

Original Research Article

Bauerenol inhibits proliferation, migration and invasion of retinoblastoma cells via induction of apoptosis, autophagy and cell cycle arrest

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Sent for review: 17 January 2020

Revised accepted: 20 June 2022

Abstract

Purpose: To determine the anticancer effects of bauerenol on human retinoblastoma cells.

Methods: The effect of bauerenol on cell proliferation was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, while cancer cell migration and invasion were determined by Transwell assay. Apoptosis of retinoblastoma cells was assessed by Annexin V/FITC/PI staining procedure. Autophagy was evaluated using TEM, while cell cycle was studied by flow cytometry.

Results: Bauerenol significantly inhibited the proliferation of retinoblastoma cells, with a half-maximal inhibitory concentration (IC_{50}) of 10 μ M ($p < 0.05$). However, bauerenol exhibited a comparatively lower antiproliferative effect on normal paediatric retina cells, with a higher IC_{50} of 100 μ M. Annexin V/PI staining results revealed that the antiproliferative effect of bauerenol was due to apoptotic cell death. The proportion of apoptotic SORB-50 cells increased from about 4 % in control to about 19 % on exposure to 20 μ M bauerenol. Western blot assay showed marked up-regulation of LC3B II protein, indicating autophagy. Cell cycle analysis showed that the arrest of SO-RB50 cells at the G_2/M phase of the cell cycle markedly contributed to the antiproliferative effects of bauerenol. Moreover, the migration and invasion of SO-RB50 cells were suppressed by bauerenol ($p < 0.05$).

Conclusion: These results indicate that bauerenol suppresses the growth of retinoblastoma cells. Therefore, it may be a beneficial lead molecule for the development of a suitable agent for the treatment of retinoblastoma.

Keywords: Retinoblastoma, triterpenoid, anticancer, apoptosis, autophagy, migration, invasion, metastasis

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INTRODUCTION

Retinoblastoma is an aggressive type of cancer of the eye in humans, with highest prevalence in children [1,2]. It has been estimated that the mortality due to this disorder may be as high as 70 % in children in developing or

underdeveloped countries [3]. The aggressiveness of retinoblastoma is increased by its metastasis to neighbouring tissues, as well as instances of intracranial neuroblastoma [4]. The treatment strategies currently employed for retinoblastoma are not very effective [5]. Therefore, there is need to improve the efficacy

of anticancer treatments for this human malignancy by identifying more potent chemotherapeutic agents.

Plants have been recognized for many years as repositories of therapeutic agents for human diseases, as well as phytochemicals for maintaining human health. Indeed, majority of drugs in use today were developed from natural products and/or from compounds derived from natural products. Owing to the failure of alternative drug discovery methods, natural product research has continued to receive increased attention in the quest to identify lead therapeutic compounds for production of novel drugs [6,7]. Bauerenol is a bioactive component of *Suregada angustifolia*, a plant which belongs to the *Euphorbiaceae* family [7]. It has been reported that the triterpenoid-like bauerenol exerted anti-tumour properties and inhibited the proliferation of a number of human cancers [8]. Given this background, the present study was designed to determine the anticancer effects of bauerenol against human retinoblastoma cells, and to identify the underlying mechanism(s).

EXPERIMENTAL

Cell culture

The retinoblastoma cell line SO-RB50, and normal paediatric retina cells were acquired from ATCC, USA. The cells were maintained in RPMI-1640 medium (Thermo Scientific) at 37 °C in a humid atmosphere containing 5 % CO₂.

Determination of cell proliferation

The MTT assay was used to determine the proliferation of SO-RB50 cells and normal human retina cells after exposure to bauerenol at doses of 5, 10, 20, 50 and 100 µM for 24 h at 37 °C in 96-well plates. Untreated cells served as control. After 24 h, MTT reagent was added to the samples and incubation at 37 °C was continued for 4 h. The resultant formazan product was solubilized in DMSO after discarding the culture medium, and the absorbance of formazan solution in each well was read at 450 nm. The OD₄₅₀ values were used for calculation of the percentage proliferation of cells in each culture sample.

Evaluation of cell migration and invasion

The transwell chamber method was used to determine the effect of bauerenol on the migration and invasion of retinoblastoma cancer cells. Cell cultures (about 10⁵ cells) and

bauerenol at doses of 5, 10 and 20 µM were added to the upper portion of the transwell chamber, while the lower portion which contained only 10 % FBS, was separated from the upper chamber with a filter paper of five-micron size. Untreated cells in the upper chamber served as control. After incubation at 37 °C, the filter paper was removed, and its upper surface was wiped carefully to remove the cells. The lower side of the filter paper was washed and the migrated cells were fixed using 70 % ethanol. The cells were stained using 0.1 % crystal violet, followed by visualization of the cells under a light microscope. Similar steps were followed for the determination of invasion of SO-RB50 cancer cells, but in this case, Matrigel was used.

Apoptosis assay

Retinoblastoma cells were seeded in 6-well plates and treated separately with bauerenol at concentrations of 5, 10 and 20 µM at 37 °C for 24 h. Untreated cells served as control. Thereafter, the cancer cells were harvested through centrifugation at 5,000 rpm. The cells collected were rinsed with PBS and fixed. Then, the cells were sequentially stained with annexin V-FITC and PI in the dark, followed by analysis of apoptosis using a flow cytometer.

Assessment of cancer cell cycle

Following co-culturing with bauerenol at doses of 5, 10 and 20 µM in 12-well plates at 37°C for 24 h, cell cultures were centrifuged, and the harvested cells were fixed with 4 % formaldehyde. Then, the cells were mixed with propidium iodide solution, after which cell cycle phase distribution was investigated using a flow cytometer.

Western blotting

Total proteins were extracted from cancer cells treated with bauerenol at concentration of 5, 10 or 20 µM using RIPA buffer. Following centrifugation of the lysates, the protein concentrations were determined with BCA method. Then, equal amounts of proteins were resolved on SDS-polyacrylamide gel electrophoresis, and the separated proteins were electro-transferred to PVDF membranes. The membranes were incubated overnight at 4 °C with appropriate primary antibodies, followed by incubation with horse radish peroxidase-linked secondary antibody at room temperature for 2 h. The specific protein bands were visualized using ECL kit, and subjected to Grayscale analysis to determine the protein expression levels.

Statistical analysis

The experiments were performed in triplicate, and the results are expressed as mean \pm SD. Student's *t*-test was used to carry out statistical analysis. Graphics were prepared with GraphPad prism 7 software. Statistical significance of difference was assumed at $p < 0.05$.

RESULTS

Bauerenol selectively inhibited the proliferation of retinoblastoma cells

Figure 1 shows the molecular structure of bauerenol. When normal human retina cells and retinoblastoma cells were treated with 5, 10, 20, 50 or 100 μ M bauerenol for 24 h, and cells were subjected to MTT assay, it was seen that cell proliferation was inhibited in a concentration-dependent manner (Figure 2). The IC_{50} of bauerenol against SO-RB50 cells was 10 μ M. However, the toxic effect of bauerenol on normal retina cells was comparatively lower, as was evident from an IC_{50} of 100 μ M.

Effect of bauerenol on the migration and invasion of retinoblastoma cancer cells

The effects of bauerenol on the migration and invasion of retinoblastoma cancer cells were investigated through transwell assay. Treatment of cancer cells with bauerenol at concentrations of 5, 10 and 20 μ M resulted in suppression of cell migration in a concentration-dependent manner (Figure 3). Similar results were obtained regarding the effect of bauerenol on the invasion of the cancer cells (Figure 4). These results indicate that bauerenol inhibited the metastasis of retinoblastoma cells.

Bauerenol induced apoptosis and autophagy of retinoblastoma cells

Flow cytometric analysis of SORB-50 cancer cells treated with 5, 10 or 20 μ M bauerenol indicated that it induced cell apoptosis (Figure 5). The percentage of apoptotic SORB-50 cells increased from about 4 % in control to about 19 % in cells exposed to 20 μ M bauerenol. In addition, results from western blot assay showed that the protein expression of LC3-II was up-regulated, whereas LC3-I protein expression was almost unaltered, as shown in Figure 6. These results revealed that bauerenol treatment led to induction of apoptosis and autophagy of retinoblastoma cells.

Bauerenol modulated cell cycle phase distribution in retinoblastoma cells

Analysis of cell cycle distribution in retinoblastoma cells treated with bauerenol at concentrations of 5, 10 and 20 μ M revealed that the number of cancer cells at G_2 phase increased with increase in concentration of the molecule (Figure 7). The G_2 phase SO-RB50 cells increased from 7.32 % in control (untreated cells) to 20.95 % in cells treated with 20 μ M bauerenol. These results indicate that bauerenol treatment induced arrest of cell cycle at the G_2/M stage.

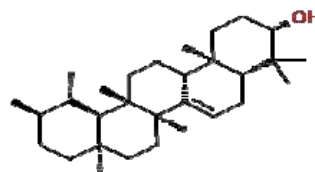


Figure 1: Molecular structure of bauerenol

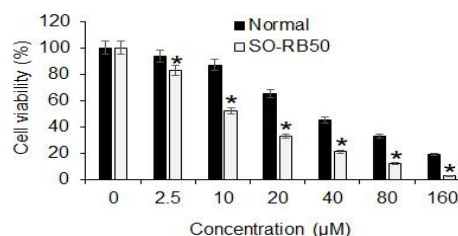


Figure 2: Bauerenol selectively inhibited the proliferation of retinoblastoma cells. The MTT assay was used for determination of proliferation of normal human retina cells and SO-RB50 retinoblastoma cells treated with bauerenol at concentrations of 5, 10, 20, 50 and 100 μ M. The experiments were performed in triplicate, and the results are expressed as mean \pm SD. * $P < 0.05$

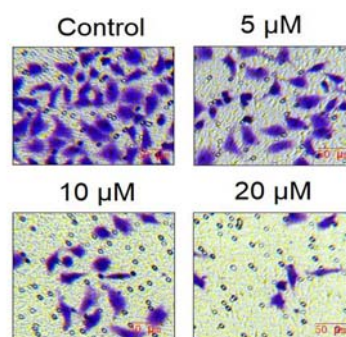


Figure 3: Bauerenol reduced the migration of retinoblastoma cancer cells. Transwell assay was used for determination of migration of SO-RB50 human retinoblastoma cells treated with bauerenol at doses of 5, 10 and 20 μ M. The experiments were performed in triplicate

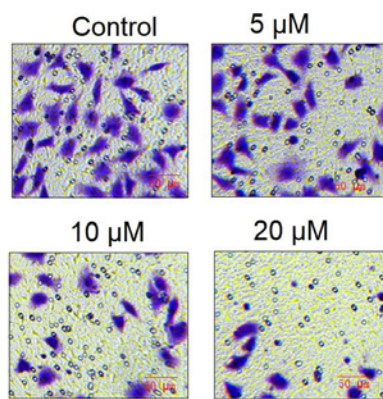


Figure 4: Bauerenol suppressed the invasion of retinoblastoma cancer cells. Transwell assay was used for determination of invasion of SO-RB50 human retinoblastoma cells treated with bauerenol at doses of 5, 10 and 20 μM . The experiments were performed in triplicate

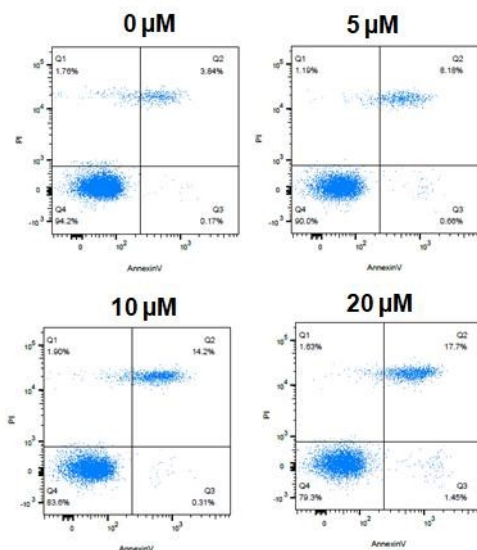


Figure 5: Bauerenol induced apoptosis of retinoblastoma cancer cells. Flow cytometric analysis showing percentages of apoptotic SO-RB50 human retinoblastoma cells treated with bauerenol at concentrations of 5, 10 and 20 μM . The experiments were performed in triplicate

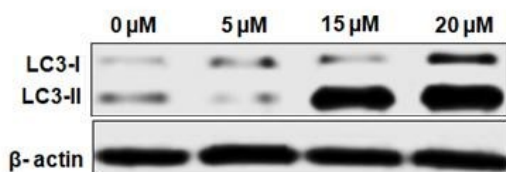


Figure 6: Bauerenol induced autophagy in retinoblastoma cancer cells. Western blot assay was used for determination of protein expressions of LC3I and LC3-II in SO-RB50 human retinoblastoma cells treated with bauerenol at concentrations of 5, 10 and 20 μM . The experiments were performed in triplicate

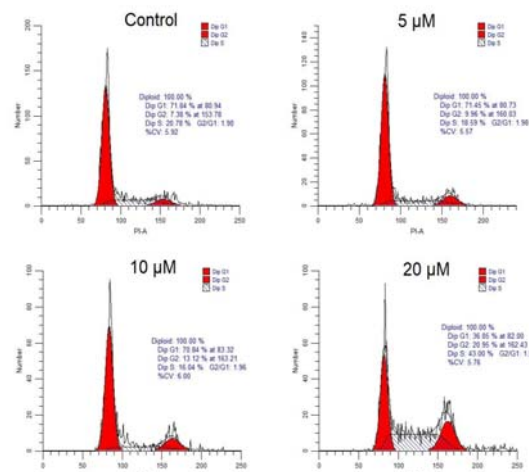


Figure 7: Bauerenol induced cell cycle arrest in retinoblastoma cancer cells at G₂/M phase. Flow cytometric analysis showing cell cycle phase distribution in SO-RB50 human retinoblastoma cells treated with bauerenol at doses of 5, 10 and 20 μM . The experiments were performed in triplicate, and the results are expressed as mean \pm SD. * $P < 0.05$

DISCUSSION

Natural compounds offer tremendous medicinal properties and exhibit a vast array of health-promoting effects in humans [9, 10]. These compounds have been evaluated for their therapeutic effects on human diseases [11]. Recently, several studies focused on the anticancer potential of natural compounds against different human cancers [12,13]. These investigations led the identification of a battery of natural compounds effective against different human cancers, and also broadened general understanding of human cancers, while identifying specific cellular targets for cancer treatment. The present study is a similar type of research work in which the anticancer potential of bauerenol was investigated in human retinoblastoma cells.

Retinoblastoma is a deadly human cancer which causes high mortality [14]. The prevalence of retinoblastoma is particularly high in children in underdeveloped and developing countries [15]. Retinoblastoma seriously affects children, and it is associated with secondary complications [16]. Studies have revealed that the current treatment methods for human retinoblastoma are relatively ineffective, and the outcomes are not very promising [17]. Taking these into consideration, researchers have focused on investigating the mechanism(s) involved in the pathogenesis of retinoblastoma, and identification of more potent anticancer agents for the malignancy. The present study has shown that bauerenol

effectively inhibited the growth and proliferation of retinoblastoma cancer cells in a concentration-dependent manner. Furthermore, bauerenol exerted comparatively lower toxic effects against normal eye cells. The migration and invasion of retinoblastoma cells were also suppressed by bauerenol, indicating the potential of this compound to arrest metastasis of human retinoblastoma. Indeed, previous studies proposed similar anti-cancer effects of bauerenol [7]. In addition, the results obtained in this study showed that bauerenol treatment led to induction of apoptosis and autophagy in retinoblastoma cancer cells. The pro-apoptotic role of the triterpenoid bauerenol has also been shown in previous research [7].

It has been reported that many natural compounds induced cell cycle arrest in cancer cells *in vitro* [18]. A similar effect was demonstrated by bauerenol on retinoblastoma cancer cells. In all, the results of this study indicate the antiproliferative influence of bauerenol against human retinoblastoma cancer cells, most likely due to induction of apoptosis, autophagy and cell cycle arrest.

CONCLUSION

This study has demonstrated the anticancer effect of bauerenol against human retinoblastoma. The anticancer effects are due to induction of apoptosis, autophagy and G₂/M cell cycle arrest. Moreover, consistent with its anticancer potential, bauerenol suppresses the migration and invasion of retinoblastoma cells. However, there is need to confirm the present findings through *in vivo* studies.

DECLARATIONS

Acknowledgements

None provided.

Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yiyi Chen, Jianjun Peng and Si Cao performed all the experiments. The whole study was designed by Yiyi Chen.

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