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ORIGINAL ARTICLE

Diosgenin inhibits the proliferation, migration and invasion of the optic nerve sheath meningioma cells via induction of mitochondrial-mediated apoptosis, autophagy and G0/G1 cell cycle arrest

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Summary

Purpose: *Diosgenin - a steroidal saponin- has been shown to exhibit wonderful anticancer potential. However, the antiproliferative effects of diosgenin have not been examined against the optic nerve sheath meningioma cells. In the current research work, the anticancer potential of diosgenin was evaluated in optic nerve sheath meningioma cells along with evaluating its effects on cancer cell invasion, migration, apoptotic cell death, autophagy and cell cycle progression.*

Methods: *The WTS-1 assay was used to determine the viability of HBL-52 cells. Autophagy was detected by transmission electron microscopy and western blot. The cell migration and invasion of HBL-52 cells was determined by wound healing and transwell assays. Apoptotic and effects on cell cycle were studied by fluorescence microscopy, western blot and flow cytometry.*

Results: *The results showed that diosgenin decreased the viability of the HBL-52 considerably and exhibited an IC_{50} of 15 μM. The antiproliferative effects were found to be due to the activation of the autophagy in the HBL-52 cells. The autophagy was also accompanied by upregulation of LC3 II and Beclin 1 expression. Diosgenin also triggered cell cycle arrest of the HBL-52 at the sub-G1 phase of the cell cycle. The cell migration and invasion of the HBL-52 cells was also suppressed by diosgenin. Diosgenin also triggered mitochondrial-dependent apoptotic cell death.*

Conclusions: *The results of the current study clearly indicate that diosgenin has anticancer potential in optic nerve sheath meningioma cells under in vitro conditions and can be a potential drug candidate provided further studies are carried out in this direction.*

Key words: *diosgenin, optic nerve sheath meningioma, apoptosis, autophagy, cell cycle*

Introduction

Plants have always played crucial role by providing not only food and spices but also medicines to treat a variety of diseases. For over 5000 years of human history, plants have been used as medicines. Even today, it is estimated that 75-85% of the people in developing countries continuously use traditional medicine to cure different diseases. The traditional Chinese Medicine (TCM) and Traditional Indian Medicine (TIM) also known as Ayurveda have furnished the majority of the current knowledge about medicinal and aromatic plants. Herbal medicines are the plants that contain useful molecules with therapeutic effect and can heal and prevent numerous diseases in humans [1,2].

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Cancer is characterised by uncontrolled multiplication of cells forming tumours of malignant cells with a tendency to spread to other parts of the body, known as metastasis. Cancer is responsible for the majority of morbidity and mortality globally. About 8.2 million cancer-related deaths and about 14 million new cancer cases were identified in 2013 and this number is expected to rise [3]. The treatment of cancer in general involves surgical resection followed by radiotherapy and chemotherapy. But radiotherapy and chemotherapy are associated with adverse side effects which affect the quality of life of the patient. This severely limits the use of chemo and radiotherapy. The treatment of some of the cancers, like breast cancer, involves very costly biological drugs. Additionally, the cancer cells acquire drug resistance which further complicates the treatment. In most cases, the patient is given a combination of drugs [4,5]. Therefore, there is an urgent need to find more effective, novel and cheaper anticancer drugs.

In the present study, our main aim was to evaluate the anticancer effects of diosgenin- a steroidal saponin - against the optic nerve sheath meningioma cells along with determining its effects on cellular apoptosis, autophagy and cell cycle phase distribution. Diosgenin is the main bioactive natural product present in dietary fenugreek seeds. Various pharmacological activities like antioxidant, gastroprotective, anti-inflammatory, hepatoprotective, anti-diabetic and antitumor have been reported for this molecule. There are numerous published reports which indicate that diosgenin exhibits both *in vitro* and *in vivo* anticancer and pro-apoptotic properties against a wide-range of cancer cells. This molecule has also been reported to sensitize cancer cells along with reversing the drug-resistance in them [6-9]. However, there are no previous reports on its anticancer potential against the optic nerve sheath meningioma cells.

Methods

CCK-8 Cell viability assay

HBL-52 optic nerve sheath meningioma cell line was purchased from the Department of Biochemistry and Biology, Chinese Academy of Sciences, Shanghai, China. The cells were kept in MDEM medium comprising of 10% fetal bovine serum (FBS), at 37°C in a 5% CO_2 incubator. The cells were firstly exposed to various doses of diosgenin, following which 30 µl CCK-8 (purchased from Dojindo Laboratories, Kumamoto, Japan) were added to the cell culture plates. Incubation of cell culture plates was carried out for 12 h at 37°C, following which the absorbance was measured at 450 nm wavelength using a microplate reader (Bio-Rad, Hercules, USA). Cell cytotoxicity was measured from the observed absorbance.

In vitro wound healing assay for cell migration

HBL-52 cells at a density of 2×10^5 cells/ml were seeded in 6-well plates and incubated to acquire 85% monolayer of confluent cells. Following treatment of HBL-52 optic nerve sheath meningioma cells with various does of diosgenin, the DMEM medium, in which cells were suspended, was removed and the cells were washed twice with phosphate buffered saline (PBS). A 100 ml sterile pipette was used to make a straight cell-free wound in the wells. The cells were then fixed and stained with 3.5% ethanol containing 1.55% crystal violet dye for 40 min. The cells were washed again and a picture was taken. The plates were cultured for 24 h and a picture was taken again under an inverted microscope (Nikon Corporation, Tokyo, Japan). Arbitrarily designated fields were photographed and the portion of cells that migrated into the scratched area was calculated.

Cell invasion assay

The effects of diosgenin on the invasion tendency of HBL-52 cells were measured by Matrigel coated transwell chambers with polyvinylpyrrolidone-free polycarbonate filter (6-mm pore size). About 150 ml cell cultures were placed onto the upper chambers and in the bottom chambers only medium was placed. After a time gap of 24 h for which time the cells were incubated, the cells were removed from the upper chamber and the cells which invaded were fixed with methanol and subsequently the cell cultures were stained with 1.55% crystal violet dye for 40 min. Inverted microscope was used to count the number of invaded cells at 200× magnification.

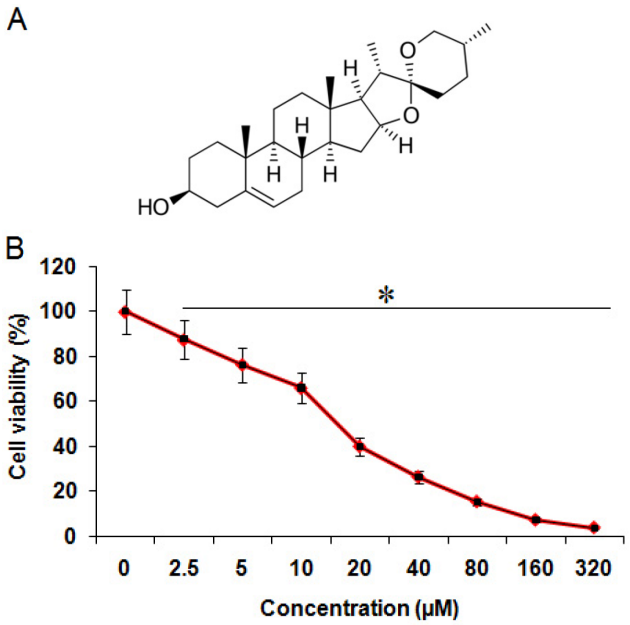

Figure 1. Chemical structure of diosgenin **(A)**. CCK-8 assay **(B)** indicates the cytotoxic effects of diosgenin on the viability of HBL-52 optic nerve sheath meningioma cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

Autophagy evaluation by electron microscopy

In this experiment, electron microscopy was used to evaluate the autophagic effects of diosgenin on HBL-52 optic nerve sheath meningioma cells. In brief, HBL-52 cells were treated with different doses of diosgenin for 24 h. Then, the cells were collected via trypsinization and washed twice with phosphate-buffered saline (PBS) and fixed in 2% glutaraldehyde in 0.1 M phosphate buffer. Then, using osmium tetraoxide (1%), the cells were post-fixed and were treated with ethanol and embedded in resin. Thin sections were then cut with an ultramicrotome and examined using a Zeiss CEM 902 electron microscope.

Apoptosis evaluation by fluorescent microscopy

The apoptotic effects exerted by diosgenin on HBL-52 optic nerve sheath meningioma cells were examined by fluorescence microscopy using DAPI as a staining agent. In brief, HBL-52 cells at a cell density of 1×10^5 cells per ml were cultured in 6-well plates and treated with diosgenin at various doses for 24 h at 37°C. Afterwards, 30 µl of cell culture were placed onto glass slides and stained with DAPI. The slides were then enclosed with cover slips and examined under fluorescence microscope (Nikon Corporation, Tokyo, Japan).

Cell cycle analysis

The HBL-52 optic nerve sheath meningioma cells (at a cell density of 1×10^6 cells/ml) cultured in DMEM medium were treated with several concentrations (0, 7.5, 15, 30 µM) of diosgenin molecule. The cells were first harvested and then fixed with ice-cold ethanol (70%) and treated with RNase A (20 µg/ml). Later, the cells were washed with PBS and stained using 20 µl (20 µg/mL) solution of propidium iodide (PI). The distribution of HBL-52 optic nerve sheath meningioma cells in various phases of the cell cycle were determined by FACSCalibur flow cytometry.

Western blot analysis

The HBL-52 optic nerve sheath meningioma cells were firstly washed with ice-cold PBS and then lysed in RIPA lysis buffer comprising the protease inhibitor. Around 50 µg of proteins from each cell culture were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands. Finally, the signal was detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalisation.

Statistics

The results are presented as mean ± standard deviation values from three independent experiments. Differences between the groups were examined by Student's *t*-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Disogenin exerted potent antiproliferative effects in HBL-52 optic nerve sheath meningioma cells

The cytotoxicity induced by diosgenin in HBL-52 cells was evaluated by CCK-8 (Cell Counting Kit-8) assay at various doses of the molecule. The chemical structure of diosgenin is shown in Figure 1A, while Figure 1B shows the cytotoxicity results. The CCK-8 assay results clearly indicated that diosgenin led to concentration-dependent antiproliferative effects in these cells showing an IC_{50} value of 8.5 µM. So this molecule had a lower value of IC_{50} indicating its high potency to cause cell cytotoxicity in cancer cells.

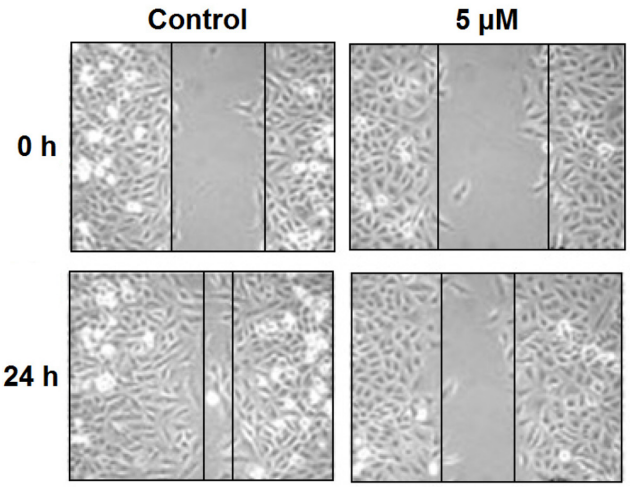

Figure 2. Diosgenin led to inhibition of cell migration in HBL-52 optic nerve sheath meningioma cells. The experiments were performed in triplicate.

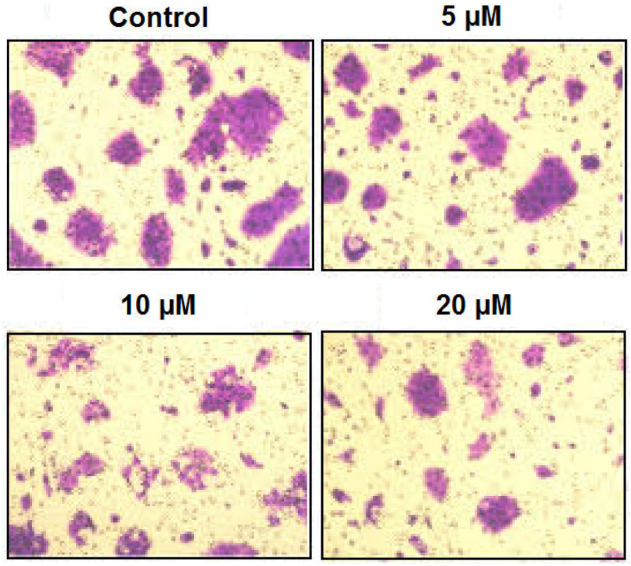

Figure 3. Diosgenin induced dose-dependent inhibition of cell invasion in HBL-52 optic nerve sheath meningioma cells. The experiments were performed in triplicate.

Diosgenin inhibited cell migration and invasion in HBL-52 cells

In vitro wound healing assay for cell migration and transwell assay for cell invasion were carried out to check the efficacy of diosgenin in inhibiting cancer cell migration and invasion. The results which are shown in Figure 2 and Figure 3 clearly indicate that diosgenin induced inhibitory effects on both cell migration as well as cell invasion in a dose-dependent manner as was clearly visible from the wound width at high doses of the molecule. Both these results suggest that diosgenin might find applications as an anti-metastatic agent which can curb the spread of cancer cells.

Diosgenin induced autophagic cell death in HBL-52 optic nerve sheath meningioma cells

Further investigation on the anticancer mode of action of diosgenin was carried out using electron microscopy in HBL-52 optic nerve sheath meningioma cells. The results which are depicted in Figure 4 indicate that at 5 µM dose of diosgenin, autophagosomes and autophagic vacuoles could be seen in treated cells as compared to the control cells. The presence of autophagosomes and vacuoles is an indication of autophagy. Western blot assay was further employed in order to confirm autophagy. The results are presented in Figure 5 and clearly indicate that the molecule had a significant effect on autophagy-associated protein expressions including LC3-I, LC3-II and p62. Disogenin led to dose-dependent increase of LC3-II and LC3-I expression while led to an inhibition of p62 expression.

Diosgenin also induced apoptotic cell death in HBL-52 optic nerve sheath meningioma cells

Diosgenin created not only autophagy but it was also able to induce apoptotic effects in HBL-52 cells. This was evaluated by fluorescence microscopy using DAPI as a staining agent which was further confirmed by western blot by checking Bax and Bcl-2 protein expressions which are apoptosis-related proteins. Diosgenin led to a significant nuclear fragmentation, chromatin condensation, and splitting of the nucleus, representative of the apoptotic cascade (Figure 6). This apoptotic cascade was seen to be increased with increasing diosgenin-concentration. Further, it was seen that diosgenin led to increase in Bax expression and subsequent decrease in Bcl-2 expression dose-dependently (Figure 7).

Diosgenin induced G0/G1 cell cycle arrest

The cytotoxic effects of diosgenin were further shown to be mediated through arrest of cell cycle phase distribution. Results obtained from flow

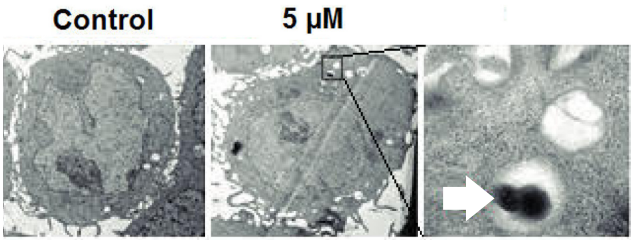

Figure 4. Diosgenin-induced formation of autophagosomes and vacuoles in HBL-25 optic nerve sheath meningioma cells which is characteristic of autophagy process. The experiments were performed in triplicate.

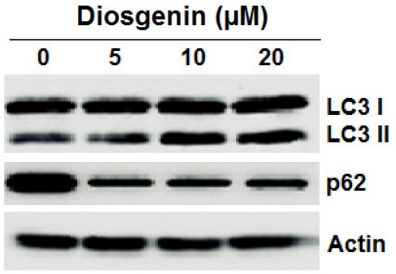

Figure 5. Effects of diosgenin on various autophagy-related protein expressions including LC3-II, LC3-I, p62 expressions. The results showed that diosgenin led to increase of LC3-II and LC3-I expressions and decrease of p62 expression.

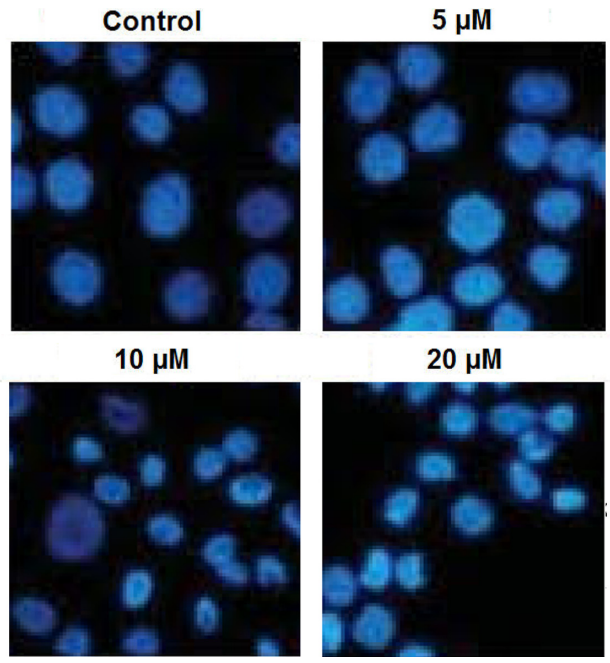

Figure 6. Fluorescence microscopy studies using DAPI (4',6-diamidino-2-phenylindole) staining showing diosgenin could induce programmed cell death as indicated by increasing chromatin condensation and nuclear fragmentation with increasing diosgenin dose. The experiments were performed in triplicate.

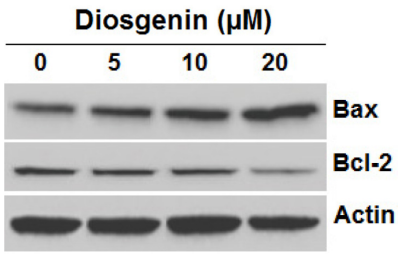

Figure 7. Effect of indicated doses of diosgenin on the expression of apoptosis-related proteins (Bax and Bcl-2) by western blot analysis. The experiments were performed in triplicate.

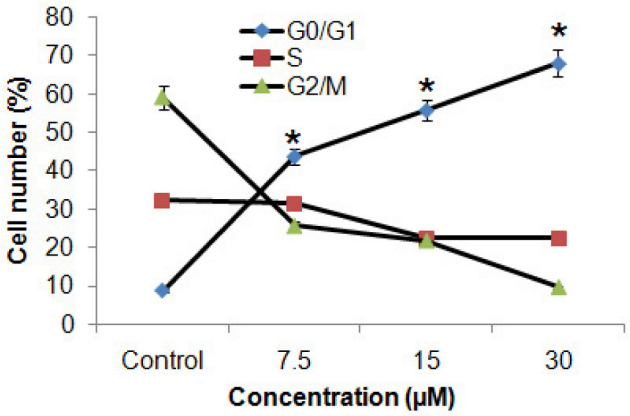

Figure 8. Diosgenin triggered G0/G1 cell cycle arrest of the HBL-52 optic nerve sheath meningioma cells as revealed by flow cytometry. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

cytometric measurements showed that diosgenin led to G0/G1 cell cycle arrest in a concentration-dependent manner. With increase in the diosgenin dose, the HBL-52 cells in G0/G1 phase were also seen to upsurge. The results of this assay are shown in Figure 8 and show that at 30 μM dose of diosgenin, the percentage of G0/G1 cells increased up to more than 70%.

Discussion

The anticancer activity of plant extracts and single molecules is well-documented. In fact plants remain the main source of anticancer and anti-infective drugs. Plant derived molecules exert their antitumor effects through a wide range of molecular mechanisms including targeting cell cycle phase distribution, inducing apoptosis (programmed cell death), generation of reactive oxygen species (ROS), collapse of mitochondrial membrane potential, inhibition of cell migration and invasion, targeting various key biochemical signalling pathways including PI3K/AKT, Nf-kB, m-TOR, MAPK/ERK pathway etc. Most of the anticancer drugs which have been clinically approved for cancer treatment are either pure natural products or their synthetic derivatives. These approved anticancer drugs include taxanes, vincristine, vinblastine, etoposide, teniposide, podophyllotoxins etc [10-13]. In the current study, we evaluated the anticancer effects of a steroidal saponin - namely diosgenin - against HBL-52 optic nerve sheath meningioma cells along with examining its mode of action by studying its impact on cell autophagy, cell cycle, programmed cell death and cell migration and invasion. Previous published reports have shown that this molecule has a strong anticancer potential and shows anticancer activity in a wide-range of cancer cells. Diosgenin has been reported to exhibit anticancer activity by reducing lipid peroxidation, and increasing antioxidant defense system in NMU-induced breast cancer [14]. In another study, the authors reported that diosgenin suppressed the growth of osteosarcoma cells by inducing G1 phase cell cycle arrest and initiating programmed cell death. It was also shown that diosgenin could activate p53 and binding of Nf-kB to DNA [15,16]. In other published reports, it has been shown that diosgenin induced cytotoxicity in human leukemic cells causing 50% of cell cytotoxicity in chronic myelogenous leukemia cells at a dose level of only 25 μM [17]. This molecule has been shown to induce anticancer effects in a wide range of cancers including osteosarcoma, leukemia, colon cancer, erythroleukemia, melanoma etc [18-20]. However, the anticancer effects of diosgenin in HBL-52 optic nerve sheath meningioma cells have not been reported so far. Hence, in the present study we reported its effects on HBL-52 optic nerve sheath meningioma cells along with deciphering its effects on cell cycle, apoptosis, cell migration and invasion and autophagy. The results showed that diosgenin led to concentration-dependent antiproliferative effects in these cells showing an IC_{50} value of 8.5 μM. Diosgenin also induced inhibitory effects on both cell migration and invasion in a dose-dependent manner as was clearly visible from the wound width at high doses of the diosgenin molecule. Diosgenin also triggered the formation of autophagosomes, characteristic of autophagy, which was also accompanied with dose-dependent increase of LC3-II and LC3-I expression and inhibition of p62 expression. This molecule also led to induction of cellular apoptosis as revealed by fluorescence microscopy and western blot. Finally diosgenin could also trigger G0/G1 cell cycle arrest.

Conclusion

To sum up, these results indicate the anticancer potential of diosgenin in HBL-52 optic nerve

sheath meningioma cells as this molecule could induce autophagy, apoptosis, cell cycle arrest as well as inhibition of cell migration and invasion.

Conflict of interests

The authors declare no conflict of interests.

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