ORIGINAL ARTICLE

The anticancer effects of 7-Methoxyheptaphylline against the human retinoblastoma cells are facilitated via S-phase cell cycle arrest, mitochondrial apoptosis and inhibition of Wnt/β-catenin signalling pathway

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Summary

Purpose: The main purpose of the current study was to evaluate the anticancer action of 7-Methoxyheptaphylline in Y-79 human retinoblastoma cells along with evaluating its effects on cellular apoptosis, cell cycle phase distribution and Wnt/β-catenin signalling pathway.

Methods: The retinoblastoma cell line RbY-79 was used in this study. Cell viability was assessed by WTS-1 assay while apoptotic studies were carried out by DAPI, acridine orange (AO)/ethidium bromide (EB), annexin V + propidium iodide (PI) staining using fluorescence microscopy and flow cytometry. Effects on cell cycle progression were studied using Annexin V/PI staining in combination with flow cytometry. Western blot assay was used to examine the effects on Bax, Bcl-2 and proteins associated with Wnt/β-catenin signalling pathway.

Results: The results indicated that 7-Methoxyheptaphylline suppressed the viability of the Y-79 cells concentration-dependently with IC_{50} value of 15.5 μM. The percentage of the DAPI-positive cells showed a significant upsurge reminiscent of the apoptosis in the Y-79 retinoblastoma cells. 7-Methoxyheptaphylline also caused considerable nuclear fragmentation of the Y-79 retinoblastoma cells, representative of apoptosis. The apoptosis percentage increased significantly as the dose of the 7-Methoxyheptaphylline increased from 0 to 12, 24 and 48 μM. The molecule caused upregulation of Bax and downregulation of Bcl-2 in Y-79 retinoblastoma cells. 7-Methoxyheptaphylline also caused S-phase cell cycle arrest with concomitant concentration-dependent decline in the expression levels of cyclin A, E and D1. It was also seen that increasing doses of 7-Methoxyheptaphylline led to a dose-dependent decline in the expression levels of wnt-13a and β-catenin.

Conclusions: In conclusion, it is believed that the molecule may prove to be a promising anticancer agent for the treatment of retinoblastoma.

Key words: 7-Methoxyheptaphylline, retinoblastoma, apoptosis, flow cytometry, cell cycle

Introduction

Retinoblastoma (Rb) is a rare malignancy which originates from the immature retinal cells and is mostly prevalent in young children. Retinoblastoma is responsible for 4% of the cancers found in children and infants. This cancer ranks first in the malignancies of eyes and recently it has been found to be on a significant rise in China [1,2]. Patients with Rb mostly lose their vision even if they happen to survive this malignancy. As far as diagnosis is concerned, Rb in children is mostly diagnosed at or before the age of 3-4 years [3]. Various causative agents have been identified which are accountable

for Rb including papilloma virus, inactivation of the bi-allelic Rb gene in cells of the retina in both sporadic and hereditary kinds of cells. Absence of a functional pRb1 initiates faulty cellular differentiation and unrestrained division of the human retinal cells, ultimately leading to retinoblastoma [4,5]. The prevailing treatment options for Rb comprise chemotherapy, radiation therapy and laser therapy in combination with chemotherapy or radiotherapy, eye enucleation in tandem with or without the use of chemotherapy. The ideal method of treatment includes chemotherapy combined with photocoagulation. Despite the various advances in Rb treatment, there are a number of vital issues which remain to be solved. These issues include the severe side effects of chemo and radiotherapy as well as development of resistance to chemotherapy, and enhanced risk of developing secondary cancers like melanoma and osteosarcoma [6,7]. Another unresolved issue is related to the loss of vision and deformity of the face following eye enucleation therapy [8]. Keeping these things in mind, there is a pressing need for the design and development of efficacious chemotherapeutic agents for the treatment of Rb. Since natural products have been at the forefront of the anticancer drugs [9], our main objective was to investigate the antitumor activity of a plant alkaloid, namely 7-Methoxyheptaphylline, along with studying its detailed mechanism of anticancer action including its effects on cell cycle phase distribution, mitochondrial-dependent apoptosis and inhibition of Wnt/β-catenin signalling pathway. To the best of our knowledge, there are no previous reports on the anticancer action of 7-Methoxyheptaphylline against human Rb.

Methods

Cell line, cell culture conditions and WST-1 cell proliferation assay

The Rb cell line Y-79 was purchased from American Type Culture Collection. The cells were kept in Dulbecco's modified Eagle's medium (DMEM). The medium was also supplemented with 10% fetal bovine serum (FBS), antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), and 2 mM glutamine. The viability of the human Rb Y-79 cells was monitored by WST-1 assay. In brief, using 96-well plates, Y-79 cells were cultured at a density of 2×10^6 cells per well and then subjected to the treatment of 7-Methoxyheptaphylline at doses of 0, 2.5, 5, 10, 20, 40, 80, 160 and 320 μM for 24 h at 37°C. Subsequently, the Y-79 cells were incubated with WST-1 cell proliferation reagent at 37°C for 3 h. At the end of the experiment, the absorbance was measured at a wavelength of 450 nm with the help of a microplate reader.

4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) staining assays

The Y-79 cells were grown in 6-well plates (0.6×10^6 cells/well) for 12 h. The cells were then subjected to 7-Methoxyheptaphylline treatment (0, 12, 24 and 48 μM) for 24 h at 37°C. Around 15 μl cell cultures were put onto a glass slide and stained with a solution of DAPI or separately. The slides were then cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [10].

Acridine orange/ethidium bromide (AO/EB) staining assay for apoptosis

The Y-79 cells were grown in 6-well plates (0.6×10^6 cells/well) and incubated for 12 h. The cells were treated with increasing doses (0, 12, 24 and 48 μM) of 7-Methoxyheptaphylline for 24 h at 37°C. About 50 μl cell culture were put onto a glass slide and then stained with AO/EB. The slides were then cover-slipped and examined with a fluorescent microscope.

Cell cycle analysis

The Y-79 Rb cells were exposed to varied concentrations of 7-Methoxyheptaphylline (0, 12, 24 and 48 μM) and incubated for 24 h and were washed with phosphate-buffered saline (PBS). The, the Y-79 cells were stained with Annexin V/PI and the distribution of the cells in cell cycle phases was assessed by FACS flow cytometer.
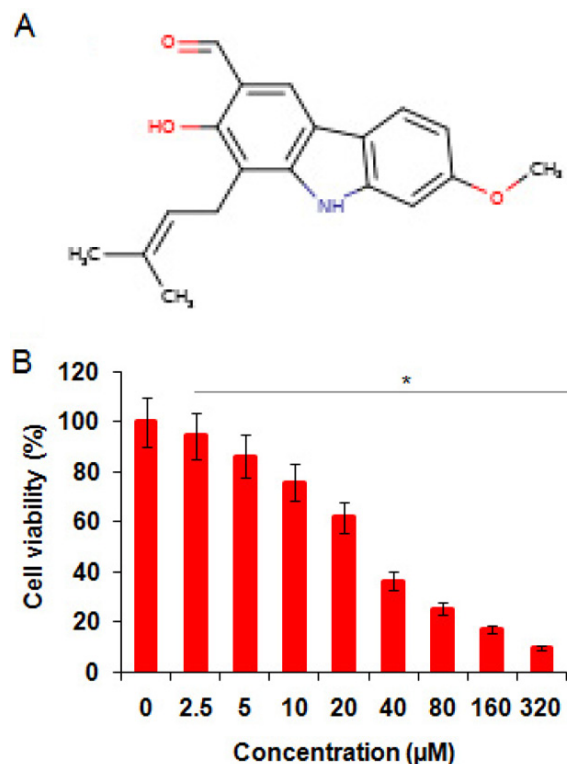

Figure 1. A: Chemical structure of 7-Methoxyheptaphylline. **B:** WTS-1 assay showing cytotoxic effects of 7-Methoxyheptaphylline on the viability of Y-79 human retinoblastoma cells. The experiments were performed in triplicate and shown as mean ± SD (*p<0.05).

Western blotting

The Y-79 Rb cells were washed with ice-cold PBS and suspended in a lysis buffer at 4°C and then shifted to 95°C. Thereafter, the protein content of each cell extract was checked by Bradford assay. About 40 µg of protein were loaded from each sample and separated by SDS-PAGE before being shifted to polyvinylidene fluoride membrane. The membranes were then subjected to treatment with tris-buffered saline (TBS) and exposed to primary antibodies at 4°C. Thereafter, the cells were treated with appropriate secondary antibodies and the proteins of interest were visualised by enhanced chemiluminescence reagent. Finally, the signal was detected by Odyssey Infrared Imaging System. Actin and GAPDH were used as control for normalization.

Statistics

Data are shown as mean±SD. Statistical analyses were done using Students *t*-test with GraphPad prism 7 software. Values of $p < 0.05$ were considered as statistically significant.

Results

7-Methoxyheptaphylline exerts growth inhibitory effects on Y-79 retinoblastoma cells

The growth inhibitory effects of Methoxyheptaphylline (Figure 1A) were assessed on the Y-79 Rb cell line by WTS-1 assay at concentration ranging from 0 to 320 µM. The molecule was shown to suppress the viability of the Y-79 cells concentration-dependently (Figure 1B). The IC_{50} of Methoxyheptaphylline against the Y-79 cells was 15.5 µM. The cell viability was much more pronounced at 320 µM dose, inhibiting more than 90% of the cell growth.

7-Methoxyheptaphylline initiates apoptosis in Y-79 retinoblastoma cells

To demonstrate whether 7-Methoxyheptaphylline antiproliferative effects on the Y-79 Rb cells are mediated via induction of apoptotics, DAPI and annexin V/PI staining assays were performed. It was shown that the percentage of the DAPI-positive cells showed a significant upregulation reminiscent of the apoptosis in the Y-79 Rb cells (Figure 2). To ascertain the underlying mechanism for the growth inhibitory property of the molecule, the Y-79 cells were treated with different doses of 7-Methoxyheptaphylline and then stained with AO/EB. The results of AO/EB showed that 7-Methoxyheptaphylline caused considerable nuclear fragmentation of the Y-79 Rb cells, representative of apoptosis (Figure 3). The annexin V/PI staining showed that Y-79 Rb apoptotic cell percentage increased in a concentration-dependent manner. The apoptosis percentage increased significantly

as the dose of the 7-Methoxyheptaphylline drug was increased from 0 to 12, 24 and 48 µM (Figure 4). Further, for the substantiation of apoptosis, the expression of apoptosis-associated proteins (Bax and Bcl-2) was examined and it was shown that 7-Methoxyheptaphylline caused upregulation of Bax and downregulation of Bcl-2 in Y-79 Rb cells (Figure 5).
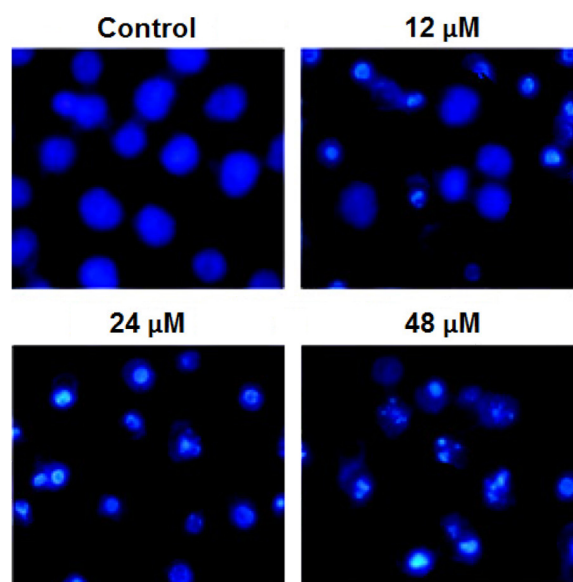

Figure 2. DAPI staining showing 7-Methoxyheptaphylline induces concentration-dependent apoptosis in Y-79 human retinoblastoma cells. The experiments were performed in triplicate.
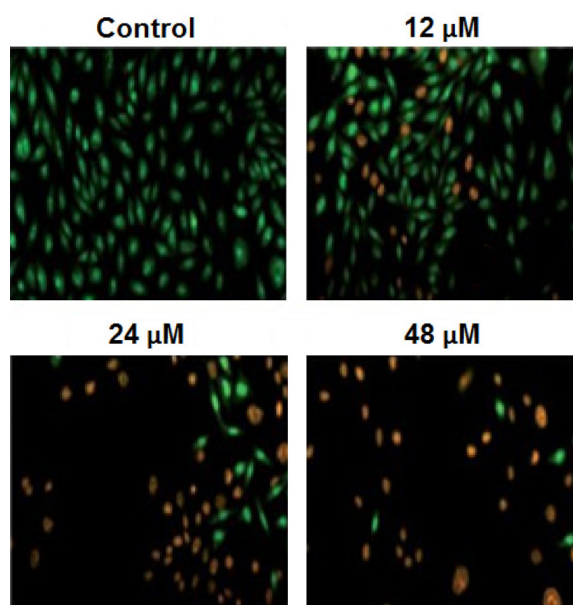

Figure 3. Acridine orange/Ethidium bromide staining showing that 7-Methoxyheptaphylline induces concentration-dependent apoptosis in Y-79 human retinoblastoma cells. Green colored cells show normal cells and orange colored cells depict the apoptotic cells. The experiments were performed in triplicate.

Putting all together, these three assays (fluorescence microscopy, flow cytometry and western blot) clearly showed that 7-Methoxyheptaphylline induces apoptosis in Y-79 Rb cells beyond doubt.

7-Methoxyheptaphylline arrests the Y-79 retinoblastoma cells at S phase

The effects of 7-Methoxyheptaphylline on the cell cycle phase distribution of Y-79 Rb cells was assessed by flow cytometry after annexin V/ PI staining at 0 to 12, 24 and 48 µM concentration of 7-Methoxyheptaphylline. It was seen that the percentage of S phase cells increased considerably with 6.7% in the control to 80.12% at 48 µM concentration of 7-Methoxyheptaphylline (Figure 6). Thus, this assay further lends support to the fact that the molecule targets Y-79 Rb cells even at a cell cycle check point. Further, in order to con-

firm whether 7-Methoxyheptaphylline targets cell cycle check point in these cells, western blot was designed to show the effects of 7-Methoxyheptaphylline on the expression and phosphorylation of cell cycle regulatory proteins including cyclin A, E and D1 and it was seen that the molecule at doses of 12, 24 and 48 µM led to a significant and
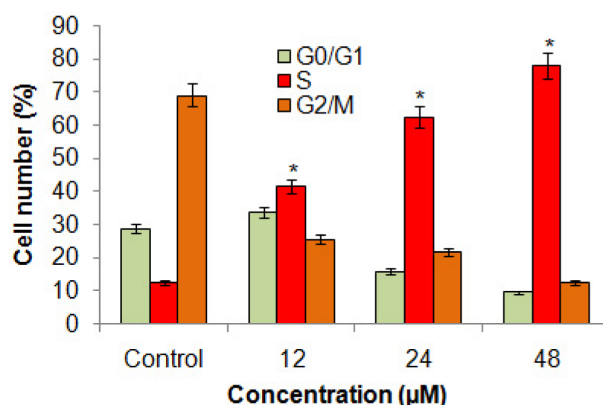

Figure 6. 7-Methoxyheptaphylline triggers S cycle phase arrest of the Y-79 retinoblastoma cells as evidenced by flow cytometry (*p<0.05). The experiments were performed in triplicate.

Figure 7. Effect of indicated concentrations of 7-Methoxyheptaphylline on the expression of cell cycle regulatory proteins by western blot analysis. The Figure shows that 7-Methoxyheptaphilline inhibits the Cyclin A, D1 and E expression dose-dependently. The experiments were performed in triplicate.
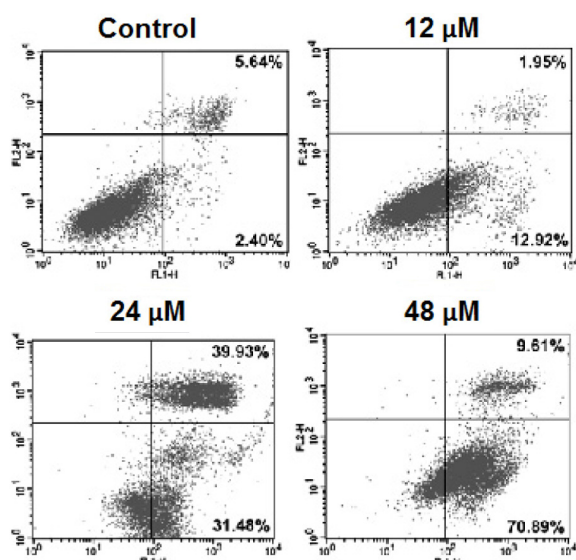

Figure 4. Annexin V/PI assay showing the percentage of Y-79 apoptotic cells at the indicated concentrations of 7-Methoxyheptaphylline. The experiments were performed in triplicate.
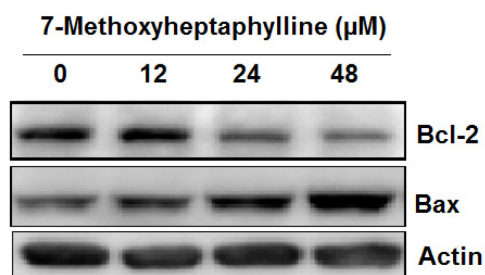

Figure 5. Effect of indicated concentrations of 7-Methoxyheptaphylline on the expression of apoptosis-related proteins by western blot analysis. The Figure shows that the expression of Bax increased and that of Bcl-2 decreased upon 7-Methoxyheptaphylline treatment of the retinoblastoma cells. The experiments were performed in triplicate.
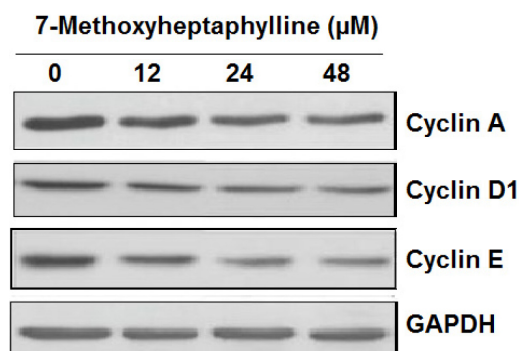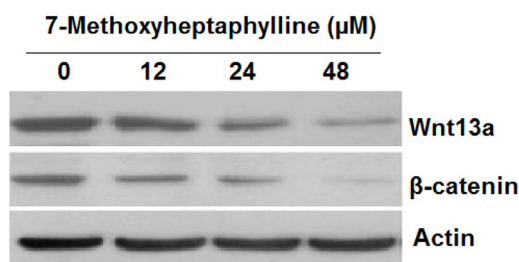

Figure 8. Effect of indicated concentrations of 7-Methoxyheptaphylline on the expression of Wnt/β-catenin signalling pathway proteins. The Figure shows that 7-Methodyheptaphylline inhibits the Wnt and β-catenin expression dose-dependently. The experiments were performed in triplicate.

concentration-dependent decline in the expression levels of cyclin A, E and D1 (Figure 7).

7-Methoxyheptaphylline inhibits Wnt/β-catenin signalling pathway in Y-79 retinoblastoma cells

Finally, it has been observed previously that wnt signalling plays a key role in cell proliferation, migration and differentiation of various cancers and as such targeting this signalling pathway could play a significant role in the development of important anticancer therapeutic agents for Rb. In this study it was seen that increasing doses of 7-Methoxyheptaphylline led to a dose-dependent decline in the expression levels of wnt-13a and β-catenin. A significant decrease of cytosolic β-catenin was observed after 7-Methoxyheptaphylline treatment (Figure 8).

Discussion

Natural products have always played a crucial role in treating different human illnesses, especially in treating different cancers. Several clinically useful anticancer drugs have been isolated from plants which have been used in treating different cancers in the past few decades. Plant-derived natural products play a significant role in drug discovery keeping in view the increasing number of new and novel drugs which are currently in different phases of clinical development [11,12]. Over the past few decades, a number of studies have clearly illustrated the overall significance of natural products, particularly in the pharmaceutic industry. Alkaloids are a class of naturally occurring compounds with diverse chemical structure, distribution, biological activity and commercial importance. Some of the pharmaceutically important alkaloids are paclitaxel, docetaxel, vincristine, vinblastine, camptothecin, huperzine-A, caffeine, theophylline, capsaicin, colchicine, galanthamine, pilocarpine, scopolamine etc [13]. Keeping in mind the high pharmaceutical potential of natural alkaloids, the main aim of this research work was to explore the anticancer activity of 7-Methoxyheptaphylline in Y-79 human Rb cells along with studying its effects on apoptosis,

cell cycle phase distribution, and Wnt/β-catenin signalling pathway. 7-methoxyheptaphylline is a carbazole alkaloid, mostly isolated from the roots *Clausena harmandiana* extract [14]. Heptaphylline and 7-Methoxyheptaphylline have been shown to exhibit cytotoxicity against human small cell lung carcinoma, human epidermoid cancer and oral cancer. These compounds showed IC_{50} value as low as 1.3 and 2.7 and more importantly showed very less cytotoxicity against normal cells (Vero cells; African green monkey kidney) [15]. In yet another study, this molecule has been shown to induce apoptosis in human colon adenocarcinoma cells through Akt/Nf-kB/bid pathways [16]. However, its effects on Y-79 human Rb cells have not been reported so far. Therefore, in the current study we demonstrated the anticancer effects of this molecule in Y-79 human Rb cells. Its mode of action was demonstrated by studying its effects on cellular apoptosis, cell cycle arrest and Wnt/β-catenin signalling pathway. Methoxyheptaphylline was shown to suppress the viability of the Y-79 cells concentration-dependently with IC_{50} value of 15.5 μM. Further, fluorescence microscopy, flow cytometry using DAPI, AO/EB and annexin V/PI as staining dyes were used to study apoptosis and it was seen to induce mitochondrial-mediated apoptosis in Y-79 Rb cells. Flow cytometry also indicated that this molecule induces S-phase cell cycle arrest in Y-79 cells. Finally western blot assay indicated that 7-Methoxyheptaphylline inhibited Wnt/β-catenin signalling pathway.

Conclusion

In brief, the current study indicated that 7-Methoxyheptaphylline inhibits Y-79 human Rb cell viability by inducing mitochondrial-mediated programmed cell death, S-phase cell cycle arrest and inhibition of Wnt/β-catenin signalling pathway.

Conflict of interests

The authors declare no conflict of interests.

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