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ORIGINAL ARTICLE

Friedelin inhibits the growth and metastasis of human leukemia cells via modulation of MEK/ERK and PI3K/AKT signalling pathways

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Summary

Purpose: *Leukemia constitutes just 3% of all malignancies but because of its high incidence and mortality in children and persons below 40 years of age, it is considered one of the devastating malignant conditions. This study was undertaken to evaluate the anticancer effects of Friedelin triterpenoid against the human AML-196leukemia cells.*

Methods: *CCK-8 assay was used to determine cell viability. DAPI staining was used for the assessment of apoptosis. Annexin V/propidium iodide (PI) analysis was employed for the detection of percentage of apoptosis. Transwell assays were used for cell migration and invasion. Western blotting was used for the determination of protein expression.*

Results: *The results showed Friedelin inhibited the proliferation of the human AML-196 cells with little effects on the normal cells. Investigation of the underlying mechanisms showed that Friedelin induced apoptosis in AML-196 cells. Friedelin-induced apoptosis was linked with upregulation of cleaved caspase-3, 8 and 9, as well as cleaved PARP. The Bax protein levels were increased and of Bcl-2 were decreased. Transwell assays showed that Friedelin suppressed the migration and invasion of the AML-196leukemia cells. Additionally, Friedelin also blocked the MEK/ERK and PI3K/AT signalling cascades dose-dependently.*

Conclusion: *Taken together, Friedelin may prove beneficial in the treatment of leukemia.*

Key words: *leukemia, apoptosis, cell cycle, Friedelin, proliferation*

Introduction

Plants are sessile organisms and in order to cope up with the changing environmental conditions, they synthesize a huge number of natural compounds. Triterpenoids have shown tremendous health promoting benefits [1]. Studies have shown that many of the triterpenoids suppress the growth of human cancers [2]. The triterpenoids derived from plants have been used in the treatment of different human disorders in Asian countries [3]. Friedelin has been isolated from several plant species such as *Azimatetracantha* and *Maytenusilicifolia* to name a few [4,5]. Studies have shown that Friedelin suppresses the growth of glioblastoma cells [6]. Although leukemia constitutes just 3% of all malignancies but because of its high incidence and mortality rate and high incidence in children and persons below 40 years of age, it is considered one of the devastating conditions [7,8]. Only few

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efficient chemotherapeutic agents are available for leukemia and they too have severe side effects [9]. With this background, the identification of novel therapeutics may enable the proper management of leukemia. Given mortality, there is high urgency to identify compounds that are safer and effective in the treatment of leukemia.

The present study reports that Friedelin significantly suppressed the growth of the human leukemia via induction of apoptosis. This molecule also suppressed the migration and invasion of AML-196 leukemia cells via blocking of the MEK/ERK and PI3K/AKT signalling pathway. Taken together, Friedelin may prove beneficial in the treatment of leukemia.

Methods

Growth and proliferation assay

Cell counting kit-8 (CCK-8, MedChemExpress) was used for estimation of the proliferative rates of AML-196 leukemia cells treated with Friedelin (Sigma-Aldrich, St. Louis, Missouri, United States) which were compared with those of HS-5 normal bone marrow cell lines. In brief, the cells were placed in 96-well plates at 1×10^6 cells/well and cultured for 24h with 0, 2.5, 5, 10, 20, 40, 80 and 160μM Friedelin, after which CCK-8 was employed to estimate the proliferation rates by the addition of 10μl of CCK-8 solution to each well, at the indicated time intervals. Following 2-h incubation at 37°C, absorbance at 450 nm was read for each sample with of microplate reader.

Apoptosis assay

DAPI staining was used to examine thse effect of Friedelin on the viability of leukemia cells and induction of cell apoptosis. The cells were placed in 12-well plates at a density of 0.6×10^6 cells/well. Friedelin at the concentrations of 0, 5, 10 and 20 μM was added to each well and cells were incubated at 37°C for 24 h. Afterwards, the cells were harvested and washed twice with phosphate buffered saline (PBS), followed by fixing with 4% paraformaldehyde. The DAPI solution was then used to stain the cells. Afterwards, the cells were examined for fluorescence measurements using fluorescent microscope. Annexin V/PI staining was used as described previously [3] to determine the percentage of apoptotic cells.

Migration and invasion assay

Transwell chamber with or without Matrigel coating was used to assess the migration and invasion of AML-196 leukemia cells. Briefly, 100μl cell culture containing 6000 cells was added to the upper chamber of the transwell and the lower chamber was added with 750μl of DMEM supplemented with 10% fetal bovine serum (FBS). After 48-h incubation at 37°C/5% CO_2, cells from the surface of membrane's upper side were removed carefully with cotton swabs while those sticked to lower side of membrane were fixed with 70% ethyl alcohol and stained with 0.1% crystal violet. Light microscope (×100) was used for visualization of cells and photographs were taken. At least seven random fields were used for counting of migratory or invasive cells.

Western blotting

Using RIPA lysis buffer (Thermo Fisher Scientific, Waltham, Massachusetts, United States), total proteins were isolated from untreated leukemia cells and cells treated with 10, 20 and 40μM Friedelin for 24 h. Bradford method was used to quantify the protein concentrations. About 45 μg of total proteins from each sample were separated electrophoretically on 10% SDS-PAGE. The gel was blotted to nitrocellulose membrane which gave the exposure of primary protein antibodies followed by exposure of secondary antibodies. Enhanced chemiluminescence (ECL) reagent was used for the detection of bands corresponding to proteins of interest. The protein expression was normalized with GADPH protein.

Statistics

The experiments were performed in triplicate and expressed as mean ± SD. One-way ANOVA followed by Turkey's test were performed using SPSS software. P value < 0.05 was considered as showing statistically significant difference.

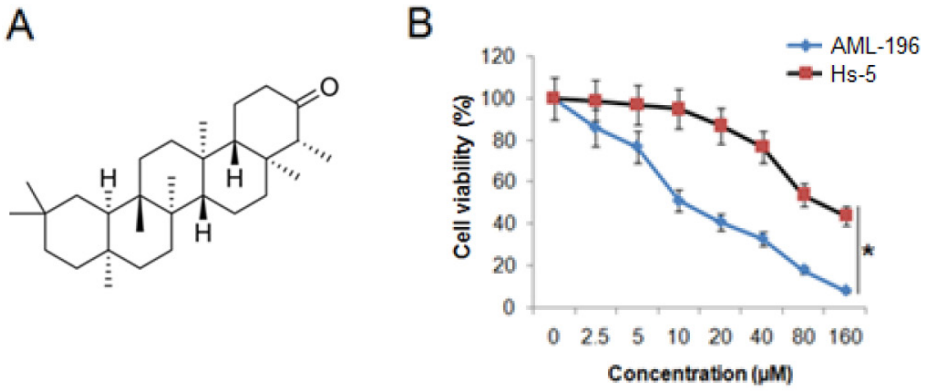

Figure 1. A: Chemical structure of Friedelin. **B:** CCK-assay showing the effect of Friedelin on the viability of Leukemia AML-196 and Hs-5 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Results

Friedelin suppresses the growth of leukemia cells

Friedelin (Figure 1A) exerted inhibitory effects on the proliferation of leukemia cells as ascertained by CCK-8 assay. The normal Hs-5 bone marrow cells and AML-196 leukemia cells were treated with 0 to 160 μM of Friedelin for 24 h and it was found that the viability of AML-196 cells decreased proportionally with the increasing doses of Friedelin with an IC_{50} of 10 μM (Figure 1B). Surprisingly, the effects of the molecule on the normal NCI-H526 cells were less severe as evidenced from the IC_{50} of around 80 μM.

Friedelin promotes apoptosis in AML-196 leukemia cells

The DAPI staining revealed that the suppressive effects of Friedelin on the proliferation of AML-196 human leukemia cells were mainly because of the initation of apoptosis as evidenced from the increase in the nuclear fragmentation (Figure 2). The extent of apoptosis induced by Friedelin was determined by annexin V/PI staining. The percentage of apoptosis increased from 3.8% to 27.8% with 20 μM Friedelin compared to control cells (Figure 3).

Friedelin alters apoptosis-related protein expression

Friedelin-induced apoptosis was also confirmed by examining the effects of the expression of the apoptosis-related proteins. The results showed a concentration-dependent increase in the proteins levels of cleaved caspsase-3, 8 and 9 as well as of the cleaved PARP in Friedelin-treated AML-196 cells. Furthermore, the expression of Bax increased while of Bcl-2 showed a dose-dependent decrease (Figure 4).

Friedelin suppresses migration and invasion of AML-196 cells

The determination of the effects of Friedelin on the migration and invasion of the human AML-196 leukemia cells was done by transwell assays.

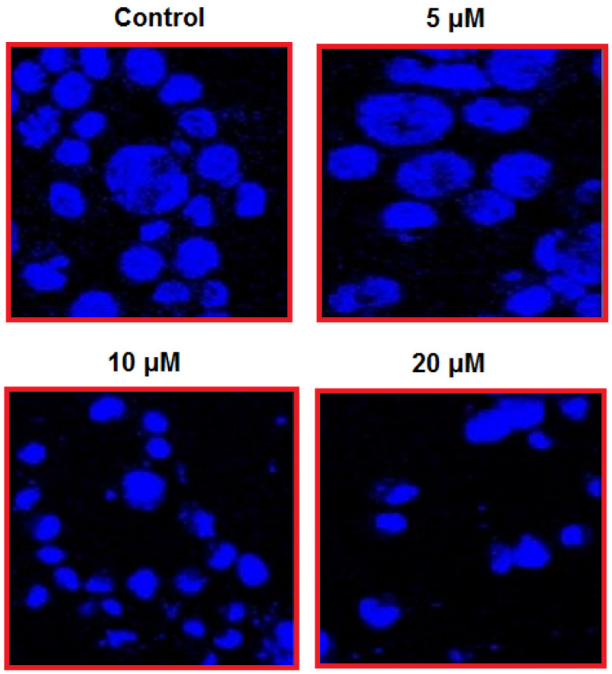

Figure 2. DAPI staining showing the effect of Friedelin on the nuclear morphology of the AML-196 cells at varied doses. The experiments were performed in triplicate.

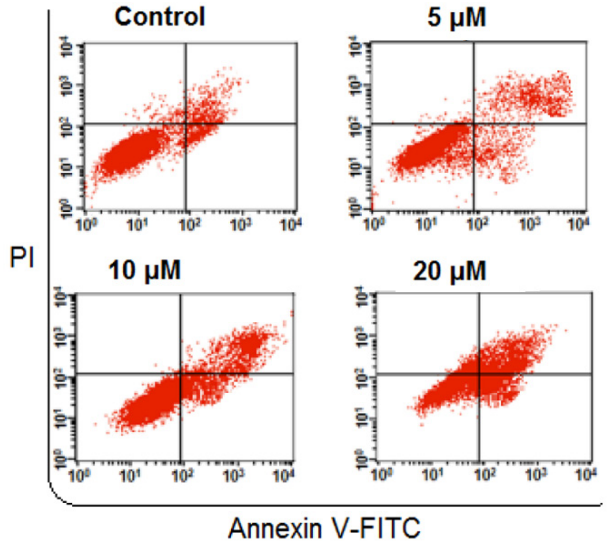

Figure 3. Annexin V/PI staining showing the effect of Friedelin on the percentage of apoptosis of the AML-196 cells at varied doses. The experiments were performed in triplicate.

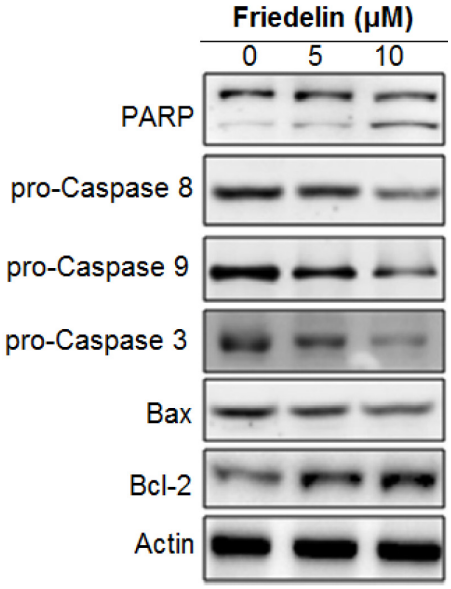

Figure 4. Western blot analysis showing the effects of Friedelin on the expression of apoptosis-related proteins. The experiments were performed in triplicate.

The results revealed that the molecule suppressed the migration of the AML-196 cells concentration-dependently (Figure 5). The effects of this molecule were also determined on AML-196 cell invasion and the results were similar to cell migration (Figure 6).

Friedelin blocks MEK/ERK and PI3K/AKT signalling

The impact of Friedelin were also evaluated on the MEK/ERK signalling pathway. It was found that the protein levels of p-MEK and p-ERK declined significantly and concentration-dependently (Figure 7). Additionally, it was revealed that the molecule caused concentration-dependent inhibition of phosphorylation of PI3K and AKT (Figure 8). Nonetheless, there was no apparent effect on the total PI3K and AKT.

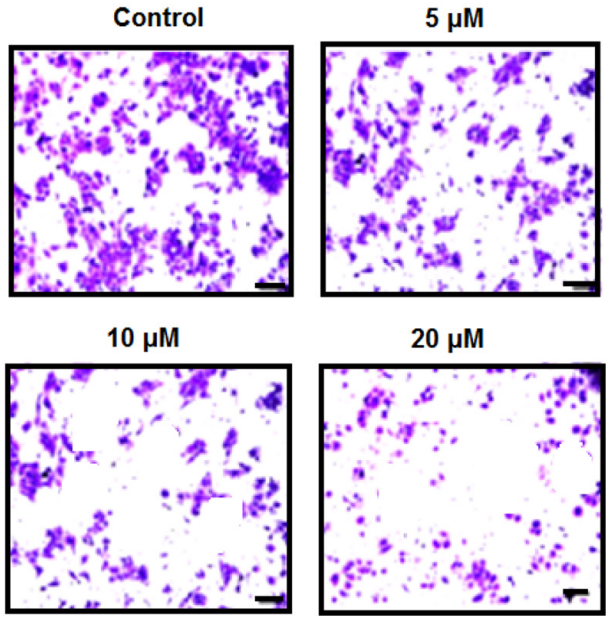

Figure 5. Effect of Friedelin on the migration of the AML-196 leukemia cells. The experiments were performed in triplicate.

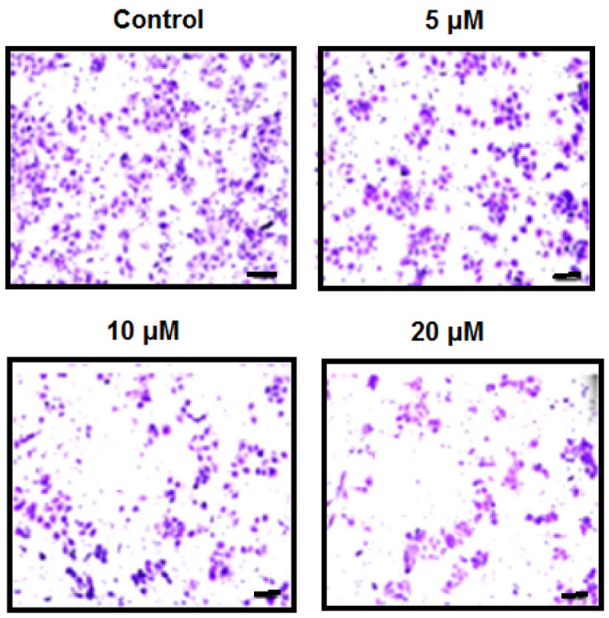

Figure 6. Effect of Friedelin on the invasion of the AML-196 leukemia cells. The experiments were performed in triplicate.

Discussion

During the course of evolution plants have learned to defend themselves by synthesising a wide array of molecules which also include trit-

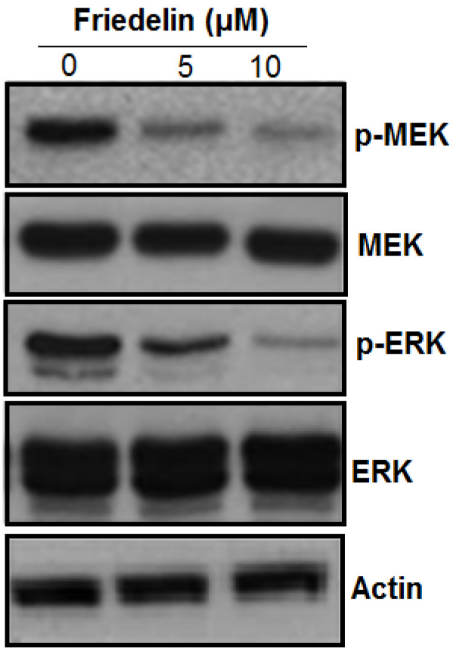

Figure 7. Western blots showing the effect of Friedelin on the MEK/ERK signalling pathway. The experiments were performed in triplicate.

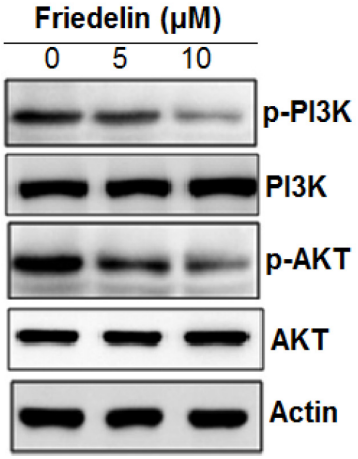

Figure 8. Western blots showing the effect of Friedelin on the PI3K/AKT signalling pathway. The experiments were performed in triplicate.

erpenoids. Triterpenoids constitute a large and diverse group of plant-derived compounds with enormous pharmacological potential [10] and they ubiquitously present across the plant kingdom. These plant-derived metabolites have a wide array of biological roles in plants which include defense against the biotic and abiotic stresses. Accordingly, active research is going on to examine the anticancer effects of triterpenoids against different human cancers. This study was undertaken to evaluate the anticancer effects of Friedelin, a naturally occurring triterpenoid of plant origin against the human leukemia cells. The cell proliferation assay showed significant inhibition of the leukemia cells growth upon Friedelin treatment. Previous studies have shown that triterpenoids have the potential to trigger apoptosis in the leukemia and neuroblastoma cells [11].More precisely, Friedelin has been shown to suppress the growth of breast cancer cells via induction of apoptosis [12].The expression of cleaved caspase-3, 8 and 9 as well as that of cleaved PARP was remarkably increased. Additionally, the Bax/Bcl-2 ratio was also increased which is an important indicator of apoptosis [13]. Apoptosis plays key role in eliminating the defective cells and thus drugs that promote apoptosis are currently being studied extensively [14]. This study also examined the effects of Friedelin on migration and invasion of the AML-196 cells and found that this molecule suppressed their migration and invasion. These findings are in agreement with a previous study wherein a triterpenoid Ursolic acid has been found to suppress the migration and invasion of cancer cells [15]. MEK/ERK and PI3K/AKT signalling pathways are among the critical signalling cascades that are dysregulated in cancer cells [16]. Herein we observed that Friedelin blocks both the MEK/ERK and PI3K/AKT signalling pathway. Hence, more studies are required to establish Friedelin as a lead molecule for the development of new therapeutic agents for leukemia.

Conclusion

Taken together, the results of the present study showed that Friedelin inhibited the growth of the human leukemia cells via induction of apoptosis and G2/M cell cycle arrest. Friedelin also suppresses the migration and invasion of the leukemia cells and may therefore prove beneficial in the treatment of leukemia.

Funding support

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Conflict of interests

The authors declare no conflict of interests.

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