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ORIGINAL ARTICLE

Anticancer effects of Daidzein against the human melanoma cell lines involves cell cycle arrest, autophagy and deactivation of PI3K/AKT signalling pathways

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Summary

Purpose: *Melanoma is one of the fatal human malignancies. Its incidence in humans is increasing constantly and therefore there is urgent need to develop effective therapies for its management. This study was therefore undertaken to investigate the anticancer effects of Daidzein on human melanoma cells and also an attempt was made to decipher the underlying mechanisms.*

Methods: *MTT assay was used to determine the melanoma A-375 cells viability. Acridine orange (AO)/ Ethidium bromide (EB) and Annexin V/propidium iodide (PI) assays were used to detect the cell apoptosis. Autophagy was detected by electron microscopy and cell cycle analysis was performed by flow cytometry. The protein expression was determined by western blot analysis.*

Results: *The results of MTT assay showed that Daidzein causes significant decrease in the proliferation of the melanoma A-375 cells and showed an IC_{50} of 18 μM. However, the IC_{50} of Daidzein was very high against the normal HEMn-LP cells, indicative of low cytotoxicity. Flow cytometry showed significant arrest of the A-375 cells at the G0/G1 phase of the cell cycle. Western blot analysis showed that the molecule suppressed the expression cell cycle regulatory proteins such as cyclin D1, CDK4, CDK6 and p27. DAPI and annexin V/PI staining assays showed that Daidzein prompted apoptosis in A-375 melanoma cells which was concomitant with depletion of Bcl-2, increase of Bax and activation of cleavage of caspase-3 and caspase-9. Electron microscopic analysis showed that the molecule led to the development of autophagosomes in A-375 cells, which was also concomitant with increase in the expression of LC3B II and decrease in the expression of p62. Finally, Daidzein also suppressed the phosphorylation of PI3K and AKT, causing deactivation of the PI3K/AKT signalling pathway.*

Conclusion: *Daidzein may prove beneficial in the development of melanoma systemic therapy.*

Key words: *melanoma, Daidzein, apoptosis, autophagy, cell cycle arrest*

Introduction

Melanoma is one of the fatal malignancies world over. Although the incidence of many cancers is decreasing, the incidence of melanoma is still continuously increasing [1]. Patients with localized melanoma are treated with surgical interventions followed by chemotherapy [2]. However, most of the patients are diagnosed at the time when the tumor has already metastasized. With increasing incidence, melanoma is now the 5th and 6th most prevalent type of cancer in men and women in United States [4]. Melanoma is believed to be multi-factorial disease arising as a result of interaction between both genetic and environmental factors. The exposure to ultraviolet (UV) radiation

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is considered as one of the critical factors owing to its genotoxic effect [5]. Nature has always been a great source of exceptional chemical scaffololds for the development of drugs. Researchers continue to explore natural products for the treatment of deadly diseases [6]. Among natural products, isoflavones have gained considerable attention for their anticancer properties. They have been reported to decrease the proliferation of several types of cancer cells via multiple mechanisms [7]. Daidzein is an important isoflavone with potent anticancer effects [8]. This molecule has been reported to inhibit the growth of colon cancer cells [9]. In another study, daidzein has been shown to suppress the growth of breast cancer cells by causing arrest of the cancer cells at different check points of the cell cycle [10]. Diadzein also been reported to suppress the growth of ovarian cancer cells [11]. Nonetheless, the anticancer potential of Daidzein has not been explored against human melanoma cells. This study was therefore designed to explore the anticancer effects of Diadzein against human melanoma cells and an attempt was made to decipher the underlying mechanisms.

Methods

Cell viability

The A-375 melanoma cells and HEMn-LP normal cells were cultured and treated with Daidzein at concentrations ranging from 0 to 200 µM for 24 h. Subsequently, the cells were treated with MTT (500 µg/mL) for 4 h. DMSO (10%) was then added to dissolve the blue formazan crystals formed. Finally, the optical density (OD) was taken at 570 nm by a spectrophotometer to monitor the cell viability.

Cell cycle analysis

The cultured human melanoma A-375 cells were firstly treated with varied concentrations of Daidzein for 24 h at 37°C. The cells were then washed with phosphate buffered saline (PBS) and stained with Annexin V/propidium iodide (PI) and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

Apoptosis detection assays

The A-375 cells (0.6×10^6) were cultured in 6-well plates and treated with Daidzein at concentrations 0, 9, 18 and 36 µM for 24 h at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with AO/EB. The slides were then cover-slipped and examined under fluorescence microscope. ApoScan kit was used to determine the apoptotic A-375 cell percentage. In brief, Daidzein-treated A-375 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC/PI. The percentage of apoptotic A-375 cells at each concentration was then determined by flow cytometry.

Detection of autophagy

Autophagy in Daidzein-treated melanoma cells was studied by electron microscopy. In brief, A-375 melanoma cells were treated with 0, 9, 18 and 36 µM Daidzein for 24 h. The cells were collected by trypsinization and washed with phosphate-buffered saline (PBS) which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by the treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with an ultramicrotome and subjected to electron microscopy.

Western blot analysis

The A-375 cells were then lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were subjected to separation on 10% SDS-PAGE, followed by transferring the proteins to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally the protein signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalisation.

Statistics

All the experimental procedures were performed in triplicate. The values obtained are presented as mean of these three replicates ± SD. *P<0.05 and **p<0.01 were considered statistically significant. The statistical analysis was performed by Student's *t*-test using GraphPad prism 7 software.

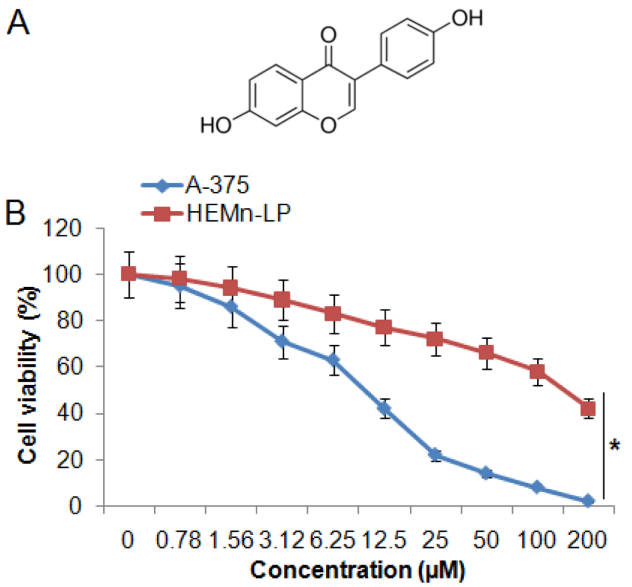

Figure 1. A: Chemical structure of Daidzein. **B:** MTT assay showing the effect of Daidzein on the viability of the A-375 melanoma and normal HEMn-LP cells. The experiments were performed in triplicate and values are mean±SD (*p<0.01).

Results

Inhibition of the human melanoma cells by Daidzein

The A-375 human melanoma and normal HEMn-LP normal cells were used to assess the anti-proliferative effects of Daidzein (Figure 1A) by MTT assay. Daidzein caused a remarkable decrease in the viability of the A-375 cells which was concentration-dependent and IC_{50} of 18 μM was reported for Daidzein against the A-375 cells (Figure 1A). On the contrary, the anti-proliferative effects of Daidzein on the normal CDD-18Co cells showed an IC_{50} of >100 μM (Figure 1B).

Daidzein causes G0/G1 arrest of human melanoma cells

The effects on the cell cycle phase distribution of the A-375 cells was assessed by flow cytometry.

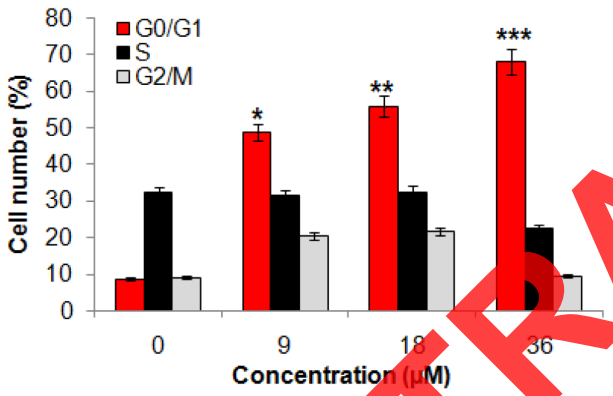

Figure 2. Cell cycle analysis of Daidzein-treated A-375 melanoma cells by flow cytometry, showing the induction of G0/G1 cell cycle arrest dose-dependently. The experiments were performed in triplicate and values are mean±SD (*p<0.05, **p<0.01, ***p<0.001).

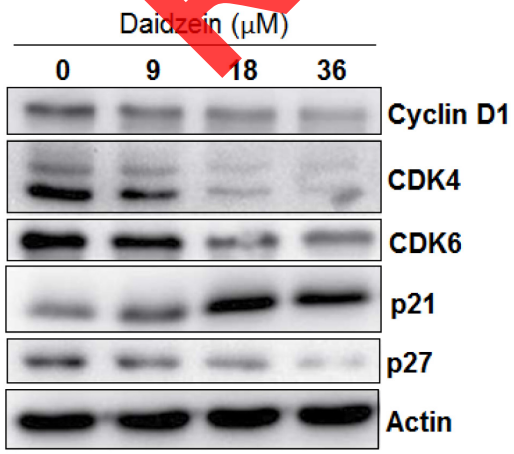

Figure 3. Western blot analysis showing that Daidzein inhibits the expression of cyclin D1, CDK4, CDK6, and p27 and enhances the p21 dose-dependently. The experiments were performed in triplicate.

The percentage of the G0/G1 phase cells was enhanced considerably upon Daidzein treatment. The percentage of G0/G1 phase cells were 8.7, 48.7, 55.8 and 68% at 0, 9, 18 and 36 μM of Daidzein respectively, indicating G0/G1 arrest of the A-375 cells (Figure 2). Western blot analysis showed that Daidzein exerted remarkable effects on the protein expression of the cell cycle regulatory proteins in A-375 cells. It was found that the expression of CDK4, CDK6, cyclin D1 and p27 decreased and that of p21 increased concentration-dependently (Figure 3).

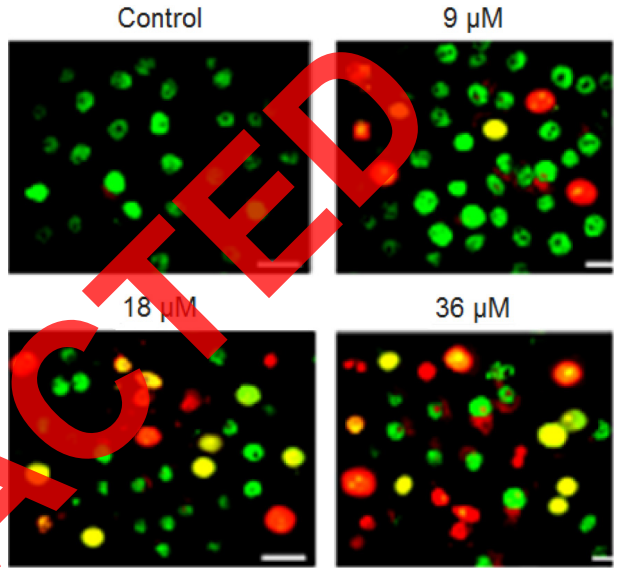

Figure 4. AO/EB staining showing the induction of apoptosis in the A-375 cells at indicated concentrations of Daidzein. Green color depicts normal cells, orange depicts normal cells, and red late apoptotic cells. The experiments were performed in triplicate.

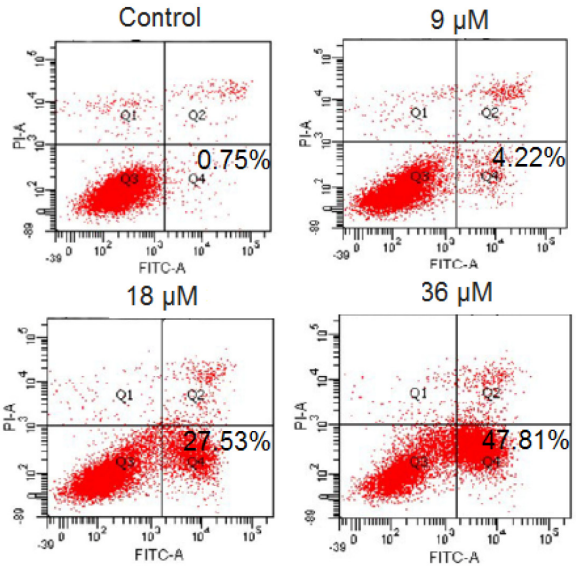

Figure 5. Annexin V/PI staining showing the percentage of apoptotic A-375 cells at indicated concentrations of Daidzein. The experiments were performed in triplicate.

Induction of both apoptosis and autophagy by Daidzein in A-375 melanoma cells

G0/G1 arrest alone of the A-375 cells may not be responsible for the low IC_{50} of Daidzein against the A-375 cells. Therefore, A-375 cells were treated with different doses of Daidzein and then stained with AO/EB. The results of AO/EB assay showed that the molecule caused nuclear fragmentation of the A-375 cells, characteristic of apoptosis (Figure 4). Annexin V/PI staining was also carried out and the apoptotic A-37 cell percentage was determined at different concentrations of Daidzein. The apoptotic cell percentage was 0.75, 4.22, 27.53 and 47.81% at Daidzein concentrations of 0, 9, 18 and 36 μM (Figure 5). Western blot analysis showed that the molecule caused increase of Bax and decrease of Bcl-2 expression. Moreover, Daidzein increased the expression of caspase-3 and 9 and also promoted their cleavage (Figure 6).

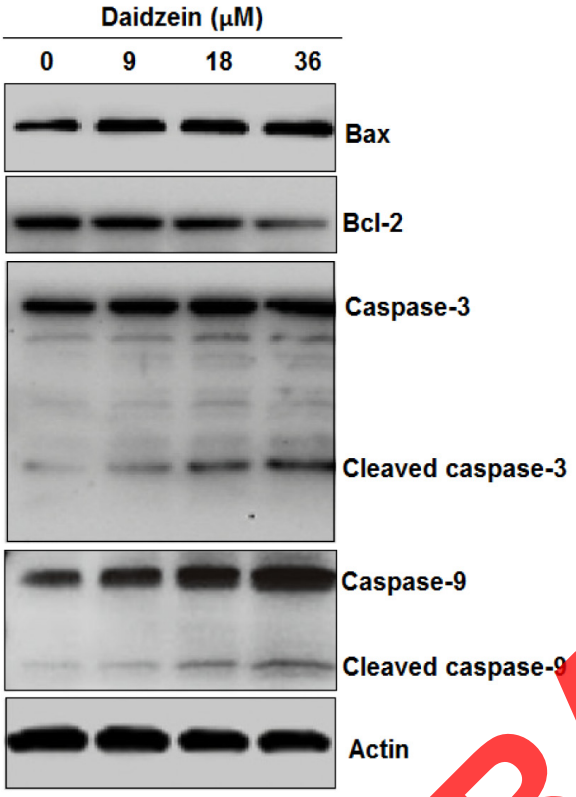

Figure 6. Western blot analysis showing that Daidzein inhibits the expression of Bcl-2, increases the expression of Bax and activates the cleavage of Caspase-3 and 9 dose-dependently. The experiments were performed in triplicate.

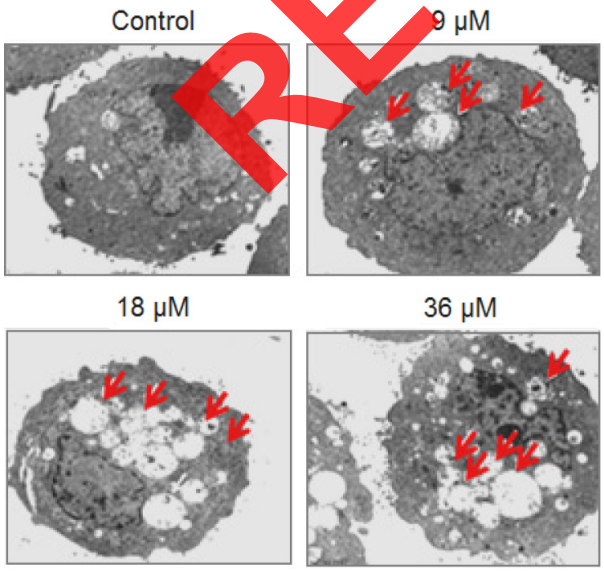

Figure 7. Electron microscopy showing that Daidzein induces autophagy in the A-375 cells at indicated concentrations (arrows depict the autophagosomes). The experiments were performed in triplicate.

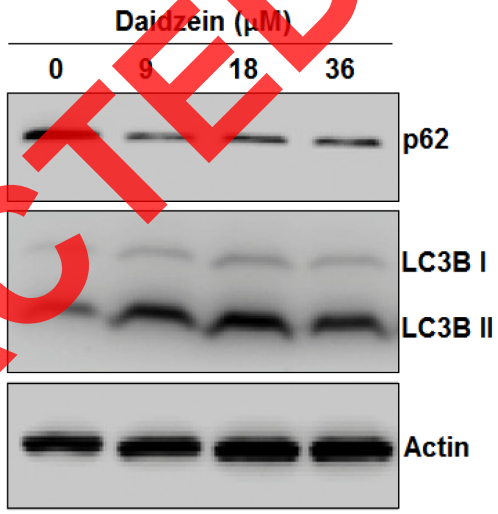

Figure 8. Western blot analysis showing the effect of Daidzein on the expression of the autophagy-related proteins. The Figure shows that Daidzein decreases p62 and increases the LC3B II expression. The experiments were performed in triplicate.

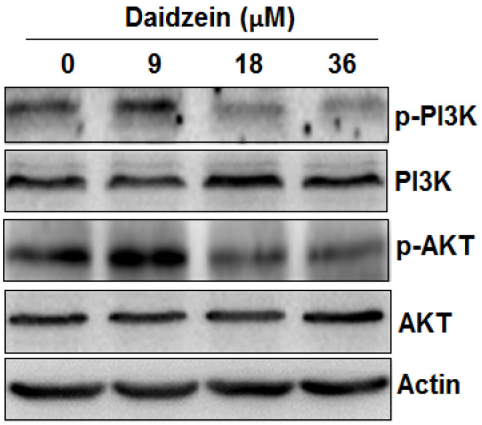

Figure 9. Western blot showing the effect of Daidzein on the PI3K/AKT signalling pathway at indicated concentrations. The Figure reveals that Daidzein inhibits the phosphorylation of PI3K and AKT concentration-dependently. The experiments were performed in triplicate.

Electron microscopic analysis was also performed to investigate if Daidzein also induces autophagy in the A-375 melanoma cells. The results showed that Daidzein led to development of autophagosomes in the A-375 cells, indicative of autophagy (Figure 7). Western blot analysis showed that the molecule caused increase in the expression of LC3B II and decrease in the expression of p62 in a concentration-dependent pattern (Figure 8).

Deactivation of PI3K/AKT signalling pathway by Daidzein

The effects of Daidzein on the PI3K/AKT signalling pathway were examined by western blot analysis. The results showed that Daidzein caused remarkable decrease in the phosphorylation of PI3K as well as AKT. However, the total expression of both PI3K/AKT remained unchanged (Figure 9).

Discussion

Over the years a lot of progress has been made in the field of cancer research resulting in a significant decrease in the incidence of many cancers. Nonetheless, the incidence of melanoma is still increasing at an alarming rate making it one of the difficult-to-treat human malignancies [12]. Therefore, studies are being directed to develop new safe and effective systemic therapies for melanoma. Consistently, this study was undertaken to investigate the anticancer effects of a naturally occurring isoflavone Daidzein against human melanoma cells. The results showed that Daidzein selectively suppressed the growth of the human melanoma cells with very low cytotoxicity against the normal human cells. These observations are in line with previous studies wherein Daidzein has been shown to suppress the growth of breast cancer cells [13]. In another study Daidzein caused apoptosis of the MCF7 breast cancer cells [14]. Diadzein has also been reported to cause cell cycle arrest of various cancer types, such as prostate cancer and cervical cancer cells [15,16]. Therefore, we also investigated the effects of Daidzein on the cell cycle phase distribution of A-375 melanoma cells which revealed that this molecule caused arrest of these cells in the G0/G1 phase of the cell cycle. This was also accompanied by suppression of the expression of cyclin D1, CDK4, CDK6 and p27. Daidzein has also been reported to trigger apoptosis in the SKOV-3 ovarian cancer and colon cancer cells [17,18]. Therefore, DAPI and annexinV/PI staining assays were performed, which showed that Daidzein also caused apoptosis in the A-375 melanoma cells by increasing the Bax/Bcl-2 ratio and activation of caspases-3 and 9. Many isoflavones of plant origin have been shown to induce autophagy in cancer cells [19] and herein we found that the isoflavone Daidzein also activates autophagy in the A-375 cells by increasing LC3B II and suppressing p62 expression. Previous studies have shown that Daidzein targets PI3K/AKT in breast and ovarian cancer cells [20,21]. Therefore, western blot analysis was performed to investigate the effects of the molecule on the therapeutically important PI3K/AKT signalling pathway [22] which revealed that Daidzein blocked this pathway by suppressing the phosphorylation of PI3K and AKT, indicative of its potent anticancer effects.

Conclusion

To conclude, the findings of the present study suggest that Daidzein exerts potent anticancer effects on the human melanoma cells via multiple mechanisms which include apoptosis, autophagy and cell cycle arrest. Daidzein also deactivates the PI3K/AKT signalling pathway in human melanoma cells, indicative of its potential as a lead molecule for the development of new systemic therapies in melanoma and other malignancies.

Acknowledgement

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Conflict of interests

The authors declare no conflict of interests.

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