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ORIGINAL ARTICLE

Anticancer effects of Carvone in myeloma cells is mediated through the inhibition of p38 MAPK signalling pathway, apoptosis induction and inhibition of cell invasion

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Summary

Purpose: *There is accumulating evidence indicating that plant-derived monoterpenes have impressive health-promoting effects, among them anticancer, antimicrobial and many other activities. Carvone is an important monoterpene with pharmacological potential. In the current study we investigated the anticancer effects of carvone in myeloma KMS-5 cells.*

Methods: *The cell viability was estimated by MTT assay. Apoptosis was detected by DAPI, acridine orange/ethidium bromide (AO/EB) and annexin V/propidium iodide (PI) staining. Cell cycle analysis was done by flow cytometry. Cell invasion was determined by martigel invasion assay and protein expression was checked by western blotting.*

Results: *The results of the present study indicated carvone exerts significant anticancer effects on the myeloma cancer cells in a dose-dependent manner. The IC_{50} of carvone was found to be 20 μM against the myeloma KMS-5 cells. The antiproliferative effects were due to induction of apoptosis and G2/M cell cycle arrest. Moreover, carvone could inhibit the cell invasion and the expression of p-P38 protein at IC_{50}.*

Conclusions: *In conclusion, the results indicate that carvone could prove to be an important molecule for the development of natural product-based anticancer therapy, especially against myeloma.*

Key words: *apoptosis, carvone, cell cycle, monoterpenes, myeloma*

Introduction

Plants are natural chemical factories as they possess the capacity to synthesize a wide array of secondary metabolites [1]. In plants these secondary metabolites play vital roles to defend the plant against the harsh environmental conditions such as pathogen attacks [2]. Since these metabolites selectively kill or prevent the growth of pathogens, it is believed that they may exhibit these properties against cancer cells as well [3]. A number of plant-derived molecules has been reported to exhibit anticancer activities. In fact, a number of plant-derived metabolites are being currently used as standard drugs which include vincristine, vinblastine and taxanes [4]. Nowadays exhaustive research efforts are being carried out on screening plant-derived natural products against cancer cells [5]. Among plant-derived natural products, monoterpenes have been reported to exhibit significant pharmacological properties [6], including antimicrobial, anticancer, antiinflammatory and several other activities. These monoterpenes are basically the components of our diet, and hence considered safe [6,7]. They are prevalent in the essential oils of several plants and mainly function as chemo-attractants and repellents at physiological level and are also responsible for the distinct fragrance of the

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plants. Chemically, monoterpenes are 10-carbon isoprenoids that are derived from the mevalonate pathway in plants. Among the monoterpenes, carvone has a distinct place as it has been reported to exert a wide array of pharmacological properties [8]; however, the activity of carvone (Figure 1) against the myeloma cells has not been evaluated [9]. Against this background the purpose of the present study was to evalutate the anticancer activity of carvone against myeloma KMS-5 cells.

Methods

Chemicals, reagents and cell cultures

The chemicals and the reagents used in the present study were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Fetal bovine serum (FBS), RPMI-1640 medium, L-glutamine and antibiotics were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Human myeloma cell line KMS-5 was obtained from Type Culture Collection of Chinese Academy of Sciences, Shanghai, China. The cells were grown in RPMI-1640 medium containing 10% FBS and 100 U/mL penicillin and 100 mg/mL streptomycin and kept in a humidified atmosphere containing 5% CO_2.

Cell viability assay

Cell viability was assessed spectrophotometrically by using MTT assay. Untreated and treated (0-100 µM) KMS-5 myeloma cells were seeded at a density of 1.2×10^4 to 1.5×10^4 in 96-well microtiter plates. This was followed by the addition of MTT solution in all the wells and then the absorbance at 570 nm was determined using an ELISA plate reader.

Apoptotic assays

The untreated and the carvone-treated myeloma KMS-5 cells were seeded at the density of 2×10^5 cells/well in 6-well plates. The cells were then stained with a mixture of AO and EB and were examined under fluorescent microscopy. Similarly, DAPI staining was carried out by incubating the cells with DAPI and then washed with PBS and finally fixed in 10% formaldehyde. The DAPI-stained cells were then examined by fluorescence microscope. For annexin V/PI a similar procedure as that of DAPI was followed except for the cells stained with annexin V/PI and investigated by flow cytometry.

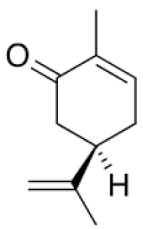

Figure 1. Chemical structure of carvone.

Cell cycle analysis

For estimating the distribution of myeloma KMS-5 cells in different phases of the cell cycle, the cells were treated with carvone at IC_{50}. Thereafter, the cells were fixed with 70% ethanol for about an hr and then washed again by PBS. The cells were finally resuspended in a solution of PI (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and fluorescence-activated cell sorting cater-plus cytometer using 10,000 cells/group.

Matrigel invasion assay

Invasion was determined using Matrigel®-coated invasion chambers (BD Biosciences, New Jersey, USA). The untreated and carvone-treated myeloma KMS-5 cells that reached the lower surface of the membrane were stained with crystal violet (CV). Images of CV-stained cells were captured by a phase contrast microscope .The resultant CV complex was dissolved in 10% acetic acid and the absorbance was measured at 600 nm, to determine the extent of invasion.

Western blot analysis

Total protein from myeloma cells was isolated in RIPA lysis buffer. Equal protein extracts from each group were run on SDS PAGE and then transferred to a polyvinylidene fluoride membrane. This was followed by blocking with 5% non-fat milk and incubation at room temperature for 1 hr. Following this, the membranes were incubated with a specific primary antibody at 4°C overnight. This was followed by washing in washing buffer and incubation for 1 hr with the suitable secondary antibody. The protein bands of interest were visualised by an ECL Advanced Western Blot Detection Kit (Amersham, USA).

Statistics

The experiments were carried out in triplicate and presented as mean ± SD. Statistically significant differences between groups were determined using one way ANOVA and Tukey's *post hoc* test using GraphPad prism 7 software. A p value <0.05 was considered statistically significant.

Results

Carvone inhibits the proliferation of the myeloma KMS-5 cells

To investigate the antiproliferative effects of carvone, the myeloma KMS-5 cells were treated with carvone at different concentrations ranging from 0-100 µM and were subjected to MTT assay. The results revealed that carvone could decrease the cell viability of the myeloma cells in a concentration-dependent manner (Figure 2). The IC_{50} of carvone against the myeloma cells was found to be 20 µM, indicative of the potent anticancer activity of carvone.

Carvone induces apoptosis in myeloma cells

Since, carvone could decrease the cell viability of the myeloma KMS-5 cells in a concentration-dependent manner, we wanted to know about the reason behind this antiproliferative activity. In order to investigate the underlying mechanism, we carried out DAPI (Figure 3), EB/OR (Figure 4) and Annexin-V/PI staining (Figure 3). In all these assays, it was observed that treatment of the myeloma KMS-5 cells with carvone triggered apoptosis. Moreover the apoptotic cell populations increased from 2.71% in controls to 32.5% at IC_{50} of carvone (Figure 5). Taken together, these results indicate carvone decreases the cell viability through induction of apoptosis.

Carvone triggers G2/M cell cycle arrest in myeloma KMS-5 cells

Besides apoptosis, cell cycle arrest is another mechanism by which anticancer agents exert their effects. Therefore we also investigated whether carvone could induce apoptosis in myeloma cells at IC_{50}. It was observed that the myeloma cells treated with carvone showed significantly increased number of cells in G2/M phase of the cell cycle as compared to the untreated cells (Figure 6). These results suggested carvone exerts antiproliferative effects in part via induction of G2/M cell arrest of myeloma cells.

Carvone inhibits migration of KMS-5 myeloma cells

Anticancer effects that inhibit the cell migration are considered efficient as they help prevent metastasis. The results of Martigel invasion assay indicated that carvone significantly reduced the cell migration of the myeloma cells after 20 hrs. After 20 hrs the relative cell migration in the control untreated myeloma cells was 38% as compared to 15% cell migration in carvone-treated myeloma cells (Figure 7).

Carvone inhibits the P38MAPK signalling

We further investigated the effect of carvone on P38 MAPK signalling pathway. The results indicated that carvone could inhibit the expression of p-P38 at IC_{50} concentration. However the expression of P38 remained more or less unaltered (Figure 8).

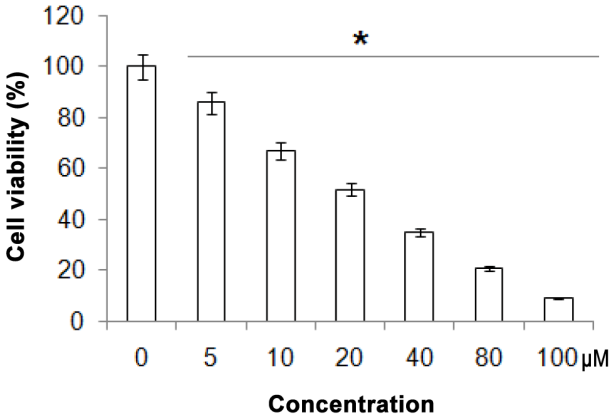

Figure 2. Effect of carvone on the cell viability of myeloma KMS-5 cells. The experiments were carried out in triplicates and expressed as mean ± SD (*p<0.01 vs control).

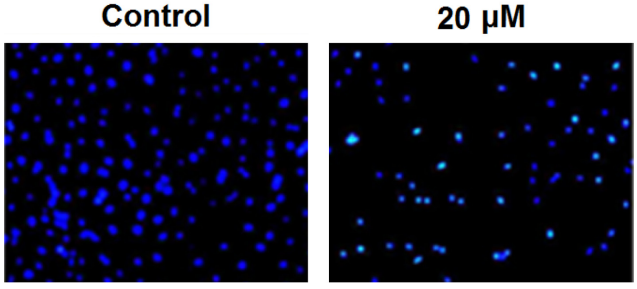

Figure 3. Carvone at IC_{50} triggers apoptosis in KMS-5 myeloma cells as indicated by DAPI staining. The experiments were carried out in triplicate. The bright fluorescent cells on the right image are apoptotic cells after carbone exposure.

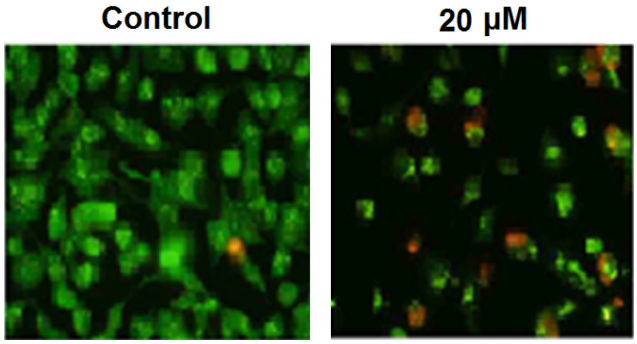

Figure 4. Carvone at IC_{50} triggers apoptosis in KMS-5 myeloma cells as indicated by AO/EB staining. The experiments were carried out in triplicate. The green stained cells are normal cells while the orange stained cells are apoptotic cells.

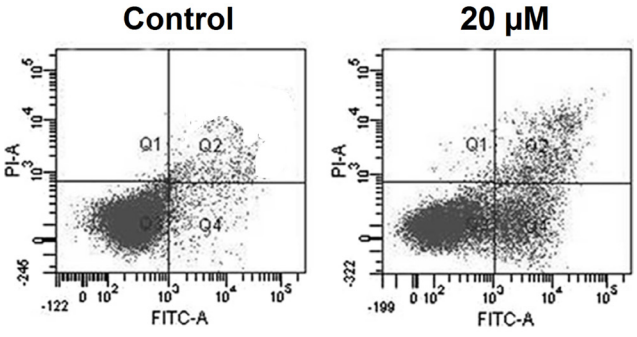

Figure 5. Carvone induces apoptosis in KMS-5 myeloma cells as indicated by annexin V/PI staining. The experiments were carried out in triplicate. The results indicate that exposure to 20 µM of carbone leads to significant increase of apoptotic cells.

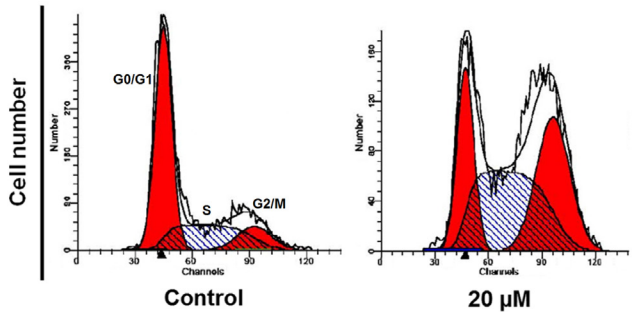

Figure 6. The results of this flow cytometry Figure suggest that 20μM of carbone led to G2/M cell cycle arrest of KMS-5 myeloma cell line. The experiments were carried out in triplicate.

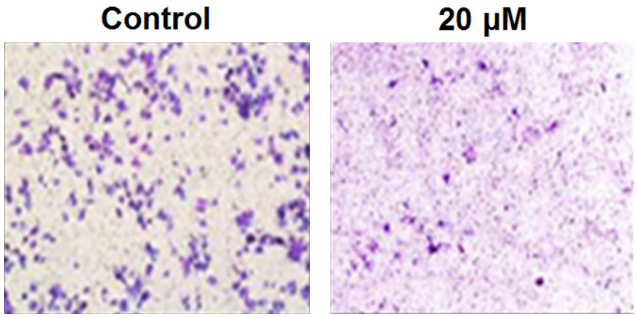

Figure 7. Effect of carvone on cell migration as determined by Boyden chamber assay. The experiments were carried out in triplicate. Exposure to 20μM dose of carbone led to significant migration inhibition of myeloma cells.

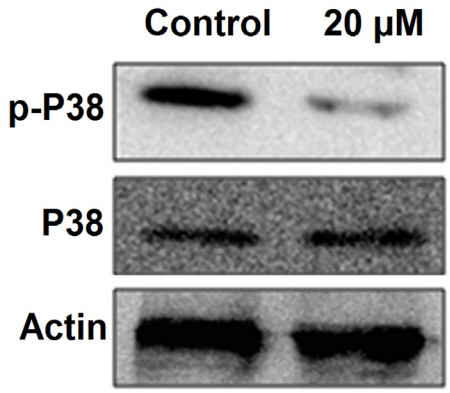

Figure 8. Effect of carvone at IC_{50} on the expression of P38MAPK. The results indicate that carbone at 20μM could inhibit the expression of p-P38. However, the expression of P38 remained more or less unchanged. The experiments were carried out in triplicate.

Discussion

Myeloma is a deadly disease responsible for significant morbidity and mortality around the world [10]. The treatment options for myeloma are limited and the need to look for new drug options that could prove beneficial in the treatment of myeloma is urgent [11]. Plants are the sources of a wide array of chemical compounds. Many of the plant-derived compounds have been used in the treatment of different types of cancers [12]. In this study we evaluated the anticancer effects of a plant-derived monoterpene, carvone, against a myeloma cell line. The results revealed that carvone could inhibit the growth of myeloma cells in a concentration-dependent manner with an IC_{50} of 20 μM. In previous studies monoterpenes have also been reported to inhibit the cancer cell growth, for instance perillyl alcohol inhibits the proliferation of breast cancer cells [13].

Next, we wanted to know the reason behind the anticancer effects of carvone. The results of DAPI staining showed that carvone could induce apoptosis in myeloma cells at IC_{50}. Similar results were obtained by AO/EB staining. Furthermore, the apoptotic cell populations increased from 2.71% to 32.5 % with carvone administration. Our results are in agreement with previous investigations showing that perillic acid, which is a monoterpene, induces apoptosis in non-small lung cancer cells [14,15]. Cell cycle arrest is another mechanism by which anticancer agents curb the proliferation of the cancer cells [16]. In this study we also observed that carvone could prompt G2/M cell cycle arrest in the myeloma cells. These results are well supported by a previous work wherein monoterpenes have been reported to induce cell cycle arrest in cancer cells, e.g. β-ionone induces cell cycle arrest in MCF-7 breast cancer [17]. Anticancer agents that inhibit cell migration are considered important for prevention of metastasis. In the present study we observed that 20 μM of carvone could inhibit the migration of the KMS-5 myeloma cells. P38MAPK signalling pathway is considered important and has been implicated in the proliferation of a wide array of cancers [18]. Our results showed that carvone could inhibit the expression of p-P38 at IC_{50} concentration. However, the expression of P38 remained almost unaltered. These results indicated that carvone could prove an efficient molecule in the treatment of myeloma.

Conclusion

In conclusion, the results of the present study indicate that the plant-derived monoterpene carvone could inhibit the proliferation of the KMS-5 cells through induction of apoptosis, cell cycle arrest and inhibition of cell invasion. However, further research efforts are required for the evaluation of this molecule *in vivo*.

Conflict of interests

The authors declare no conflict of interests.

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