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ORIGINAL ARTICLE

Pelargonidin induces antitumor effects in human osteosarcoma cells via autophagy induction, loss of mitochondrial membrane potential, G2/M cell cycle arrest and downregulation of PI3K/AKT signalling pathway

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Summary

Purpose: *Osteosarcoma is the most common type of primary malignancy of bone in children and adults. The treatment options for osteosarcoma are limited and are associated with a number of drawbacks. Therefore there is an urgent need to look for more efficient options for the treatment of this disease. Flavonoids have been considered as important anticancer agents due to their efficacy and lower cytotoxicity. In the present study we evaluated the anticancer effects of pelargonidin in U2OS osteosarcoma cell line.*

Methods: *Cell viability was assessed by MTT assay. Reactive oxygen species (ROS), mitochondrial membrane potential (MMP) and cell cycle analysis was done by flow cytometry. Expression of proteins was examined by western blotting.*

Results: *Pelargonidin exhibited significant anticancer effects on osteosarcoma U2OS cell line with an IC_{50} of 15 μM. The anticancer effects of pelargonidin were due to induction of autophagy as evidenced from the expression of LC3-I, LC-3II and Beclin-1. Moreover, pelargonidin triggered ROS-induced reduction in MMP and triggered G2/M cell cycle arrest. In addition, pelargonidin inhibited the expression of p-PI3K and p-AKT in a concentration-dependent manner.*

Conclusions: *Taken together, these results indicated that pelargonidin may prove a potential lead drug for the treatment of osteosarcoma.*

Key words: *apoptosis, cell cycle, osteosarcoma, pelargonidin, ROS*

Introduction

Use of plant natural products that can halt, reverse or delay the progression of cancer has been an interesting and attractive strategy to fight the increasing incidence and prevalence of malignancies across the globe [1]. A number of epidemiological studies have clearly indicated a direct relation between the flavonoid-rich diets and the comparatively lower risk for development of cancer [2]. Flavonoids are commonly found across the plant kingdom and constitute a very large group of plant secondary metabolites. They are found in leaves, flowers, bark, seed, stem and all other parts of the plants. In plants flavonoids perform a plethora of functions which include but are not limited to attraction of pollinators, dispersal of seeds, repelling of predators and tolerance to biotic and abiotic stress. It has been reported that there are more than 4,000 types of flavonoids with a widest frame of functions in plants [3]. With recent advances in science and technology, flavonoids are being evaluated every now and then for their health promoting properties. Several of the flavonoids have shown amazing pharmacological properties. These include antioxidant, anticancer,

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anticancer and anti-inflammatory activities that are attributed to their ability to interact with a wide array of cellular entities [4]. Pelargonidin is an anthocyanin that is biosynthesized from flavonoid precursors and is responsible for the color of several fruits and flowers [5] and it has been shown to possess impressive pharmacological potential. However, its anticancer activity has not been evaluated up until today. Against this background, the present study was designed to evaluate the anticancer activity of pelargonidin (Figure 1) against an osteosarcoma cell line. Osteosarcoma is the most prevalent and commonly detected malignancy of bones [6]. Although its incidence is higher in children and adolescents, it is considered as a rare type of cancer and constitutes only about 1% of all the cancers detected in United States. It has been reported that osteosarcoma arises mostly in the metaphyses of long bones like femur [7]. Even though most of the osteosarcomas arise during adolescence, they are also common in elderly people aged 70-80 years [7]. The treatment of osteosarcoma involves surgery, radiotherapy and chemotherapy. However, the results are far from satisfactory and frequent relapses have been observed. The current chemotherapeutic drugs used for the treatment of osteosarcoma have a number of side effects which adversely affect the patient quality of life [8]. Keeping this in mind, there is a demand to isolate molecules from plants that are efficient, effective, with lesser side effects and are cost-effective. In the current study we investigated the anticancer effects of pelargonidin against the osteosarcoma cell line U2OS. The results showed that pelargonidin induced autophagy in U2OS cells, altered the MMP, triggered G2/M cell cycle arrest and downregulated PI3K/AKT signalling pathway. Our results unequivocally indicate that pelargonidin may prove a lead molecule in the treatment of osteosarcoma.

Methods

Chemicals, reagents and cell culture conditions

3-methyladenine (3-MA), 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), mouse anti-human BNIP3 and rabbit anti-human light chain 3 (LC3) were purchased from Sigma-Aldrich (St.Louis, Missouri, USA) or otherwise mentioned. The antibodies for rabbit anti-human β-actin and rabbit anti-human Beclin-1 were purchased from Cell Signalling Technology (Beverly, MA, USA). The osteosarcoma U2OS cells were cultured in RPMI-1640 medium containing 10% bovine serum albumin (BSA), and penicillin and streptomycin (both 100 U/ml). The cells were then cultured at 37°C in a humid atmosphere containing 5 % CO_2 and 95 % air.

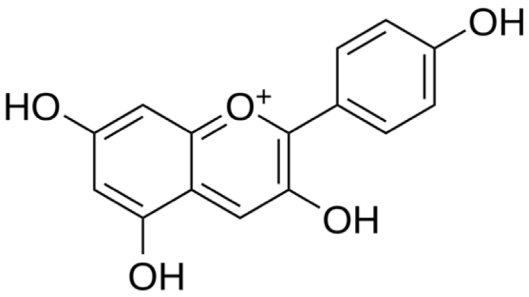

Figure 1. Chemical structure of pelargonidin.

Determination of cell viability

The viability of U2OS cells was assessed by MTT assay. In brief, U2OS cells were cultured in a 96-well plates at a density of 5×10^3 cells in each well. Then the cells were incubated overnight and afterwards the RPMI-1640 medium was removed and replaced with a fresh RPMI-1640 medium with pelargonidin at different concentrations (0, 7.5, 15 and 30 μM) for 24 hrs. Thereafter, MTT solution at a concentration of 0.5 mg/ml was added for the last 4 hrs of incubation and the absorbance was measured at 570 nm.

Determination ROS and MMP

For the measurement of ROS levels, U2OS cells were cultured in a 6-well plate at a density of 5×10^5 cells per well. The plates were then incubated at 37°C overnight. The RPMI-1640 medium was removed and the cells were cultured in a fresh RPMI-1640 medium containing pelargonidin at varying doses (0, 7.5, 15 and 30 μM) for 24 hrs or at 15 μM concentration for 0, 12, 24 and 48 hrs of incubation. Afterwards, the cells were collected and incubated with 5 μM dihydroethidium (for ROS) or 5 μM JC-1 (for MMP) in phosphate buffered saline (PBS) for 25 min at 37°C. Finally, PBS was used to wash twice the cells before fluorescence-activated cell sorting (FACS) analysis.

Investigation of cell cycle phase distribution

In order to assess the distribution of U2OS cells in different phases of the cell cycle, the pelargonidin-treated cells were collected and washed twice with PBS. Then the cells were fixed with ethanol (70%) for about 50 min and then washed again by PBS. The cells were finally resuspended in a solution of propidium iodide (PI) at a concentration of 50 μl/ml and RNase1 at a concentration of 250 μg/ml. This was followed by incubation for 30 min at room temperature and then FACS caterplus cytometer was used for examining the cell phase distribution.

Determination of protein expression by western blot analysis

For protein expression, the cells treated with pelargonidin were lysed in lysis buffer (Cell Signaling Technology, Danvers, MA, USA). The cell lysates were then run and separated on a SDS-PAGE (12%). Then, the proteins were transferred to PVDF membrane (Millipore, Billerica, MA, USA) in the transfer buffer. Then, block-

ing was carried out by non-fat dried milk (5%) for 2 hrs. Afterwards, the membrane was incubated overnight at 4°C with the primary antibody. Finally, the immunoreactive bands were observed by chemiluminescence with the help of HRP-conjugated secondary antibodies and blots were developed by an enhanced chemiluminescence detection system (Amersham-Pharmacia Biotech, Little Chalfont, UK).

Statistics

The experiments were carried out in triplicate and the data were presented as mean ± SD. The data were analyzed by Tukey's test and one way ANOVA and the values were considered significant at *p<0.01, **p<0.001 and ***p<0.0001.

Results

Pelargonidin decreases cell viability via induction of autophagy

The effect of pelargonidin (Figure 1) on cell viability was determined by MTT assay. The results revealed that pelargonidin significantly reduced the viability of U2OS cells. With increasing concentration of pelargonidin the viability of U2OS cells decreased, indicative of a concentration-dependent activity of pelargonidin (Figure 2). To clarify whether the antiproliferative effects of pelargonidin were due to autophagy, U2OS cells were treated with the autophagy inhibitor 3-MA. The results showed that treatment of the U2OS cells with the autophagy inhibitor reversed the effects of pelargonidin on the viability of U2OS cells (Figure 3). This provided a strong clue that pelargonidin caused autophagic cell death in U2OS cells. To further confirm, the autophagic death of U2OS cells we determined the expression of autophagy-related proteins L3-I, LC3-II and Beclin-1. It was observed that the expression of L3-II was increased while that of LC3-I was slightly decreased (Figure 4). Moreover, Beclin-1 has been reported to be involved in the formation of autophagosomes and in the present study it was observed that the expression of Beclin-1 was significantly increased, suggesting that pelargonidin induced cell death via induction of autophagy.

Pelargonidin causes ROS-mediated alterations in MMP

Previously several studies had reported that some anticancer drugs induce autophagy via ROS-mediated alterations in MMP [9]. Therefore, we determined the ROS and MMP levels in pelargonidin U2OS-treated cells. The results showed that pelargonidin caused time- and concentration-dependent increase of ROS in U2OS cells (Figure

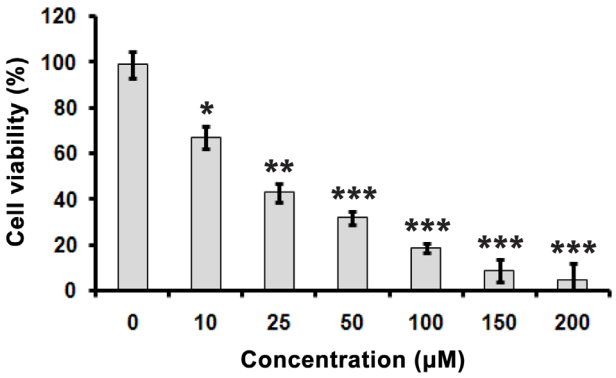

Figure 2. Effect of pelargonidin on the viability U2OS cells as determined by the MTT assay at the indicated concentrations. The experiments were carried out in triplicate and expressed as mean ± SD. The Figure depicts that pelargonidin inhibits the cell viability in a concentration-dependent manner (*p<0.01, **p<0.001 and ***p<0.0001).

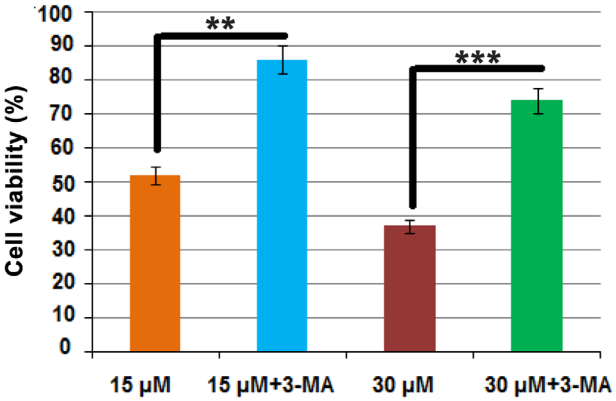

Figure 3. Effect of autophagy inhibitor 3-MA on the pelargonidin-induced autophagy in U2OS osteosarcoma cells. The experiments were carried out in triplicate and expressed as mean ± SD. The Figure depicts that pelargonidin triggers autophagy in U2OS cells. However, addition of the autophagy inhibitor prevents the autophagic cell death in U2OS cells (**p<0.001 and ***p<0.0001).

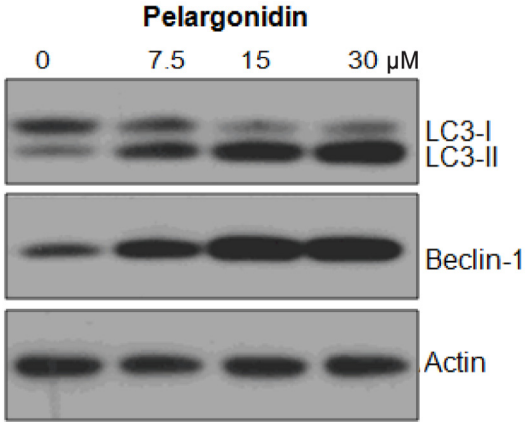

Figure 4. Effect of pelargonidin on the expression of autophagy-related proteins LC3-I, LC3-II and Beclin-1 in U2OS osteosarcoma cells as determined by western blotting. The experiments were carried out in triplicate. The Figure depicts that pelargonidin causes downregulation of LC3-I and Beclin-1.

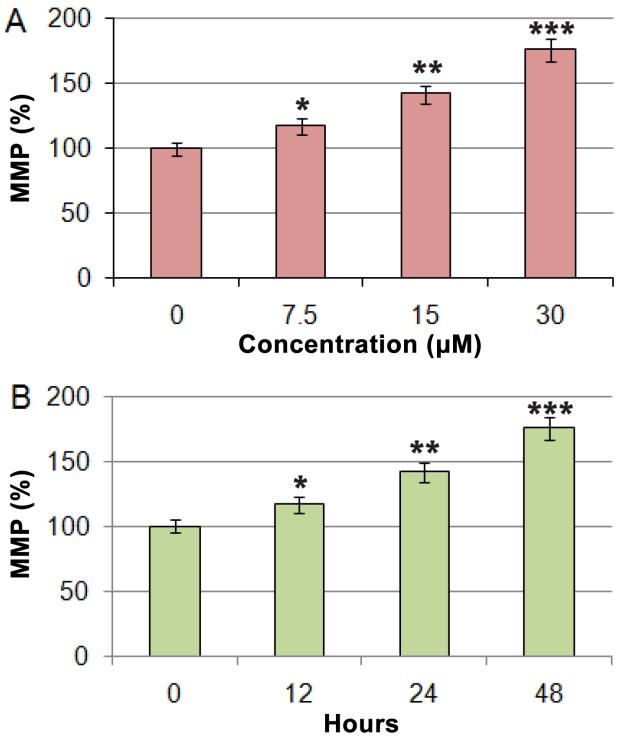

Figure 5. Effect of pelargonidin on ROS of U2OS osteosarcoma cells at indicated concentrations **(A)** and at indicated time points **(B)**. The experiments were carried out in triplicate and expressed as mean ± SD (*p<0.01, **p<0.001 and ***p<0.0001).

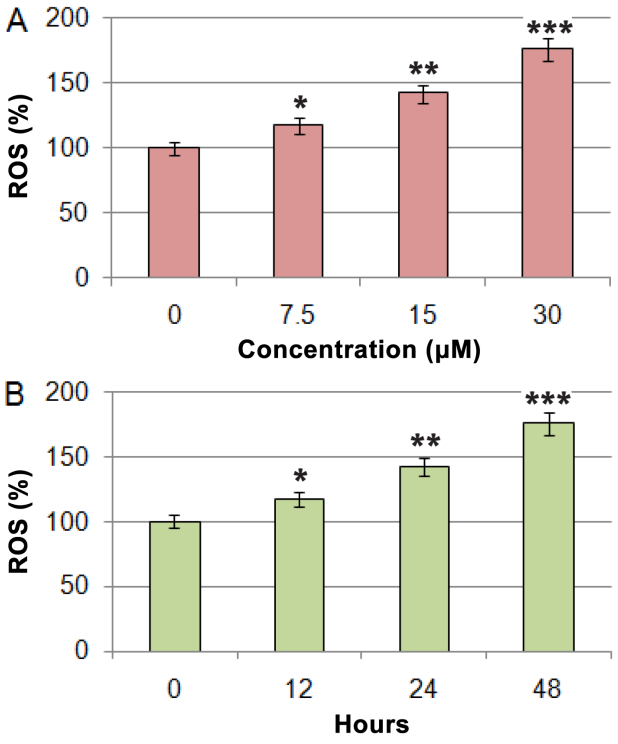

Figure 6. Effect of pelargonidin on MMP of U2OS osteosarcoma cells at indicated concentrations **(A)** and at indicated time points **(B)**. The experiments were carried out in triplicates and expressed as mean ± SD. The Figure shows that pelargonidin causes generation of ROS in a concentration and time-dependent manner (*p<0.01, **p<0.001 and ***p<0.0001).

5A, B), while at the same time pelargonidin caused time and concentration-dependent reduction of the MMP (Figure 6A, B).

Pelargonidin triggers G2/M cell cycle arrest

Cell cycle arrest is one of the important mechanisms by which anticancer drugs inhibit the growth of cancer cells [10]. To investigate whether the inhibitory effects of pelargonidin on U2OS cell growth were in part due to cell cycle arrest, U2OS cells were treated with different concentrations of pelargonidin and the percentage of cells in each cell cycle phase was estimated by flow cytometry. The results showed that pelargonidin caused marked increase of the U2OS cells in the G2 phase of the cell cycle, triggering G2/M cell cycle arrest (Figure 7). The effects of pelargonidin were found to be concentration-dependent and the percentage of the G2 cells increased with increasing concentration of pelargonidin.

Pelargonidin downregulates PI3K/AKT signalling pathway

PI3K/AKT pathway has been reported to play an important role in the autophagic cell death of several types of cancer cells [11]. This pathway has been shown to be involved in the tumorigenesis and progression of several types of cancers. The anticancer drugs which target this pathway are considered potential lead molecules for cancer chemotherapy [12]. Therefore we investigated the effect of pelargonidin on the expression of p-PI3K,

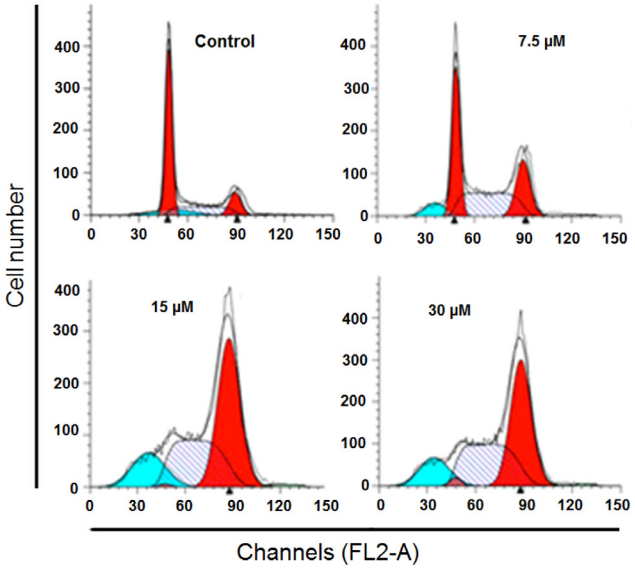

Figure 7. Effect of pelargonidin on cell cycle distribution of U2OS osteosarcoma cells at indicated doses. The experiments were carried out in triplicate. The Figure shows that pelargonidin triggers G2/M cell cycle arrest in a concentration-dependent manner.

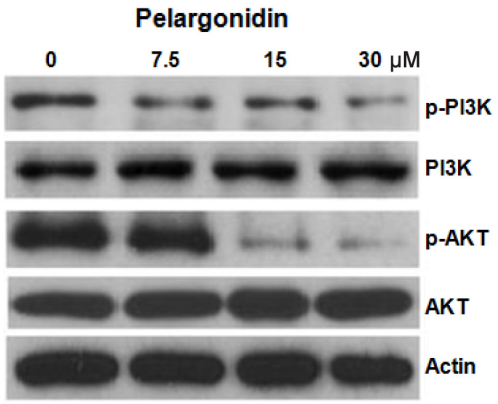

Figure 8. Effect of pelargonidin on the expression PI3K/AKT protein expression in U2OS osteosarcoma cells as determined by western blotting. The experiments were carried out in triplicate. The Figure depicts that pelargonidin decreases the expression of p-PI3K and p-AKT with no change on the expression of PI3K and AKT.

PI3K, p-AKT and AKT proteins (Figure 8). The results showed that pelargonidin caused reduction in the protein expression of p-PI3K and p-AKT, while the expression of PI3K and AKT proteins remained more or less unchanged.

Discussion

Osteosarcoma is one of the prevalent primary bone malignancy in children and adolescents. The treatment options currently used for OS have a lot of side effects and are too costly [6,7]. Therefore, it is believed that exploration of plant natural products might help to overcome these issues associated with the currently used drugs. Flavonoids and flavonoid-derived metabolites have been evaluated against different types of cancers. Flavonoids, like genistein, quercetin and naringenin, have shown promising anticancer effects [2-4]. In this connection, the present study was designed to evaluate the anticancer activity of pelargonidin against osteosarcoma U2OS cell line. The results of our study showed that pelargonidin exhibited significant anticancer activity against the U2OS cells with an IC_{50} of 15 μM. Previously, it has been reported that several flavonoids trigger autophagy in cancer cells. For instance silibinin, which is a natural flavonoid, has been reported to induce autophagy in breast cancer cells [13]. Similarly, quercetin triggers autophagy in gastric cancer cells [14]. Therefore, we examined whether pelargonidin could trigger autophagy in U2OS cells. For this we treated U2OS cells with the autophagy inhibitor 3-MA which has been reported to inhibit autophagy by blocking the initial steps of autophagy via suppression of PI3 kinases. What we observed was that the treatment of U2OS cells with 3-MA inhibitor led to inhibition of pelargonidin-induced autophagy. To further confirm that pelargonidin induced cell death via autophagy, we examined the expression of autophagy-related proteins, such as LC3-I, LC3-II and Beclin-1, in U2OS cells and noticed that the expression of L3-II was increased, while that of LC3-I was slightly decreased. Moreover, Beclin-1 has been reported to be involved in the formation of autophagosomes [15] and in the present study it was observed that the expression of Beclin-1 was significantly increased, suggesting that pelargonidin induces cell death via induction of autophagy. Previously, it has been reported that ROS-mediated MMP plays important role in the induction of autophagy [9]. Therefore, we determined we determined the ROS and MMP levels in pelargonidin-treated U2OS cells. The results showed that pelargonidin caused concentration-dependent increase in ROS and at the same time caused significant reduction in the MMP. These results indicate that pelargonidin-induced cell death is due to mitochondrial membrane potential mediated autophagy. Some flavonoids with anticancer effects have been reported to induce cell cycle arrest [16-18]. Consistent with this, in our study we observed that pelargonidin triggered G2/M cell cycle arrest in U2OS cells. Several lines of evidence suggest that inhibition of PI3K/AKT signalling cascade play an important role in the induction of autophagy [11]. In the present study, it was observed that pelargonidin caused significant reduction in the levels of p-PI3K and p-AKT, while the levels of PI3K and AKT remained more or less unaltered. These results indicate that pelargonidin-induced autophagy involves the PI3K/AKT pathway.

Conclusion

Our results show that pelargonidin induces cell death in U2OS cells via autophagy induction, loss of MMP, G2/M cell cycle arrest and downregulation of PI3K/AKT signalling pathway. Therefore, pelargonidin may prove to be a handy molecule in the treatment of osteosarcoma. However, further research is needed to evaluate this molecule *in vivo*.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

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