JBUON 2018; 23(4): 1174-1178
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Methyl eugenol induces potent anticancer effects in RB355 human retinoblastoma cells by inducing autophagy, cell cycle arrest and inhibition of PI3K/mTOR/Akt signalling pathway

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Summary

Purpose: *Retinoblastoma is one of the lethal malignancies in children. Approximately half of the children that are diagnosed with retinoblastoma die of this disease. Enucleation is commonly used for the treatment of retinoblastoma but this leads to loss of vision. Therefore there is an urgent need to look for viable chemotherapeutic agents for the treatment of retinoblastoma. Consistent with this, natural products can produce efficient anticancer agents and in the present study a plant-derived phenylpropene methyl eugenol (ME) was evaluated against retinoblastoma RB355 cells.*

Methods: *The cytotoxic activity of this molecule was evaluated by MTT cell viability assay, while autophagic effects were evaluated by flow cytometry using acridine orange (AO)/monodansylcadaverine (AO/MDC) staining dyes. The effects on the cell cycle progression were analyzed by flow cytometry while the effects on mTOR/PI3K/Akt signalling pathway were assessed by western blot method.*

Results: *The results indicated that ME exhibited an IC_{50} value of 50 μM and exerted its cytotoxic effects in a dose-dependent manner in RB355 cells. Moreover, it was observed that the ME lessens the cell viability and triggers G2/M cell cycle arrest. It was also observed that ME induced autophagy dose-dependently in retinoblastoma RB355 cells. Western blot analysis revealed that ME could modulate the mTOR/PI3K/Akt signalling pathway in RB355 cells at the IC_{50} concentration.*

Conclusions: *The above results clearly indicate that ME is a potent anticancer agent and may be developed further as a possible anticancer lead molecule against retinoblastoma provided further in depth in vivo studies are carried out.*

Key words: *autophagy, cell cycle arrest, flow cytometry, methyl eugenol, retinoblastoma*

Introduction

Retinoblastoma is the most frequent primary intraocular malignancy in children. The incidence of retinoblastoma ranges from 1 in every 18,000 live births [1,2], representing around 4% of all pediatric malignancies [3]. It has been reported that around 5,000 new cases of retinoblastoma are detected every year around the globe [3]. However, half of these children survive from retinoblastoma [3]. The main treatment for retinoblastoma is enucleation [4], but it may cause loss of vision and a poor cosmetic look [5]. Chemotherapy may prove efficient without causing any harm to the appearance. However, there are only limited drug options available for retinoblastoma, which are also accompanied with lot of side effects [6,7]. Therefore, there is pressing need to look for more viable drug options. Natural products have long been considered as good source of important drugs and about more

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Received: 04/03/2018; Accepted: 18/03/2018

than half of the currently available drugs are natural products. For instance, taxanes and podophyllotoxins are both isolated from plants [8]. Consistent with this, in the current study a plant-derived compound 4-O-methylhonokiol was evaluated against retinoblastoma cells. Methyl eugenol (ME) is a plant-derived bioactive molecule mainly isolated from *Eugenia caryophyllata*. It and its analogs have been reported to exhibit tremendous pharmacological potentials which include, but are not limited, to anti-microbial and anticancer activities [9-11].

The present study revealed that ME exhibits the minimum inhibitory concentration (MIC) of 50 µM against retinoblastoma cells. The antiproliferative effect was found to be concentration-dependent. Moreover, ME caused G2/M cell cycle arrest and induced autophagy in retinoblastoma RB355 cells in a concentration-dependent manner. Protein expression analysis revealed that ME inhibits the expression of key proteins of PI3K/mTOR/Akt signalling pathway. These findings, therefore, suggest that ME may prove beneficial in the treatment of retinoblastoma and may act a lead molecule for anticancer drug development.

Methods

Chemicals, reagents and cell culture conditions

All the chemicals and reagents used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Retinoblastoma RB355 cell line was procured from Cancer Research Institute of Beijing, China, and it was maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in an incubator at 37°C (5% CO_2 and 95% air).

MTT assay for antiproliferative activity

The antiproliferative effect of ME on RB355 retinoblastoma cell line was evaluated by MTT assay. RB355 cells were grown at $1x10^6$ cells per well in 96-well plates for 12 hrs and then exposed to 0-200 µM of ME for 48 hrs. To each well, MTT solution (20 µl) was added. Prior to the addition of 500 µl of DMSO, the DMEM as completely removed. To solubilize MTT formazan crystals, 500 µl DMSO were added. ELISA plate reader was used for the determination of optical density (OD) at 570 nm.

Cell cycle analysis

For cell cycle analysis the RB355 cells were treated with 0, 25, 50 and 100 µM of ME and the percentage of cells in each of the cell cycle phases were estimated by using Muse™ Cell Analyzer and Muse™ Cell Cycle Kit according to the manufacturer's protocol (Merck Millipore, Burlington, Massachusetts, United States).

Detection of autophagy

Cells at a density of 1.5×10^5 cells/well were treated with either DMSO or 50 µM (IC_{50}), of ME for 24 hrs and successively stained with monodansylcadaverine (MDC) or acridine orange (AO). All cell samples were observed under microscope and images were captured for at least three independent experiments.

Autophagy related protein expression and inhibitor treatment

RB355 cells were seeded in 6-well plates at the density of 1.5×10^5 cells/well and kept for 24 hrs. The cells were then administered ME at its IC_{50} concentration. Untreated cells were also included as controls. Following 48-h treatment, cells were collected and lysed for quantification of proteins and expression analysis. For inhibitor treatment, RB355 cells were seeded at the density of 1.5×10^5 cells/well in 6-well plates and left to adhere for 24 hrs. Cells were then exposed to 15 µg/mL of the liposomal inhibitor pepstatin A for 1 hr and were then exposed to IC_{50} concentration of ME for 48 hrs. Control cells were not exposed to ME. The protein expression was examined by western blotting.

Protein expression by western blotting analysis

After administration with various concentrations of ME, RB355 cells were harvested and lysed in lysis buffer. Out of the total protein samples 20 µg aliquot was separated on 10% SDS-PAGE gel. The gel was then transferred to nitrocellulose membranes, blocked with 5% bovine serum albumin (BSA) and probed with a primary antibody. This was followed by probing with the required secondary antibody. Finally, the signal was perceived with WEST-SAVE Up™ luminal-based ECL reagent (ABFrontier, Korea).

Statistics

All experiments were carried out in triplicates and presented as mean values ± SD. Student's t-test was used for statistical analyses using GraphPad prism 7 software. Results were considered significant at $p< 0.05$.

Results

Cytotoxic potential of ME on RB355 cell line

The growth inhibitory role of ME on RB355 cells was detected by treating these cells with varying ME concentrations. ME displayed potent antiproliferative effect against RB355 cells with an IC_{50} 50 µM (Figure 1). The antiproliferative activity of the natural phenylpropanoid compound was found be concentration-dependent.

ME induces autophagy in RB355 cells

In order to investigate whether ME triggers autophagy in RB355 cells, ME-administered cells were stained with MDC. Vital staining of ME-treated RB355 cells with MDC, an autophagolysosome

marker, indicated an increased buildup of the dye as compared to the control cells (Figure 2). These observations provided a strong evidence that ME triggered autophagy in RB355 cells. To quantify the increase of the acidic vesicular organelles, ME-treated cells were treated with AO dye, which revealed accumulation of AO in the ME-treated cells.

To further confirm autophagy, the expression of several autophagy associated proteins was evaluated. The results indicated that treatment with the extract induced expression of several autophagy-associated proteins as indicated in Figure 3. It was observed there was no change in the expression of several proteins including Vps34, Beclin-1, and LC3-I. However, the expression of LC3-II was significantly increased in a time-dependent manner, while slight reduction in the expression of p62 was observed. The autophagy inducing potential of ME was further confirmed by the use of autophagy inhibitor, pepstatin. However, the results indicated the ME abridged the effect of the inhibitor (Figure 4).

ME triggers G2/M cell cycle arrest

To investigate the effect of ME on cell cycle distribution of retinoblastoma RB355 cells, the cells were treated with different concentrations of ME (0, 25, 50 and 100 µM). It was observed that treatment of the cells with ME caused significant increase in the cells at G2/M phase of the cell cycle

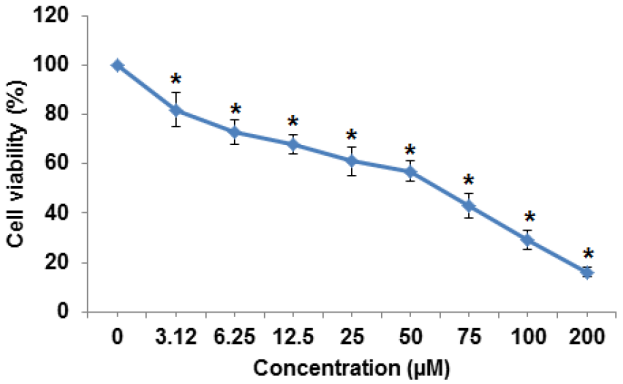

Figure 1. Effect of indicated doses of ME cell viability. All experiments were carried out in triplicate and shown as mean ± SD. The values were considered significant at *p<0.05.

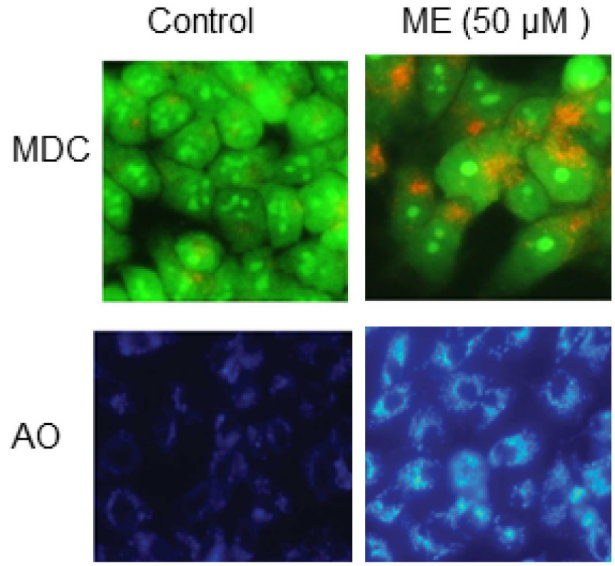

Figure 2. Induction of autophagy by ME at IC_{50} concentration. Experiments are representative of three biological replicates. MDC denotes monodansylcadaverine and AO denotes acridine orange. The figure depicts that ME triggers autophagy in the retinoblastoma cells.

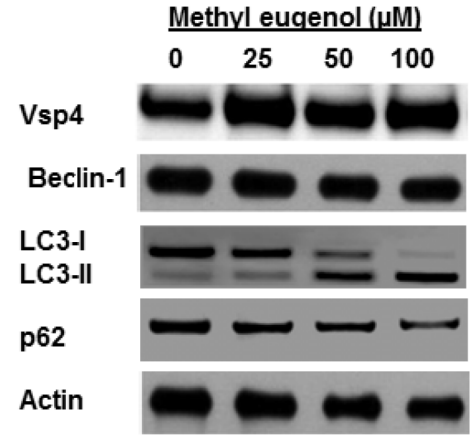

Figure 3. Expression pattern of autophagy-associated proteins at indicated concentrations of ME by western blotting. All the experiments were performed in triplicate. The Figure shows that ME increases the expression of autophagy-related protein LC3-II and decreases the expression LC3-I and p62, while the expression of other proteins remains unaffected.

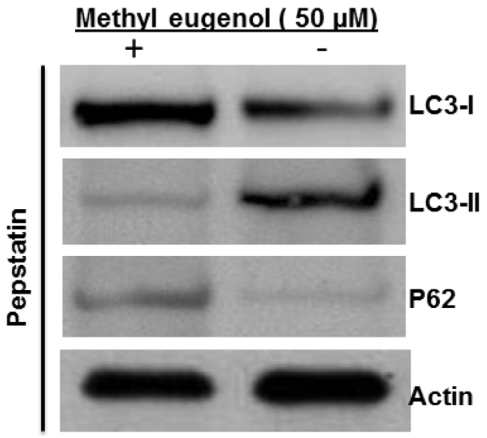

Figure 4. ME abolishes the effect of autophagy inhibitor at IC_{50} concentrations as depicted by western blotting. All experiments were carried out in triplicate. The results indicate that ME abrogates the effects of autophagy inhibitor on the autophagy of the retinoblastoma cells.

which was concentration-dependent with highest increase observed at the highest tested dose of ME (Figure 5).

ME targets m-TOR/PI3K/Akt signalling pathway

The effect of ME on the protein expressions of m-TOR/PI3K/Akt signalling pathway was investigated by using western blot assay. The findings are shown in Figure 6 and indicate an interesting outcome. As compared to the untreated control cells, ME-treated RB355 cells showed a concentration-dependent downregulation of m-TOR and pm-TOR proteins. Moreover, it was also observed downregulation of PI3K/Akt protein expressions. Thus it may be concluded that ME induced anticancer and autophagy effects by targeting m-TOR/PI3K/Akt signalling pathway.

Discussion

Retinoblastoma is one of the leading causes of cancer-related mortality in children. Around 5,000 new cases are diagnosed every year across the world. Additionally, treatments for this type of cancer are limited and enucleating the tumor affects the physical appearance of the patients [1-5]. Therefore, the present study aimed at determining the anticancer activity of the ME, a natural product, against retinoblastoma RB355 cells. The results indicated that the test molecule ME showed considerable anticancer effects on retinoblastoma RB355 cells in a dose-dependent manner with an IC_{50} of 50 µM. The IC_{50} value of ME was lower than the IC_{50} value observed for several other anticancer compounds [12]. Therefore, these results suggest that the ME is a potential cytotoxic agent.

It has been reported that many anticancer drugs exert their anticancer effects through cell cycle arrest and this mechanism is considered important to create inhibition of cancer cells growth [13]. As a result, we also investigated the effect of ME on the cell cycle distribution of RB355 retinoblastoma cells. The results of the study indicated that ME caused a significant increase in the population of RB355 cells at G2/M phase of the cell cycle, ultimately inducing cell cycle arrest. There was a marginal increase in the G2/M cells with the lower concentrations of ME, but the G2/M populations increased drastically as the dose of ME was increased.

Furthermore, the investigations indicated that ME exerts its anticancer activity through induction of autophagy as evidenced from the accumulation of MDC and AO in ME-treated retinoblastoma RB355 cells. The expression of several of the autophagy-associated proteins was evaluated and it was found that only the expression of LC3-II was highly induced by the ME in ME-treated retinoblastoma RB355 cells. Additionally, ME exhibited

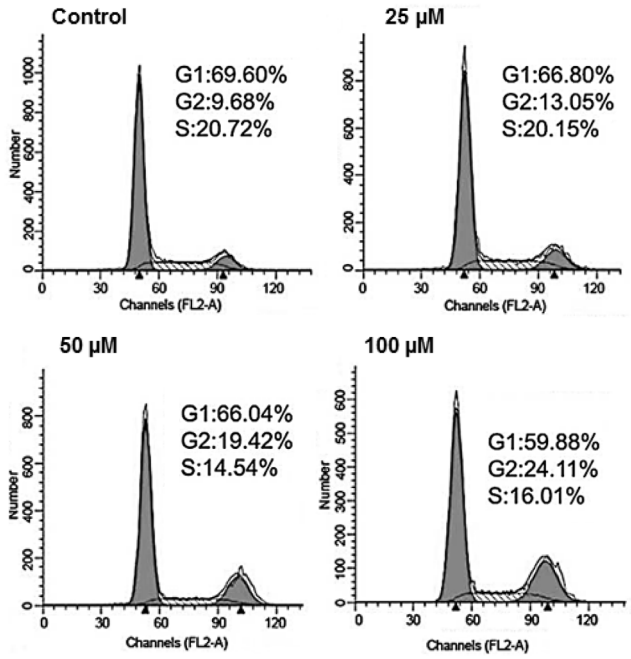

Figure 5. Effect of indicated concentration of ME cell cycle distribution of RB355 cells as determined by western blotting. All experiments were performed in triplicate. The results of this Figure indicate that ME triggers G2/M cell cycle arrest in RB355 cells in a concentration-dependent manner.

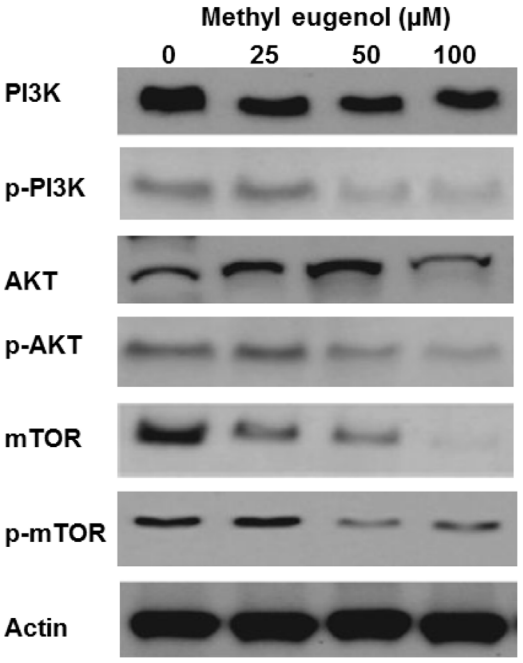

Figure 6. Effect of indicated concentrations of ME on the protein expression levels of mTOR/PI3/Akt pathway as shown in the western blots. Experiments were performed in triplicate. This Figure shows that the MED decreases the expression of PI3K, p-PI3K, AKT, p-AKT, m-TOR and p-mTOR in a concentration-dependent manner.

a strong potential to reduce the expression of autophagy inhibitors, providing a strong clue towards the role of this compound in triggering autophagy.

The effects of the expression levels of various proteins including m-TOR, pm-TOR, PI3K, p-PI3K and Akt were studied using western blot assay. This signalling pathway is considered as an important target for development of anticancer drugs [7,14,15] and several mTOR inhibitors are currently tested in clinical trials. Our results revealed ME-treated RB355 cells showed a concentration-dependent downregulation of m-TOR and pm-TOR proteins, causing also reduction in the protein expression levels of PI3K/Akt.

Conclusion

In conclusion, we propose that ME exhibits significant anticancer activity against retinoblastoma RB355 cells by inducing autophagy and modulating the expression of m-TOR/PI3K/Akt signalling pathway which is considered critical target for development of anticancer systemic therapy. Thus, ME may prove a lead molecule against retinoblastoma and therefore requires further research *in vivo*.

Conflict of interests

The authors declare no conflict of interests.

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