

ORIGINAL ARTICLE

Antitumor activity of 4-O-Methylhonokiol in human oral cancer cells is mediated via ROS generation, disruption of mitochondrial potential, cell cycle arrest and modulation of Bcl-2/Bax proteins

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Summary

Purpose: The plant-derived natural product 4-O-methylhonokiol (MH) has been reported to possess tremendous pharmacological potential ranging from neuroprotection to anticancer activity. However, the anticancer activity of MH in oral squamous cell carcinoma (OSCC) cells has not been evaluated. In the present study, MH was evaluated for its anticancer activity against OSSC PE/CA-PJ41 cells and the possible underlying mechanism was determined.

Methods: Cell cytotoxicity was evaluated by colorimetry-based MTT assay while the effects on cell cycle phase distribution were assessed by flow cytometry. Effects of MH on reactive oxygen species (ROS) production and mitochondrial membrane potential (MMP) were evaluated by flow cytometry. Western blot assay was finally utilized to study the effects of MH on key cancer and apoptosis-linked proteins including Bax and Bcl-2.

Results: MH induced cytotoxicity in OSSC PE/CA-PJ41 cells with an observed IC_{50} of 1.25 μ M. It also caused significant increase in the production of ROS and disrupted the MMP in a dose-dependent manner. The reduction in MMP favored mitochondrial apoptotic pathway which was further confirmed by determining the expression of Bax and Bcl-2. It was observed that MH downregulated the expression of Bax and upregulated the expression of MMP, ultimately leading to apoptosis of OSSC PE/CA-PJ41 cells. Additionally, MH also caused G2/M cell cycle arrest in a dose-dependent manner.

Conclusion: Taken together, our results indicate that 4-O-methylhonokiol may prove a potential natural anticancer molecule against human oral carcinoma cells.

Key words: cell cycle arrest, mitochondrial apoptosis, oral squamous cell carcinoma

Introduction

Despite recent advancements in science and technology, cancer is still known to be the most deadly disease and is one of the main reasons for mortality across the world [1]. With more than 100 known types of cancers, any organ of the body may be affected by this lethal disease with devastating outcomes [2,3]. Among all cancers, OSCC is one of the major cause of cancer related deaths and is responsible for approximately 90% of all oral malignancies. It has been reported that OSCC is the third most prevalent type of cancer in de-

veloping countries and the sixth most reported tumor worldwide [4]. Although treatment options for OSCC often involve surgery or radiation therapy, they may also include chemotherapy. Regardless of these treatment options, there has hardly been any improvement in the survival of OSCC patients with 5-year overall survival rate that still remains below 50% [5]. Consistently, there is an urgent need for the development of new, effective and efficient anticancer molecules for cancer treatment in general and OSCC in particular.

Plant-derived natural products offer an opportunity for developing anticancer agents against OSCC due to their promising activities and lower toxicities [6-8]. MH is a plant-derived bioactive molecule mainly isolated from *Magnolia officinalis* (Figure 1). MH and its analogs have been reported to exert strong pharmacological potential with its reported bioactivities ranging from anti-microbial to anticancer properties [9-11]. In the current study, MH was evaluated against a human OSCC cell line. The results of the study indicated that MH exerts significant anticancer effects on human PE/CA-PJ41 cells. It was observed that MH has an IC_{50} of 2.5 μ M against human OSCC PE/CA-PJ41 cell line. The effects were found to be due to G2/M cell cycle arrest and ROS-mediated mitochondrial apoptosis. Taken together, we strongly believe that MH may prove a promising candidate for the management of OSCC.

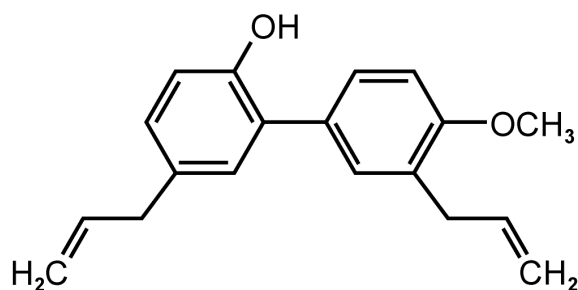


Figure 1. Chemical structure of 4-O-methylhonokiol (MH).

Methods

Chemicals and reagents

The chemicals and reagents used in the present study included (i) RPMI-1640, streptomycin, penicillin G, MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide), DMSO (dimethyl sulfoxide), MH, DCFH-DA (2,7-Dichlorodihydrofluorescein diacetate), all procured from Sigma (St. Louis, USA); (ii) fetal bovine serum (FBS) obtained from GIBCO (Fisher Scientific, Burlington, ON, Canada) and (iii) all antibodies, β actin and annexin V/PI purchased from Santa Cruz Biotechnology. (Delaware Ave Santa Cruz, CA, USA).

Cell culture conditions

OSCC PE/CA-PJ41 cell line was procured from Cancer Research Institute of Beijing, China, and routinely maintained in RPMI-60 containing 10% FBS and antibiotics (100 μ g/ml streptomycin and 100 U/ml penicillin G) kept in an incubator with atmosphere containing 5% CO_2 and relative humidity of 90% at 37°C.

Determination of cytotoxicity

The cytotoxic effect of MH on OSCC PE/CA-PJ41 cells was evaluated by colorimetry-based MTT assay. OSCC PE/CA-PJ41 cells were grown at 1×10^6 cells per

well in 96 well plates for 12 hrs and then exposed to 0-10 μ M MH for 24 and 48 hrs. To each well, MTT solution (20 μ l) was added. Prior to the addition of 500 μ l of DMSO, the medium was completely removed. To solubilize MTT formazan crystals, 500 μ l DMSO was added. Afterwards, the absorbance was determined in a microplate reader. IC_{50} was considered as the concentration of MH that caused 50% inhibition of cancer cell growth.

Cell cycle phase distribution of OSCC PE/CA-PJ41 cells

For determination of cell cycle distribution of OSCC PE/CA-PJ41 cells, they were plated at a density of 2×10^5 cells/well in 6-well plates and MH was administered to the cells at 0, 1, 2.5 and 5 μ M followed by 24-hr incubation. DMSO was used as control. For determination of DNA content, PBS was used to wash the cells and later the cells were fixed in ethanol at -20°C. Afterwards cells were re-suspended in PBS holding 40 μ g/ml PI and, RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in the dark at 37°C. Afterwards, analysis was carried out by flow cytometry as reported elsewhere [12].

Evaluation of ROS and MMP

OSCC PE/CA-PJ41 cells were plated at a density of 2×10^5 cells/well in a 6-well plate for 24 hrs and treated with 40 μ M MH for 6-72 hrs at 37°C in an atmosphere with 5% CO_2 and 95% air. Thereafter, cells from all samples were collected, washed twice by PBS and re-suspended in 500 μ l of DCFH-DA (10 μ M) for ROS estimation and DiOC6 (1 μ mol/l) for MMP at 37°C in the dark for 30 min. The samples were then examined immediately using flow cytometer as described previously [12].

Western blot analysis

Western blot analysis was used to determine the expression of mitochondrial apoptosis pathway related proteins. Briefly, MH-administered cells were harvested and lysed. The protein concentrations of the lysates were quantified by BCA assay using specific antibodies. β -actin was used as control. From each sample equal amounts of protein were loaded and separated by electrophoresis on a 12% denaturing SDS gel. Afterwards, the proteins were electroblotted on polyvinylidene difluoride membranes of 0.45 m pore size.

Statistics

All experiments were carried out in triplicate and expressed as mean \pm SD. One-way ANOVA and Tukey's test were performed using GraphPad prism 6 software and the results were considered significant at $p < 0.05$.

Results

MH exhibits significant cytotoxicity against PE/CA-PJ41 cells

In the colorimetry-based MTT assay, the cytotoxicity of MH was evaluated at varied concentrations ranging from 0 to 10 μ M in OSCC PE/

CA-PJ41 cells. It was observed that administration of PE/CA-PJ41 with MH caused considerable cytotoxicity with an IC_{50} of 2.5 μ M as depicted in Figure 2. Moreover, the cytotoxic effect of MH was found to be concentration-dependent.

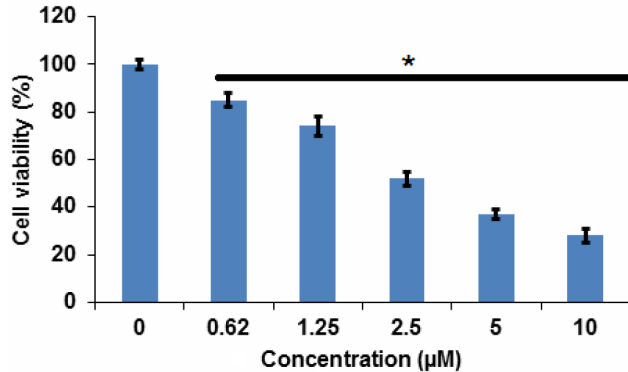


Figure 2. Effect of MH on cell viability of OSSC PE/CA-PJ41 cells at the indicated concentrations. Cell viability was carried out using MTT assay. All values are mean \pm SD of three experiments. * p <0.05

MH induces G2/M cell cycle arrest in PE/CA-PJ41 cells

Cell cycle phase distribution of PE/CA-PJ41 cells was estimated at different concentrations from 1.25 to 5 μ M and the results of the study indicated that the percentage of PE/CA-PJ41 cells significantly increased in G2 phase as the concentration of MH was increased, ultimately causing G2/M cell cycle phase arrest (Figure 3). Therefore, MH-induced G2/M increase of PE/CA-PJ41 cancer cells was seen to follow a dose-dependent manner.

MH causes production of ROS in PE/CA-PJ41 cells

The cytotoxic potential of MH observed through MTT assay suggested that MH might induce generation of intracellular ROS. Therefore, we calculated the ROS level at concentrations of 0, 1.25, 2.5 and 5 μ M for 48 hrs. The results showed that the intracellular ROS levels of treated cells increased significantly in a dose-dependent manner as compared to untreated cells (Figure 4).

MH-induced cell death in PE/CA-PJ41 cells is due mitochondrial apoptosis

We determined MMP in PE/CA-PJ41 cells treated with MH at concentrations of 0, 1.25, 2.5 and 5 μ M and the results indicated that MH reduced MMP in a dose-dependent manner (Figure 5). Mitochondrial mutilation expedited cytochrome C discharge from mitochondria into the cytoplasm and triggered apoptotic factors (Bcl-2 family proteins), which caused stimulation of the caspase signalling and mitochondrial facilitated apoptosis [5]. Beginning of caspase signal-

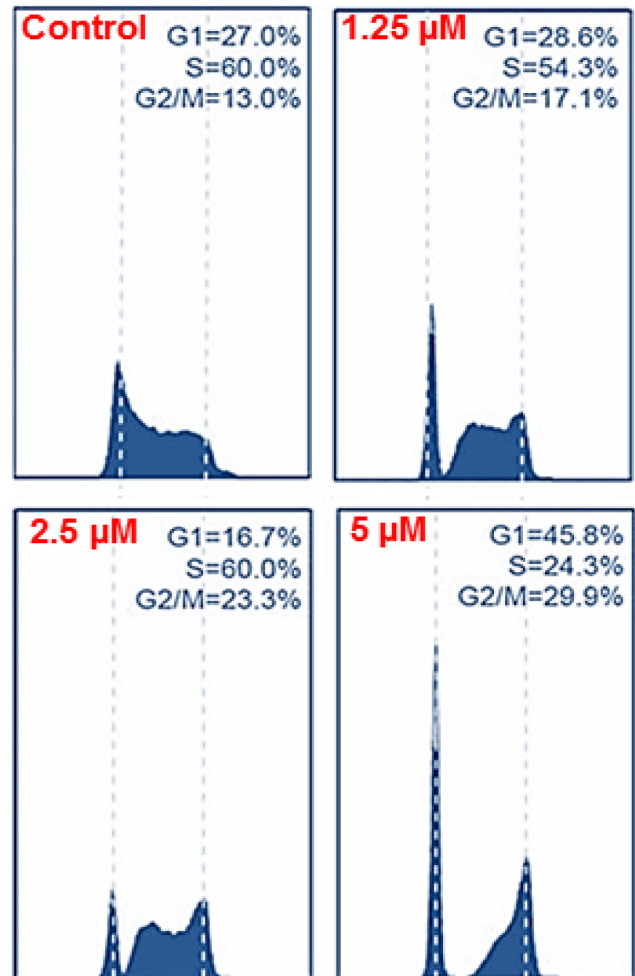


Figure 3. Effect of indicated doses of MH on cell cycle distribution of OSSC PE/CA-PJ41 cells by flow cytometry. As shown in the Figure, MH caused G2/M cell cycle arrest of the cells. All images represent the results of three experiments.

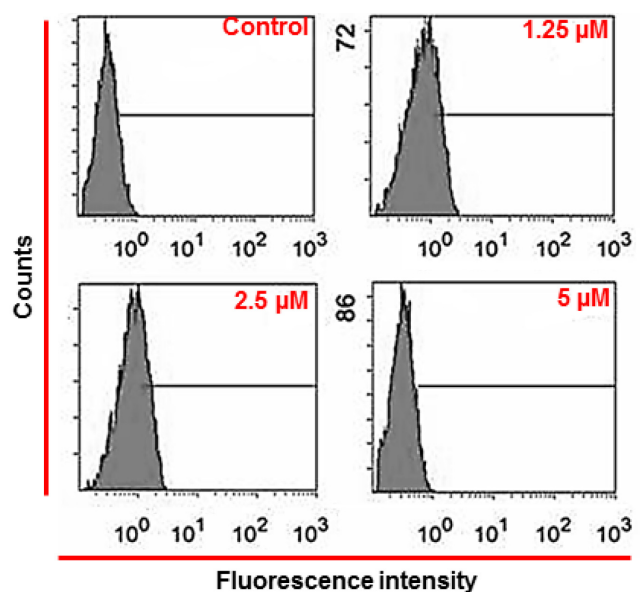


Figure 4. Effect of indicated doses of MH on ROS generation in OSSC PE/CA-PJ41 cells. As shown in the Figure, MH enhanced the production of ROS in a dose-dependent manner. All images represent the results of three experiments.

ling caused PARP cleavage which is considered as the main pathway in triggering apoptosis. To assess whether MH triggers apoptosis via this mechanism in PE/CA-PJ41 cells, we investigated the expression levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 through Western blot analysis. The results revealed that that MH upregulated Bax and downregulated Bcl-2 expression in comparison to the untreated cells kept as control (Figure 6).

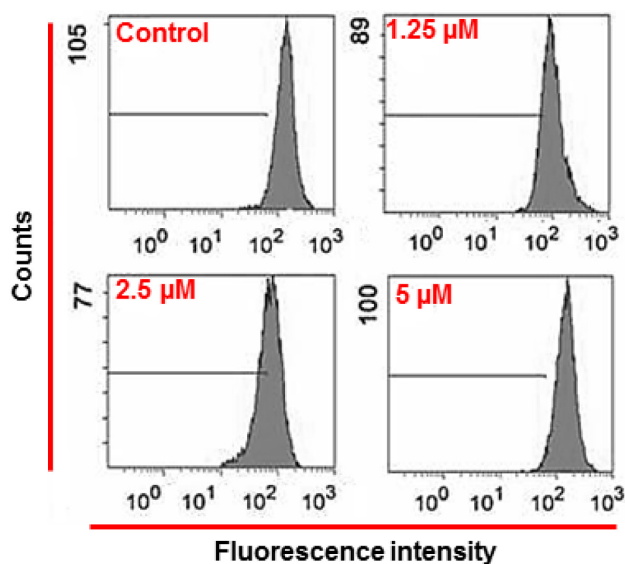


Figure 5. Effect of indicated doses of MH on MMP reduction in OSCC PE/CA-PJ41 cells, showing significant MMP reduction. All images represent the results of three experiments.

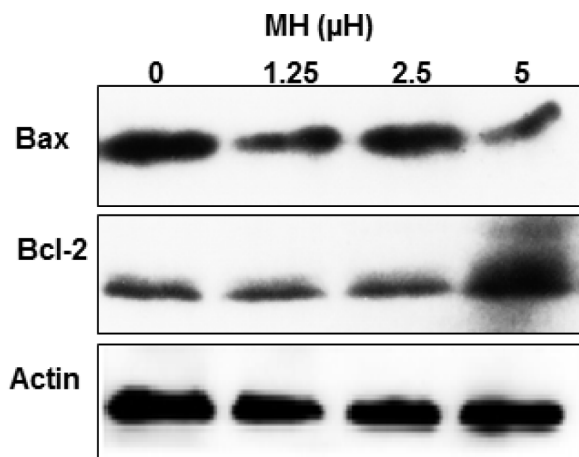


Figure 6. Effect of indicated doses of MH on the expression of Bax and Bcl-2 in OSCC PE/CA-PJ41. As depicted in the Figure, the expression of Bax was upregulated and of Bcl-2 downregulated. β -actin was used as positive control and all experiments were carried out in triplicate.

Discussion

OSCC is considered as one of the most prevalent types of cancer and includes about 90% of all oral malignancies across the globe [1]. The sharp

increase in the incidence of OSCC, lack of proper treatment and the severe side effects associated with the current treatment options has made it necessary to search for new and more effective molecules. Since plant-derived natural products have low toxicity, they are being considered as potential anticancer agents. In the current study, MH was evaluated against the OSCC cell line PE/CA-PJ41 for its potential anticancer activity and the results indicated that the molecule exhibits significant anticancer activity against this cell line. The cytotoxic effect of MH was found to be dose-dependent and IC_{50} of MH was found to be $2.5 \mu\text{M}$ against PE/CA-PJ41 cell line. One of the reasons for apoptosis might be the observed capacity of MH to cause cell cycle arrest as it induced the G2/M phase increase of PE/CA-PJ41 cancer cells in a dose-dependent manner. Cell cycle and apoptosis are known as the main controlling mechanisms for cell growth and proliferation. Apoptotic cell death is triggered when explicit checkpoints are arrested during the cell cycle [13,14]. Consistent with this, several anticancer agents lead to cell cycle arrest and have been found to be clinically effective for cancer treatment [15]. Although, apoptosis is triggered via different routes, the mitochondrial pathway is a crucial signalling pathway in the induction of apoptosis. It is well established that Bcl-2 family proteins are frequently the main players in the mitochondrial apoptotic pathway. The anti-apoptotic and pro-apoptotic protein members of Bcl-2 protein family control apoptosis by regulating mitochondrial membrane permeability [15]. While Bcl-2 is a strong antiapoptotic protein, Bax is inducer of apoptosis. Bax is present in the outer membrane of the mitochondria, facilitating the discharge of cytochrome C and stimulating caspase 9. Caspase-3 is activated by proteolytic cleavage of caspase 9 and is a key apoptotic executive caspase. In the present study, involvement of the mitochondrial apoptotic pathway in MH-induced cell death was first detected by the observed reduction in the MMP. This was further strengthened by the changes observed in the Bcl-2 and Bax expression levels, since mitochondrial malfunction is often due to MMP loss and discharge of cytochrome C into the cytosol. We observed MH lessened the MMP, leading to cytochrome C release from the mitochondria into the cytoplasm. Protein expression analysis revealed that MH caused considerable downregulation of Bcl-2 expression and upregulation of Bax protein, therefore ultimately favoring apoptosis. The results suggest that MH may trigger apoptosis through ROS-mediated reduction in MMP. Our

results are in agreement with previous studies where a number of anticancer agents induce apoptosis in cancer cells by generating high levels of intracellular ROS [16-20].

Conclusion

Taken together, we conclude that MH exerts significant anticancer activity against OSCC PE/

CA-PJ41 cell line. The anticancer activity is due to its capacity to induce ROS-mediated alteration of MMP, mitochondrial apoptosis and cell cycle arrest. The present study paves way for *in vivo* evaluation of this molecule against OSCC.

Conflict of interests

The authors declare no conflict of interests.

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