. The stores of cellular energy are maintained by AMPK through regulation of oxidative metabolism, switching off consumption of ATP, and biogenesis of mitochondria [18].

The biogenesis of mitochondria is also regulated by AMPK by directly upregulating PGC-1α level [18]. Moreover, AMPK exhibits anti-tumor effect by promoting p53 and FOXO3a activation, thereby either increasing cell apoptosis or arresting cell cycle [19,20]. The activation of AMPK arrests cell cycle by inducing expression of inhibitors of cyclin-dependent kinases, p21cip1 and p27kip1 [19,21]. In the present study, florofangchinoline induced AMPKα activation in KHOS and Saos-2 cells in a concentration-based manner. A marked elevation of p-AMPKα level by florofangchinoline was observed in KHOS and Saos-2 cells. Therefore, florofangchinoline inhibits osteosarcoma by activation of AMPK/PGC-1α/TFAM pathway, leading to mitochondrial biogenesis. In breast and pancreatic carcinomas, anti-cancer agents suppress the transcription level of EZH2 which is believed to be linked to elevation of miR-26a and miR-101 [22].

The reduction of migratory ability of prostate carcinoma cells by metformin is associated with suppression of histone methyltransferase of H3 Lys9 [22]. In the present study, florofangchinoline treatment suppressed the level of H3K27me3 in KHOS and Saos-2 cells in dose-based manner. In florofangchinoline-treated KHOS and Saos-2 cells, the expressions of EED, EZH2 and SUZ12 were also suppressed to a marked extent. Thus, the targeting of histone methylation by florofangchinoline plays anti-proliferative role in osteosarcoma cells.

CONCLUSION

The findings of this study indicate that florofangchinoline acts as an anti-proliferative agent for osteosarcoma cells *via* upregulation of AMPK phosphorylation and increases in the expressions of PGC-1α and TFAM. Moreover, it increases the biogenesis of mitochondria, but suppresses the level of H3K27me3 in osteosarcoma cells. Therefore, florofangchinoline may be a potent molecule for osteosarcoma

treatment. However, *in vivo* studies need to be performed to confirm this potential role.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Zhili Zhao - conceived and designed the study; Liyan Zhao, Xiongtao Liu, Weina Zhu, Pei Yang, Jie Qin - collected and analyzed the data; Ru Gu, Liyan Zhao, Weina Zhu, Pei Yang -wrote the manuscript. Zhili Zhao -Approved final version of the manuscript. All authors read and approved the manuscript for publication.

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