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ORIGINAL ARTICLE

β-Aescin shows potent antiproliferative activity in osteosarcoma cells by inducing autophagy, ROS generation and mitochondrial membrane potential loss

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Summary

Purpose: *Osteosarcoma is one of the frequent bone tumor affecting mainly children and is associated with considerable mortality. The limited availability of anticancer drugs and less efficacious treatment options have led to poor survival rates of patients with osteosarcoma. Therefore, there is need to look for more viable treatment options and against this backdrop, natural products may prove handy. Therefore the aim of the present study was to evaluate the anticancer activity of a natural product of plant origin, β-aescin, against U2OS human osteosarcoma cells.*

Methods: *U205 human osteosarcoma cell line was used in this study. Antiproliferative activity was determined by MTT assay. Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) were evaluated by flow cytometry. Autophagy was detected by monodansylcadaverine (MDC) staining and immunofluorescence. Protein expression was examined by western blotting.*

Results: *The results indicated that β-aescin showed significant anticancer activity against U2OS human osteosarcoma cells and exhibited an IC_{50} of 40 μM. β-aescin treatment caused significant increase in ROS and decrease in the MMP. The anticancer effect of β-aescin was found to be due mainly to autophagic cell death as evidenced from MDC staining and immunofluorescence. Moreover, β-aescin caused significant increase in the expression levels of LC3-II protein in U2OS osteosarcoma cells in a time and dose-dependent manner.*

Conclusion: *Taken together we propose that β-aescin may prove a lead molecule in the management of osteosarcoma and deserves further research efforts.*

Key words: *antiproliferative activity, autophagy, β-Aescin, osteosarcoma, ROS*

Introduction

Osteosarcoma is the one of the frequently occurring primary bone malignancies. It is mainly detected among children and young adults [1,2]. Osteosarcoma involves the malignant growth of mesenchymal cells which exhibit the ability to produce osteoid or immature bones [3]. Osteosarcoma is accompanied by prompt metastasis and very high morbidity and mortality [4-6]. It has been reported that despite the availability of chemo- and radiotherapy, as well as surgery, the survival rate of osteosarcoma is still very poor [7]. Additionally, surgical interventions are not able to delay the metastasis and chemotherapy is associated with the development of drug resistance and even tremendous side effects [7]. Therefore, the immediate need is to look for efficient and more viable options for the management of osteosarcoma. In this regard natural products are considered good options and about more than half of the currently used anticancer agents are of natural

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origin [8]. Consistent with this, the present study was designed to evaluate the anticancer effects of β-aescin (Figure 1) against osteosarcoma cells *in vitro*. Aescin is commercially isolated from *Aesculus hippocastanum*, commonly known as horse chestnut [9]. A mixture of saponins is found the seeds of this plant and two crystalline substances called aescin prosapogenin are found in this saponin mixture [9]. Moreover, aescin is a natural mixture of α- and β-aescin [9]. There is concrete evidence that have indicated that β-aescin is one of the main constituents of several pharmaceutical products [9-11]. Moreover, β-aescin has been reported to exhibit anticancer effects against a number of cancer types [11,12]. In the current study we observed that β-aescin exhibits significant and dose-dependent antiproliferative effects on U2OS human osteosarcoma cells. The results indicated that β-aescin caused significant increase in ROS which was associated with concomitant decrease in the MMP. Furthermore, the antiproliferative effects of β-aescin were found to be due to the induction of autophagy in human U2OS osteosarcoma cells. Given these results, we propose that β-aescin may prove a lead molecule in the management of osteosarcoma and therefore deserves further research efforts.

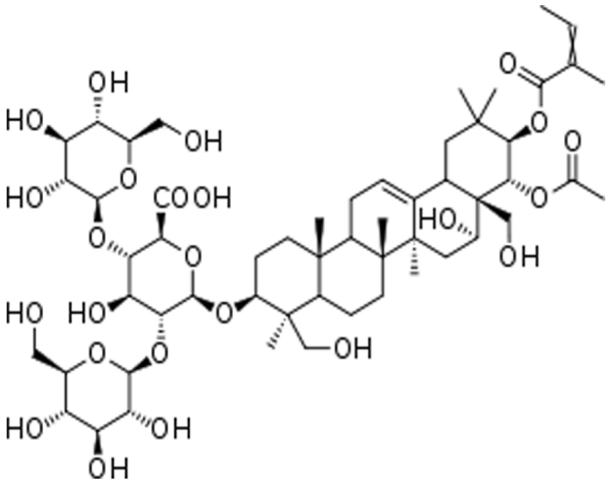

Figure 1. Chemical structure of β-Aescin.

Methods

Chemicals, reagents and cell culture

All chemicals and reagents used in the present study were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human osteosarcoma cell line U2OS was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100U/mL penicillin and 100μg/mL streptomycin and maintained in a humidified atmosphere containing 5% CO_2.

Antiproliferative activity by MTT assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] assay was used to determine the antiproliferative activity of β-aescin against the human osteosarcoma UOS2 cells. The UOS2 cells in 100 μL culture medium were seeded in a 96-well plate at a density of 3×10^3 cells/mL and kept at 37°C in 5% CO_2 for 24 hrs. After 24 hrs, an additional 100 μL of complete medium with either no additions or different concentrations (0-100 μM) of β-aescin were added and the cells were incubated for 24 hrs. This was followed by the addition of 20 μL of MTT solution (5mg/mL) and the cells were incubated for another 24 hrs. Afterward, the medium was removed and 150 μL DMSO were added. The optical density (OD) of each well was measured at 490 nm using a Tunable Mi-85 croplate Reader (EL-x 800, BioTek Instruments, USA).

Evaluation of ROS and MMP

U2OS cells were platted at a density of 2×10^5 cells/well in a 6-well plate and kept for 24 hrs and treated with 0, 20, 40 and 80 μM β-aescin for 24 hrs at 37°C in 5% CO_2 and 95% air. Afterward, cells from all samples were collected, washed twice by phosphate buffered saline (PBS) and re-suspended in 500 μl of DCFH-DA (10 μM) for ROS estimation and DiOC6 (1 μmol/l) for MMP at 37°C in the dark for 30 min. The samples were then examined instantly using flow cytometer.

MDC staining

To investigate whether β-aescin could induce autophagy in U20S human osteosarcoma cells MDC staining and immunofluorescence for LC3-II protein was carried out by fluorescence microscopy. Briefly, the β-aescin-treated cells were stained with MDC (0.05 mM) for 20 min in the dark. After MDC treatment U2OS cells were washed twice with PBS and instantly examined under inverted fluorescence microscope.

Immunofluorescence

The human U2OS osteosarcoma cells were first treated with 0, 20, 40 and 80 μM β-aescin and then fixed with paraformaldehyde (4%) followed by blocking with 2% bovine serum albumin for 30 min Thereafter the cells were treated at 4°C overnight with primary antibody for LC3-II. Afterward, the treated U2OS cells were washed twice with PBS and were then incubated at room temperature with fluo-conjugated secondary antibody for about one hour. Finally, the U2OS cells were stained with DAPI and studied by inverted fluorescence microscopy.

Western blot analysis

The β-aescin-treated cells were washed with cold PBS and then lysed to prepare the total protein extract. Equal amounts of the protein extract from each sample was run and separated by SDS-PAGE, followed by transfer to a PVDF membrane. After treatment with PBS containing Tween-20 (0.1%) and nonfat milk (5%) for one hour, the transferred membrane was treated

with a specific primary antibody and then subjected to treatment with the corresponding secondary antibody. The specific protein bands were visualized by an ECL Advanced Western Blot detection Kit (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Statistics

All experiments were carried out in triplicate and the values were expressed as means ± SD. Statistical analysis was carried out by one-way ANOVA with Tukey's posthoc tests and the values were considered significant at *p<0.05 or **p<0.01. SPSS 11.5 software was used for all statistical analyses.

Results

Antiproliferative activity of β-aescin against human U2OS osteosarcoma cells

To examine the antiproliferative effects of β-aescin, human U2OS osteosarcoma cells were treated with different concentrations (0-100 μM) of β-aescin. The results indicated that that β-aescin exerted potent antiproliferative activity against human U2OS cells (Figure 2). The antiproliferative effects were found to be concentration-dependent and increased with increase in the concentration of β-aescin. The IC_{50} of β-aescin against human osteosarcoma U2OS cells was found to be 40 μM.

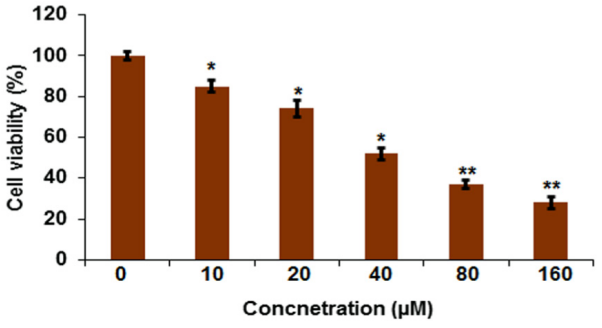

Figure 2. Effect of indicated doses of β-aescin on U2OS cell viability. All experiments were carried out in triplicate and expressed as mean±SD. The values were considered significant at *p<0.05 or **p<0.01.

β-aescin triggered the ROS generation in human U2OS osteosarcoma cells.

Since several natural products have been reported to exert their anticancer effects through generation of substantial amounts of ROS, we investigated whether β-aescin could induce accretion of ROS in U2OS human osteosarcoma cells. For determination of ROS levels, the U2OS cells were treated with 0, 20, 40 and 80 μM concentrations of β-aescin. The results indicated that β-aescin triggered the production of substantial amounts of ROS in U2OS cells (Figure 3). Moreover, the effects of β-aescin showed a concentration-dependent trend. These results suggested that β-aescin is a potent molecule for activating ROS in U2OS cells.

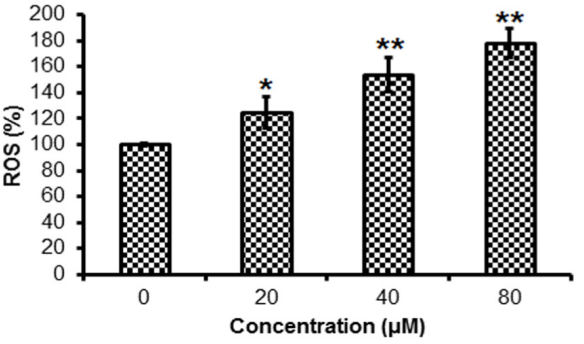

Figure 3. Effect of indicated doses of β-aescin on reactive oxygen species (ROS) levels in U2OS cells. All experiments were carried out in triplicate and expressed as mean±SD. The values were considered significant at *p<0.05 or **p<0.01.

β-aescin decreases MMP in human U2OS osteosarcoma cells.

The production of ROS in cancer cells causes mitochondrial dysfunction. ROS disrupts the outer mitochondrial potential to release death-promoting proteins. Therefore, in the present study we investigated whether β-aescin has an influence on MMP of U2OS cells. The cells were treated with 0, 20, 40 and 80 μM concentrations of β-aescin and MMP was determined by flow cytometry (Figure 4). Our results indicated that β-aescin-treated U2OS cells showed a significant reduction in MMP. Moreover, this reduction in MMP exhibited a β-aescin concentration-dependent pattern.

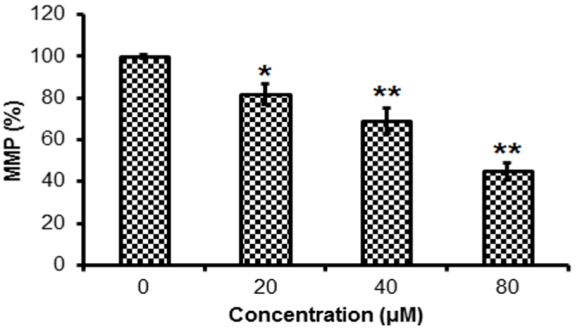

Figure 4. Effect of indicated doses of β-aescin on mitochondrial membrane potential (MMP) of U2OS cells. All experiments were carried out in triplicate and expressed as mean±SD. The values were considered significant at *p<0.05 or **p<0.01.

β-aescin triggered autophagic cell death in human U2OS osteosarcoma cells.

To examine whether β-aescin could trigger autophagic cell death in human U2OS osteosarcoma cells, these cells were firstly treated with 0, 20, 40 and 80 μM concentrations of β-aescin. Thereafter, the β-aescin-treated U2OS cells were subjected to

MDC staining (Figure 5). The results indicated that while the control cells exhibited low fluorescence, the β-aescin-treated cells showed considerable accumulation of MDC in the granular structures of high fluorescence intensity.

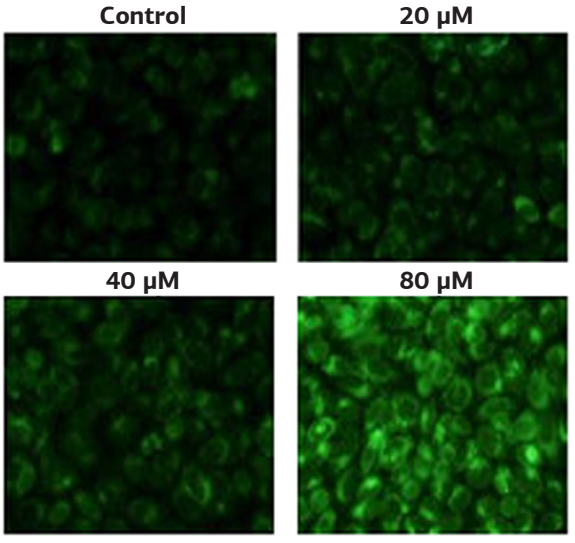

Figure 5. Effect of indicated doses of β-aescin on induction of autophagy in U2OS cells by MDC staining. All experiments were carried out in triplicate. The Figure shows that β-aescin induced autophagy in a concentration-dependent manner.

β-aescin enhances the expression of LC3-II in human U2OS osteosarcoma cells.

Immunofluorescence was carried out for LC3-II to further investigate the generation of autophagic vesicles. In comparison with the untreated cells, U2OS osteosarcoma cells treated with β-aescin showed significant increase in both size and number of LC3-II-postive puncta (Figure 6).

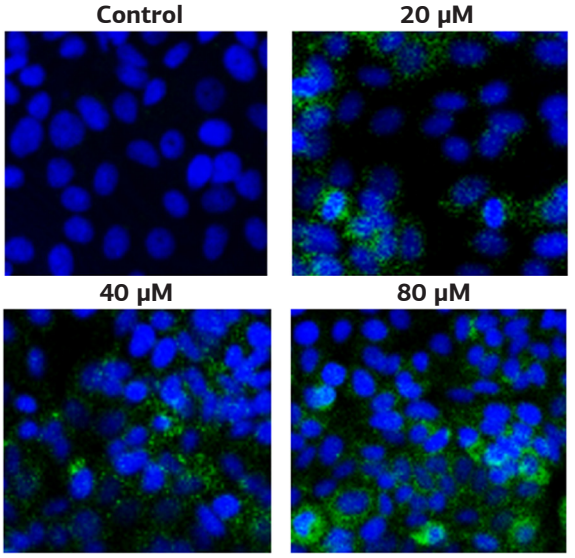

Figure 6. Effect of indicated doses of β-aescin on endogenous LC3 expression in U2OS cells by immunofluorescence analysis. All experiments were carried out in triplicate. β-aescin enhanced the expression of LC3-II protein in a concentration-dependent manner.

The effect of β-aescin on the protein expression of LC3-II in U2OS cells was also investigated at different concentrations (0, 20, 40 and 80 μM) and at different time intervals (0, 6, 12 and 24 hrs). The results of our study indicated that β-aescin enhanced the protein expression levels of LC3-II in U2OS human osteosarcoma cells in a concentration- and time-dependent manner (Figure 7).

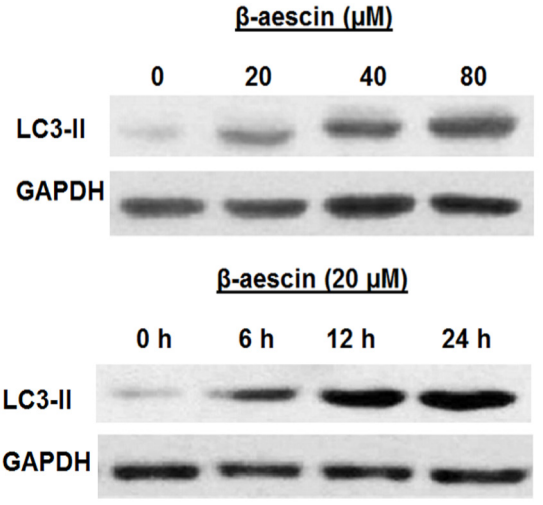

Figure 7. Effect of indicated doses of β-aescin and time intervals on induction of LC3-II expression by western blotting. All experiments were carried out in triplicate. The Figure shows that β-aescin increased LC3-II expression in a concentration-dependent manner.

Discussion

Osteosarcoma is one of the frequent bone tumors affecting mainly children and is associated with considerable mortality. The limited availability and less efficacious treatment options have led to poor survival rates of patients suffering from osteosarcoma [1]. Currently, the overall survival rate of conventionally treated osteosarcoma patients is only 50-60% while for the recurrent osteosarcoma patients the survival is about 20-30% [2]. Therefore, there is an urgent need to explore more viable and effective treatment options for the treatment of osteosarcoma. Consistent with this, natural products are considered good options due to their lower side effects and the fact that about more than half of the anticancer agents are natural products [13]. Against this backdrop, the present study was designed to evaluate the anticancer activity of β-aescin, a natural product of plant origin, against U2OS human osteosarcoma cells. The results of the present study indicated that aescin exerted significant anticancer effects on U2OS human cells and reduced the viability of these cells in a concentration-dependent manner. Additionally, our results indicated that

β-aescin treatment lead to generation of significant amounts of ROS in U2OS human osteosarcoma cells. This ROS-activating β-aescin property was concentration-dependent. Our results are in agreement with previous studies wherein several anticancer agents of natural origin have been reported to induce considerable amounts of ROS [14]. For instance, the anticancer molecule psoralidin induces autophagic cell death in lung cancer cells via generation of significant amounts of ROS [15]. It has been reported that accretion of ROS lead to mitochondrial dysfunction by altering the MMP [16]. Therefore, we also investigated the MMP in treated U2OS cells and we observed that β-aescin caused significant reduction in MMP. These results are also supported by other studies, for instance, capsaicin disturbs MMP and mediates oxidative stress resulting in cell death of pancreatic cancer cells [17]. Finally, we evaluated the potential of β-aescin to induce autophagic cell death. The results indicated that β-aescin induced autophagy in U2OS cells as evidenced by the MDC staining and immunofluorescence assay. These autophagic effects were due to the increase in the expression of LC3-II in a concentration- and time-dependent manner. Both MDC staining and the protein expression levels of LC3-II are considered strong hallmarks for autophagy and are commonly used to evaluate the autophagic activities of compounds [15].

Taken together, these results indicate that β-aescin shows potent antiproliferative activity in osteosarcoma cells by inducing autophagy, ROS generation and MMP loss and therefore may prove to a be a potent lead molecule. This study also paves the way for *in vivo* studies of this molecule.

Conflict of interests

The authors declare no conflict of interests.

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