

## ORIGINAL ARTICLE

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# Marrubenol inhibits osteosarcoma cancer cell growth by inducing autophagic cell death and inhibiting cancer cell migration and invasion

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## Summary

**Purpose:** The present study aimed at evaluating the anticancer activity of marrubenol against osteosarcoma cells along with evaluating its effects on autophagic cell death, reactive oxygen species (ROS) generation and cell migration and invasion tendency.

**Methods:** In this study the Saos-2 osteosarcoma cell line was used. Cell cytotoxicity effects were evaluated by MTT cell viability assay, while clonogenic assay assessed the effects on cancer cell colony formation. In vitro wound healing assay was used to evaluate the effects on cell migration. To confirm autophagy, we evaluated the expression of several autophagy-associated proteins Western blot assay along with transmission electron microscopy (TEM).

**Results:** The results indicated that the marrubenol exhibited an IC<sub>50</sub> value of 45 μM and exerted its cytotoxic effects in a dose-dependent manner. Moreover, it was observed that the drug inhibited colony formation and induced autophagy dose-dependently. The underlying mechanism for the induction of autophagy was found to be ROS-mediated and significant inhibition of cell migration as well as cell invasion potential of osteosarcoma cells at the IC<sub>50</sub> was observed.

**Conclusions:** These results strongly indicate that marrubenol may be considered as a potent drug lead molecule for the treatment and management of osteosarcoma.

**Key words:** autophagy, cell cytotoxicity, marrubenol, osteosarcoma, ROS

## Introduction

Plants and their wide spectrum of bioactive metabolites have been used in the management of a number of diseases from the very past [1]. This has in turn enforced researchers to isolate molecules from plants and to evaluate them against deadly diseases such as cancer [2]. A number of drugs that are currently being used for the treatment of cancers actually come from plants [3]. Marrubenol is an important plant-derived molecule that could prove to be a prospective molecule for the treatment of diseases including cancer. Chemi-

cally, marrubenol is a diterpenoid, mainly isolated from the plants belonging to the *Marrubium* genus of *Lamiaceae* family [4,5]. Although the bioactivity of several plant extracts has been attributed to the presence of the diterpenoid marrubenol, its anticancer activity has not been evaluated so far. In the current study, the anticancer potential of marrubenol was assessed against the osteosarcoma cell line Saos-2. Osteosarcoma is one of the rarest types of malignancies accounting for about 2.4% of all the human cancers [6]. Despite its rarity, it

has been found to be the 3<sup>rd</sup> most prevalent type of cancer in adolescent and is responsible for significant morbidity and mortality. The treatment for osteosarcoma involves preoperative chemotherapy and subsequent removal of the affected tissue and finally postoperative chemotherapy [7]. However, the results of the currently available treatments are still far from satisfactory [7]. In this study we evaluated the antiproliferative effects of marrubenol against the Saos-2 osteosarcoma cells.

## Methods

### Chemicals, reagents and cell cultures

All chemicals and reagents were procured from Sigma-Aldrich Co., Darmstadt, Germany or otherwise specified. Antibodies were purchased from Santa Cruz Biotechnology Inc. (Dallas, TX, USA). Human osteosarcoma Saos-2 cell line was procured from American Type Cell Culture. The cells were grown in RPMI-1640 medium having 10% fetal bovine serum (FBS), penicillin and streptomycin (100 Ug/mL and 100 µg/mL, respectively) and kept at 37°C in atmosphere containing 5% CO<sub>2</sub> till further experimentation.

### MTT assay

For assessment of the cell viability, the Saos-2 cells were cultured in a 96-well plates (5×10<sup>5</sup> cells per well). The cells were kept overnight at 37°C and afterwards RPMI-1640 medium was removed and replaced with fresh medium with marrubenol at varying doses (0-200 µM) for 24 hrs. Thereafter, MTT solution of 0.5 mg/ml was added for the last 4 hrs of incubation and finally the absorbance was evaluated at optical density (OD) 570 nm.

### Clonogenic assay

To elucidate the impact of marrubenol on the colony formation potential of Saos-2 cells, the cells were collected at the exponential growth phase and then counted using a hemocytometer. The cells were plated at 200 cells /well and the plates were incubated at 37°C for 48 hrs to permit the cells to attach. This was followed by the addition of 45 µM of marrubenol. Following treatment with marrubenol, the cells plates were again incubated for 6 days at 37°C. After 6 days of incubation, the cells were washed with PBS and fixed with methanol. Afterwards, the cells were treated with crystal violet for 25 min and then counted under light microscope.

### Transmission electron microscopy (TEM)

The samples for TEM were prepared as described previously [8]. In brief, the untreated and marrubenol-treated Saos-2 cells were fixed in glutaraldehyde (2.5%) in phosphate buffer for 35 min and post-fixed in 1% osmium tetroxide in the same buffer for 35 min. This was followed by dehydration of the cells in molecular graded ethanol and subsequent washing with propylene

oxide and then embedded in Epon. This was followed by sectioning on a Reichert-Jung ultramicrotome at 90-nm thickness. The sections were then stained with 5% uranyl acetate and 5% lead citrate and observed on a Hitachi H7100 transmission electron microscope at 75 kV.

### Determination of ROS and MMP

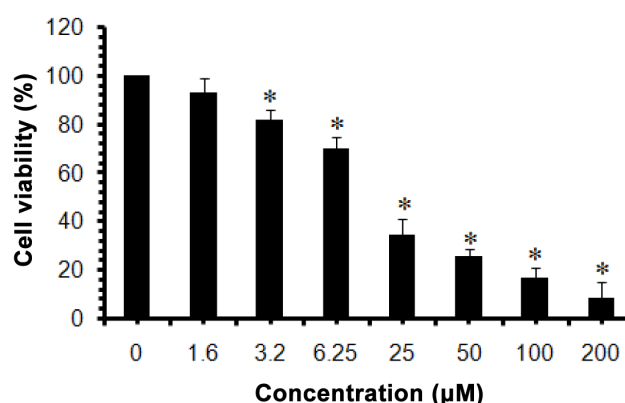
Saos-2 cells were cultured in 96-well plates (2×10<sup>5</sup> cells in each well), kept at 37°C for 24 hrs and treated with 45 µM of marrubenol for 0, 12, 24 and 48 hrs at 37°C in CO<sub>2</sub> (5%) and air (95%). Afterwards, the marrubenol-treated cells were harvested, PBS-washed and then suspended in 500 µl of 10 µM DCFH-DA for the estimation of ROS and 1 µmol/l of DiOC<sub>6</sub> 3,3' (dihexyloxycarbocyanine iodide) for evaluation of the MMP level. The samples were then examined instantly using flow cytometer.

### Wound healing assay

The cell migration potential of marrubenol-treated osteosarcoma Saos-2 cells was investigated by wound healing assay. In brief, 5×10<sup>4</sup> cells/well were cultured in 96-well plates. Afterwards, the plates were kept for 24 hrs at 37°C to allow the cells to adhere. Then a wound was scratched using a sterile pipette tip after the cells reached 80% confluence. The cells were then PBS-washed to clear the detached cells. Saos-2 cells were monitored after 20-h interval and photographed.

### Western blotting

Saos-2 cells were cultured (2×10<sup>5</sup> cells/well) in 6-well plates. The cells were treated with 45 µM of marrubenol and incubated for 24 hrs and then the cells were treated with DAPI. The cells were then PBS-washed, fixed with 10% formaldehyde and were subjected to fluorescence microscopy. For the estimation of apoptotic cell populations, a similar procedure was carried out except for the cells stained with annexin V/PI, and analyzed by flow cytometry.



**Figure 1.** Effect of marrubenol on the cell viability of osteosarcoma Saos-2 cells as indicated by MTT assay. The values are mean ± SD of three biological experiments (\*p<0.05 vs control).

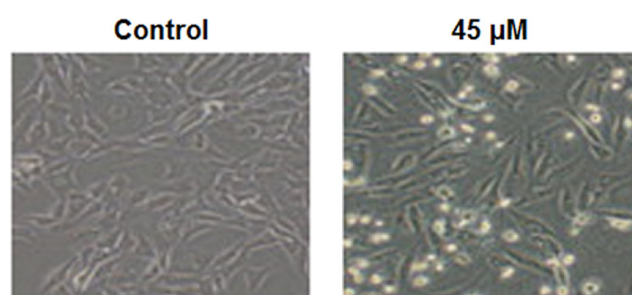
### Statistics

Data are shown as mean  $\pm$  standard error of the mean (SEM) and were statistically analyzed using Student's Newman Keul's test or t-test. A p value  $<0.05$  was considered to be statistically significant.

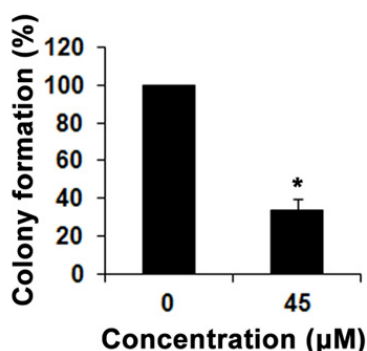
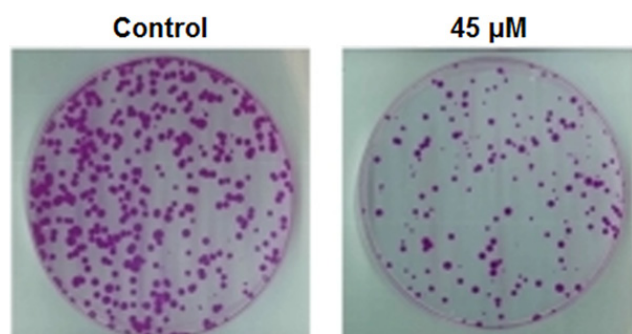
## Results

### Marrubenol affected the viability of Saos-2 osteosarcoma cells

The effects of marrubenol on Saos-2 cell viability were determined by MTT assay which revealed that marrubenol exhibited considerable antiproliferative effects on Saos-2 cells, which effects were concentration-dependent (Figure 1). The  $IC_{50}$



**Figure 2.** Effect of marrubenol on the morphology of osteosarcoma Saos-2 cells as indicated by phase contrast microscopy. The experiments were performed in triplicate.

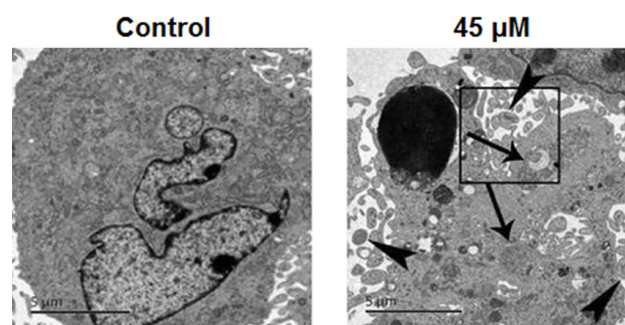


**Figure 3.** Effect of marrubenol on the morphology of Saos-2 cells as indicated by clonogenic assay. The experiments were carried out in triplicate and the values are mean  $\pm$  SD (\* $p < 0.05$  vs control).

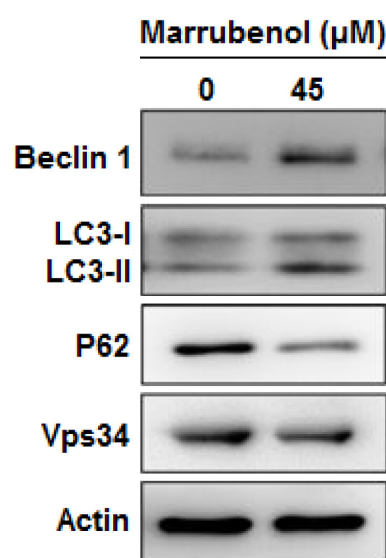
of marrubenol against Saos-2 osteosarcoma cells was  $45 \mu\text{M}$ . Additionally, marrubenol also triggered changes in the morphology of the Saos-2 cells (Figure 2). Moreover, marrubenol reduced the colony forming potential of the Saos-2 cells at  $IC_{50}$  concentration (Figure 3). These results unequivocally suggest that marrubenol exerts anticancer effects on osteosarcoma cells.

### Marrubenol induced autophagy in Saos-2 osteosarcoma cells

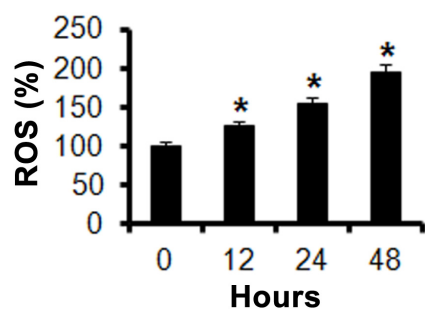
To gain insights about the underlying mechanism of the marrubenol-induced anticancer effects we carried out TEM of the untreated and marrubenol-treated Saos-2 osteosarcoma cells. The results showed that treatment of these cells led to generation of autophagic vesicles in these cells (Figure 4). To further validate the autophagic potential



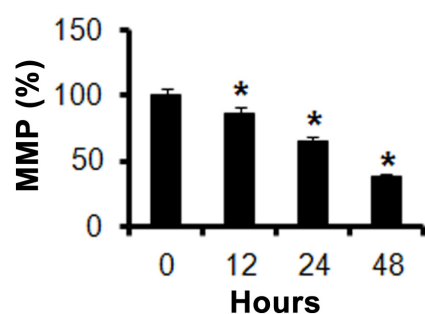
**Figure 4.** Transmission electron microscopy. The Figure shows that marrubenol causes formation of autophagic vesicles (arrows) on Saos-2 cells. Arrows indicate the autophagic vesicles.



**Figure 5.** Effect of marrubenol at  $IC_{50}$  expression of autophagy-associated proteins as determined by western blot analysis. The figure indicates that marrubenol alters the expression of autophagy-related proteins, especially LC3-II.



**Figure 6.** Effect of marrubenol at  $IC_{50}$  on ROS levels of Saos-2 cells at indicated time intervals. The values are mean  $\pm$  SD of 3 biological experiments (\* $p < 0.05$  vs control).



**Figure 7.** Effect of marrubenol at  $IC_{50}$  on the MMP levels of Saos-2 cells at indicated time intervals. The values are mean of three biological experiments  $\pm$  SD (\* $p < 0.05$  vs control).

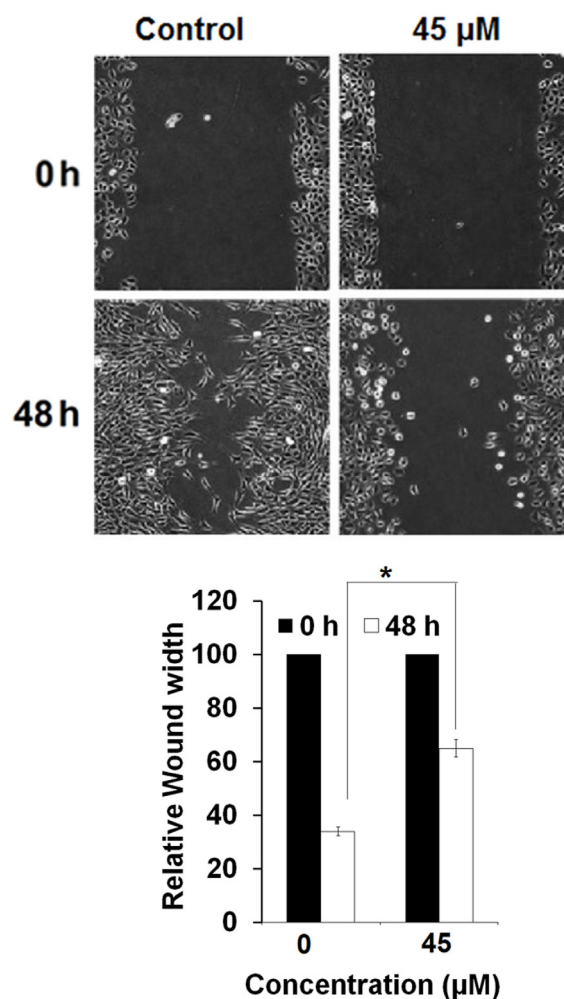
of marrubenol we determined the expression of autophagy-associated proteins in the untreated and marrubenol-treated cells. It was observed that the expression of Beclin-1 and LC3-II proteins was significantly upregulated in the marrubenol-administered Saos-2 cells. Moreover, the expression of p62 was found to be significantly decreased in the marrubenol-treated Saos-2 osteosarcoma cells. However, no visible alterations were observed on the expression of LC3-I and Vps34 (Figure 5).

#### Effect of marrubenol on ROS and MMP

Next, we determined the effect of marrubenol on the ROS and MMP levels of the Saos-2 cells at  $IC_{50}$  at different time intervals (0, 12, 24 and 48 hrs). The results revealed that marrubenol treatment enhanced the production of ROS in a time-dependent manner (Figure 6). However, the MMP levels significantly decreased time-dependently in Saos-2 cells (Figure 7)

#### Marrubenol inhibited cell migration of Saos-2 osteosarcoma cells

The effects of marrubenol at  $IC_{50}$  on migration of Saos-2 cells were evaluated by wound healing assay and the results showed that marrubenol inhibited considerably the migration of the Saos-2 cells (Figure 8).



**Figure 8.** Effect of marrubenol at  $IC_{50}$  on the migration of Saos-2 osteosarcoma cells as indicated by wound healing assay. The values are mean of three biological experiments  $\pm$  SD (\* $p < 0.05$  vs control).

## Discussion

Osteosarcoma is one of the lethal but rarest types of cancers, accounting for a significant number of deaths across the world. It has been reported to be the third most prevalent type of all cancers in adolescents [9]. The current treatments for osteosarcoma include chemotherapy, surgical interventions or radiotherapy but the results are far from satisfactory [10]. In this study marrubenol exerted significant anticancer effects on the osteosarcoma Saos-2 cells. These effects of marrubenol were dose-dependent and were exhibited at  $IC_{50}$  45 against the Saos-2 cells. The results of the MTT assay were further confirmed by the clonogenic assay wherein marrubenol significantly inhibited the colony forming potential of Saos-2 cancer cells. These outcomes are also backed by earlier investigations wherein plant extracts containing marrubenol have been reported to suppress the growth

and proliferation of cancer cells. For instance, *Marrubium persicum*, which is a rich source of diterpenoids such as marrubenol, has been reported to inhibit the proliferation of the breast cancer MCF-7 cells [4]. Similarly, *Marrubium vulgare* has been reported to exert cytotoxic effects on different cancer cells [11]. Next, to investigate the underlying mechanisms of the anticancer activity of marrubenol, we carried out TEM of untreated and marrubenol-treated Saos-2 osteosarcoma cells and found that marrubenol treatment led to the formation of autophagic vesicles in these cells. To further confirm that marrubenol causes autophagic cell death in Saos-2 cells, we determined the expression of autophagy-related proteins, such as LC3-I, LC3-II, Beclin-1, Vps34 and p62, and observed that marrubenol treatment caused enhancement in the expression of LC3-II and beclin-1 with concomitant downregulation of the expression of p62. These changes in the expression of autophagy-related proteins, especially due to the upregulation of LC3-II, are characteristic of autophagy [12]. Previously, several plant-derived diterpenoids have also been reported to induce autophagy in cancer cells. For example, a plant-derived diterpenoid oridonin causes autophagy in cervical cancer cells [13]. Several studies have shown that increase in the ROS and concomitant decrease in the MMP could be one of the important reasons for the induction of autophagy [14,15]. For example a natural product

bufalin induces autophagy in colon cancer cells through accretion of ROS [16]. In the current investigation, it was observed that marrubenol triggered the production of significant amounts of ROS in a time-dependent manner. This was also associated with reduction in the MMP of Saos-2 cells, indicating that marrubenol might be inducing autophagy these cells via generation of ROS and loss of MMP. We finally evaluated the effects of marrubenol on the cell migration potential of the Saos-2 osteosarcoma cells and found that marrubenol inhibits the migration of the osteosarcoma cells suggesting that it could potentially prevent metastasis of the osteosarcoma cells *in vivo*.

## Conclusion

To sum up, the current study revealed that marrubenol exerts anticancer effects on the osteosarcoma Saos-2 cells through induction of autophagy via ROS generation. Moreover, marrubenol could clearly inhibit the migration of the Saos-2 cells indicating that this compound could prove to be an imperative lead molecule for the management of osteosarcoma and deserves further *in vivo* evaluation.

## Conflict of interests

The authors declare no conflict of interests.

## References

1. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. *Environ Health Perspect* 2001;109:69-74.
2. Clark AM. Natural products as a resource for new drugs. *Pharmaceut Res* 1996;13:1133-41.
3. Gordaliza M. Natural products as leads to anticancer drugs. *Clin Trans Oncol* 2007;9:767-76.
4. Hamedeyazdan S, Fathiazad F, Sharifi S, Nazemiyeh H. Antiproliferative activity of *Marrubium persicum* extract in the MCF-7 human breast cancer cell line. *Asian Pac J Cancer Prev* 2012;13:5843-8.
5. Cseke LJ, Kirakosyan A, Kaufman PB, Warber S, Duke JA, Briemann HL. Natural products from plants. CRC Press; 2016 Apr 19.
6. Ottaviani G, Jaffe N (Eds): The epidemiology of osteosarcoma. In: *Pediatric and Adolescent Osteosarcoma 2009* (pp 3-13). Springer US.
7. Luetke A, Meyers PA, Lewis I, Juergens H. Osteosarcoma treatment—where do we stand? A state of the art review. *Cancer Treat Rev*. 2014;40:523-32.
8. Chan WY.; Huang H.; Tam SC. Receptor-mediated endocytosis of trichosanthin in choriocarcinoma cells. *Toxicology* 2003;186:191-203.
9. Sampo M, Koivikko M, Taskinen M et al. Incidence, epidemiology and treatment results of osteosarcoma in Finland—a nationwide population-based study. *Acta Oncol* 2011;50:1206-14.
10. Geller DS, Gorlick R. Osteosarcoma: a review of diagnosis, management, and treatment strategies. *Clin Adv Hematol Oncol* 2010;8:705-18.
11. Zarai Z, Kadri A, Chobba IB et al. The in-vitro evaluation of antibacterial, antifungal and cytotoxic properties of *Marrubium vulgare* L. essential oil grown in Tunisia. *Lipids Health Disease*. 2011;10:161.
12. Oh SY, Choi SJ, Kim KH, Cho E, Kim JH, Roh CR. Autophagy-related proteins, LC3 and Beclin-1, in placentas from pregnancies complicated by preeclampsia. *Reprod Sci* 2008;15:912-20.
13. Zhang YH, Wu YL, Tashiro SI, Onodera S, Ikejima T. Reactive oxygen species contribute to oridonin-induced apoptosis and autophagy in human cervical carcinoma HeLa cells. *Acta Pharmacol Sin* 2011;32:1266.

14. Khan MI, Mohammad A, Patil G, Naqvi SA, Chauhan LK, Ahmad I. Induction of ROS, mitochondrial damage and autophagy in lung epithelial cancer cells by iron oxide nanoparticles. *Biomaterials* 2012;33:1477-88.
15. Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. *Cell Death Differ* 2008;15:171-82.
16. Xie CM, Chan WY, Yu S, Zhao J, Cheng CH. Bufalin induces autophagy-mediated cell death in human colon cancer cells through reactive oxygen species generation and JNK activation. *Free Rad Biol and Med* 2011;51:1365-75.