Polyphyllin VI induces apoptosis and autophagy in human osteosarcoma cells by modulation of ROS/JNK activation

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Purpose: Polyphyllin VI, a main active saponin isolated from traditional medicinal plant Paris polyphylla, has exhibited antitumor activities in several cancer cell lines. In the present study, we investigated the antitumor effect of Polyphyllin VI against human osteosarcoma cells (U2OS) and the underlying molecular mechanisms.

Methods: The U2OS cell lines were used to determine the antiproliferative effect of Polyphyllin VI by CCK8 assay. Cell cycle was analyzed by flow cytometry. The Polyphyllin VI-induced apoptosis was determined by Annexin V-APC/7-AAD apoptosis detection kit and JC-1 staining. Meanwhile, the autophagy was determined by acridine orange staining. The apoptosis and autophagy-related proteins were monitored by Western blot assay. Subsequently, intracellular hydrogen peroxide (H₂O₂) and the activation of ROS/JNK pathway were detected.

Results: Polyphyllin VI could potently inhibit cell proliferation by causing G2/M phase arrest. Polyphyllin VI induced mitochondria-mediated apoptosis with the upregulation of proapoptotic proteins Bax and poly ADP-ribose polymerase, and downregulation of anti-apoptotic protein Bcl-2 in U2OS cells. Concomitantly, Polyphyllin VI provoked autophagy with the upregulation of critical Atg proteins and accumulation of LC3B-II. Intracellular H₂O₂ production was triggered upon exposure to Polyphyllin VI, which could be blocked by ROS scavenger. Polyphyllin VI dramatically promoted JNK phosphorylation, whereas it decreased the levels of phospho-p38 and ERK.

Conclusion: Our results reveal that Polyphyllin VI may effectively induce apoptosis and autophagy to suppress cell growth via ROS/JNK activation in U2OS cells, suggesting that Polyphyllin VI is a potential drug candidate for the treatment of osteosarcomas.

Keywords: Polyphyllin VI, osteosarcoma, apoptosis, autophagy, JNK activation

Introduction

Osteosarcoma is regarded as the most common primary bone tumor, which stems from primitive bone-forming mesenchymal cells that produce osteoid and/or immature bone.¹,² The annual osteosarcoma incidence was at around 2–3 patients per million and that of adolescence was high at approximately 8–11 per million at 15–19 years of age.³ Although current therapies incorporate surgical resection and combinational chemotherapy, the 5-year survival rate for patients with metastatic or relapsed osteosarcoma has remained unchanged over the past 30 years.⁴ Therefore, new treatment approaches for osteosarcoma are urgently needed to be developed.
Cell death is a trait of cancer that can be classified by morphological differences. Programmed cell death, including apoptosis, autophagy and programmed necrosis, is proposed to be the death of a cell in any pathological format, which is a new target for anticancer therapeutic strategies. Apoptosis, type I programmed cell death, contains several well-characterized morphological and biochemical changes of dying cells, such as cell volume loss, chromatic condensation, dynamic membrane blebbing and loss of adhesion to the extracellular matrix. Many studies have reported that apoptosis can be induced by diverse proapoptosis stimuli converging on mitochondria, leading to mitochondrial dysfunction, the activation of caspase enzymes and eventually cell apoptosis. In particular, it is now well established that numerous chemotherapeutic agents induce tumor cell apoptosis via mitochondria-dependent pathway, which is highly regulated by Bcl-2 family members. The Bcl-2 family of proteins is regulated to be the key factors regulating mitochondrial membrane permeabilization. The Bcl-2 family is composed of proapoptotic and antiapoptotic members, such as Bax and Bcl-2. Autophagy, type II programmed cell death, is a dynamic degradation procedure involving cytoplasmic vesicles and intracellular organelles are transported into vacuoles called autophagosomes. These organelles or/and vesicles are fused by lysosomes to generate autophagolysosomes, where the mixed material is degraded, leading to cell death. More importantly, a recent study has highlighted the persistent autophagy served as a potent death signal, which is triggered off in response to the knockout growth factors or on exposure to antitumor agents. Apoptosis and autophagy are considered as potent therapeutic targets for the treatment of tumor.

Recently, more and more traditional Chinese medicine (TCM) has been applied in osteosarcoma prevention and treatment because of the advantages of curative effect and lower toxicity. Polyphyllin VI, isolated from Paris polyphylla, showed strong anticancer activity among several cancer cell lines. However, the role of Polyphyllin VI in human osteosarcoma cells and the potential mechanisms underlying its antitumor activity remain largely unclear. In this work, we aimed to investigate the molecular mechanism of Polyphyllin VI-induced cell death of human osteosarcoma cells. Exposure of osteosarcoma cells to Polyphyllin VI induced cell apoptosis and autophagy. Moreover, we found the involvement of H2O2 formation and ROS/JNK activation in the autophagic pathway. Hence, our results revealed the multifunctionality of Polyphyllin VI in its anticancer effect via autophagy and apoptosis in osteosarcoma cells, and it could be a promising drug candidate.

Materials and methods
Materials and reagents
Purified Polyphyllin VI (>98%) was purchased from Chengdu Must Bio-Technology Co., Ltd (Chengdu, China). Stock solution was dissolved in DMSO (Sigma, St. Louis, MO, USA) and was stored in the dark at –20°C. Then, the diluting stock solution was diluted into working concentrations by culture medium immediately before use. CCK8 (Cell Counting Kit-8) was purchased from Dojindo Molecular Technologies, Inc (Japan). N-acetyl-L-cysteine (NAC) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Antibodies against poly ADP-ribose polymerase (PARP), Bax, Bcl-2, LC3B-I, LC3B-II, p62, Atg-7, Atg-5, Atg-12-Atg-5, Atg-12, Atg-3, JNK, phospho-JNK, p38, phospho-p38, p-ERK, ERK, caspase-3, caspase-7, and GAPDH were purchased from Cell Signaling Technology (Beverly, MA, USA).

Cell lines and cell culture
The human osteosarcoma cell lines U2OS were purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, People’s Republic of China). The cells were cultured in minimum essential medium (MEM, Gibco Life Technologies) supplemented with 10% FBS (Sijiqing, Hangzhou, China), 100 μg/mL streptomycin and 100 U/mL penicillin at 37°C with 5% CO2.

CCK8 assay for cell viability
The effects of Polyphyllin VI on U2OS cells were analyzed by CCK8 assay. The U2OS cells were seeded into 96-well plate at the density of 5×104 cells/well. After incubation overnight, the cells were treated with Polyphyllin VI (0, 1, 2, 4, 6, 8, 12 and 30 μM) for 24 hrs and 48 hrs. Following the treatment, cells were incubated with 0.5% CCK8 for 4 hrs. The formed formazan crystals were dissolved in DMSO (200 μL/well). The absorbance was measured at a test wavelength of 570 nm and a reference wavelength of 650 nm.

APC annexin V/7-AAD staining assay for cell apoptosis
Apoptotic cells were evaluated by low cytomtery. U2OS cells were treated with various concentrations of Polyphyllin VI or...
0.1% DMSO for 24 hrs. For flow cytometry, 1×10^6 U2OS cells in 500 μL binding buffer were stained with 5 μL Annexin V-APC and 5 μL 7-AAD at room temperature for 15 mins, respectively. Cells were then analyzed by flow cytometry using BD FACSCalibur flow cytometer (Becton & Dickinson Company, Franklin Lakes, NJ, USA).

**Mitochondrial membrane potential measurement**

The mitochondrial membrane potential (MMP) was determined using a JC-1 assay kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cells were treated with Polyphyllin VI (0, 2.5, 5, and 7.5 μM) for 24 hrs. After collection, the U2OS cells were washed with PBS twice, stained with 500 mL JC-1 in a 37°C, 5% CO2 incubator for 25 mins, protected from light. And the cells were then centrifuged at 2000 rpm×5 mins and washed twice. The cells were resuspended in 500 mL 1× incubation buffer for flow cytometry analysis (488 nm excitation and 525 nm emission filters).

**Acridine orange staining for cell autophagy**

For the detection of autophagy, U2OS cells were treated with Polyphyllin VI or 0.1% DMSO for 24 hrs. Following treatment, the cells were stained with 1 μg/mL acridine orange (Amresco, Solon, OH, USA) in PBS for 30 mins at 37°C. Cells were then observed using the BD Accuri C6 flow cytometry.

**Hydrogen peroxide (H₂O₂) measurement**

For quantitative detection of intracellular H₂O₂ levels, U2OS cells were pretreated with treated with Polyphyllin VI (0, 2.5, 5, and 7.5 μM) for 24 hrs and then incubated with 2',7'-dichlorofluorescin-diacetate (DCHF-DA) (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocols. After incubation with 10 μM DCHF-DA for 30 mins at 37°C in dark, DCFH-DA was cleaved by intracellular esterase to liberate free DCFH. The relative H₂O₂ levels of the cells were measured by flow cytometry.

**Western blotting analysis**

After U2OS cells were incubated with Polyphyllin VI (0, 2.5, 5, and 7.5 μM) for 24 hrs, the total proteins were extracted and quantified using a BCA Protein Assay Kit (Beyotime, Shanghai, China). The proteins were separated on SDS-PAGE gels with 10% Tris-SDS gel, and wet-transferred to NC membrane (BioRad Laboratories, Hercules, CA, USA) and blotted with primary antibodies specific for PARP, Bax, Bcl-2, LC3B-I, LC3B-II, p62, Atg-7, Atg-5, Atg-12-Atg-5, Atg-12, Atg-3, JNK, phospho-JNK, p38, phospho-p38, ERK, p-ERK, caspase-3, caspase-7, GAPDH, etc. The proteins were probed with using anti-rabbit (1:5,000) or anti-mouse (1:5,000) secondary antibodies tagged with horseradish peroxidase. Bound immunocomplexes were detected using the ChemiDoc XRS (BioRad).

**Statistical analysis**

All experiments were performed at least three times. The results were analyzed using one-way ANOVA with Tukey multiple comparison. All data were expressed as the mean ± SD. P<0.05 was regarded as a statistically significant difference.

**Results**

**Polyphyllin VI inhibits the proliferation and induces cell cycle G2/M arrest in OS cells**

Figure 1A shows the chemical structure and molecular weight of the Polyphyllin VI. To investigate the inhibitory effects of Polyphyllin VI on growth, U2OS cells were exposed to various concentrations of Polyphyllin VI for 24 hrs and 48 hrs by CCK8 assay. The IC₅₀ values of Polyphyllin VI were 7.33±2.85 μM for 24 hrs and 6.02±1.42 μM for 48 hrs (Figure 1B). The results showed that Polyphyllin VI had a significant proliferation inhibitory effect on osteosarcoma cell lines in a dose- and time-dependent manner. In addition, in order to investigate the causal relationship between cell proliferation inhibition and cell cycle arrest by Polyphyllin VI, we performed the cell cycle distribution detection. As shown in Figure S1, G2/M phase cell number after 24-hr treatment by Polyphyllin VI was increased; on the contrary, the number of cells in S and G0/G1 phase was decreased. The above results indicated that Polyphyllin VI inhibited cell proliferation by trigging the G2/M-phase arrest.

**Polyphyllin VI induces mitochondria-mediated apoptosis**

To determine whether apoptosis is responsible for the cell growth inhibition, we used an Annexin V-APC/7-AAD doubled-labeled flow cytometry analysis to ascertain whether Polyphyllin VI treatment elicited apoptosis in
**Figure 1** (A) the structure and molecular weight of Polyphyllin VI. (B) U2OS cells were treated with Polyphyllin VI, and then the cell viability was determined by the CCK8 assay at the indicated time.

**Figure 2** Polyphyllin VI induces apoptosis in osteosarcomas cells. (A) U2OS cells were incubated with the indicated concentrations of Polyphyllin VI for 24 h and stained with Annexin V-APC and 7-AAD. (B) Flow cytometric measurement of Δψm in U2OS cells after treatment with Polyphyllin VI (2.5, 5, 7.5 μM) for 24 h. (C) Western blot analysis with antibodies specific for cleaved PARP, Bcl-2, and Bax was used as the loading control. Results are presented as the mean ± SD from three independent experiments. *P<0.05, **P<0.01, compared to control.
osteosarcoma cells. The results showed that the proportion of apoptosis was negligible for control cells, whereas the apoptotic rate of osteosarcomas cells increased in a dose-dependent manner after being treated with Polyphyllin VI for 24 hrs (Figure 2A).

Since loss of MMP was associated with the initiation and activation of the apoptotic process in cells, we investigated the effect of Polyphyllin VI on the integrity of the mitochondrial membrane (Figure 2B). The results revealed that MMP significantly decreased following Polyphyllin VI treatment, suggesting that Polyphyllin VI induces the apoptosis in osteosarcoma cells through disruption of their mitochondrial dysfunction.

We then investigated the levels of proteins related to apoptosis by Western blot. As shown in Figure 2C, the expression of antiapoptotic protein Bcl-2 in U2OS dramatically decreased in a dose-dependent manner, whereas the protein levels of proapoptotic proteins Bax and cleaved PARP gradually increased. Besides, Figure S2 showed an obvious decrease in the activation of Caspase-3 and Caspase-7 in U2OS cells. These results suggested that Polyphyllin VI induces apoptotic cell death in U2OS cells via the mitochondrial mediated apoptotic pathway.

Polyphyllin VI triggers autophagy in osteosarcomas cells
Autophagy is a form of programmed cell survival, which affects tumor initiation and progression. To further understand whether autophagy is involved in the Polyphyllin VI-induced cell growth inhibition, we measured the autophagic response to Polyphyllin VI in osteosarcomas cells. After exposure to Polyphyllin VI, the cells were stained with acridine orange and analyzed by flow cytometry (Figure 3A). The results showed that fluorescence intensity was significantly increased in U2OS cells after 24-hr exposure to Polyphyllin VI, which indicated Polyphyllin VI triggered autophagy in osteosarcomas cells. During autophagy, microtubule-associated protein 1 light chain 3 (LC3) is cleaved to LC3-I, which is further modified to another form, called LC3-II (Figure 3B). LC3-II is localized to the autophagosomal membrane, which is now identified as a marker for autophagosome and autophagy activity. P62 is an autophagy substrate that is also used as a marker of autophagy activity. The increased expression of IC3-II and p62 was observed in a dose-dependent manner up to 7.5 μM Polyphyllin VI (Figure 3B). These data were consistent with the results of other autophagy marker proteins, including Atg-5, Atg-7 and Atg-3 (Figure 3C).

Polyphyllin VI increased the expression of Atg-5 and Atg-7 in a dose-dependent manner, whereas the increasing trend of the expression of Atg-3 was terminated when cells were treated with 7.5 μM Polyphyllin VI. Interestingly, the expression of Atg-5 was increased while that of Atg-12 was decreased, which indicated an increased expression of Atg-5–Atg-12 conjugation in osteosarcomas cells after 24-hr exposure to Polyphyllin VI. In brief, our data suggest that Polyphyllin VI caused autophagy in osteosarcoma cells.

Intracellular H₂O₂ and JNK activation involve in polyphyllin VI-induced autophagy and apoptosis
We further investigated the molecular mechanisms underlying the ability of Polyphyllin VI to induce autophagy and apoptosis in osteosarcomas cells. Both autophagy and apoptosis can be triggered by common upstream signals, such as excessive intracellular H₂O₂, which was involved in the anticancer activity of many chemotherapy drugs. Thus, we examined the H₂O₂ activation of osteosarcomas cells after exposure to Polyphyllin VI. As expected, Polyphyllin VI resulted in a significant increase in intracellular H₂O₂ production, as shown by FACS detection assay (Figure 4A). Preincubation of cells with the ROS scavenger NAC completely blocked the increase in intracellular H₂O₂ production (Figure 4B), suggesting ROS species controlled by Polyphyllin VI-induced autophagy and apoptosis.

ROS has been shown as a strong signal for the regulator of mitogen-activated protein kinases (MAPK) family members including ERK, p38 and JNK, which may subsequently mediate antioxidant responses to induce autophagy and apoptosis (Figure 4C). To determine the role of these kinases in Polyphyllin VI-induced autophagy and apoptosis, we evaluated the levels of these proteins by Western blot. The results showed that levels of JNK phosphorylation was dramatically increased in Polyphyllin VI-treated osteosarcomas cells in a dose-dependent manner, whereas the levels of phospho-p38 and ERK decreased when cells were treated with 7.5 μM Polyphyllin VI. In brief, these results showed that JNK activation involved in Polyphyllin VI-induced autophagy and apoptosis in osteosarcoma cells.

Discussion
Polyphyllin VI is a main active saponin isolated from traditional medicinal plant Paris polyphyllin, which
shows antitumor activity against lung cancer A549 and NCI-H1299 cell lines,\(^\text{17}\) breast cancer cell MCF-7 and MDA-MB-231 cell lines,\(^\text{18}\) and hepatocellular carcinoma HepG2 cell line.\(^\text{19}\) The present study for the first time investigated the cell growth inhibition of Polyphyllin VI on human osteosarcoma cancer cells.

As shown in Figure 5, it is well displayed that the mechanism of Polyphyllin VI-induced apoptosis and autophagy in U2OS cells. Polyphyllin VI-induced cell death was associated with both apoptosis (loss of MMP, decreased levels of antiapoptotic protein Bcl-2 and increased levels of proapoptotic proteins Bax and cleaved PARP) and autophagy (the notable induction of autophagic markers such as conversion of LC3-I to LC3-II, accumulation of Atg-5, Atg-7 and incorporation of Atg-12–Atg-5 protein complex).

Apoptosis is one of the most important forms of cell death and crucially involved in tumor formation and treatment response. Most conventional chemotherapies and
Figure 4 Intracellular H2O2 and JNK activation involved in Polyphyllin VI-induced autophagy and apoptosis. (A) U2OS cells were incubated with the indicated concentrations of Polyphyllin VI for 24 h. The H2O2 level was measured by flow cytometry analysis. (B) Cells were treated with 7.5 µM Polyphyllin VI alone or in combination with the ROS scavenger NAC (10 mM), and then cells were subjected to flow cytometry analysis of intracellular H2O2. (C) Western blot analysis of p-JNK, JNK, p-ERK, ERK, p-p38 and p38 in cells treated with Polyphyllin VI at the indicated doses for 24 h. *P<0.05, **P<0.01, compared to control.
radiotherapy rely on activation of apoptosis pathways to provoke apoptotic cell death in cancer cells.\textsuperscript{21} Poly PARP is a nuclear DNA-binding protein, which plays an important in DNA repair.\textsuperscript{22} Its cleavage by caspase-3 is inactivated and is a hallmark of apoptosis.\textsuperscript{23} Our results showed that Polyphyllin VI induced apoptosis by observation of PARP cleavage in osteosarcomas cells. This finding is in agreement with flow cytometry analysis demonstrating that the number of apoptotic cells is increased in Polyphyllin VI-treated cells. Analysis of other proteins in apoptosis pathways, such as Bcl-2 and Bax, also confirmed the occurrence of apoptosis. Downregulation of Bcl-2 is companied by overexpression of Bax in osteosarcoma cell with treatment of Polyphyllin VI. As proapoptotic Bax activation can lead to mitochondrial membrane permeabilization, our results suggest that Polyphyllin VI initiates apoptotic cascade through the mitochondrial pathway. The results are consistent with finding that Polyphyllin VI disrupted the integrity of the mitochondrial membrane in osteosarcomas cells. These data indicated that Polyphyllin VI regulated apoptosis by the balance of pro- and antiapoptotic proteins in human osteosarcomas U2OS cell line.

Autophagy is an essential process by which cellular materials are degraded to provide energy and macromolecular precursors, as well as avoid cytotoxic product accumulation.\textsuperscript{24,25} Thus, autophagy critically regulates cellular homeostasis with a balance between cell survival and cell death. In tumor cells, some chemotherapies and

\begin{figure}
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\caption{The mechanism of Polyphyllin VI-induced apoptosis and autophagy in U2OS cells.}
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Radiotherapies induce autophagy, which results in the emission of danger signals and the initiation of a therapeutically relevant anticancer immune response. Autophagy involves a sequential recruitment of many proteins that orchestrate the different steps of autophagy. The ability of Polyphyllin VI to trigger autophagy was confirmed by the enhanced conversion of LC3B-I to LC3B-II and increased expression of p62, Atg-3, Atg-5 and Atg-7. In addition, Polyphyllin VI decreased the expression of Atg12 and increased expression of Atg12-Atg5, which is an important ubiquitin-like conjugate. Thus, our results showed that Polyphyllin VI-induced autophagy might be responsible for the inhibition of cell growth in human osteosarcomas U2OS cell line.

A lot of studies have demonstrated a very complex association between apoptosis and autophagy, which may promote each other, inhibit each other, or unilaterally promote or inhibit. Apoptosis-related caspase family proteins interact with autophagy-related proteins, and some studies have shown that caspase-3 inhibits autophagy to promote apoptosis by cleavage of Beclin-1. Bcl-2 family proteins play a key dual regulatory role in apoptosis and autophagy. Studies have shown that the antiapoptotic proteins Bcl-2 and Bcl-xL do not directly affect autophagy, but indirectly through inhibition of Bax and Bak. Autophagosome formation is dependent on the coherent attachment of a series of Atg proteins during protein ubiquitination. Atg5 and Atg12 are known as the “core” of autophagy and are required for autophagosome formation. They have also been found to be involved in the regulation of apoptosis. Atg5 can be cleaved by Caplains, causing the Atg5 N-terminal fragment to translocate to the mitochondria in an unknown mechanism, binding to the antiapoptotic protein Bcl-xL, promoting mitochondrial cytochrome C release and inducing apoptosis. Atg12 was developed to have a proapoptotic function due to its BH3-like domain that binds to Bcl-2 and Mcl-1. According to the relevant references, the upregulated expression of LC3B-II and Atg5-Atg12 showed that cell death caused by autophagic pathway when apoptosis was blocked. In contrast, when suppression of autophagy diminished, the increased expression of Bax and decreased expression of Bcl-2 indicated that apoptosis is promoted to lead to cell death. Further experimental research was needed to study about the molecular mechanism between apoptosis and autophagy.

ROS has been described as the chemical species that are produced by incomplete redox reaction and includes the superoxide anion (O2–), hydrogen peroxide (H2O2) and the hydroxyl radical (HO•). In this study, we found that Polyphyllin VI increased intracellular H2O2 production, which could be blocked by ROS scavenger NAC. In apoptosis-inducing processes, ROS production is due to the electron transport chain in mitochondria and to the MMP decrease, leading to the mitochondrial translocation of Bax, Bcl-2 and Cytochrome C. Besides, the autophagy-inducing activity processes are proved to be related to the accumulation of intracellular ROS, which have been functioning as signaling molecules in the autophagic pathway through reversibly oxidizing crucial signaling components. Thus, our results suggested that H2O2 was involved in Polyphyllin VI-induced autophagy and apoptosis.

ROS has been shown as an inducer or mediator for the activation of MAPK family members, which play an important role in autophagy and apoptosis. Exposure of osteosarcomas cells to Polyphyllin VI led to strong activation of JNK (phosphorylation), suggesting that Polyphyllin VI-induced cell growth inhibition might be JNK independent, as phospho-p38 and ERK had no significant enhancement. JNK activation has been implicated in apoptosis and autophagy in response to a variety of stress signals. JNK could mediate antiapoptotic proteins Bcl-2/Bcl-xL phosphorylation to change the MMP; in this regard, Polyphyllin VI-induced disruption of mitochondrial dysfunction might be associated with JNK-mediated apoptotic response. JNK activation also contributed to autophagic induction, which is a key part for upregulation of LC3. Consistently, we also found that Polyphyllin VI-induced activation of JNK pathway was accompanied by upregulation of LC3B-II in human osteosarcomas U2OS cell line. ROS is the upstream pathway of JNK and is probably the core of the effect of Polyphyllin VI, which mediates G2/M arrest, apoptosis and autophagy by activating ROS/JNK pathway.

In summary, our study demonstrated that Polyphyllin VI could inhibit cell growth of human osteosarcomas cancer cell line by induction of autophagy and apoptosis, which was associated with accumulation of intracellular H2O2 and activation of JNK pathway. This study revealed antitumor effects of Polyphyllin VI and its potential for the treatment of osteosarcomas.

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Disclosure
The authors report no conflicts of interest in this work.

References


Supplementary materials

**Figure S1** Polyphyllin VI induces G2/M cell cycle arrest. (A) The alterations in cell cycle distribution of U2OS cells were determined by flow cytometry after treatment with control and Polyphyllin VI (2.5, 5, and 7.5 μM) for 24 h. (B) Ratios of cells in different cell cycle phases in U2OS cells. The percentage of cells in each phase is shown as mean ± SD from three independent experiments. *P<0.05 and **P<0.01, significantly different compared with control.

**Figure S2** U2OS cells were incubated with control and Polyphyllin VI (2.5, 5, and 7.5 μM) for 24 h. Cell lysates were prepared and analyzed by Western blotting for Caspase-3 and Caspase-7. Results are presented as the mean ± SD from three independent experiments. *P<0.05 and **P<0.01, significantly different compared with control.