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 IC₅₀ of 25 µM. The toxic effects of Heptaphylline were comparatively lower on the normal Hs172.T cells, as evidenced from the IC₅₀ of 95 µM. The wound healing assay 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
Heptaphylline is an agent against bladder cancer

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 µM 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; IC50 of 25 µM was reported for Heptaphylline against the RT4 cells.

**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 µM 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; IC50 of 25 µM was reported for Heptaphylline against the RT4 cells.
Heptaphylline is an agent against bladder cancer (Figure 1B). The antiproliferative effects of Heptaphylline were also assessed on the normal Hs172.T cells and its IC_{50} was found 95 µM (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 µM). 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 cancer cells (Figure 4). 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 µM 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).

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-
liferative effects of Heptaphylline were found to be cancer cell-specific, as evidenced from the high IC<sub>50</sub> 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.

**References**

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