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ORIGINAL ARTICLE

Heptazoline exerts antiproliferative effects on human melanoma cells by inducing apoptosis, cell cycle arrest and targeting MAPK signalling pathway

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Summary

Purpose: *Melanoma is one of the prevalent types of cancer and ranks 6th major cause of cancer associated mortality. In this study the anticancer effects of the carbazole alkaloid Heptazoline were investigated against a panel of melanoma cells.*

Methods: *The normal BJ-5TA and melanoma cell lines MEL-CLS-1M MEL-CLS-2, MEL-CLS-3 were used in this study. MTT and colony formation assays were used to determine the proliferation rate of melanoma cells Aciridine orange (AO)/ ethidium bromide (EB) and annexin V/propidium iodide (PI) staining were used to check the apoptotic cell death. Cell cycle analysis was performed by flow cytometry and protein expression was checked by western blotting.*

Results: *Heptazoline inhibited the growth of all the melanoma cell lines, exhibiting an IC_{50} of 15 to 40 μM against the melanoma cells. However, the normal skin cells had IC_{50} 125 μM. The anticancer effects were found to be due to induction of apoptotic cell death which was associated with the upregulation of Bax, cleaved caspase 3, 9 and PARP and downregulation of Bcl-2. Furthermore, Heptazoline also triggered the G0/G1 arrest of melanoma cells. The effects of Heptazoline on the MAPK signalling pathway revealed that this molecule could inhibit the expression of p-p38 concentration-dependently.*

Conclusion: *Taken together, Heptazoline may prove a lead molecule in the development of systemic therapy of melanoma.*

Key words: *melanoma, heptazoline, apoptosis, MAPK, caspases*

Introduction

Melanoma is one of the devastating diseases and around 4% of the cancer-related deaths are attributed to melanoma. It is the sixth most prevalent type of cancer in United States and has been reported that one in every five individuals develops skin cancer in this country[1]. There is concrete evidence that exposure to ultraviolet radiation (UVR) initiates the development of melanoma and during the recent past a lot of attention has been paid to explore the potential of natural products to confer protection in UVR induced skin cancer [2]. Several of the natural products which include flavonoids, alkaloids and other secondary metabolites have shown promising results in the preliminary studies [3]. Plants have served as rich sources of anticancer agents such as campothecins, podophyllotoxins to name a few [4]. Heptazoline is an important carbazole alkaloid that has been isolated from several plant species and is also synthesized by organic chemists [5,6]. It has been reported to exhibit anticancer effects against a range of cancer cell lines. Besides Heptazoline, other carbazole alkaloids have also been reported to halt the growth of cancer cells via different mechanisms [7]. For example Maha-

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nine, a carbazole alkaloid, has been found to inhibit the cancer cell growth by prompting cell cycle arrest and apoptosis [8]. However, the anticancer potential of Heptazoline is yet largely unexplored. Hence, the present study was undertaken to screen Heptazoline against human melanoma cell lines and to explore the underlying mechanism. MAPK pathway is being considered as an imperative therapeutic target for cancer treatment [9] and herein the effects of Heptazoline were also examined on this pathway in melanoma cells.

Methods

Cell lines and culturing conditions

The normal (BJ-5TA) and melanoma cell lines (MEL-CLS-1, MEL-CLS-2, MEL-CLS-3, MML-1) were procured from American Type Culture Collection. The cells were maintained in Dulbecco's modified Eagle's medium in incubator (Thermo Scientific) at 37°C with 98% humidity and 5% CO_2.

MTT and colony formation assay

The antiproliferative effect of Heptazoline was assessed on melanoma cell lines by MTT assay as described previously by Mosmann [10]. The melanoma cells were subjected to treatment with varied concentrations of Heptazoline and the proliferation rate was determined by taking the absorbance at 570 nm. Colony formation assay was performed as described previously [11].

Apoptosis assay

The apoptosis-inducing effects of Heptazoline were determined by AO/EB staining as described previously [12]. In brief, the melanoma cells (0.6×10^6) were grown in 6-well plates. Following 12 h of incubation, the cells were subjected to Heptazoline treatment for 24 h at 37°C. The cell cultures were then centrifuged and the pellets were washed with phosphate-buffered saline (PBS). Thereafter, the cells were DAPI stained, centrifuged and PBS-washed. Finally, the nuclear morphology of the stained cells was examined by fluorescence microscopy. The percentage of the apoptotic cells was estimated by annexin V/PI staining by flow cytometry. DNA damage was assessed by comet assay as described previously [13].

Table 1. Antiproliferative effect of Heptazoline on different melanoma cells as depicted by MTT assay. The data are mean of three experiments ± SD

No.	*Cell lines*	*IC_{50} (μM)*
1	MEL-CLS-1	40
2	MEL-CLS-2	30
3	MML-1	15
4	MEL-CLS-3	30
5	BJ-5TA	125

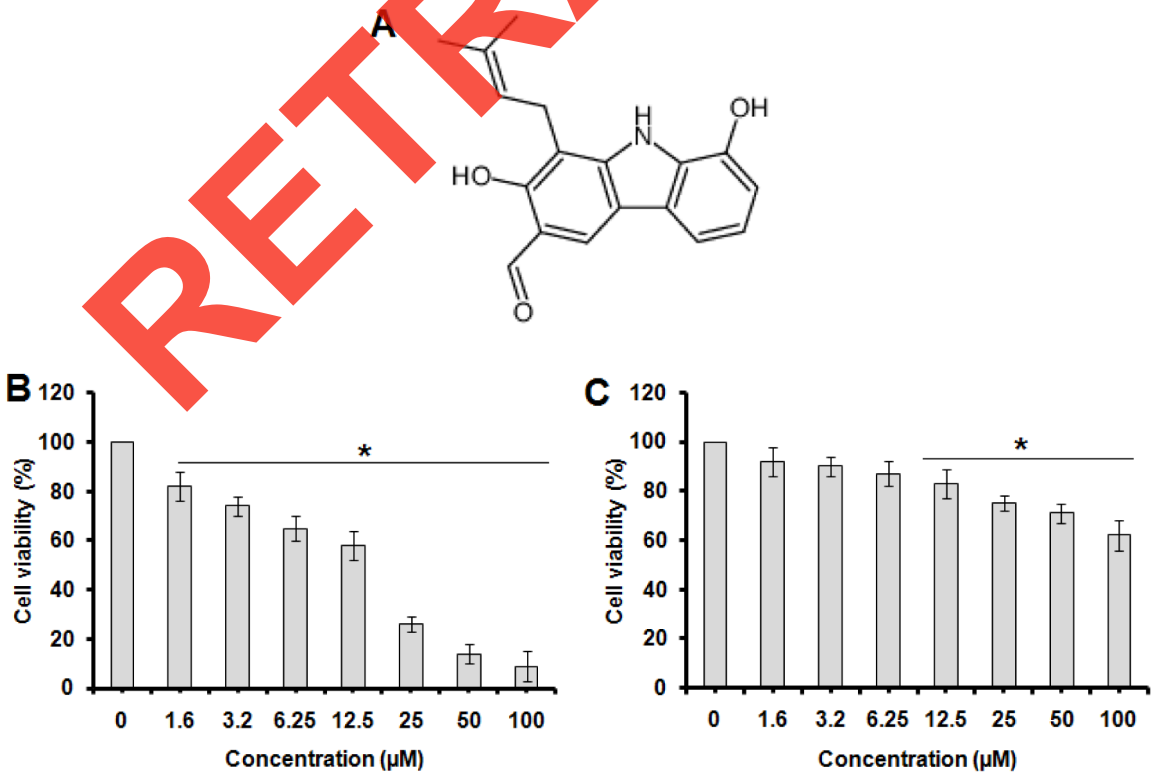

Figure 1. (A): Chemical structure of Heptazoline; **(B):** Effect of Heptazoline on the proliferation of MML-1 melanoma cells; and **(C):** Effect of Heptazoline on the proliferation of normal BG-5TA cells. The experiments were repeated in triplicate and expressed as mean ± SD (*p < 0.01).

Cell cycle analysis

The distribution of the melanoma cells in different cycle phases was performed by flow cytometry after PI staining. In brief, the melanoma cells were grown in 6-well plates and treated with Heptazoline for 24 h. The cells then collected and PBS-washed followed by fixation in ethanol (70%). After overnight incubation at 4°C, the cells were subjected to PI staining and subjected to flow cytometry.

Western blotting

After lysis of the melanoma cells in RIPA lysis buffer, the protein content of each lysate was estimated by bicinchoninic acid (BCA) assay. The samples were then loaded on the SDS-PAGE. The gels were then transferred to nitrocellulose membranes and subjected to treatment with primary antibody at 4°C for a period of 24 h. After this, the membranes were incubated with HRP-conjugated secondary antibody (1:1000) for 50 min at 25°C. Enhanced chemi-luminescence reagent was used to visualise the protein bands.

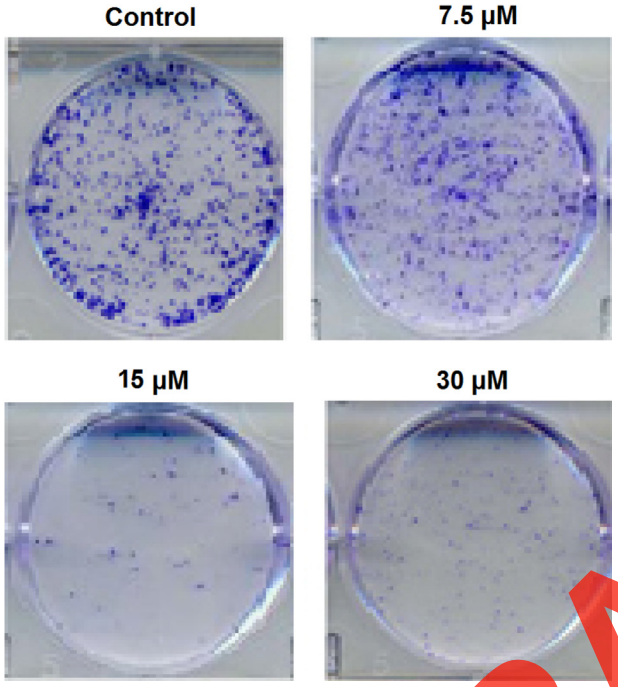

Figure 2. Colony formation assay showing inhibition of colony formation of MML-1 melanoma cells at indicated concentrations of Heptazoline. The experiments were repeated in triplicate.

Results

Heptazoline inhibits the growth of melanoma cells

The effects of Heptazoline (Figure 1A) on the proliferation of the melanoma cells were examined on a panel melanoma and normal cell lines by MTT assay. It was found that that Heptazoline exerted antiproliferative effects on all the melanoma cell lines (Table 1). The lowest IC_{50} of 15 μM was observed for the MML-1 cell line (Figure 1B). However, the IC_{50} of Heptazoline was significantly higher against the normal BJ-5TA skin cells (IC_{50} 125 μM) (Figure 1B). In addition, it was found that the anticancer effects of Heptazoline on the melanoma cells were concentration-dependent. Further, the colony formation assay found that Heptazoline inhibited the formation of colonies of the melanoma cells in a concentration-dependent manner (Figure 2).

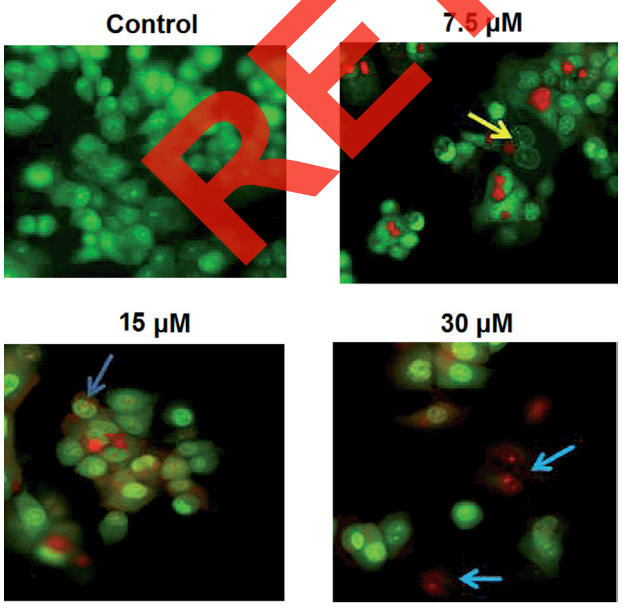

Figure 3. AO/EB staining showing induction of apoptotic cell death of the MML-1 cells at varied concentrations of Heptazoline (arrows show apoptotic cells). The experiments were repeated in triplicate.

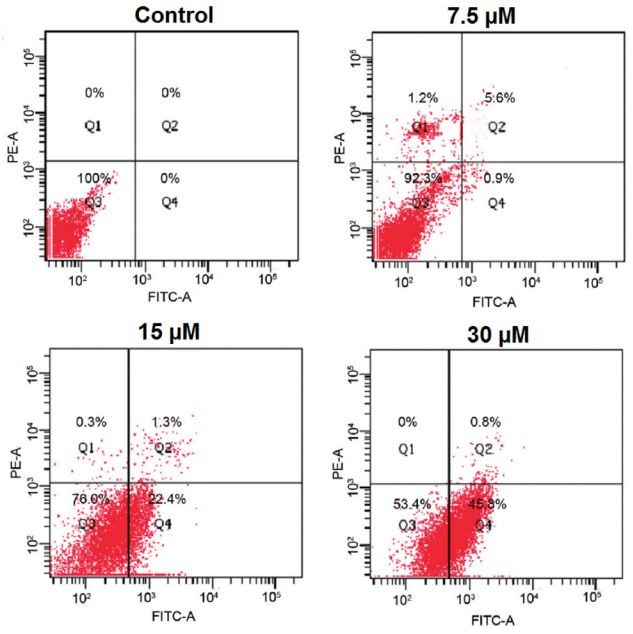

Figure 4. Annexin V/PI staining showing Heptazoline increases the percentage of apoptotic cells dose-dependently. The experiments were performed in triplicate.

Heptazoline induces apoptosis in melanoma cells

The effects of Heptazoline on the MML-1 melanoma cells were firstly evaluated by AO/EB staining. It was found that the number of orange colour cells increased with increase in the concentration of Heptazoline, indicative of apoptosis (Figure 3). Therefore, annexin V/PI staining was performed which showed that the apoptotic cell populations increased from 2.11% in control to 48.66% at 30 μM concentration of Heptazoline (Figure 4). The apoptosis was further confirmed by the increased expression of Bax, cleaved caspase 3, 9 and cleaved PARP and decreased the expression of the Bcl-2 in MML-1 melanoma cells (Figure 5). Next, the comet assay was performed to examine the effects of Heptazoline on melanoma cells and it was found that this molecule caused tail formation in the melanoma cells, indicative of DNA damage (Figure 6).

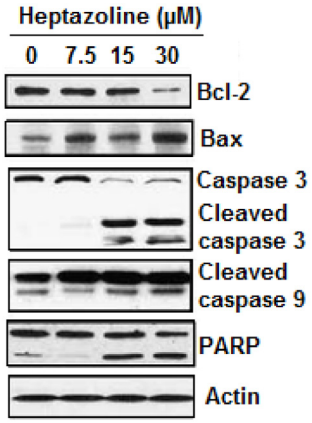

Figure 5. Effect of Heptazoline on the apoptosis-related protein expression as depicted by western blotting. The Figure shows that the expression of Bcl-2 decreased and that of Bax increased. In addition, Heptazoline activated the cleavage of Caspase-3, 9 and PARP. The experiments were performed in triplicate.

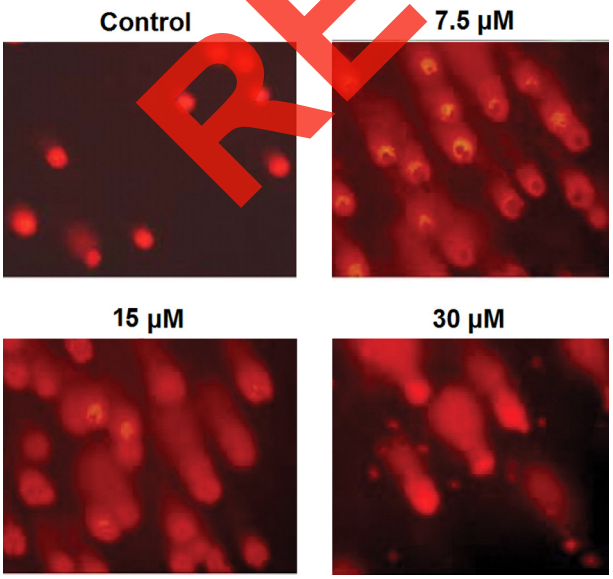

Figure 6. Comet assay showing induction of DNA damage by Heptazoline in MML-1 cells at indicated doses. The experiments were performed in triplicate.

Heptazoline causes the G0/G1 arrest of melanoma cells

The effects of Heptazoline on the distribution of MML-1 cells in various cell cycle phases was assessed by flow cytometry. It was found that Heptazoline caused remarkable increase in the percentage of the MML-1 cells in the G0 phase of the cell cycle. The percentage of MML-1 cells in the G0 phase increased from 10.12% to 35.25% upon treatment with Heptazoline (Figure 7). These results clearly indicate that Heptazoline induces G0/G1cell cycle arrest of the melanoma cells.

Heptazoline inhibits the MAPK signalling pathway

The effect of Heptazoline was also investigated on the p38 MAPK signalling pathway. It was found

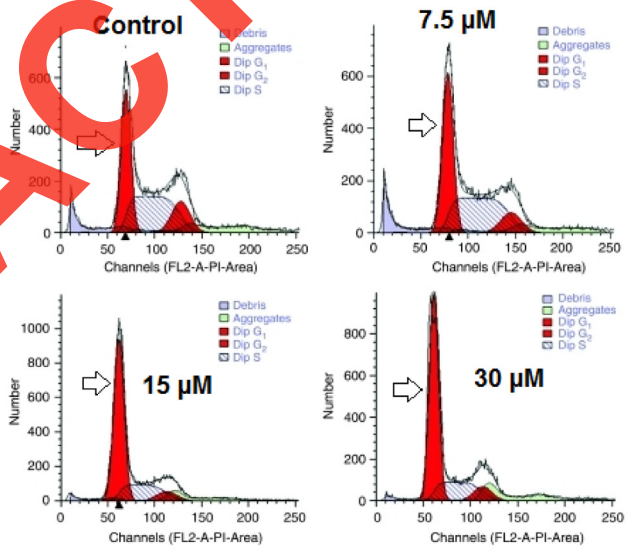

Figure 7. Cell cycle analysis by flow cytometry showing induction of G0/G1 cell cycle arrest of MML-1 cells at indicated concentrations of Heptazoline. The experiments were performed in triplicate.

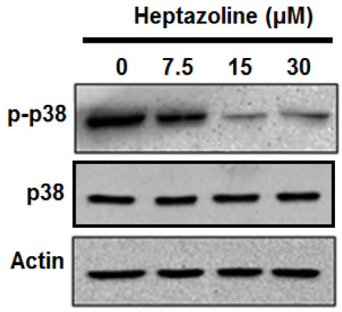

Figure 8. Western blot showing Heptazoline inhibits the expression of p-p38 dose-dependently. The experiments were performed in triplicate.

that this molecule suppressed the expression of p-p-38 in a dose-dependent manner. However, the expression of p-38 was enhanced as depicted in the western blot (Figure 8).

Discussion

Plant-derived anticancer agents have attained remarkable attention in the recent past due to their minimal toxic effects. As such, an ever increasing number of plant-derived natural products are being evaluated against cancer cells for their anticancer activity [14]. Melanoma is one of the deadliest cancers and the treatment options are limited and inefficient [15] and in this study, the anticancer effects of Heptazoline were examined against melanoma cell lines. It was found that Heptazoline exerts considerable anticancer effects on all the melanoma cell lines. However, its effects were negligible on the normal cell lines. These results were further complemented by the results of the colony formation assay wherein Heptazoline was found to suppress the colony formation potential of the melanoma cells. Previous investigations have also shown the anticancer effects of several carbazole alkaloids, further supporting our results [16]. What's more is that Heptazoline inhibited the growth of the melanoma cells more effectively which indicates that this molecule selectively targets cancer cells. Different anticancer agents halt the growth of cancer cells via different routes or mechanisms [17]. In this study we found that Heptazoline induces apoptotic cell death in melanoma cells.

Apoptosis is one of vital mechanisms which eliminates the defective and harmful cells from the body of an organism [18]. Several of the anticancer drugs have been reported to induce apoptosis in cancer cells. For example, cisplatin has been reported to induce apoptotic cell death of cancer cells [19]. Apoptosis prevents the development of drug resistance in cancer cells and as such the molecules that exhibit apoptotic inducing potential are considered important for the development of systemic therapy for cancer treatment [20]. Heptazoline-induced apoptosis was also associated with upregulation of Bax, cleaved caspase 3, 9 and PARP which are important biomarkers for apoptosis [21].

Next, it was also found that Heptazoline could induce G0/G1 cell cycle arrest of the melanoma cells which confirms previous studies wherein several of the carbazole alkaloids have been shown to induce cell cycle arrest of cancer cells [22]. MAPK signalling pathway has been found to play a vital role in the proliferation, progression and tumorigenesis of cancer cells and may serve as a therapeutic target for the treatment of cancer [23]. Therefore, the effects of Heptazoline were also investigated on the MAPK signalling pathway and the results showed that Heptazoline could efficiently inhibit this pathway, indicative of the potential of Heptazoline in the treatment of melanoma.

Conclusion

Taken together, it is concluded that Heptazoline inhibits the growth of melanoma cells by induction of apoptosis and cell cycle arrest. Therefore, Heptazoline may prove a lead molecule in the development of systemic therapy of melanoma and warrants *in vivo* evaluation.

Conflict of interests

The authors declare no conflict of interests.

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