ORIGINAL ARTICLE

In vitro and *in vivo* growth inhibition of human leukemia cells by Nodakenetin are mediated via mitochondrial apoptosis, cell cycle arrest and inhibition of cell migration and invasion

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Summary

Purpose: *To study the anticancer potential of a plant-derived coumarin, Nodakenetin, against acute lymphocytic leukemia HL-60 cells.*

Methods: *The proliferation rate of the leukemia cells was checked by CCK-8 assay. Apoptotic cell death was studied by acridine orange (AO)/ethidium bromide (EB) staining. Cell cycle analysis was performed by flow cytometery. Cell migration and invasion were checked by transwell assay. Protein expression were determined by immuno blotting. Xenografted mice models were used for in vivo evaluation of Nodakenetin.*

Results: *Nodakenetin could significantly inhibit the proliferation of the all the leukemia cells. The anticancer activity of Nodakenetin against the HL-60 cells was found to be due to G2/M cell cycle arrest and induction of apoptosis. Nod-*

akenetin prompted mitochondrial apoptosis which was also associated with alteration in the apoptosis-related protein expression (Bax and Bcl-2). It was also observed that Nodakenetin could inhibit the migration and invasion of the leukemia cells in a concentration-dependent manner. The effects of the Nodakenetin were also investigated in vivo in xenografted mice models and it was found that this molecule could inhibit the growth of xenografted tumors.

Conclusions: *These results indicate Nodakenetin significantly inhibits the growth of leukemia cells in vitro and in vivo and may be a valuable molecule in the management of leukemia, and as such needs further in depth investigations.*

Key words: *nodakenetin, leukemia, apoptosis, flow cytometry, cell migration, proliferation*

Introduction

Leukemia is one of the destructive malignancies and as per the American Cancer Society estimates around 0.315 million people develop any form of leukemia every year in USA and out of these approximately 0.215 million die of this dreadful disease in USA [1,2]. Although accounting for less than 3% of all cancers, leukemia is still one on the leading causes of death due to its occurence in children and persons below the age of 40 [3]. Late diagnosis and lack of potent and safe chemotherapeutic drugs form an obstacle in the treatment of leukemia [4]. Plant-deriving chemical scaffololds form an excellent source of drugs for the treatment

of human diseases and diseases [5]. Herbal extracts prepared from different plant parts have been used since times immemorial, but use of pure isolated compounds started only in the 19th century [6]. Since then a wide array of molecules have been isolated, evaluated and used for the treatment of several malignancies and other diseases [7]. Among plant metabolites, the ubiquitous flavonoids have shown promising potential for drug development. Coumarins are one of the widespread plant metabolites and exhibit an exceptional pharmacological potential [8]. Nodakenetin is an important coumarin that is also believed to carry important

pharmacological potentials [9]. It is prevalently isolated from the many plant species such as *Aegle marmelos* and has been reported to halt the growth of several types of cancer cells [8,9]. For example, Nodakenetin, also known as marmesin, has been shown to inhibit the growth of leukemia cells [10]. However, the anticancer effects of Nodakenetin are yet largely unknown. Herein, we report the anticancer effects of Nodakenetin against acute lymphocytic leukemia HL-60 cell line.

The purpose of this study was to evaluate the *in vitro* and *in vivo* anticancer activities of Nodakenetin against human leukemia cells by assessing its effects on apoptosis, cell cycle, and cell migration and invasion.

Methods

Cell counting kit-8 (CCK-8) assay

Acute lymphocytic leukemia HL-60 cell line was inoculated in 96-well plate, and subjected to treatment with Nodakenetin at various concentrations (0, 3.06, 6.12, 12.5, 25, 50 and 100μM). The number of HL-60 cells was measured at a each concentration. The procedures were as follows: the culture medium (Dulbecco's Modified Eagle Medium, DMEM) was discarded and 100 μL CCK-8 reagent (Beyotime Institute of Biotechnology, Shanghai, China) were added to a fresh DMEM medium. The 96-well plate was incubated in a carbon dioxide incubator for 2 h. The optical density (OD) values were measured by a microplate reader at the wavelength of 450 nm. The cell proliferation rate (%) was calculated as follows: OD value of experimental well - OD value of control well/OD value of control well ×100%.

AO/EB staining for apoptosis

For detection of apoptosis, the acute lymphocytic leukemia HL-60 cells ($0.6×10^6$) were grown in 6-well plates. Following an incubation of around 12 h, the HL-60 cells were subjected to Nodakenetin treatment for 24 h at 37°C. As the cells sloughed off, 25 μl cell cultures were put onto glass slides and subjected to staining with a solution (1 μl) of AO and EB. The slides were covered with a cover slip and examined under a fluorescent microscope.

Cell migration and invasion assay

The migration and invasion abilities of the HL-60 cells were examined by transwell chamber assay. In brief, $1×10^4$ HL-60 cells were seeded in upper chamber of the transwell (8 μm pore size polycarbonate filters). This was followed by the placement of the chambers into 24-well plates and subjected to incubation at 37°C for 48 h. However, in invasion assay, the inserts were coated with extracellular matrix gel (50 μl) (ECM, Sigma, USA). Swabbing was performed to remove the non-migrated and non-invaded cells from the upper surface. Then, the migrated and the invaded cells on the lower surface were subjected to fixation with methanol for about 35 min and

followed by staining with crystal violet (0.5%) for about 50 min, subjected to washing with phosphate buffered saline (PBS) and finally counted under light microscope (5 fields).

Determination of the reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) levels

For determination of the endogenous ROS levels, the HL-60 cells were treated with 0, 8, 16 and 32 μM of Nodakenetin for 24 h and then the MMP and ROS levels of the cells were determined as described previously [11].

Western blotting

To determine the expression of the selected proteins in the Nodakenetin treated HL-60 cells, the cells were subjected to lysis with RIPA buffer and the protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples 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. Following 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.

In vivo study

The *in vivo* evaluation of Nodakenetin was performed in xenografted mice models. The mice were injected
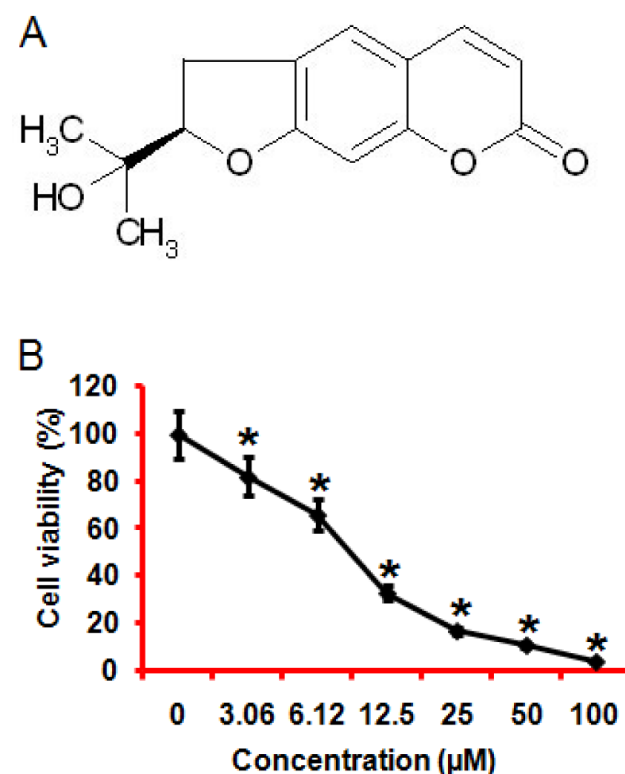

Figure 1. A: Structure of Nodakenetin. **B:** Nodakenetin inhibited the proliferation rate of the HL-60 leukemia cells as depicted by the cell counting kit-8 (CCK-8) assay. The values are mean of three biological experiments (*p<0.01).
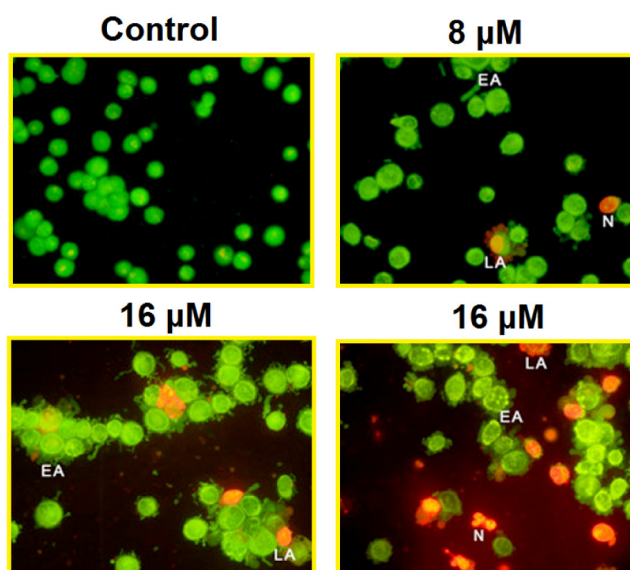
Figure 2. AO/EB staining showing the induction of apoptosis in the HL-60 cells by Nodakenetin in a dose-dependent manner. EA and LA represent early apoptotic and late apoptotic cells respectively, while N indicates necrotic cells. The experiments were performed in triplicate.
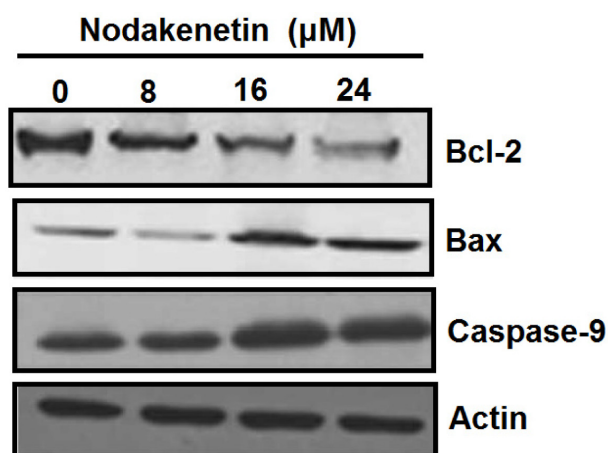

Figure 3. Impact of Nodakenetin on the expression of Bax, Bcl-2 and caspase 9 in the HL-60 leukemia cells as indicated by western blot analysis. The Figure shows that Nodakenetin caused upregulation of Bax and Caspase-9 and downregulation of Bcl-2. The experiments were performed in triplicate.
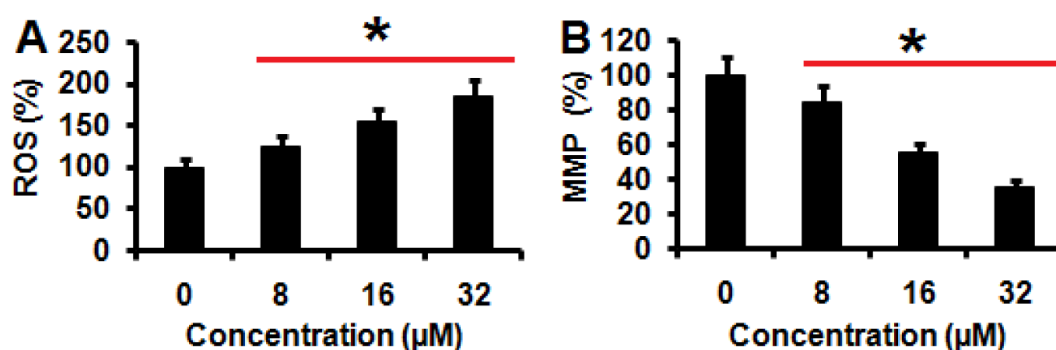
with 5×10^6 HL-60 cells sub-cutaneously at the left flank. As the tumors were apparent (~4 mm after about 2 weeks), the mice (n=5) of each group were injected intraperitoneally with DMSO (0.1 %) dissolved Nodakenetin and diluted with 100 μL normal saline at 20, 40 and 80 mg/kg and taken as the day one of the experiment. Nodakenetin was administered to the mice three times a week, while the control mice were administered DMSO (0.1%) in normal saline only. At the end of the study, the mice were euthanized and tumors were harvested for assessment of tumor growth and other investigations.

Statistics

Data are shown as mean±SD. Statistical analyses were done using Student's *t*-test with GraphPad prism 7 software. P values <0.05 were considered as statistically significant.

Results

Nodakenetin inhibits the growth of leukemia cells

The effects of Nodakenetin on the proliferation of the leukemia HL-60 line were examined by CCK-8 assay (Figure 1A) and it was found that Nodakenetin exerted antiproliferative effects on these cells with IC_{50} of 16 μM. In addition, it was found that the anticancer effects of Nodakenetin on the leukemia cells were concentration-dependent (Figure 1B).

Nodakenetin induces apoptosis in leukemia cells

The effects of Nodakenetin on the HL-60 leukemia cells were firstly evaluated by fluorescent microscopy after AO/EB staining. It was found that Nodakenetin triggered apoptosis as indicated by the nuclear morphology and membrane blebbing of HL-60 leukemia cells (Figure 2). For the confirmation of apoptosis the expression of apoptosis-associated proteins was examined and it was found that Nodakenetin caused upregulatin of Bax and Caspase 9 and downregulation of Bcl-2 (Figure 3).
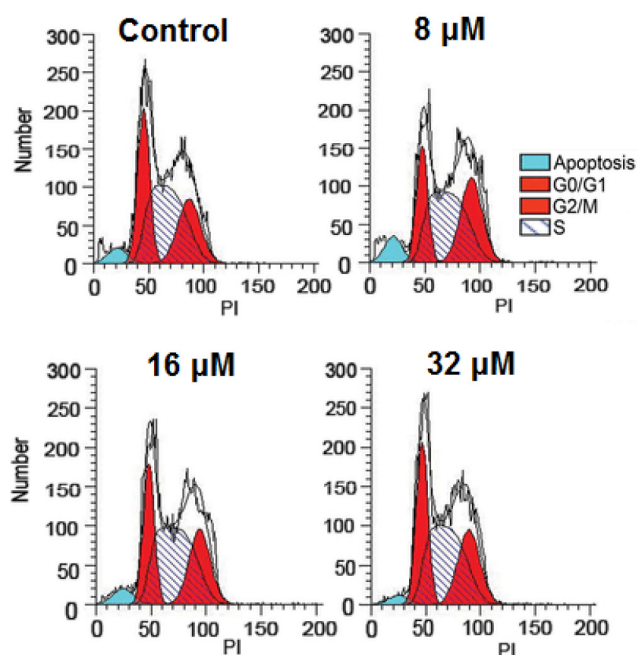

Figure 4. Nodakenetin caused increase in the ROS levels **(A)** and decrease in the MMP levels of the HL-60 leukemia cells **(B)**. The values are mean of three biological experiments (*p<0.01).

Figure 5. Nodakenetin triggered the G2/M cell cycle arrest of HL-60 leukemia cells in a dose-dependent manner as evidenced from flow cytometry. The experiments were performed in triplicate.

Nodakenetin affects the MMP and ROS levels in the HL-60 cells

The effect of the Nodakenetin was also examined on the endogenous ROS levels of HL-60 cells. The results showed that the levels of the endogenous ROS increased concentration-dependently in the HL-60 cells upon treatment with Nodakenetin (Figure 4A). However, unlike ROS, the MMP levels decreased concentration-dependently upon Nodakenetin treatment of HL-60 cells (Figure 4B)

Nodakenetin causes the G2/M arrest of HL-60 leukemia cells

The effects of Nodakenetin on the distribution of HL-60 cells in various cell cycle phases was assessed by flow cytometry. It was found that Nodakenetin caused remarkable increase in the percentage of the HL-60 cells in the G2 phase of the cell cycle. The percentage of HL-60 cells in the G2 phase increased from 10.12 to 48.15% upon treatment with Nodakenetin (Figure 5). These results clearly indicate that Nodakenetin induces G2/M cell cycle arrest of the leukemia cells.
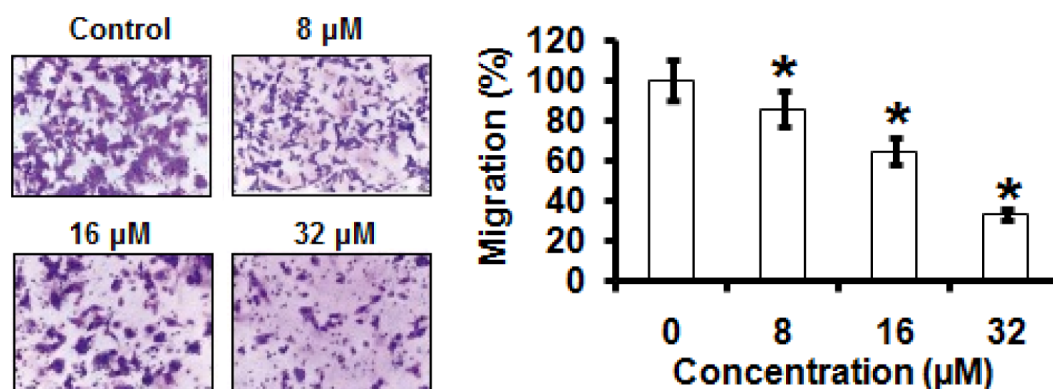

Figure 6. Inhibition of cell migration of the HL-60 leukemia cells upon treatment with Nodakenetin at indicated concentrations (Transwell chamber assay). The experiments were performed in triplicate and the values are expressed as mean ± SD (*p<0.01).
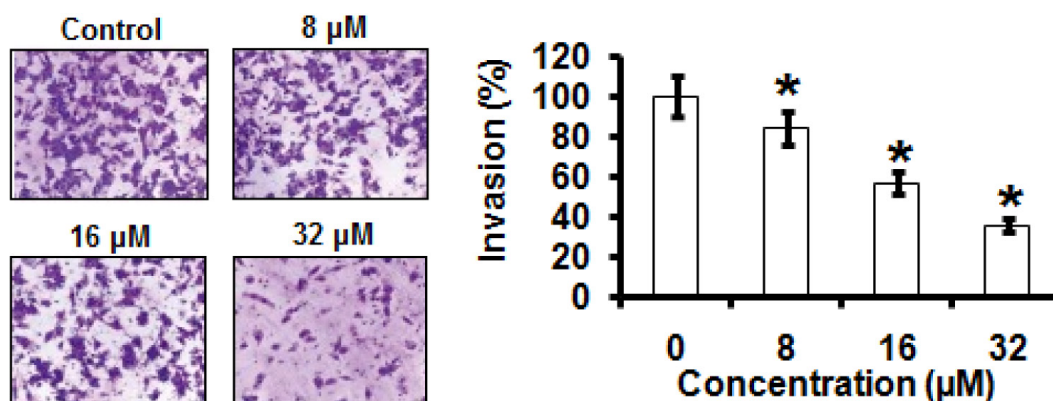

Figure 7. Suppression of cell invasion of HL-60 leukemia cells upon treatment with Nodakenetin at indicated concentrations (Transwell chamber assay). The experiments were performed in triplicate and the values are expressed as mean±SD (*p<0.01).
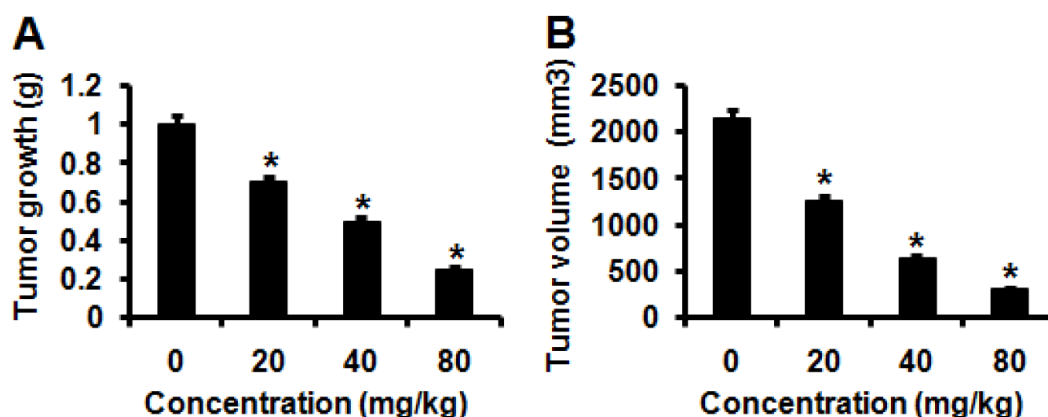
Figure 8. Nodakenetin inhibits the tumor growth **(A)** and tumor volume *in vivo* **(B)** in xenografted mice models at indicated concentrations. The experiments were performed in triplicate and the values are expressed as mean±SD (*p<0.01).

Nodakenetin inhibits the migration and invasion of HL-60 leukemia cells

The effects of Nodakenetin on the migration and invasion of the HL-60 cells were checked by transwell chamber assay. It was found that Nodakenetin treatment could considerably inhibit the migration of leukemia cells in a dose-dependent fashion (Figure 6). Similar effects were also found by Nodakenetin on the invasion of the HL-60 cells (Figure 7).

Nodakenetin inhibits the tumor growth in vivo

The effects of Nodakenetin were also examined in xenografted mice models. It was found that Nodakenetin could significantly inhibit the tumor growth and volume of the xenografted tumors in mice (Figure 8A and B).

Discussion

Leukemia accounts for 3% of all malignancies detected around the world, but it causes significant morbidity and mortality [11]. The chemotherapeutic agents used for the management of leukemia are generally inefficient and create severe adverse effects on the overall health of the patients [12]. Plant-derived anticancer agents have gained significant attention in the recent past due to their minimal toxic effects. Hence, many plant-derived natural products have been evaluated against a plethora of cancer cells and the process still continues [13]. Herein, the anticancer effects of Nodakenetin were examined against human leukemia HL-60 cell line and showed that Nodakenetin decreased the viability of these cells. A previous study has shown that Nodakenetin could inhibit the growth of non-small lung cancer cells [10]. Apoptosis eliminates the harmful cells from the body of an organism [14]. In this study the investigation of mechanism of action of Nodakenetin revealed that it prompts apoptotic cell death of the HL-60 cells. This was also associated with changes in the expression of apoptosis-related protein expression such as Bax, Bcl-2 and Caspase-9. Previously Nodakenetin (marmesin) has been reported to induce apoptosis in U937 human leukemia cells by inducing apoptosis [10]. ROS has been reported to be implicated in the induction of the apoptotic cell death [15] and herein we observed that Nodakenetin caused the generation of significant amounts of ROS in the HL-60 cells, which was also associated with reduction in the MMP levels. Furthermore, Nodakenetin caused arrest of the HL-60 cells at the G2/M check point and thereby halted their growth. A previous study has indicated that several of the anticancer molecules induce autophagy, apoptosis and cell cycle arrest of cancer cells [16]. The migration and invasion of cancer cells is an important determinant of their metastatic potential [19]. Herein, we observed that Nodakenetin inhibited both the cell invasion and migration of HL-60 cells. Because of the potent *in vitro* anticancer activity, we sought to know the antiproliferative effects of Nodakenetin *in vivo*. What was found was that Nodakenetin could inhibit the growth of xenografted tumors, indicative of the potential of Nodakenetin in the treatment of leukemia.

Conclusion

In summary, it is concluded that Nodakenetin inhibits the proliferation of human leukemia cells by apoptotic cell death. In addition, it can also induce cell cycle arrest and inhibition of tumor growth *in vivo*. As such, Nodakenetin could be an important therapeutic agent and warrants further investigation.

Conflict of interests

The authors declare no conflict of interests.

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