Original Article
Ferulic acid inhibits proliferation and promotes apoptosis via blockage of PI3K/Akt pathway in osteosarcoma cell

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Abstract: Ferulic acid, a ubiquitous phenolic acid abundant in corn, wheat and flax, has potent anti-tumor effect in various cancer cell lines. However, the anti-tumor effect of ferulic acid on osteosarcoma remains unclear. Therefore, we conduct current study to examine the effect of ferulic acid on osteosarcoma cells and explore the underlying mechanisms. In present study, ferulic acid inhibited proliferation and induced apoptosis in both 143B and MG63 osteosarcoma cells dose-dependently, indicated by MTT assay and Annexin V-FITC apoptosis detection. Additionally, ferulic acid induced G0/G1 phase arrest and down-regulated the expression of cell cycle-related protein, CDK 2, CDK 4, CDK 6, confirmed by flow cytometry assay and western blotting. Moreover, ferulic acid upregulated Bax, down-regulated Bcl-2, and subsequently enhanced caspase-3 activity. More importantly, ferulic acid dose-dependently inhibited PI3K/Akt activation. Using adenoviruses expressing active Akt, the anti-proliferation and pro-apoptosis of ferulic acid were reverted. Our results demonstrated that ferulic acid might inhibit proliferation and induce apoptosis via inhibiting PI3K/Akt pathway in osteosarcoma cells. Ferulic acid is a novel therapeutic agent for osteosarcoma.

Keywords: Osteosarcoma, ferulic acid, proliferation, apoptosis, PI3K/AKT

Introduction
Osteosarcoma is the most frequent primary malignant bone tumor, approximately 20% of all primary sarcomas in bone, which predominantly occurs in childhood and adolescence [1]. Since 1980s, the survival of osteosarcoma has been improved due to the application of intensive multi-agent chemotherapy coupled with advanced surgery [2]. However, despite advancements in surgery and multi-agent chemotherapy, the survival rate of localized osteosarcoma remains in the plateau over the past decades, what is more, the osteosarcoma with metastases maintains to be dismal with a poor survival rate of 10-20% [3]. The plateau might partially attribute to lack of better therapeutic agents, for some types of osteosarcoma are low response and/or chemotherapy resistance to the currently used agents [4]. Additionally, the agents used today have been confirmed to be associated with acute and long-term toxicities, including hearing lose, hypomagnesemia, cardiomyopathy, encephalopathy and hemorrhagic cystitis [2, 5]. In view of current situation, stagnation state of osteosarcoma survival rate, poor outcome of patients with low response and/or chemotherapy resistance and side-effect of current chemotherapeutic agents, the developments of novel therapeutic agents for osteosarcoma treatment are desperately needed.

Ferulic acid (4-hydroxy-3-methoxycinnamic acid, Figure 1), a ubiquitous phenolic acid, is abundant in corn, wheat and flax [6]. Due to its superior antioxidant, features of long residue time in blood and easy absorption, ferulic acid
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has been widely used in health food and nutrition restoratives [7]. It is also reported that ferulic acid benefited diabetic via alleviating oxidative stress and lowering blood glucose levels [8]. Additionally, accumulating studies demonstrate that ferulic acid possesses a neuroprotective effect against cerebral ischemic injury-induced disease [9-11] and attenuates ischemia/reperfusion-induced cell apoptosis [12, 13]. Recent years, emerging studies find a chemopreventive effect of ferulic acid in 7,12-dimethylbenz(a)anthracene (DMBA) induced carcinogenesis in rats [14-16]. Based on above, ferulic acid can be a promising agent for the treatment of cancer. Though, a promising agent against carcinogenesis, the anti-tumor effect of ferulic acid on osteosarcoma remains unacknowledged. We, therefore, conduct current study to verify the anti-tumor effect of ferulic acid on osteosarcoma and explore the underlying mechanisms by using human osteosarcoma cells.

**Chemical and reagents**

Ferulic acid (C_{10}H_{10}O_{4}, MW: 194.19, purity≥98%) was purchased from Nanjing Debiochem Co. Ltd. (Nanjing, China). Bicinchoninic acid (BCA) protein assay kit and was obtained from Pierce (Rockford, IL, USA). RPMI 1640 medium and FBS (fetal bovine serum) were obtained from HyClone Laboratories (Logan, UT, USA). Monoclonal antibodies anti-β actin, anti-Bax, anti-Bcl-2, anti-p-Akt, anti-CDK2, anti-CDK4, anti-CDK6, and anti-p-Rb were purchased from Abcam (Cambridge, UK), LY294002 (inhibitor of PI3K), Dimethyl sulfoxide (DMSO), propidium iodide (PI), and MTT were obtained from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals and solvents obtained from local area were of the highest analytical grade available.
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Cell culture and treatment

Osteosarcoma cells 143B and MG63 were obtained from American Type Culture Collection (ATCC, Rockville, MD), and cultured in RPMI-1640 supplemented with 10% FBS and 1% penicillin/streptomycin. All cells were routinely incubated in an atmosphere of 5% CO₂ at 37°C. All cell experiments were done using cells in exponential cell growth. Incubated for twenty-four hours after seeding, cells were treated with culture medium containing various concentrations of Ferulic acid (30, 100, 150 μM, respectively).

MTT assay for proliferation assay

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide) Assay was performed to evaluate the proliferation of osteosarcoma cells. MTT assay is a rapid and sensitive procedure for accessing cellular toxicity of compounds in-vitro. Cells were seeded into 96-well plates at a concentration of 10⁵/ml and plates were sealed and cultured for 24 hours before treatment of Ferulic acid. Cells were incubated for various times after treatment. Following incubation, 20 μl of MTT was added to each well, and the cells were incubated for an additional four hours. Subsequently, media was carefully discarded and 100 μl of dimethyl sulfoxide were added to dissolve the formazan crystals, then the 96-well plates were put on a horizontal oscillator for ten minutes. The absorbance values were measured with the plate reader at a wavelength of 570 nm and a reference wavelength of 620 nm. Each experiment was conducted in triplicate, and the data are presented as mean values.

Flow cytometry assay for cell cycle analysis

Cell cycle analysis was performed by detecting DNA content with propidium iodide (PI) staining. Briefly, the cells were incubated for 48 h before treating with different concentrations of ferulic acid for 24 h. At the end of treatment, the collected cells were incubated for 24 h before treatment with different concentrations of ferulic acid for 24 h. At the end of treatment, the collected cells were lysed using Cell Quest Software (BD Biosciences, San Jose, CA).

Flow cytometry assay of apoptosis

For apoptosis analysis, Annexin V-FITC/propidium iodide (PI) staining using an Annexin V-FITC apoptosis detection kit (KeyGEN Biotech, Nanjing, China) was performed by flow cytometry according to the manufacturer's guidelines. Briefly, after 48 h of Ferulic acid treatment, the cells were washed with cold phosphate buffered saline (PBS) twice, incubated with Annexin V-FITC/PI at room temperature (RT) for 5 min in the dark. The fluorescence of the cells was detected by the flow cytometry using a FITC signal detector (FL1) and a PI signal detector (FL2). According to the method, Annexin V-FITC (-)/PI (-) indicates survived cells, Annexin V-FITC (+)/PI (-) indicates cells of apoptosis in the early stage, and Annexin V-FITC (+)/PI (+) indicates cells of apoptosis or necrosis in the late stage. Each experiment was performed in triplicate and reproducible results were achieved.

Caspases activities assay

The activity of caspase-3 was detected in vitro using the caspase 3 colorimetric assay kit (KeyGEN, Nanjing, China) according to the manufacturer's instructions. In short, following the treatment, the collected cells were lysed and centrifuged at 12000 × g for 15 minutes at 4°C. The supernatant were collected and the protein concentrations were measured by the Bradford method. Then supernatant containing 50 µg of total protein were incubated with 5 µl caspase substrate in the 100 µl reaction buffer at 37°C for 4 h in the dark. Fluorescence intensity of the caspase substrate was determined by a microplate reader at 405 nm, and the caspase activity was calculated as a percentage of OD in Ferulic acid treatment cells relative to the control that were not treated with the Ferulic acid.

Immunostaining

Osteosarcoma cells were incubated with ferulic acid in 12-well plates at a density of 1.2 × 10⁵ cells/well. Next, cells were washed with PBS twice and fixed with PBS containing 4% paraformaldehyde for 15 min at RT. Subsequently, cells were blocked with 5% bovine serum albumin for 2 h and washed with PBS. Next, cells
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were incubated with rabbit anti-human rabbit anti-human p-AKT monoclonal antibody overnight at 4°C. The cells were then washed with PBS and incubated with 100 μl FITC-conjugated anti-rabbit IgG (1:100) in the dark for 1 h.

Protein isolation and western blot

Cells were washed with PBS and lysed in cell lysis buffer. The lysate was centrifuged at 12000 g at 4°C for 10 min. The supernatant was collected and protein concentration was determined by BCA method. 40μg total protein from each treated cell group was fractionated on 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel and then transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% (w/v) fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20, followed by incubation with a primary polyclonal anti-body at 4°C overnight. Then after washing with TBST for three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies. Antibody binding was visualized using enhanced ECL chemiluminescence system and short exposure of the membrane to X-ray films (Kodak, Japan).

Adenovirus infection

Recombinant adenoviruses overexpressing of active AKT was used according to procedure described previous [17]. Additionally, the expression of transgene also expressed RFP as a marker for monitoring transfection efficiency. An analogous adenovirus expressing only RFP (Ad-RFP) was used as a control, and expression efficiency was determined by real-time PCR, western blotting, and functional assays of PI3K/Akt signaling pathway.

Animal experiments

The severe combined immunodeficiency (SCID) hairless mice were obtained from the Laboratory Animal Center, Chongqing Medical University and randomly divided into two groups (Control group and Ferulic acid group). The mice were injected subcutaneously with MG63 cells (2 × 10^6) in Matrigel on the right rear flanks. Body weights were monitored weekly as an indicator of overall health. After 1 week, the mice were perorally (p.o.) gavaged with either 100 μl water control (Control group) or 100 mg/kg weight ferulic acid (Ferulic acid group). The animals were gavaged daily for the duration of the experiment. Tumor diameter was measured every week, and tumor volumes were calculated with the formula: tumor volume (mm^3) = 0.5 × length (mm) × width^2 (mm^2). At the end of 3 weeks of gavage treatment, the mice were euthanized via CO₂ asphyxiation. Tumors were then removed, weighed, and sent for immunohistochemistry (IHC) analysis. All the animal studies were approved by the Animal Ethics Committee of Chongqing Medical University.

Immunohistochemistry

Briefly, sections from xenografted mouse tissues, fixed in 10% formalin were deparaffinized and rehydrated in xylene and a graded series of ethanol and blocked in 2% goat serum/PBS for 30 min. After incubation with specific primary antibody overnight and HRP-conjugated secondary antibody for 2 h, slides were counter-stained with hematoxylin and immunoreactive complexes were detected using DAB or AEC (Dako Corp., CA). Sections were visualized on microscope and images captured with a camera attached to computer.

Statistical analysis

The results were expressed as the means ± standard deviation (SD), either Student’s t-test or one-way ANOVA by Prism GraphPad 4 software (GraphPad Software, Inc.) was used to achieve data analyses. A two-tailed P value of less than 0.05 was considered significant difference.

Results

Ferulic acid inhibited the proliferation of osteosarcoma cells

To measure the effect of ferulic acid on cell viability, osteosarcoma cells lines, 143B and
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A

B

C

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Figure 3. Effect of ferulic acid on apoptosis in osteosarcoma cells. 143B and MG63 osteosarcoma cells were treated with PBS or ferulic acid (30, 100, and 150 μM, respectively) for 48 h. Cells were harvested and measured for apoptosis and caspase 3 activity. A. The apoptotic cells were determined by annexin V-FITC/PI staining using flow cytometry. B. The percentage of osteosarcoma cells apoptosis was shown in statistical analysis. C. The caspase 3 activity was measured by a microplate reader. Each value is mean ± S.D. *P<0.05, **P<0.01.

Figure 4. Effect of ferulic acid on cell cycle-related and apoptosis-related proteins expression in osteosarcoma cells. MG63 osteosarcoma cells were treated with PBS or ferulic acid (30, 100, and 150 μM, respectively) for 48 h. Cell lysate was harvested, the expression of Bcl2, Bax, CDK2, CDK4, CDK6 and p-Rb was determined by Western blotting. GAPDH was used as an internal control.

MG63, were exposed to a series of concentrations (10, 30, 100, 150 μM) of ferulic acid for 24, 48, and 72 h, and subsequently MTT assay was used to examine the cell viability. Ferulic acid treatment could significant attenuate the cell viability, with a dose-dependent manner, by comparison of control in both osteosarcoma cell lines (Figure 1B and 1C). The IC50 values at 48 h for ferulic acid were 59.88 μM in 143B and 66.47 μM in MG63.

Ferulic acid induced G0/G1 phase arrest in osteosarcoma cells

To determine whether the cell-growth suppressive effect of ferulic acid attributed to inhibited proliferation, the cell cycle distribution was detected by flow cytometric analysis after treatment. Flow cytometric analysis showed that ferulic acid treatment significantly increased percentage of cells at the G0/G1 phase but decreased percentage of cells at the S and G2/M phase in a dose-dependent manner (Figure 2A and 2C). The alteration of cell cycle distribution maintained consistent in all the two osteosarcoma cells (Figure 2B and 2D).

Ferulic acid induced apoptosis in osteosarcoma cells

In order to distinguish the repressive cell viability from inhibitory proliferation or increased apoptosis, flow cytometry with Annexin V-FITC/PI staining was used to measure the apoptosis of 143B and MG63 after treatment. In both osteosarcoma cell lines, we found that apoptosis were profoundly augmented in the groups treated with ferulic acid when compared with controls (Figure 3A and 3B). Additionally, dose-dependent effects were found among the treatment groups.

Consistent with the flow cytometry results that ferulic acid could induce apoptosis in osteosarcoma cells; caspase-3 activity assay found that ferulic acid can evidently and dose-dependently induce activation of caspase-3 in two osteosarcoma cells (Figure 3C).

Ferulic acid regulated cell cycle-related and apoptosis-related proteins expression

Since CDK4 and CDK6 are identified to be involved in the early G1, whereas CDK2 is necessary to complete the G1 phase and initiate the S phase, the CDK4, CDK6 and CDK2 protein levels were examined using western blotting. As shown in Figure 4, ferulic acid could dose-dependently down-regulate the CDK4, CDK6 and CDK2 protein levels in osteosarcoma cells. Therefore, the results reveal that inhibitory effect of ferulic acid on osteosarcoma cell proliferation results from G0/G1 phase arrest through down-regulation of CDK4, CDK6 and CDK2.
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The Bcl-2 protein family plays an important role in apoptosis, and the ratio of active anti- and pro-apoptotic Bcl-2 family controls apoptosis of cell. To verify the potential role of Bcl-2 family proteins in the apoptosis induce by ferulic acid. The effect of ferulic acid on the expression of Bcl-2 and Bax in osteosarcoma cells was examined using immunostaining. As shown in Figure 4, ferulic acid profoundly and dose-dependent-ly increased Bax and reduced Bcl-2 in all the osteosarcoma cell lines.

PI3K/Akt pathway mediated the anti-tumor ef-fect of ferulic acid in human osteosarcoma

To determine whether PI3K/Akt pathway involves in the anti-tumor effect of ferulic acid, IF and WB were used to examine the protein level of activated Akt (p-Akt) in human osteosarcoma cells. Treatment of ferulic acid obvi-ously decreased p-Akt in MG63 cells with a dose-dependent manner, as presented in Figure 5A and 5B.

To further determine the effect of inhibition of PI3K/Akt pathway for anti-tumor effect of ferulic acid, cells were treated with LY294002, a specific inhibitor of PI3K. As shown in Figure 5C and 5D, the anti-tumor effect of LY294002 also existed, which is similar to ferulic acid. Additionally, overexpression of active Akt by adenovirus system reversed the anti-tumor effect of ferulic acid, abrogating the inhibition of cell proliferation and promotion of cell apoptosis (Figure 5C and 5D).

Ferulic acid exhibited antitumor activity in vivo

MG63 xenograft model was employed to evaluate the antitumor potential of ferulic acid in
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As shown in Figure 6A and 6B, smaller tumor volumes and lower tumor weights were observed in ferulic acid-treated mice as compared with control mice. Significantly decreased proliferation (Ki-67 staining) and p-AKT were detected in the tumor specimens of ferulic acid-treated mice (Figure 6C).

Discussion

Osteosarcoma is the most common type of malignant bone tumor. Owing to the application of neoadjuvant chemotherapy, the survival of osteosarcoma has been improved. However, the survival rate of osteosarcoma reached a plateau by current drugs, which, in addition, have been reported to be associated with acute and long-term toxicities. Hence, new therapeutic agents are desperately needed for the improvement of osteosarcoma survival and prognosis.

In past decades, increasing evidences demonstrated that ferulic acid might act as an anti-tumor agent in various human cancers. It has been confirmed that ferulic acid inhibited DMBA-induced cancers, including breast and skin cancer via its antigenotoxic, antioxidant potential and modulatory effect on phase II detoxification cascade, buccal cancer via decreasing expression of PCNA and cyclin D1 [15, 18]. Additionally, ferulic acid was also reported as an inhibitor of in 12-O-tetradecanoylphorbol-13-acetate-induced tumor promotion in mouse skin [19]. Moreover, ferulic acid could enhance radiation effects by decreasing antioxidant status and increasing intracellular reactive oxygen species, lipid peroxidation and DNA damage in HeLa and ME-80 the cell lines [20]. More importantly, ferulic acid had been proved to initiate apoptosis in non-small cell lung cancer cells through modulation of p53, Bax, caspase-3 and GADD45 [21] and inhibit proliferation in colon cancer cell Caco-2 by regulating S phase-related genes expression of CEP2, CETN3, and RABGAP1 [22]. Consistent with previous studies, in present study, we proved the anti-tumor effect of ferulic acid on osteosarcoma cell. We observed that ferulic acid could dose-dependently inhibit the cell proliferation and induce cell apoptosis in human 143B and MG63...
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osteosarcoma cells. Additionally, we confirmed that PI3K/Akt pathway, cell cycle related proteins, and apoptosis related proteins were related to the anti-tumor effect of ferulic acid.

The PI3K/Akt pathway is crucial to cell growth and survival. Aberration of PI3K/Akt pathway is a very common mechanism for many human cancers including osteosarcoma, since it can mediate survival pathway and apoptosis pathway [23, 24]. PI3K/Akt pathway has been shown to be activated in osteosarcoma cell lines. A previous study demonstrated that blockade of PI3K/Akt pathway by bone morphogenetic protein (BMP-9) exerted inhibitory effect on the growth of osteosarcoma cells [25]. Additionally, a growing number of studies revealed that inhibition of PI3K/Akt pathway, using a cyclooxygenase-2 inhibitor, oxymatrine, dipsacus asperoides polysaccharide, grifolin, could induce apoptosis of human osteosarcoma cells [23, 26-28]. In our study, we found that ferulic acid could inhibit the growth and enhance apoptosis of osteosarcoma cells. As expected, we also observed ferulic acid exhibited an inhibition of PI3K/Akt pathway, indicated by downregulation of p-Akt. The findings suggested that ferulic acid obstructed growth and enhanced apoptosis of osteosarcoma cell through inhibiting the PI3K/Akt pathway.

Cyclin-dependent kinases (CDKs) play an important role in cell cycle of eukaryotes. CDK4 and CDK6 are involved in early G1, and CDK2 is necessary to complete G1 and initiate S phase. CDK4/6-cyclin D and CDK2-cyclin E complexes, activated CDKs, could phosphorylate retinoblastoma protein family, Rb [29, 30]. Subsequently, the hyperphosphorylated Rb release the transcription factors of E2F family, allowing them to initiate the G1/S phase progression. Alterations of proteins involved in cell cycle control have been confirmed to be associated with the pathogenesis in many human cancers, including osteosarcoma [31-33]. Our results revealed that ferulic acid could decrease the protein level of CDK2, CDK4 and CDK6, indicating that the anti-proliferation via decreasing CDK2, CDK4 and CDK6. Previous published studies had shown that amplified PI3K/Akt pathway could inactivate P53 through MDM2 but activate NF-KB, beta-catenin and mTOR via protein phosphorylate [33], resulted in enhanced cell proliferation. Additionally, inactivated NF-KB, beta-catenin and mTOR result in downregulation of CDKs [34-37]. Consistent with previous studies, our results showed that obstruction of PI3K/Akt pathway by ferulic acid or LY294002 was associated with decreased CDK2, CDK4 and CDK6, while over activation of PI3K/Akt pathway by adenovirus system abrogated the reduction of CDK2, CDK4 and CDK6 induced by ferulic acid, suggesting that ferulic acid down-regulated CDK2, CDK4 and CDK6 via inhibiting PI3K/Akt pathway.

Bcl-2 family proteins affect apoptosis through alteration of permeability of mitochondrial outer membrane (MOM) following homo- or hetero-association [38]. A pro-apoptotic Bcl-2 family protein Bax promotes apoptosis by the release of cytochrome c from mitochondria and downstream activation of caspase, while Bcl-2, an anti-apoptotic Bcl-2 family protein, restrains cell apoptosis via withstanding the pro-apoptotic effects of Bax and blocking the release of cytochrome c [38, 39]. Therefore, in some extent, the Bax/Bcl-2 ratio affects activation of effective cleaved caspase 3 and controls the cell apoptosis. By immunostaining, we found that ferulic acid upregulated the expression of Bax and downregulated Bcl-2 in a dose-dependent manner, and consequently enhanced the activation of caspase 3 to promote apoptosis. It demonstrated that the apoptosis of osteosarcoma cell was due to the increased Bax/Bcl-2 ratio induced by ferulic acid. PI3K/Akt pathway possesses an anti-apoptotic effect by phosphorylating and inactivating pro-apoptotic protein Bad [40]. Phosphorylated Bad dissociates Bcl-2 and Bcl-XL from Bcl-XL-Bad and Bcl-2-Bad heterodimers [41], which results in cell survival through leaving more Bcl-2 and Bcl-XL activated and Bax inactivated by binding with Bcl-2 and Bcl-XL. Namely, Bad competes with Bax for integration with Bcl-2 or Bcl-XL and this could increase Bax/Bcl-2 ratio to promote cell apoptosis. In addition, PI3K/Akt pathway could increase the transcription of Bcl-2 and suppress the Bax translocation to mitochondria to avoid cell apoptosis [42, 43]. Coincident with our study, the results revealed that downregulation of Bcl-2 and upregulation of Bax was associated with inhibition of PI3K/Akt pathway by ferulic acid, on the contrary, over activation of PI3K/Akt pathway by adenovirus system abrogate downregulation of Bcl-2 and upregulation of Bax induced by ferulic acid. It suggests that
ferulic acid downregulates of Bcl-2 and upregulates of Bax through obstructing PI3K/Akt pathway.

In conclusion, we can conclude that ferulic acid inhibits proliferation of osteosarcoma cells (143B, MG63) downregulation of CDK2, CDK4 and CDK6, and induces apoptosis via downregulation of Bcl-2 and upregulation of Bax. Moreover, the modulation of CDK2, CDK4, CDK6, Bcl-2 and Bax attributes to inhibition of PI3K/Akt pathway. Our finding suggests that ferulic acid inhibits proliferation of osteosarcoma cells and induces apoptosis via inhibiting PI3K/Akt pathway and might be new therapeutic agents for osteosarcoma.

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Disclosure of conflict of interest

None.

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