

ORIGINAL ARTICLE

Heptaphylline suppresses the proliferation and migration of human bladder cancer cells via induction of intrinsic apoptosis, autophagy and inhibition of β -catenin signalling pathway

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Summary

Purpose: Heptaphylline has been shown to suppress the growth of different types of cancer cells. Nonetheless, the anticancer effects of Heptaphylline have not been examined against human bladder cancer cells. Against this backdrop, this study was undertaken to investigate the anticancer effects of the carbazole alkaloid Heptaphylline against human bladder cancer cells.

Methods: Proliferation rate was determined by MTT assay. Apoptosis was demonstrated by DAPI and annexin V/propidium iodide (PI) assay. Electron microscopy was used for autophagy detection. Western blot analysis was used to determine protein expression.

Results: The results showed that Heptaphylline suppressed the proliferation of the RT4 bladder cancer cells and exhibited an IC_{50} of 25 μ M. The toxic effects of Heptaphylline were comparatively lower on the normal Hs172.T cells, as evidenced from the IC_{50} of 95 μ M. The wound healing as-

say showed that Heptaphylline suppressed the migration of the RT4 bladder cancer cells. The DAPI and annexin V/PI staining showed that Heptaphylline induced apoptosis in the RT4 bladder cancer cells which was also accompanied by enhancement in the cleavage of PARP, caspase-3 and caspase-9. Additionally, Heptaphylline caused increase in Bax and decrease in Bcl-2 expression. Electron microscopic analysis showed that Heptaphylline also caused autophagy in the RT4 cells which was associated with increase in LC3, Atg5, Atg7 and Beclin-1 expression and decrease in p62 expression. This molecule also blocked the β -catenin signalling pathway in the RT4 bladder cancer cells.

Conclusion: Taken together, Heptaphylline suppressed the proliferation of the bladder cancer cells and may prove beneficial in the bladder cancer treatment.

Key words: bladder cancer, heptaphylline, apoptosis, autophagy, migration

Introduction

Carbazole alkaloids have shown an amazing potential to suppress the growth of cancer cells [1]. Therefore over the last few decades they have gained lot of attention as pharmacologically important secondary metabolites [2]. They have shown potential to inhibit cancer cell growth via multiple mechanisms which include apoptosis, autophagy and cell cycle arrest to name a few [3]. Heptaphylline is an important carbazole alkaloid which has

been extracted from different plant species such as *Clausena heptaphylla* of family *Rutaceae* [4]. Studies have also reported that Heptaphylline can be synthesized through organic chemistry approaches [5]. The ability of Heptaphylline to suppress the growth of cancer cells has also been reported, for example, Heptaphylline suppresses the growth of colon adenocarcinoma cells via induction of programmed cell death [6]. Nonetheless, there is no

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report on the anticancer effects of Heptaphylline against human bladder cancer cells. This study was therefore designed to investigate the anticancer effects of Heptaphylline against human RT4 bladder cancer cells and the β -catenin signaling pathway which has therapeutic implications in different cancer types [7].

Methods

Cell viability

The RT4 bladder cancer cell line and HEMn-LP normal cell line were cultured and then treated with Heptaphylline at concentrations ranging from 0 to 200 μ M for 24 h. Subsequently, the cells were subjected to MTT assay (500 μ g/mL) for 4 h. Dimethyl sulfoxide (DMSO) (10%) was then added to dissolve the blue formazan formed. Finally, the optical density (OD) was taken at 570 nm by a spectrophotometer to monitor the cell viability.

Cell cycle analysis

The cultured human bladder RT4 cells were firstly treated with varied concentrations of Heptaphylline for 24 at 37°C. The cells were then washed with phosphate buffered saline (PBS). Afterwards, the RT4 cells were stained with Annexin V/PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.

DAPI assay

The RT4 cells (0.6×10^6) were cultured in 6-well plates and treated with Heptaphylline at the concentra-

tions of 0, 9, 18 and 36 μ M for 24 h at 37°C. Subsequently, 25 μ l of cell culture were put onto glass slides and stained with DAPI. The slides were then cover-slipped and examined under fluorescence microscope.

Annexin V/PI assay

ApoScan kit was used to determine the apoptotic RT4 cell percentage. In brief, Heptaphylline-treated RT4 cells (5×10^5 cells per well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC or PI. The percentage of apoptotic RT4 cells at each concentration was then determined by flow cytometry.

Electron microscopic analysis

Autophagy in Heptaphylline-treated bladder cancer cells was demonstrated by electron microscopy. In brief, the bladder RT4 cancer cells were treated with 0, 12.5, 25 and 50 μ M Heptaphylline for 24 h. The cells were collected by trypsinization and subjected to washing with phosphate-buffered saline (PBS) which was followed by fixation in glutaraldehyde (2%) in phosphate buffer (0.1 M). The cells were then post-fixed in osmium tetroxide (1%). This was followed by treatment of the cells with ethanol and embedding in resin. Thin sections were then cut with an ultramicrotome and subjected to electron microscopy.

Western blot analysis

The RT4 cells were then lysed in lysis buffer containing the protease inhibitor. Around 45 μ g of proteins from each sample were subjected to separation on 10% SDS-PAGE which was followed by transferring it to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally the protein signal was detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

Statistics

The experiments were performed in triplicate and the values represent the average of three replicates. $P < 0.05$ indicated statistically significant difference. Student's t-test was used for the statistical analyses.

Results

Heptaphylline suppressed the proliferation of bladder cancer cells

The proliferation rate of the RT4 bladder cancer and normal Hs172.T cells was monitored by MTT assay at concentrations ranging from 0 to 200 μ M of Heptaphylline (Figure 1A). Heptaphylline caused a remarkable decrease in the proliferation rate of RT4 cells and its effects on the viability of the RT4 cells were concentration-dependent; IC_{50} of 25 μ M was reported for Heptaphylline against the RT4 cells

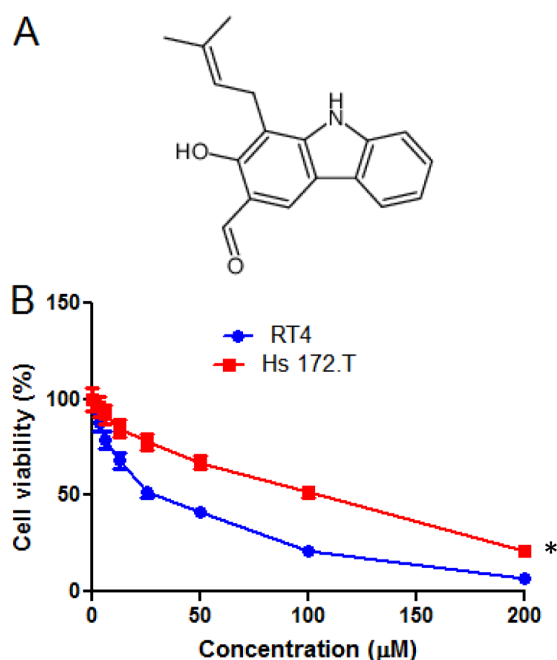


Figure 1. A: Chemical structure of Heptaphylline. **B:** MTT assay showing the effects of Heptaphylline on the viability of RT4 bladder cancer cells. The experiments were performed in triplicate and expressed as mean \pm SD ($p < 0.05$).

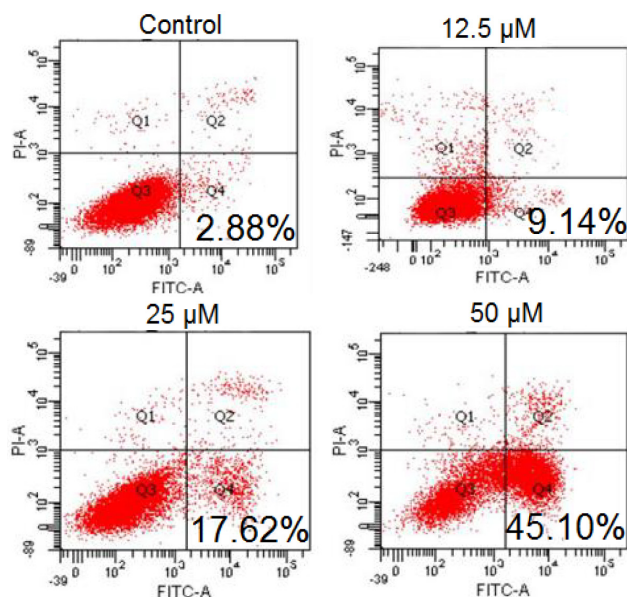


Figure 4. Annexin V/PI staining showing the percentage of RT4 cells at indicated concentrations of Heptaphylline. The experiments were performed in triplicate.

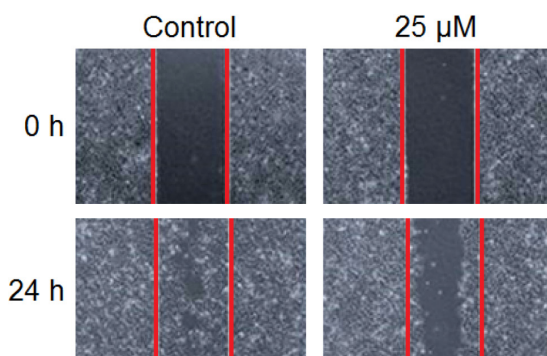


Figure 2. Wound healing assay showing that Heptaphylline inhibits the migration of RT4 bladder cancer cells. The experiments were performed in triplicate.

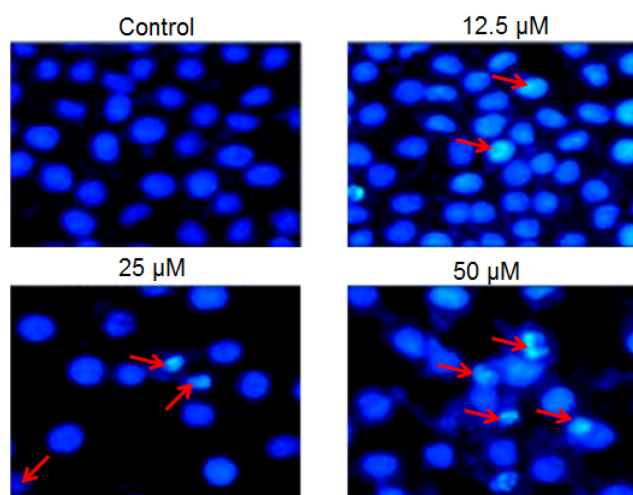


Figure 3. DAPI staining showing that Heptaphylline induces apoptosis in the RT4 bladder cancer cells concentration-dependently (arrows show apoptotic cells). The experiments were performed in triplicate.

(Figure 1B). The antiproliferative effects of Heptaphylline were also assessed on the normal Hs172.T cells and its IC_{50} was found 95 μ M (Figure 1B).

Heptaphylline suppressed the migration of bladder cancer cells

The effects of Heptaphylline on the RT4 bladder cancer cells was assessed by the wound healing assay at IC_{50} (ie., 25 μ M). The results showed that in untreated RT4 cells the wound width decreased significantly, while in Heptaphylline-treated cells, the wound width was not decreased (Figure 2). These results suggest that Heptaphylline suppressed the migration of the RT4 bladder cancer cells.

Heptaphylline induced apoptosis of RT4 bladder cancer cells

For the demonstration of the apoptotic cell death of the Heptaphylline-treated RT4 bladder cancer cells, DAPI assay was performed and the results showed that Heptaphylline caused nuclear fragmentation of these cells, indicative of apoptosis (Figure 3). Annexin V/PI showed a constant increase in the percentage of the RT4 bladder



Figure 5. Western blot analysis showing the effects of Heptaphylline on the apoptosis-related proteins. The experiments were performed in triplicate.

cancer cells upon Heptaphylline treatment. The percentage of the RT4 apoptotic cells was 2.88%, 9.14%, 17.62% and 45.10% at the 0, 12.5, 25 and 50 μ M concentrations of Heptaphylline (Figure 4). Western blot analysis showed that Heptaphylline triggered the cleavage of PARP, caspase-3 and caspase-9 in the RT-4 bladder cancer cells. Additionally, the expression of Bcl-2 was significantly decreased and the expression of Bax showed a little increase (Figure 5).

Heptaphylline induced autophagy in RT4 bladder cancer cells

Electron microscopic analysis was also performed to assess if Heptaphylline also promotes autophagy in the RT4 bladder cells. It was revealed that this molecule led to the development of autophagosomes in the RT4 cells indicative of autophagy (Figure 6). Western blot analysis showed that Heptaphylline caused increase in the expression of LC3B II, vsp34, Beclin 1, Atg5 and Atg7, while a steady decrease was observed in the expression of p62 (Figure 7).

Heptaphylline blocked β -catenin signalling pathway in RT4 bladder cancer cells

The effects of Heptaphylline were also assessed on the β -catenin signalling pathway by western blot analysis. The results showed that this the molecule caused remarkable decrease in the expression of USP9X, β -catenin, c-Myc and cyclin D1 (Figure 8).

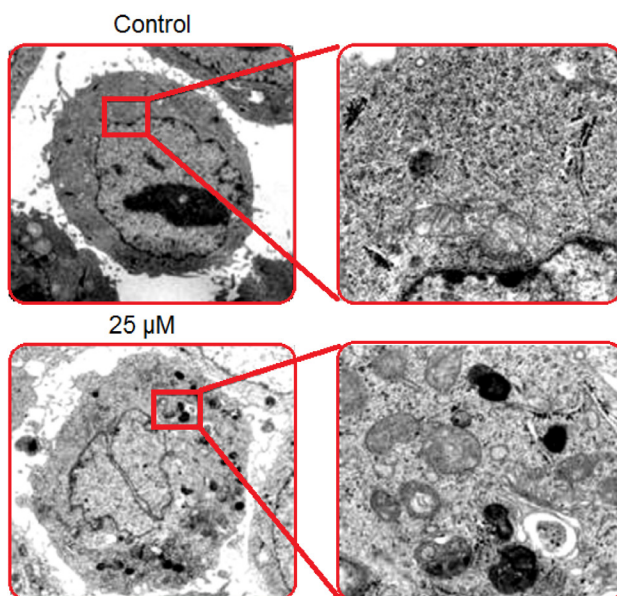


Figure 6. Electron microscopic analysis showing that Heptaphylline triggers the formation of autophagic vesicles in RT4 cells, indicative of autophagy. The experiments were performed in triplicate.

Discussion

Being the 4th prevalent type of cancer in men and 13th in women, bladder cancer causes significant morbidity and mortality across the globe [8]. Bladder cancer imposes huge economic burden on human population and is currently the 5th most expensive cancer in terms of treatment expenditure [9]. The treatment of bladder cancer involves chemotherapy, radiotherapy or surgery depending on the stage of cancer. Despite recent advancements, the clinical outcome for bladder cancer is still far from descent [10]. This study was therefore undertaken to investigate the anticancer effects of the carbazole alkaloid Heptaphylline on the human RT4 bladder cancer cells. The results showed that Heptaphylline suppressed the proliferation and migration of bladder cancer cells in a concentration-dependent manner. Additionally, the antipro-

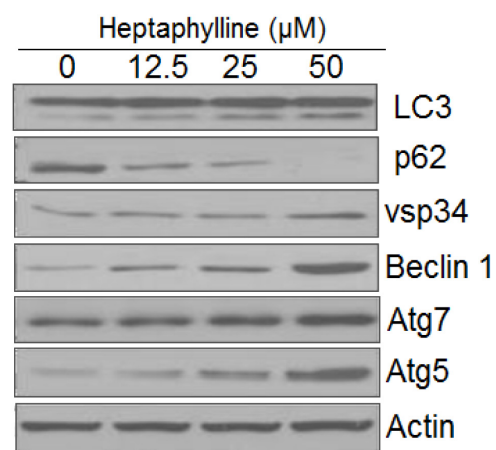


Figure 7. Western blot analysis showing the effects of Heptaphylline on the autophagy-related proteins. The Figure shows that Heptaphylline increases the expression of LC3, vsp34, Beclin 1, Atg 7 and Atg 5 and decreases the expression of p62. The experiments were performed in triplicate.

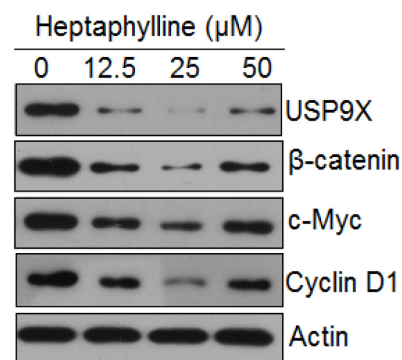


Figure 8. Western blot analysis showing that Heptaphylline decreases the expression of USP9X, β -catenin, c-Myc and Cyclin D1, indicative of inactivation of β -catenin signalling pathway in RT4 cells. The experiments were performed in triplicate.

liferative effects of Heptaphylline were found to be cancer cell-specific, as evidenced from the high IC₅₀ against the normal Hs172.T cells. These results are in agreement with previous studies wherein Heptaphylline has been reported to inhibit the growth of cancer cells [11]. Heptaphylline has also been reported to induce apoptotic cell death of colon adenocarcinoma cells [6]. Therefore, we also investigate if Heptaphylline induced apoptosis in RT4 bladder cancer cells. Interestingly, this molecule caused apoptosis in the RT4 bladder cancer cells as evidenced from both DAPI and annexin V/PI assays. The PARP, Caspases, Bax and Bcl-2 are the important biomarker proteins for apoptosis [12] and it was found that Heptaphylline increased the cleavage of PARP, Caspase-3 and Capase-9 and also increased the Bax/Bcl-2 ratio favouring apoptosis. Several of the carbazole alkaloids have been shown to induce autophagy in cancer cells, for example Isomahanin, a carbazole alkaloid, has been reported to induce autophagy in oral cancer cells [13]. We also examined if Heptaphylline induces autophagy in the bladder cancer cells and electron microscopy showed that this molecule caused development of autophagosomes in the RT4 bladder cancer cells which was also accompanied by increase in the expression of LC3, Atg5, Atg7 and Beclin 1 and

suppression of p62, indicative of autophagy. The β -catenin signalling pathway has been reported to be aberrantly overexpressed in cancer cells [14] and has been reported to play a role in the development and progression of different cancers [15] and it is therefore believed to exhibit therapeutic implications in the treatment of several cancers [16]. In this study we found that Heptaphylline suppressed this pathway in bladder cancer cells, indicative of the potential of Heptaphylline as potentially therapeutic agent in bladder cancer.

Conclusion

The findings of the present study indicate that Heptaphylline may prove beneficial in the treatment of bladder cancer. Heptaphylline induces apoptosis and autophagy in bladder cancer cells and may prove a lead molecule for the development of systemic therapy for bladder cancer. However, *in vivo* evaluation of Heptaphylline is urgently required.

Conflict of interests

The authors declare no conflict of interests.

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