

ORIGINAL ARTICLE

Inhibition of anticancer growth in Retinoblastoma cells by naturally occurring sesquiterpene nootkatone is mediated via autophagy, endogenous ROS production, cell cycle arrest and inhibition of NF- κ B signalling pathway

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Summary

Purpose: The main aim of the present study was to determine the antiproliferative effects induced by nootkatone-a plant sesquiterpene ketone along with determining its effects on autophagy, reactive oxygen species (ROS) production, cell cycle, cell migration and NF- κ B signalling pathway.

Methods: Cell proliferation of HXO-Rb44 human retinoblastoma cell line was evaluated by CCK-8 assay, while autophagic effects were evaluated by electron microscopy and western blot. Effects on cell cycle and ROS production were evaluated by flow cytometry. In vitro wound healing assay was used to determine the effects on cell migration.

Results: The results indicated that nootkatone induced significant and dose-dependent cytotoxicity in HXO-Rb44 retinoblastoma cells with an IC₅₀ value of 10.2 μ M. Electron microscopy and western blot showed that nootkatone

could induce autophagy as autophagosomes and vacuoles were seen to develop after nootkatone treatment. Autophagy was confirmed by observing the expression levels of LC3B-II, LC3B-I and p62. Nootkatone led to an increase of LC3B-II and LC3B-I but also led to inhibition of p62 expression. Nootkatone also led to increase of ROS production dose-dependently along with inducing S-phase cell cycle arrest. Nootkatone also led to inhibition of cell migration along with inhibiting NF- κ B signalling pathway.

Conclusions: In conclusion, nootkatone molecule inhibits retinoblastoma by inhibiting NF- κ B signalling pathway and cell migration, autophagy induction, ROS generation and S-phase cell cycle arrest.

Key words: nootkatone, retinoblastoma, autophagy, cell cycle, flow cytometry

Introduction

Retinoblastoma which initiates from the immature retinal cells is a rare malignancy and mostly targets young children. About 4% of cancers found in children and infants belong to retinoblastoma. Retinoblastoma has been ranked number one cancer affecting eyes. In China, this cancer has recently seen a continuous increase [1,2]. It is reported that retinoblastoma is triggered by inactivation of the retinoblastoma Rb1 gene in retinal cells. Uncon-

trolled proliferation and faulty differentiation of human retinal cells arises due to lack of a functional pRb1 gene and this ultimately leads to development of retinoblastoma. Various risk factors have been identified which are responsible to cause retinoblastoma and include inactivation of the bi-allelic Rb gene in retinal cells, papilloma virus etc. [3,4]. Chemotherapy, radiotherapy, laser photocoagulation and eyeball enucleation are the currently

employed treatment options for retinoblastoma. However, chemotherapy in combination with local consolidation therapy like photocoagulation is the desired treatment option for retinoblastoma. Nevertheless, for advanced retinoblastoma, this treatment shows discouraging results coupled with the fact that chemotherapy is associated with side effects and multidrug resistance [5]. There is also an increased risk of developing secondary malignancies like osteosarcoma and melanoma. Further, following eyeball enucleation there are chances of vision loss and face disfiguration [6]. So keeping all these growing problems in mind, there is an urgent need to find alternative treatment modalities for retinoblastoma which are effective and devoid of any such issues.

Plant-derived natural products have always played key role in alleviating human diseases. Plant natural products have been used since times immemorial in treating a range of human disorders including cancer. About 60% of the anticancer drugs which are currently in clinical use are either pure natural products or their synthetic derivatives [7].

The main objective of the present study was to examine the antitumor effects of nootkatone-a plant sesquiterpene mostly used in perfumes. We also studied its effects on autophagy, endogenous ROS production, cell cycle phase distribution and Nf- κ B signalling pathway. To the best of our knowledge, this study reports for the first time ever the properties of this sesquiterpene against retinoblastoma along with studying its mode of action in great detail.

Methods

CCK-8 cell proliferation assay

HXO-Rb44 human retinoblastoma cell line was purchased from Chinese Academy of Sciences, Shanghai, China (Department of Biochemistry and Biology). The cells were kept in RMPI-1640 medium containing 10% fetal bovine serum (FBS), at 37°C in a 5% CO₂ incubator. The HXO-Rb44 cells were seeded in 96-well plates and firstly exposed to numerous doses of nootkatone after which 30 μ l CCK-8 (purchased from Dojindo Laboratories, Kumamoto, Japan) were added. Afterwards, incubation of cell culture plates was done for 12 h at 37°C. At the end of the experiment, the absorbance was measured at 450 nm wavelength using a microplate reader (Bio-Rad, Hercules, USA). The observed absorbance was used to measure cytotoxicity.

Transmission electron microscopy for autophagy detection

The HXO-Rb44 cells were treated with 0, 5, 10 and 20 μ M concentrations of nootkatone. After treatment the cells were fixed with a solution of 4% glutaraldehyde

with 0.05 M sodium cacodylate and then post-fixed in 2% osmium tetroxide and then dehydrated in ethanol. The HXO-Rb44 cells were then prepared for flat embedding in Epon 812 and then examined using a Zeiss CEM 902 electron microscope.

Cell cycle analysis and ROS generation

The HXO-Rb44 retinoblastoma cells (at a cell density of 2×10^5 cells/ml) cultured in RMPI-1640 medium were exposed to various concentrations (0, 5, 10, 20 μ M) of nootkatone molecule. Subsequently the cells were washed with phosphate buffered saline (PBS) and then stained using 10 μ l (20 μ g/mL) solution of propidium iodide (PI). The distribution of HXO-Rb44 cells in different phases of the cell cycle was determined by FACSCalibur flow cytometry. Estimation of ROS was measured according to a method previously described [8].

In vitro wound healing assay

After treatment of the HXO-Rb44 retinoblastoma cells with nootkatone, the RMPI-1640 medium was removed and the cells were subjected to PBS washing twice. A sterile pipette tip (10 μ l) was employed to scratch a wound in each well, the cells were washed again and a picture was captured. The plates were cultured for 24 h and a picture was taken again under an inverted microscope (Leica, Germany).

Western blot analysis

Western blot method was used to measure protein expressions. The nootkatone-treated HXO-Rb44 retinoblastoma cells (0, 5, 10 and 20 μ M) were harvested following centrifugation and then subjected to lysis using a RIPA-lysis buffer containing the protease inhibitor.

The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and treated 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 normalization.

Statistics

Data are shown as mean \pm SD. Statistical analysis was done using Students *t*-test with GraphPad prism 7 software. P value < 0.05 was considered as statistically significant.

Results

Nootkatone sesquiterpene induced significant cytotoxicity

Initially the HXO-Rb44 retinoblastoma cytotoxicity was determined using Cell Counting Kit-8 (CCK-8) assay. The cytotoxicity was measured at 0, 3.12, 6.25, 12.5, 25, 50, 100 and 200 μ M. The results indicated that nootkatone induced significant and

dose-dependent cytotoxicity in HXO-Rb44 retinoblastoma cells (Figure 1) showing an IC_{50} value of 10.2 μ M, indicating its high potential as a cytotoxic agent.

Nootkatone induced autophagic cell death in HXO-Rb44 retinoblastoma cells

Electron microscopy along with western blot assay was employed to investigate the autophagic effects of nootkatone in HXO-Rb44 retinoblastoma cells. The results showed that treating these cells with increasing doses of nootkatone led to the development of autophagosomes (Figure 2) and vacuoles which is an indication of autophagy. For the confirmation of nootkatone-induced autophagy, the

expression of autophagy-associated proteins was investigated and it was observed that nootkatone led to an increase of LC3B-II and LC3B-I, but also led to inhibition of p62 expression (Figure 3).

Nootkatone led to endogenous reactive oxygen species (ROS) production

Many molecules have the tendency to induce ROS generation in human cancer cells. Therefore, the ROS levels were examined in HXO-Rb44 retinoblastoma cells after treatment with various concentrations of this molecule and the results indicated that nootkatone treatment led to a concentration-dependent increase of ROS production. The effects of nootkatone on ROS production in HXO-Rb44 cells are depicted in Figure 4.

Nootkatone induced S-phase cell cycle arrest

The effects of nootkatone on cell cycle were evaluated by using PI staining and flow cytometry. The results which are shown in Figure 5 reveal that nootkatone at the tested doses led to an accumulation of cells in S-phase of the cell cycle. With increase in nootkatone dose from 0, 5, 10 and 20 μ M the S-phase cells increased significantly and dose-

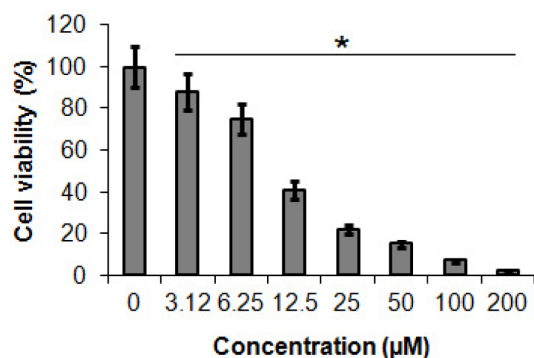


Figure 1. CCK-8 assay showing the effects of nootkatone on the viability of the HXO-Rb44 human retinoblastoma cells. The experiments were performed in triplicate and shown as mean \pm SD (* p < 0.05).

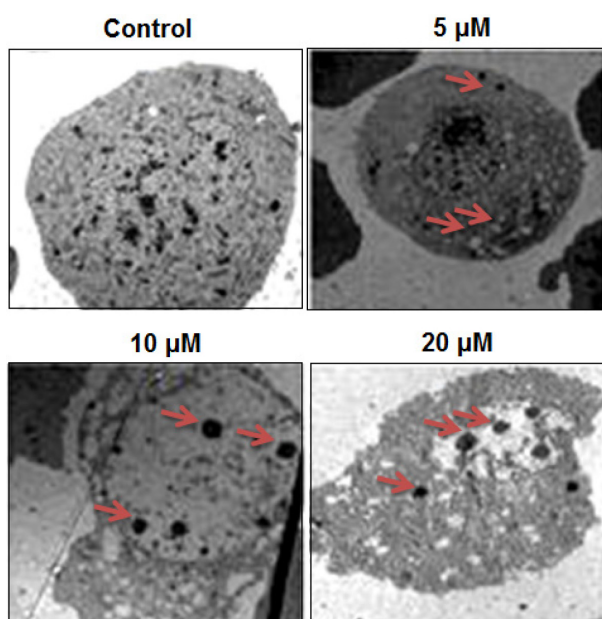


Figure 2. Electron microscopic analysis showing nootkatone inducing autophagy in HXO-Rb44 human retinoblastoma cells (Arrows depicting autophagosomes). The experiments were repeated thrice.

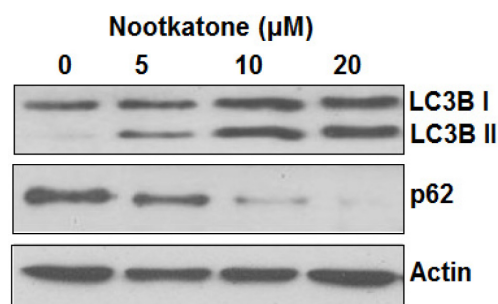


Figure 3. Effects of nootkatone on the expression of LC3B-I, LC3B-II and p62 in the HXO-Rb44 human retinoblastoma cells as shown by western blot analysis. The Figure shows that nootkatone increased the LC3B II and decreased the p62 expression. The experiments were repeated thrice.

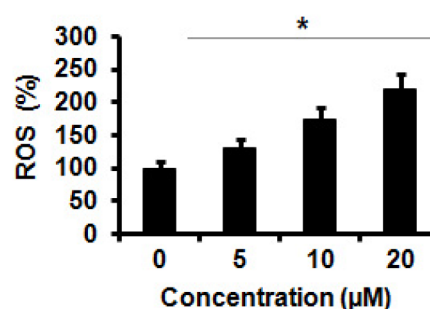


Figure 4. Effect of nootkatone on the reactive oxygen species (ROS) in HXO-Rb44 human retinoblastoma cells as revealed by flow cytometry. The experiments were repeated thrice (p < 0.05).

dependently, accompanied with a corresponding decrease in cells in G2/M phase of the cell cycle. This increase in S-phase cells was also accompanied with a concomitant concentration decrease in the expression of cyclin-B1 (Figure 6).

Nootkatone treatment led to suppression of cell migration and Nf-κB signalling pathway

The effects of this molecule on cancer cell migration in HXO-Rb44 retinoblastoma cells was examined by *in vitro* wound healing assay by measuring the scratch width. The results of this assay are shown in Figure 7 and reveal that nootkatone treatment at its IC₅₀ value led to a significant inhibition of cancer cell migration, indicating the potential of this molecule to curb cancer metastasis. Figure 8 also showed that nootkatone treatment at increasing doses led to a concentration-dependent inhibition of NF-κB signalling pathway.

Discussion

The standard treatment for retinoblastoma has produced unsatisfactory results coupled with the fact that chemotherapy and radiotherapy are associated with serious sideeffects. Additionally, these

treatment options also result in development of multidrug resistance in retinoblastoma cells along with the risk that it may lead to development of secondary cancers including osteosarcoma and melanoma [9]. Accordingly, there is a pressing need for alternative treatment options, especially the development of novel chemotherapeutic agents. Plants synthesize a huge array of molecules which can be classified into many categories like terpenoids, alkaloids, flavonoids, quinones etc. Many of these plant-derived molecules have been reported to exhibit a range of pharmacological activities including their ability to induce cytotoxic effects in cancer cells. These plant-derived molecules exert their anticancer effects by initiating a cascade of biochemical processes including cellular apoptosis, autophagy, cell cycle arrest, inhibition of cell migration and invasion along with targeting various biochemical signalling pathways [10-13]. In the current study, the anticancer effects of a naturally occurring sesquiterpene ketone, namely nootkatone, are reported along with its effects on

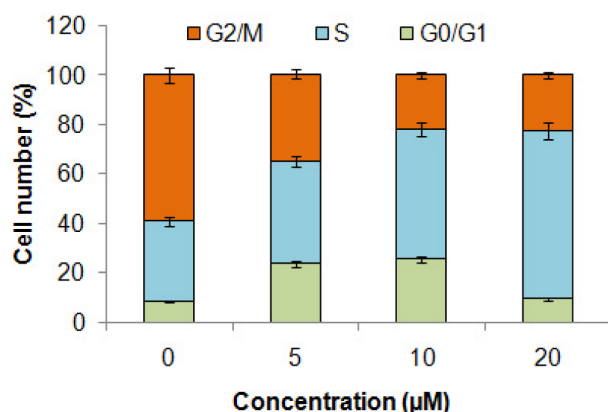


Figure 5. Effect of nootkatone on the cell cycle phase distribution in HXO-Rb44 human retinoblastoma cells as revealed by flow cytometry. The results showed that nootkatone led to S-phase cell cycle arrest in a dose-dependent manner. The experiments were repeated thrice ($p < 0.05$).

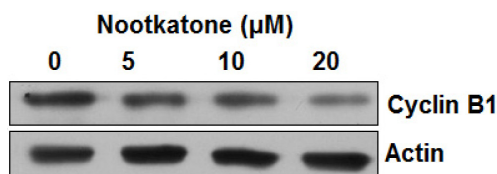


Figure 6. Effect of nootkatone on the cell cycle related protein cyclin-B1 in HXO-Rb44 human retinoblastoma cells. The Figure depicts that nootkatone suppresses cyclin B1 expression dose-dependently. The experiments were repeated thrice.

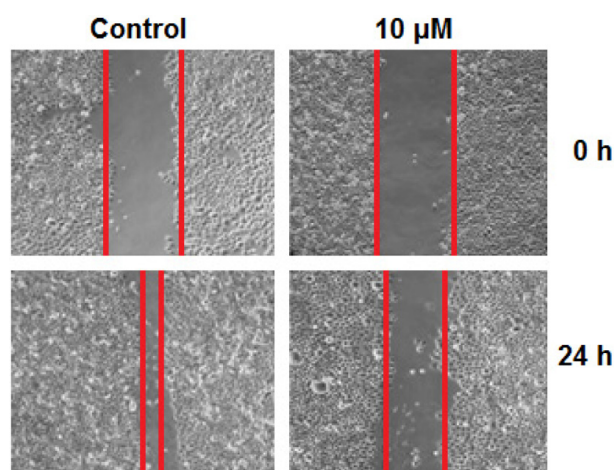


Figure 7. Effects of nootkatone on the migration of the HXO-Rb44 human retinoblastoma cells as depicted by *in vitro* wound healing assay. Nootkatone led to a significant inhibition of cell migration at 10 μM nootkatone dose. The experiments were performed in triplicate.

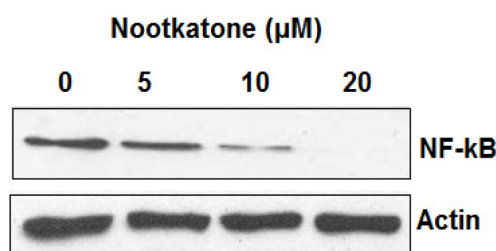


Figure 8. Effects of nootkatone on the NF-κB signalling pathway in HXO-Rb44 human retinoblastoma cells as depicted by western blotting. Nootkatone led to dose-dependent down-regulation of NF-κB protein expression. The experiments were performed in triplicate.

cell autophagy, cell cycle arrest, cell migration and invasion. Nootkatone has been reported to exhibit protective effects against particle-induced lung injury caused by diesel exhaust and this effect was mediated via the NF- κ B signalling pathway [14]. Nootkatone has also been shown to exert hepatoprotective and anti-fibrotic effects in a murine model of liver fibrosis by inhibiting oxidative stress and apoptosis [15]. There are however no reports on the anticancer activity of nootkatone in human retinoblastoma cells. In the current study it was shown that this molecule induced significant and dose-dependent cytotoxicity in HXO-Rb44 retinoblastoma cells with an IC₅₀ value of 10.2 μ M. Electron microscopy and western blot showed that nootkatone could induce autophagy as autophagosomes were seen to develop after nootkatone treatment. Autophagy was confirmed by observing the expression levels of LC3B-II, LC3B-I and p62. Noot-

katone led to an increase of LC3B-II and LC3B-I but also led to inhibition of p62 expression. This molecule also led to increase of ROS production dose-dependently along with inducing S-phase cell cycle arrest. Nootkatone also led to inhibition of cell migration along with inhibiting NF- κ B signalling pathway.

Conclusion

The results of this study indicate that nootkatone could inhibit the growth of HXO-Rb44 retinoblastoma cells by inducing autophagy, S-phase cell cycle arrest, ROS production and inhibiting cell migration and NF- κ B signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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