Erianin induces G2/M-phase arrest, apoptosis, and autophagy via the ROS/JNK signaling pathway in human osteosarcoma cells in vitro and in vivo

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Erianin, a natural product derived from Dendrobium chrysotoxum, has exhibited potential antitumor activity in various malignancies, including hepatocarcinoma, melanoma, and promyelocytic leukemia. Here we explored the effects of erianin on osteosarcoma (OS) in vitro and in vivo and further elucidated the underlying molecular mechanisms. In this study, we found that erianin potently suppressed cell viability in various OS cell lines. Treatment with erianin induced G2/M-phase arrest, apoptosis, and autophagy in OS cells. Further studies showed that erianin-induced apoptosis and autophagy was attributed to reactive oxygen species (ROS), as N-acetyl cysteine (NAC), an ROS scavenger, attenuated them. Moreover, we found that erianin induced activation of c-Jun N-terminal kinase (JNK) signal pathway, which was also blocked by NAC. Downregulation of JNK by its specific inhibitor SP600125 could attenuate apoptosis and autophagy induced by erianin. Finally, erianin in vivo markedly reduced the growth with little organ-related toxicity. In conclusion, erianin induced cell cycle G2/M-phase arrest, apoptosis, and autophagy via the ROS/JNK signaling pathway in human OS. In light of these results, erianin may be a promising agent for anticancer therapy against OS.

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Osteosarcoma (OS), the most common primary bone tumor, is derived from primitive bone-forming mesenchymal cells, which produces osteoid and/or immature bone. 1 Approximately 2–3 patients per million were diagnosed with OS annually. The prevalence is predominant in adolescence, of which the annual incidence is 8–11 per million at 15–19 years of age. 2 Because of the multi-agent, dose-intensive chemotherapy in conjunction with improved surgical techniques, the 5-year survival rate of patients with OS has been improved to 60–70%. 3 Unfortunately, this cure rate has not increased over the past 25–30 years. Therefore, continuing research into new treatment approaches and drugs is urgently needed.

The combretastatins are a group of antimitotic agents isolated from the bark of the South African tree Combretum caffrum. Structurally, combretastatins consist of two substituted aromatic (aryl) rings (rings A and B) linked by a two-carbon alkene bridge. 4 The combretastatins are naturally very potent anticancer agents with activity in the low nanomolar range. 5 Combretastatin A-4 (CA-4) is one of the most potent antitumor agents with activity in the low nanomolar range. 5 It inhibits tumor cell proliferation by causing cell cycle G0-, S-, or G2/M-phase arrest. 11–13 The G2 checkpoint prevents cells from entering mitosis when DNA is damaged and ensures the propagation of error-free copies of the genome to each daughter cell. Cdk1/Cyclin B1 complex controls the cell cycle progression from G2 phase to the M phase by regulating the phosphorylation or dephosphorylation of proteins. 14 In addition, actin remodeling in coordination can ensure proper execution of G2/M checkpoint arrest and is crucial for entry into mitosis. 15,16

Cell death is a hallmark of cancer that can be classified according to morphological differences. Apoptosis, the best defined form of programmed cell death (PCD), is characterized by specific morphological and biochemical changes of dying cells, including cell shrinkage, nuclear condensation, DNA fragmentation, chromatin condensation, and membranes blebbing. 17 PCD can occur via two major pathways, the caspase-dependent and caspase-independent pathway. 17 Because of the multi-agent, dose-intensive chemotherapy used in cancer treatment, the occurrence of PCD and autophagy is common in cancer cells. 18 Autophagy is a complex self-degradative process in eukaryotic cells, which is involved in the degradation of cytosolic organelles, proteins, and aggregates. 19 Autophagy is activated under the conditions of nutrient deprivation, such as amino acid starvation and energy deficiency. 20,21 However, an imbalance or overactivation of autophagy can lead to cell death. 22 The role of autophagy in cancer therapy is controversial. 23

Structurally similar to CA-4, erianin can induce microtubule disassembly resulting in a dramatic cytotoxic effect on various human cancer cells. Erarianin is a natural product derived from Dendrobium chrysotoxum and has been used as an analgesic in traditional Chinese medicine. Previous studies have demonstrated the antitumor activity of erianin against a variety of human cancer cells, including human hepatocarcinoma Bel7402 cells, 9 human melanoma A375 cells, 9 and human promyelocytic leukemia HL-60 cells. 10 However, whether erianin suppresses the growth of human OS and its related molecular mechanism have not yet been investigated.

Many cytotoxic agents and/or microtubule-targeting agents inhibit tumor cell proliferation by causing cell cycle G0-, S-, or G2/M-phase arrest. 11–13 The G2 checkpoint prevents cells from entering mitosis when DNA is damaged and ensures the propagation of error-free copies of the genome to each daughter cell. Cdk1/Cyclin B1 complex controls the cell cycle progression from G2 phase to the M phase by regulating the phosphorylation or dephosphorylation of proteins. 14 In addition, actin remodeling in coordination can ensure proper execution of G2/M checkpoint arrest and is crucial for entry into mitosis. 15,16

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Erianin inhibits cells proliferation and induces G2/M arrest in human OS cells. (a) CCK8 assay was used to assessed OS cell proliferation. OS cell viability following treatment with the various concentrations of erianin for 24, 48, and 72 h. (b) Colony-formation assay was performed in 143B and MG63.2 cells with and without erianin treatment. (c) Erianin induces G2/M cell cycle arrest. 143B and MG63.2 cells were treated with control and erianin (10, 25, and 50 nM) for 24 h. The distribution of cell cycle was assessed by flow cytometry. The percentage of cells in each phase is showed as mean ± S.D. from three independent experiments. (d) 143B and MG63.2 cells were exposed to erianin for 24 h. The expression of cell cycle-regulated proteins were analyzed by western blotting. *P< 0.05, significantly different compared with control.
and fragmentation, dynamic membrane blebbing, and loss of
adhesion to neighbors or to extracellular matrix.\textsuperscript{17, 18} Autophagy,
or type II PCD, is a lysosomal degradation procedure by
which excessive or dysfunctional eukaryotic cellular compo-
nents are transported into lysosomes to be digested.\textsuperscript{18, 19} The
functional relationship between apoptosis and autophagy is
complex, and the two phenomena jointly seal the fate of the
cell.\textsuperscript{20} Therefore, further investigations are required for
the apoptosis–autophagy crosstalk, which may provide novel
corcepts and new targeted agents for cancer therapy.

Reactive oxygen species (ROS) has been described as a
heterogeneous group of diatomic oxygen from free and non-
free radical species and has important roles in biochemical
functions, including apoptosis and autophagy. ROS triggers
apoptosis by causing various cellular stresses, including DNA
damage and microtubule disruption mediated by various signal
transducers.\textsuperscript{21, 22} Several apoptotic effectors are redox-sensi-
tive, such as caspases, Bcl-2, and cytochrome c, and their
functions are markedly regulated by cellular ROS.\textsuperscript{23} Recent
studies have showed that antioxidative agents abolished the
formation of autophagosomes and the consequent degradation
of proteins.\textsuperscript{24} Starvation-induced autophagy, in turn, resulted in
ROS production and DNA damage by targeting the poly (ADP-
ribose) polymerase (PARP) signaling pathway.\textsuperscript{25}

ROS have been demonstrated as an inducer or mediator for
the activation of MAPK family members, including c-Jun
N-terminal kinase (JNK), p38, and ERK1/2.\textsuperscript{26} JNK, also known as
stress-activated protein kinase of MAPK family, is a key
regulator of a variety of cellular events, including apoptosis
and autophagy. The function of JNK in apoptosis is complex,
depending on cell type, nature of the death stimulus, duration
of its activation and the activity of other signaling
pathways.\textsuperscript{27–29} In addition to apoptosis, JNK also contributes
to autophagic induction in response to stress signals, for
instance, incidences of nutrition deficiency,\textsuperscript{30} cytokine and
growth factor decreases,\textsuperscript{31} and chemotherapy drugs.\textsuperscript{32}

In the current study, we elucidated that erianin induced
G2/M-phase arrest, apoptosis, and autophagy in human OS
cells, which was mediated by ROS induction, leading to
activation of JNK/c-Jun signaling cascades. Furthermore, we
investigated that erianin administration inhibited tumor growth
using in vivo tumor xenograft model. Collectively, our data
suggest that erianin is a promising antitumor agent by
modulating the ROS/JNK signaling pathway for OS.

Results

Erianin inhibits cell proliferation and induces cell cycle
G2/M arrest in OS cells. To investigate the inhibitory effects
and cytotoxicity of erianin in OS cells, 143B, MG63.2, Saos2,
and CCHO were treated by various concentrations of erianin
for 24, 48, and 72 h, followed by Cell Counting Kit-8 (CCK8)
assay. We found that erianin decreased OS cell viability in a
time and dose-dependent manner (Figure 1a). The IC50
values were 58.19 nM (24 h), 40.97 nM (48 h), and 26.77 nM
(72 h) for 143B cells, while the IC50 values for MG63.2 were
88.69 nM (24 h), 44.26 nM (48 h), and 17.20 nM (72 h). In
addition, the antiproliferation effect of erianin in OS cells 143B
and MG63.2 was confirmed by colony-formation assay.

Results demonstrated that erianin treatment significantly
reduced the number of colonies in a dose-dependent manner
when compared with untreated cells (Figure 1b). These
results demonstrated that erianin treatment inhibited the
proliferation of OS cells.

To verify the causal relation of cell proliferation inhibition and
cell cycle arrest, the cell cycle distribution was analyzed.
Erianin increased cell number at G2/M phase after 24 h
treated with raising concentration, accompanied by decreased
cell number at S and G0/G1 phases in 143B and MG63.2 cells
(Figure 1c). Furthermore, cell cycle-regulating pathways were
measured by western blotting, the expression of Cyclin B1,
phospho-Cdk1, phospho-Cdc25c, p21, and p27 were upre-
gulated, and the level of Cdk1 was downregulated (Figure 1d).
All these data indicated that erianin triggered G2/M-phase
arrest by regulating cell cycle-related proteins.

Erianin induces apoptosis of OS cells. We further
explored the effects of erianin on apoptosis and cell death in
OS cells. Hoechst 33342 staining was used to estimate
erianin-dependent changes in cell morphology. Results
demonstrated the occurrence of cell shrinkage, chromatin
condensation, and nuclei fragmentation after treatment with
erianin for 24 h (Figure 2a). Flow cytometric analysis via
Annexim V-PE/7-AAD was performed. We found that a
significant increase of the number of apoptotic cells was
observed in cells after exposed to erianin in a dose-
dependent manner (Figure 2b). Next we used the fluorescent
mitochondrial probe JC-1 to measure mitochondrial mem-
brane potential to confirm the involvement of mitochondrial in
the induction of apoptosis by erianin. After erianin treatment,
an obvious shift from red to green was observed (Figure 2c),
indicating that mitochondrial depolarization was induced by
erianin in OS cells. To further determine whether extrinsic or
intrinsic pathway mediated erianin-induced apoptosis, we
investigated the expression of downstream apoptotic proteins
by western blotting. Caspase-8 and -9 are initiator caspases
in the extrinsic and intrinsic (mitochondrial) apoptosis path-
ways, respectively. As shown in Figure 2d, an obvious
increase in the activation of cleavage caspase-3, -8, and -9
and of PARP and the expression of Bcl-2, Bcl-xl, and survivin
were decreased (Figure 2d). Taken together, these data
indicated that erianin provoked cell apoptosis by activating
both the extrinsic and intrinsic pathways.

Erianin induces autophagy of OS cells. As autophagy
contributes to cell death, we then investigated whether
erianin induces autophagy in OS cells. Autophagy is
characterized by the increased acidic vesicular organelles,
which are correlated with increased autophagosomes.\textsuperscript{33} We
used the membrane acidotropic dye probe LysoTracker Red
label cellular acidic compartments, including lysosomes and
autolysosomes. Upon exposure to erianin for 24 h, the
fluorescence intensity in 143B and MG63.2 cells exhibited
an obvious increase (Figure 3a). Furthermore, we found that
treating cells with erianin resulted in a significant increase in
GFP-LC3 puncta formation in MG63.2 cells in both dose- and
time-dependent manner (Figure 3b). To verify the above
finding, we further tested the expression of several marker
protein of autophagy by western blotting. Erianin treatment
Figure 2  Erianin induces apoptosis in OS cells. (a) Apoptotic nuclear morphology changes induced by erianin were assessed by Hoechst 33342 staining and visualized by fluorescence microscopy. Arrows indicate chromatin condensation and nuclear fragmentation. (b) Cells were treated with increased concentrations of erianin for 24 h. Cells were processed by flow cytometry using Annexin V-PE/7-AAD (7-aminoactinomycin D) staining and analyzed by flow cytometry. The histograms indicate that the percentage of early apoptosis, late apoptosis, and total apoptosis. The percentage of apoptosis cells is shown as mean ± S.D. from three independent experiments. (c) The mitochondrial membrane potential after erianin treatment were measured using JC-1 staining by flow cytometry. The histograms indicate the ratio of green in JC-1 fluorescence. Results are shown as mean ± S.D. from three independent experiments. (d) 143B and MG63.2 cells were incubated with erianin for 24 h. Cell lysates were prepared and analyzed by western blotting for cleaved PARP, caspase-8, -9, and -3, Bcl-2, Bcl-xl, and survivin. *P < 0.05 and **P < 0.001, significantly different compared with control.
increased the amount of LC3B II protein and upregulated the expression of p62 and Beclin-1 (Figure 3c). Overwhelmingly, evidences show that autophagy has a dual role for therapeutic purpose in cancer, with response to protect cell survival or contribute to cell death. We used the autophagy inhibitor 3-methyladenine (3-MA) to block erianin-induced autophagy in OS cells. Consistently, the administration of 3-MA increased OS cells’ sensitivity to erianin by reducing its autophagic effects and enhancing its apoptotic effects (Figures 3d–f). These data suggested that cellular reactive autophagy after erianin treatment may be protective in OS cells.

Erianin activates JNK/c-Jun signaling pathway by inducing ROS production. ROS functions as signaling molecules, which has important roles in biochemical functions, including apoptosis and autophagy. Therefore, the production of ROS was analyzed in erianin-treated cells by DCFH–DA staining by fluorescence microscope and flow cytometry. Figure 4a demonstrated that exposure of cells to erianin resulted in a dramatic increase in the fluorescent signal as compared with the control. We used the antioxidant N-acetyl cysteine (NAC) to further confirm the elevation of ROS. Addition of NAC effectively blocked erianin-induced ROS in OS cells (Figure 4b). Next we investigated the effect of erianin on JNK/c-Jun pathway. As shown in Figure 4c, erianin induced phosphorylation of JNK and c-Jun in a concentration-dependent manner. However, the presence of JNK inhibitor, SP600125, potently inhibited the activation of JNK pathway (Figure 4d). ROS has been demonstrated as an inducer or mediator for the activation of JNK/c-Jun signaling pathway. We further found that pretreatment with NAC significantly reversed the phosphorylation of JNK and c-Jun in OS cells (Figure 4e). These results revealed that erianin activated the ROS/JNK signaling pathway.

Erianin induces apoptosis and autophagy via the activation of ROS/JNK pathway. We examined whether erianin-induced apoptosis and autophagy involve ROS generation and JNK activation in OS cells. Cells were first pretreated with NAC and SP600125, respectively, before they were treated with erianin for additional 24 h. Notably, CCK8 analysis showed that NAC and SP600125 could attenuate the cell-killing effect of erianin on the growth of OS cells (Figure 5a). Flow cytometric analysis demonstrated that NAC and SP600125 attenuated the erianin-induced apoptosis (Figure 5b), and western blotting analysis showed that both of them reduced the levels of apoptosis-related proteins (Figures 5c and d). Next we investigated the role of ROS generation and JNK activation in erianin-induced autophagy. Results showed that NAC and SP600125 decreased the number of GFP-LC3 puncta (Figure 5e) and the expression of LC3-II, Beclin-1, and p62 proteins (Figures 5f and g). Taken together, ROS/JNK pathway activation by erianin participated in the induction of apoptosis and autophagy.

Erianin inhibits growth of OS in vivo. To evaluate the antitumor effect of erianin in vivo, an orthotopic OS model was established by intra-tibial injection of 143B cells. The mice were injected with erianin (2 mg/kg) while control group were injected with 5% dimethyl sulfoxide (DMSO) intraperitoneally every other day for seven times in total. Erianin inhibited the growth of tumor (Figures 6a and b). However, there was no significant loss in body weight in the experimental mice (Figure 6c). As shown in Figure 6d, erianin-treated tumor tissues showed significant increase of terminal dUTP nick end labeling (TUNEL)-positive cells and the level of cleaved caspase-3 and JNK phosphorylation, whereas the level of PCNA was decreased. To investigate any potential cytotoxic effects of erianin on normal tissues, non-tumor-bearing mice were intraperitoneally treated with erianin, and hematoxylin and eosin (H&E) staining of organs collected at the end of the study also suggested no major organ-related toxicities (Figure 6e). These data showed that erianin exhibited potent antitumor activity with less toxicity in vivo.

Discussion

Erianin, a natural product derived from D. chrysotoxum, has been associated with potent antitumor activity against human cancer cell lines tested with IC50 < 100 nM. Previous studies for erianin anti-tumor effects were described as followed: induced vascular shutdown, inhibited angiogenesis, disrupted endothelial tube formation, and perturbed cityscape’s architectural.3,34 However, for proliferating and fragile tumor cells and normal tissue endothelial cells, the mechanism of action of erianin is different. Erianin was shown to be cytotoxic toward proliferation and induction of apoptosis but not quiescent endothelial cells. Non-cytotoxic concentrations of erianin resulted in the disruption of endothelial cytoskeleton, implying that there is a cell-type specificity in erianin-induced cytoskeletal impairment.9 These studies suggest that erianin may have future value in the treatment of some non-cancer diseases. In the current study, we undertook a comprehensive analysis of the effect of erianin on OS using both in vitro and in vivo models. Our data showed that erianin could suppress cell proliferation, cause G2/M-phase arrest, and induce apoptosis and autophagy via the ROS/JNK signaling pathway in human OS cells.

The G2 checkpoint prevents cells from entering mitosis when DNA is damaged, providing an opportunity for repair and stopping the proliferation of damage cells.14 According to flow cytometric analysis, erianin increased the proportion in G2/M phase and decreased the cell proportion in G0/G1 and S phases in OS cells. Western blotting analysis showed that erianin led to an increase in the accumulation and activation of G2/M-phase-related cycle regulator Cyclin B1. However, whether erianin inhibited G2/M transition or induced M arrest has been uncertain. The complex of Cdk1/Cyclin B1 has a crucial role in promoting the G2/M-phase transition. Further analysis revealed that erianin increased the expression levels of phospho-Cdk1. In addition, we examined the effects on p21, p27, and the phospho-Cdc25c. P21 and p27 have a critical role in blocking activation of Cdk1/Cyclin B1,35–37 while Cdc25c is activated for dephosphorylation of cdc2 at the onset of mitosis.38,39 Increased expression of p21, p27 and phospho-Cdc25c were observed after erianin treatment. These data indicated that erianin inhibited the G2/M transition, rather than causing M-phase arrest. Existing evidence shows that antitubulin agents are associated with inappropriate activation of Cdc25c and Cyclin B1, resulting in the induction
of G2/M phase arrest. However, the mechanism underlying this phenomenon has largely remained elusive and need to be further explored.

Apoptosis is crucially involved in the regulation of tumor formation and treatment response. Current cancer therapy, including chemotherapy, γ-irradiation, gene therapy, and immunotherapy, has linked to activation of apoptosis signal transduction pathway. Caspases, closely associated with apoptosis, are widely expressed in an inactive proenzyme form in most cells and, once activated, can often activate other proapoptases, allowing initiation of a protease cascade. Present study found that erianin induced generation of apoptotic cells through the induction of DNA fragmentation and the activation of PARP, caspase-3, -8, and -9. Immuno-histochemical analysis confirmed that erianin increased cleaved caspase-3 level, and the TUNEL assay demonstrated an obvious increase in apoptosis proportion in erianin-treated tumor tissues. These data indicated that erianin induced cell apoptosis by activating both the extrinsic and intrinsic (mitochondrial) pathways.

Autophagy, which is regarded as a promising, novel strategy for enhancing antitumor efficacy of chemotherapy drugs, has been under extensive investigation. A growing body of evidence implicates a dual role of autophagy for therapeutic purposes in cancer, with response protecting cell survival or contributing to cell death. We found that erianin-induced autophagy in OS cells was evidenced by an increase in the number of autophagic vesicles and enhanced conversion of LC3B-I to LC3B-II. A growing number of researches demonstrated that small compounds activate apoptosis and autophagy for antitumor chemotherapy. However, the functional relationship between apoptosis and autophagy is complex, and the two phenomena that jointly decide the fate of the cell. In our study, inhibition of autophagy by treatment with 3-MA increased erianin-induced apoptosis, indicating that autophagy induced by erianin contributed to the cell survival. ROS is considered as an important upstream molecule in the regulation of cell death and survival in cancer. Basic levels of ROS may function as signals to promote cell proliferation and survival, whereas high levels of ROS can damage cellular components such as DNA, protein and lipids, leading to cell apoptosis and autophagy. Alterations in ROS levels have a crucial role in tumorigenesis and is recognized as the promising strategy for cancer treatment. In the present study, erianin induced a significant increase in ROS generation, while pretreatment with ROS inhibitor NAC remarkably reversed the erianin-induced inhibition of cell proliferation, apoptosis, and autophagy. Our results indicated that the induction of ROS might activate DNA damage and lead to apoptosis and autophagy in OS cells.

Growing evidence in recent years demonstrates that activation of the JNK pathway transduces oxidative stress signal to promote cell apoptosis and autophagy in response to various stress signals. JNK is primarily activated by various environmental stress, including oxidative stress, chemotherapeutic agents, and heat shock. We found that treatment with erianin induced a significant increase in JNK and c-Jun phosphorylation. Confirmed by the use of the JNK inhibitor SP600125, JNK activation is associated with the regulation of erianin-induced apoptosis and autophagy. Furthermore, ROS accumulation is involved in the activation of JNK/c-Jun pathway, while pretreatment with NAC nearly attenuated the phosphorylation of JNK and c-Jun. Together, these data showed that erianin induced apoptosis and autophagy through activation of ROS-dependent JNK/c-Jun pathway.

Our study, for the first time, identifies antitumor effects of erianin on OS in vitro and in vivo and the potential molecular mechanisms. We found that erianin significantly induced G2/M cell cycle arrest and caused cell apoptosis and autophagy regulated via ROS/JNK signaling pathway. We also demonstrated that erianin significantly decreased tumor growth in mice bearing xenografts without obvious toxicity. This study indicates that erianin is a novel antitumor drug candidate that has great potential as a promising agent for anticancer therapy in OS.

Materials and Methods

Cell culture. Human OS cell lines 143B and Saos2 were obtained from American Type Culture Collection (Manassas, VA, USA). MG63.2 cell was derived from the metastasis of parental MG63, as previously reported. Human OS cell lines CCHO were established by Pediatric Research in MD Anderson Cancer Center. All cells were finger-printed to exclude possible contamination. All cells were cultured in high-glucose Dulbecco’s Modified Eagle’s Medium (Thermo, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Thermo), 100 U/ml penicillin, and 100 μg/ml streptomycin (Thermo) in a humidified incubator at 37 °C in 5% CO2.

Reagents and antibodies. Purified erianin (> 98%) was purchased from Shanghai Tauto Biotech Co., Ltd (Shanghai, China). Stock solution at 100 mM was made in DMSO (Sigma, St. Louis, MO, USA) and stored in the dark at −20°C. NAC and SP600125 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 3-MA were purchased from Selleckchem (Houston, TX, USA). Antibodies against caspase-3, -8, -9, PARP, Bcl-2, Bcl-xl, survivin, JNK, phospho-JNK, c-Jun, phospho-c-Jun, p38, phospho-p38, Cyclin D1, Cyclin E1, Cyclin B1, Cdk1, phospho-Cdk1, phospho-Cdc25c, p27, p21, LC3B, p62, Beclin-1, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell viability assay. The effect of erianin on cell viability was determined with the CCK8 (Dojindo, Kumamoto, Japan). Cell suspensions (3 × 105/ml) were seeded into 96-well plates overnight and then treated with various concentration of erianin (0, 10, 25, 50, 100 nM). Erianin was dissolved in DMSO, and the concentration of
Erianin increases ROS generation and activates JNK signal pathway. (a) Cells were treated with increased concentrations of erianin 12 h, followed by loading with 10 μM of DCFH-DA for 30 min. The level of ROS was determined by fluorescence microscopy. (b) Cells were preincubated with NAC (5 mM) for 2 h and then treated with erianin for 12 h. Fluorescent intensity was detected using flow cytometry. Results are presented as mean ± S.D. from three independent experiments. *P<0.05, significantly different compared with the control and NAC-treated group. (c) Cells were treated with various concentrations of erianin for 24 h. The expression of p-JNK, JNK, p-c-Jun, and Jun were analyzed by western blotting. (d and e) 143B was preincubated with SP600125 (30 μM) or NAC (5 mM) for 2 h and then treated with erianin for 24 h. Levels of p-JNK, JNK, p-c-Jun, and Jun were analyzed by western blotting.

Figure 4
Figure 5  ROS/JNK pathway mediate erianin-induced apoptosis and autophagy in OS cells. Cells were preincubated with SP600125 (30 μM) or NAC (5 mM) for 2 h and then treated with erianin for 24 h. (a) Cell viability was measured by CCK8 assay. (b) Cell apoptosis was evaluated by flow cytometry. Results are presented as mean ± S.D. from three independent experiments. *P<0.05, significantly different compared with the control, SP600125-treated, and NAC-treated groups. (c and d) The expression of apoptosis-related proteins were measured by western blotting. (e) Representative micrographs of cells that shows GFP-LC3 localization. (f and g) The level of autophagy-related proteins were analyzed by western blotting.
Figure 6. Erianin inhibits OS xenograft growth in vivo. 143B cells were orthotopically inoculated into the left tibia of BALB/c-nu mice. One week after tumor inoculation, mice were randomly divided into two groups for treatment. Intraperitoneal administration of vehicle or erianin (2 mg/kg) every other day for seven times. (a) Tumor volume was measured every week. (b) Erianin treatment resulted in significantly lower tumor volume than control group. (c) Body weights were measured every week. (d) Detection of apoptosis in tumor tissues by TUNEL assay. The expression of PCNA (proliferating cell nuclear antigen), cleaved caspase-3, and p-JNK were examined by immunohistochemistry. (e) No major organ-related toxicities was observed. H&E staining was used to evaluate the histology. Data represent mean ± S.D. *P < 0.05, significantly different compared with control.
Cells were seeded in six-well plates at a density of 5 × 10^5/ml and then treated with erianin at different concentrations for 24 h. After erianin treatment, the cells were harvested, washed with phosphate-buffered saline (PBS) and fixed with cold 70% ethanol alcohol at 4 °C overnight. The cells were then again washed with PBS and incubated with RNase A for 30 min followed by staining with 400 μl propidium iodide for 30 min at room temperature. Cell cycle analysis was performed on the Accuri C6 (BD Biosciences, Mountain View, CA, USA).

**Apoptosis analysis by flow cytometry.** Cells were seeded in six-well plates with a density of 5 × 10^5/ml and then treated with erianin at concentrations ranging from 0 to 50 nM for 24 h. Then 100 μl of JC-1 staining solution was added into 1 ml of culture medium and incubated for 20 min at 37 °C in a CO_2_ incubator. The samples were analyzed by flow cytometry, and JC-1 aggregate was measured at the FL-2 channel and green fluorescent (both JC-1 monomer) at the FL-1 channel (BD Biosciences).

**Measurement of ROS.** Intracellular ROS production was detected by using the peroxide-sensitive fluorescent probe DCFH-DA. Cells were plated in six-well plates and treated with erianin at different concentrations in the absence or presence of 5 mM NAC. Cells were then incubated with DCFH-DA at a final concentration of 10 μM in DMEM-h without FBS for 30 min at 37 °C and washed three times with DMEM. The level of ROS was determined by fluorescence microscopy (Leica, Wetzlar, Germany) and flow cytometer (BD Biosciences; San Jose, CA, USA).

**Histopathology and immunohistochemistry.** Formalin-fixed tissue samples were embedded in paraffin and 4-μm sections were cut. Primary tumors, heart, liver, spleen, lung, and kidney sections were stained with H&E for routine histological examinations and morphometric analysis. For immunohistochemical staining, slides were deparaffinized in xylene and rehydrated with graded alcohol and incubated in 3% hydrogen peroxide to block the endogenous peroxidase activity. Antigen retrieval was performed by boiling the slides in 10 mM sodium citrate (pH 6.0) for 30 min. Then slides were blocked in 10% normal goat serum for 15 min, followed by incubation with primary antibodies at 4 °C overnight in a humid chamber. On the next day, slides were washed in PBS and incubated with the secondary antibody for 1 h at room temperature. Immunoreactivity was detected using the Vectorstain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA).

**Statistical analysis.** Statistical analysis was performed using the SPSS version 18.0 software (IBM Corporation, Chicago, USA). Student's t-test, Fisher's Exact test, and one-way ANOVA were used for calculating the significance between different groups. Statistical significance is indicated by P < 0.05. All data were expressed as mean ± S.D. of three independent experiments.

**Conflict of Interest**

The authors declare no conflict of interest.

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