Original Paper

Deoxyelephantopin Induces Reactive Oxygen Species-Mediated Apoptosis and Autophagy in Human Osteosarcoma Cells

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Key Words

Deoxyelephantopin • Apoptosis • Autophagy • ROS • Osteosarcoma • Proliferation

Abstract

Background/Aims: Osteosarcoma is the predominant form of primary bone malignancy. Although the combinational application of neoadjuvant chemotherapy and surgical resection significantly increases the survival rate, the therapeutic outcome remains unsatisfactory. Deoxyelephantopin (DET), an active ingredient of *Elephantopus scaber*, has been reported to have an anti-tumor effect in recent publications. This study aimed to investigate whether DET has antineoplastic effects on osteosarcoma cells and its underlying mechanism. **Methods:** Cell viability and morphological changes were assessed by MTT and Live/dead assays. Cell apoptosis, reactive oxygen species (ROS) and mitochondrial membrane potential were detected utilizing Annexin V-FITC/PI double staining, DCFH-DA and JC-1 probes, respectively. Autophagy was detected by mRFP-GFP-LC3 adenovirus transfection and western blot. **Results:** DET dose-dependently reduced the viability of osteosarcoma cells following the increase in intracellular ROS levels. Pretreatment with N-acetylcysteine (NAC) reversed this effect. Furthermore, DET induced mitochondrial apoptosis. Depolarized cells were increased, and apoptosis-related proteins, such as Bax, Bcl-2, cleaved caspase-9, cleaved caspase-3 and cleaved poly ADP-ribose polymerase, were activated. Additionally, we found that DET could induce autophagy in osteosarcoma cells, but autophagy inhibition did not affect the decrease in cell viability. **Conclusion:** DET induced apoptosis in osteosarcoma cells through ROS generation, mitochondrial dysfunction and caspase activation; in addition, autophagy was involved in the effects of DET on osteosarcoma cells.

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Introduction

Osteosarcoma is the major form of primary bone malignancy and predominantly occurs in children and adolescents [1]. This disease is characterized by the generation of immature osteoid matrix through mesenchymal stem cell-derived tumor cells, which primarily occurs

in the distal end of long bones, such as the femur and humerus [2]. The current treatments for osteosarcoma include surgery, chemotherapy and radiotherapy. A combinational application of neoadjuvant chemotherapy and consequent surgical resection significantly increased the five-year survival rate of patients without metastasis to approximately 60%-70% [3]. However, the outcome is disappointing in metastatic patients, with a five-year survival rate of only 5%-20% [4, 5]. Additionally, severe side effects and drug resistance are common. Thus, novel agents and treatments with higher efficacy and fewer side effects are urgently required.

Recent studies have shed light on the naturally occurring bioactive compounds, sesquiterpene lactones, which were shown to have antineoplastic and anti-inflammatory effects in multiple diseases [6, 7]. Deoxyelephantopin (DET), derived from *Elephantopus scaber*, is one of them. An increasing number of reports have revealed the cytotoxic effect of DET on cancer cells, such as hepatocellular carcinoma, colorectal carcinoma, cervical carcinoma and lung cancer cells [8-10]. Nevertheless, the effects of DET on osteosarcoma have not been investigated, and the underlying molecular mechanism of the inhibitory effect on tumor cells is still unclear.

In the present study, different osteosarcoma cell lines were used to determine the effects of DET and explore the possible mechanisms involved. We found that DET induced apoptosis in osteosarcoma cell lines through reactive oxygen species (ROS) generation, mitochondrial dysfunction and caspase activation; furthermore, autophagy was induced in a ROS-dependent manner. Based on these results, we recommend DET as a potential effective agent for osteosarcoma treatment.

Materials and Methods

Reagents and antibodies

DET (Fig. 1) was purchased from BioBioPha Co., Ltd. (Kunming, China) and its purity was >97% tested by high-performance liquid chromatography. Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone (Logan, UT, USA). McCoy's 5A Medium was obtained from Boster (Wuhan, China). Fetal bovine serum (FBS) was purchased from Roya Bio-Technology Co., Ltd. (Lanzhou, China). Dimethyl sulfoxide (DMSO) and [3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) were obtained from Amresco (Solon, OH, USA). The LIVE/DEAD® Viability/Cytotoxicity Assay Kit was purchased from Invitrogen (Eugene, OR, USA). The Annexin V-Fluorescein Isothiocyanate (FITC) Apoptosis Detection Kit was acquired from Becton Dickinson (Franklin Lakes, NJ, USA). The Reactive Oxygen Species Assay Kit, the Mitochondrial Membrane Potential Assay Kit with JC-1 and the inhibitor N-acetylcysteine (NAC) were obtained from Beyotime Biotechnology (Nanjing, China). The z-VAD-fmk and chloroquine (CQ) were purchased from Selleckchem (Houston, USA). The mRFP-GFP-LC3 adenovirus was purchased from HanBio Technology Co. Ltd. (Shanghai, China). Anti-Bcl-2, anti-Bax, anti-cleaved caspase-9, anti-cleaved caspase-3, anti-cleaved poly ADP-ribose polymerase (PARP), anti-LC3II and anti-GAPDH antibodies were obtained from Protein tech (Wuhan, China).

Cell culture

Human osteosarcoma cell lines MG-63, U2OS and Saos2 were obtained from QiaoDu Biotechnology Co., Ltd (Shanghai, China). MG-63 was cultured in DMEM medium, while U2OS and Saos2 were cultured in McCoy's 5A Medium. The medium was supplemented with 10% FBS, 100 U/ml penicillin and 1% streptomycin. The cells were maintained at 37°C in a humidified atmosphere containing 5 % CO₂ and kept in exponential growth phase during experiments.

Cell viability assay

The effects of DET on osteosarcoma cells were analysed by MTT assays. Briefly, the cells were seeded in 96-well plates at a density of 5×10^3 cells per well. After 24 h incubation, cells were treated with various concentrations of DET for 24 h or 48 h. Following the treatment, 10 µl MTT (5 mg/ml) was added to each well, and 4 h later, the supernatant was replaced with 100 µl DMSO to dissolve the formazan crystals.

Absorbance was tested at a wavelength of 570 nm using a fluorescence spectrophotometer (BioTek).

Observation of morphological changes

MG-63 and U2OS cells were exposed to different concentrations of DET for 24 h. Cell morphological changes were observed by microscopy (Olympus).

Live/dead assay

The assay was performed using a LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen). MG-63 and U2OS cells were treated with different concentrations of DET for 24 h. Then, the cells were washed twice with phosphate buffered saline (PBS) and were incubated for another 30 min at 37°C; the cells were protected from light with DMEM solution containing 2.5 µM calcein AM and 4 µM ethidium homodimer-1 (EthD-1). Finally, the results were observed using fluorescence microscopy (Olympus).

Cell apoptosis analysis

Annexin V-FITC/PI double staining was used to confirm the apoptotic effect of DET on MG-63 and U2OS cells. Briefly, cells were treated with different concentrations of DET for various time periods. After the treatment, the cells were harvested, washed twice with cold PBS, resuspended with 1X binding buffer and then stained with 5 µl Annexin V-FITC and 5 µl PI following the manufacturer's instruction. Finally, the samples were analyzed by a flow cytometer (Becton Dickinson) using BD FACSDiva™ software.

Measurement of intracellular ROS levels

The intracellular ROS levels were measured using a Reactive Oxygen Species Assay Kit (Beyotime Biotechnology, China); 2', 7'-dichlorofluorescein-diacetate (DCFH-DA), which is easily oxidized to fluorescent dichlorofluorescein (DCF) by intracellular ROS, is its principal component, and therefore, the ROS levels were quantified. Briefly, the cells were seeded in 96-well plates as described above and exposed to various concentrations of DET for different time intervals. Following the treatment, the cells were incubated with DCFH-DA for 20min at 37°C and then observed using fluorescence microscopy (Olympus) and measured at 488 nm excitation and 525 nm emission by a fluorescence spectrophotometer (BioTek).

Measurement of mitochondrial membrane potential (MMP) with fluorescent JC-1

MMP changes were confirmed by the fluorescent probe JC-1. Normally, JC-1 is agglomerated into J-aggregates, which emit red fluorescence, in the mitochondrial matrix. When the MMP decreases, the J-aggregates are decomposed into a monomer form, and the emitted fluorescence changes from red to green. Thus, the percentage of depolarized cells can be measured through statistical analysis of the different fluorescence signals. Briefly, after treatment with different concentrations of DET, cells were collected, resuspended using DMEM and then incubated with JC-1 at room temperature for 20 min. Finally the samples were tested with a flow cytometer (Becton Dickinson).

Evaluation of autophagic flux

mRFP-GFP-LC3 adenovirus transfection was used to monitor the autophagic flux through marking and tracking LC3. Briefly, MG-63 cells were transfected with mRFP-GFP-LC3 adenovirus for 24 h following the manufacturer's instruction and then treated with DET for another 24 h. Finally, the cells were observed and images were acquired using confocal fluorescence microscopy (Nikon).

Immunoblotting

After treatment with different concentrations of DET, MG-63 and U2OS cells were lysed with RIPA buffer containing 1% phenylmethylsulfonyl fluoride and 10% phosphatase inhibitor. Cells were centrifuged at 13,500 rpm at 4°C for 15 min. The supernatants were collected, and protein concentrations were determined by BCA Protein Assay Kit (Beyotime). The proteins were separated by 10% to 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The

Fig. 1. The chemical structure of DET.

membranes were blocked using 5% nonfat milk for 1 h and then incubated with primary antibodies at 4°C overnight and fluorescence-conjugated secondary antibodies for 1 h at 25°C. Finally, the protein bands were acquired by the Odyssey infrared imaging system (LI-COR) and analyzed using Odyssey v3.0 software.

Statistical analysis

The data are expressed as the mean $\pm$ SEM. Statistical analysis was conducted using GraphPad Prism version 5.0 (GraphPad Software). Differences were assessed by one-way ANOVA followed by Tukey's multiple comparison test. $P < 0.05$ was considered statistically significant.

Results

DET inhibits the proliferation of MG-63, U2OS and Saos2 cancer cells

The cytotoxicity of DET on different human osteosarcoma cell lines was confirmed by MTT assays. As shown in Fig. 2A and 2B, cell viability was reduced in a dose-dependent manner after exposure to various concentrations of DET (0, 4, 8, 16 and 32 μ M) for 24 h and 48 h. The inhibitory effect was further determined using optical photography and the LIVE/DEAD® Viability/Cytotoxicity Assay. As shown in Fig. 2C and 2D, DET changed MG-63 and U2OS cells' morphology from a long spindle to round shape and significantly reduced the density of live cells (green) and increased the number of dead cells (red) with increasing concentrations.

DET increases ROS generation and disturbs mitochondrial function in MG-63 and U2OS cells

Intracellular ROS, which are predominantly derived from the mitochondria, play an important role in determining the fate of cancer cells [11]. Since DET was reported to induce ROS generation in HepG2 and TS/A cells [10, 12], the ROS levels of MG-63 and U2OS

Fig. 2. DET reduced the viability of osteosarcoma cells. (A and B), DET dose-dependently reduced the viability of MG-63, U2OS and Saos2 cancer cells after 24 h and 48 h treatment ($n = 12$). $*P < 0.05$, $**P < 0.01$ versus CTL. CTL, control. (C and D), DET changed MG-63 and U2OS cells' morphology from a long spindle to round shape and significantly reduced the density of live cells (green) and increased the number of dead cells (red) following the ascending concentrations. $*P < 0.05$, $**P < 0.01$ versus CTL. CTL, control.

Fig. 3. DET increased ROS generation and disturbed mitochondrial function in MG-63 and U2OS cells. (A and B), DET time-dependently increased intracellular ROS levels at concentrations of 16 μ M for MG-63 and 32 μ M for U2OS cancer cells ($n = 6$). * $P < 0.05$, ** $P < 0.01$. (C and D), DET dose-dependently increased the number of depolarized cancer cells. The decrease in MMP is a potent indicator of mitochondrial dysfunction. ($n = 3$), * $P < 0.05$, ** $P < 0.01$ versus CTL. CTL, control.

Fig. 4. DET-induced apoptosis was confirmed by Annexin V-FITC/PI double staining. The detected cells were divided into 4 types using flow cytometer. Annexin V-FITC (-)/PI (-) cells were alive, Annexin V-FITC (+)/PI (-) cells were considered in the early stage of apoptosis, while Annexin V-FITC (+)/PI (+) cells were in the late stage. Annexin V-FITC (-)/PI (+) cells were necrotic. The apoptosis rate was calculated and analyzed using early apoptotic and late apoptotic cells ($n = 3$). * $P < 0.05$, ** $P < 0.01$ versus CTL. CTL, control.

cells were measured after DET exposure. As shown in Fig. 3A and 3B, ROS generation was significantly enhanced in a time-dependent manner after treatment with 16 μ M DET for MG-63 and 32 μ M DET for U2OS cells, respectively. To further confirm the effect of ROS, we used a ROS scavenger, NAC, in the LIVE/DEAD® Viability/Cytotoxicity Assay. As shown in Fig. 6A, pretreatment with NAC (1 mM) for 1 h abolished the cytotoxic effect of DET (8 μ M) in MG-63 cells. Similar results were also obtained in MTT assays (Fig. 6B). These results indicate that DET-induced cell death is ROS-dependent in osteosarcoma cells.

Previous studies showed that ROS generation was closely related to MMP changes [13, 14]. Thus, we measured the MMP levels in MG-63 and U2OS cells using fluorescent JC-1. As shown in Fig. 3C and 3D, the number of depolarized cells was increased massively after exposure to various concentrations of DET, which indicated that mitochondrial function was dose-dependently disturbed by DET treatment.

DET induces apoptosis in MG-63 and U2OS cells

Since ROS overexpression and MMP disruption are two important steps in mitochondrial apoptosis [15, 16], we used Annexin V-FITC/PI double staining to confirm the existence of apoptosis in MG-63 and U2OS cells. As shown in Fig. 4, apoptotic cells, including early apoptotic (Annexin V+/PI -) and late apoptotic (Annexin V+/PI +) cells, were dose-

Fig. 5. DET activated apoptosis-related proteins. (A and B), DET dose-dependently activated apoptosis-related proteins in MG-63 and U2OS cells. Quantification of Western blot data for Bax activity (C and D), Bcl-2 activity (E and F), cleaved caspase-9 (G and H), cleaved caspase-3 (I and J), cleaved PARP (K and L). (n = 4), * $P < 0.05$, ** $P < 0.01$ versus CTL. CTL, control.

dependently increased after DET treatment in MG-63 and U2OS cells. Consistent with the above results, the apoptosis-related proteins were also altered in MG-63 and U2OS cells (Fig. 5A and 5B), including a decrease in the anti-apoptotic protein Bcl-2 (Fig. 5E and 5F) and increases in the pro-apoptotic proteins Bax (Fig. 5C and 5D), cleaved caspase-9 (Fig. 5G and 5H), cleaved caspase-3 (Fig. 5I and 5J) and cleaved PARP (Fig. 5K and 5L). To further elucidate the DET-induced apoptosis, we investigated the effect of z-VAD-fmk, a pan-caspase inhibitor, using the LIVE/DEAD® Viability/Cytotoxicity Assay and MTT assay. As shown in Fig. 6A and 6B, z-VAD-fmk treatment reduced the percentage of dead cells and attenuated the DET-induced cell viability decrease. Taken together, these results indicate that DET-induced cell death in osteosarcoma cells is apoptosis-dependent.

DET induces autophagy in MG-63 and U2OS cells

The above results demonstrated the participation of apoptosis in DET-induced cell death; however, it was unclear whether autophagy, an important process that maintains cellular homeostasis, was involved. Hence, the protein levels of LC3II were measured. As shown in Fig. 7, LC3II levels were upregulated due to DET treatment in MG-63 cells (Fig. 7A, 7B) and U2OS cells (Fig. 7C, 7D). However, LC3II accumulation could result from the increased upstream autophagosome formation or the damage from downstream autophagosome-lysosome fusion [17]. If autophagic flux is occurring, the levels of LC3II will be higher under conditions of lysosomal dysfunction [18]. Therefore, chloroquine, which could prevent lysosomal degradation by neutralizing the lysosomal pH, was used in further analysis of LC3II levels in MG-63 cells. As shown in Fig. 7E and 7F, LC3II levels were increased due to the addition of chloroquine in the DET-treated group. Moreover, mRFP-GFP-LC3 adenovirus transfection was also performed to detect autophagic flux in the present study. As shown in Fig. 7G, the levels of autophagosomes (yellow dots in merged images) and autophagosome-lysosomes (red dots in merged images) were significantly increased after DET exposure. The above results indicated that autophagy was activated due to DET treatment. Additionally, the DET-induced LC3II increase could be partly abrogated by the ROS scavenger NAC (Fig. 7E, 7F), which revealed that the DET-induced autophagy was ROS-dependent. Since autophagy can

Fig. 6. NAC and z-VAD-fmk attenuated DET-induced MG-63 cell apoptosis. (A), NAC and z-VAD-fmk treatment alleviated DET-induced cell death in MG-63 cells, shown using the LIVE/DEAD staining assay. Cells were pretreated with NAC (1 mM) or z-VAD-fmk (100 μ M) for 1 h, and then, DET (8 μ M) was added until 24 h. $**P < 0.01$ versus CTL. $##P < 0.01$ versus DET (8 μ M). CTL, control. (B), NAC and z-VAD-fmk treatment attenuated the decrease in MG-63 cell viability. Cells were pretreated with NAC (1 mM) or z-VAD-fmk (100 μ M) for 1 h, and then both DET (8 μ M) and NAC (1 mM) or z-VAD-fmk (100 μ M) were added for 24 h ($n = 12$). $*P < 0.05$, $**P < 0.01$ versus CTL. $#P < 0.05$, $##P < 0.01$ versus DET (8 μ M). CTL, control.

Fig. 7. DET induced autophagy in osteosarcoma cells. (A, B and C, D), LC3II protein expression was dose-dependently upregulated after DET treatment in MG-63 and U2OS cells ($n=4$). $**P < 0.01$ versus CTL. CTL, control. (E and F) NAC and chloroquine treatment alleviated and enhanced DET-induced LC3II upregulation, respectively, in MG-63 cells. Cells were pretreated with NAC (1 mM) or chloroquine (10 μ M) for 1 h, and then, DET (8 μ M) was added until 24 h ($n=3$). $**P < 0.01$ versus CTL. $#P < 0.05$ versus DET (8 μ M). CTL, control. CQ, chloroquine. (G) DET induced autophagic flux in MG-63 cells. After transfection with mRFP-GFP-LC3 adenovirus, MG-63 cells were exposed to DET (8 μ M) for 24 h, and then, the cells were observed with confocal fluorescence microscopy. (H) Chloroquine treatment did not alter the decrease in MG-63 cell viability. Cells were pretreated with chloroquine (10 μ M) for 1 h and then treated with both DET (8 μ M) and chloroquine (10 μ M) for 24 h ($n = 10$). $**P < 0.01$ versus CTL. ns, no significance versus DET (8 μ M). CTL, control. CQ, chloroquine.

play both positive and negative roles in tumor cell growth [19, 20], the autophagy inhibitor, chloroquine, was also used in MTT assays to elucidate its role. Nevertheless, autophagy inhibition did not alter the decrease in cell viability (Fig. 7H). These results indicate that autophagy is present but is not the major mechanism in DET's effect on osteosarcoma cells.

Discussion

Osteosarcoma is a highly malignant bone tumor and is characterized by early metastasis and poor prognosis [21]. Combined with consequent surgical resection, neoadjuvant chemotherapy has become the standard treatment [4]. However, the accompanying drug resistance and side effects substantially weaken the therapeutic effectiveness [22]. Hence, novel agents that target malignant behavior of osteosarcoma cells are urgently needed. Recently, accumulating studies revealed the anti-tumor effect of DET on multiple cancer cells. However, the effects of DET on osteosarcoma cells have never been investigated, and the underlying mechanism of the anti-tumor effect is still unclear. In the present study, we found that DET induces apoptosis in osteosarcoma cells via ROS generation, mitochondrial dysfunction and caspase activation; meanwhile, autophagy is activated in a ROS-dependent manner.

Previous studies have found that cancer cells contain higher levels of ROS than those of normal cells as a result of their hypermetabolism, which is closely associated with cell proliferation, differentiation and cell death [23, 24]. Due to the higher levels of ROS, cancer cells show increased injuries by oxidative stress, and this characteristic can be utilized as a drug target for cancer therapy. Our results showed that DET increased ROS levels of osteosarcoma cells in a time-dependent manner. Mitochondria are the major source of ROS generation through respiratory chain leakage [25]. The elevated ROS expression could damage mtDNA followed by transcriptional impairment of relevant mtRNAs involved in respiratory chain, which further enhances the ROS generation [26]. This vicious cycle ends with respiratory chain disruption and dysfunctional ATP synthesis, which results in the loss of MMP [23]. Additionally, ROS could more directly damage MMP by provoking mitochondrial membrane hyperpolarisation [27]. Consistent with these findings, we demonstrated that DET indeed decreased MMP levels and dose-dependently increased the percentage of depolarized cancer cells, indicating that mitochondrial function was injured due to DET treatment.

Apoptosis is a normal physiologic process, which plays an important role in maintenance of cellular homeostasis by eliminating malicious cells [28]. Many anti-tumor drugs exhibit cytotoxic effects on cancer cells through induction of apoptosis [29, 30]. Recent studies have shown that ROS overexpression and MMP disruption are two early steps involved in mitochondria-mediated apoptosis [15, 16]. Oxidative stress could activate pro-apoptotic proteins, such as Bax, and suppress the function of anti-apoptotic factors from Bcl-2 family [23]. In the present study, our results also verified the above findings with the discovery that DET treatment increased Bax expression and reduced the level of Bcl-2. The activated Bax, together with MMP disruption, facilitates the opening of the mitochondrial permeability transition pore, which leads to the release of cytochrome C, a vital pro-apoptotic factor, into the cytoplasm [31]. Once released into the cytoplasm, cytochrome C, combined with apoptosis activating factor-1 (Apaf-1) and procaspase-9, in turn activates caspase cascades. Cleaved caspase-3 acts as the cardinal executioner to activate downstream effector proteins, such as PARP [26, 31]. Our results showed that caspase-9 and 3 and PARP were activated due to DET treatment, and apoptosis inhibition could partly reverse the cell viability decrease, which confirmed apoptosis to be the major mechanism by which DET induced cell death.

Similar to apoptosis, autophagy is another vital process associated with cellular homeostasis via elimination of harmful cellular components [32], but autophagy exerts more complicated responses under different circumstances [33]. Autophagy plays a protective role when cells encounter environmental stresses, such as starvation or pathogen infection [34, 35]. This type of autophagy benefits cell survival, but excessive autophagy can result in autophagic cell death [36]. Accordingly, induction of excessive autophagy has been regarded as a new therapeutic strategy [37]. Since apoptosis has been identified as a crucial mechanism in DET-induced cell death, we further investigated whether autophagy was involved. In the present study, we found that DET activated autophagic responses, and the autophagic responses could be inhibited by NAC and chloroquine. Nevertheless, pretreatment with chloroquine did not change the cell viability decrease. Based on these results, we conclude

that although autophagy was induced by DET in on osteosarcoma cells, it did not play a major role. Moreover, apoptosis is primarily responsible for the induction of cell death as confirmed by our results. The specific effects of autophagy should be further investigated.

Conclusions

In the present study, our results demonstrated that DET induced apoptosis in osteosarcoma cells through ROS generation, mitochondrial dysfunction and caspase activation. In addition, autophagy was activated by DET in osteosarcoma cells; however, more studies should be conducted to elucidate its specific function. Taken together, our findings provide a foundation for DET to become a potential anti-tumor agent in osteosarcoma treatment.

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Disclosure Statement

The authors declare no conflict of interest in relation to this article.

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