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ORIGINAL ARTICLE

Berbamine exerts anticancer effects on human colon cancer cells via induction of autophagy and apoptosis, inhibition of cell migration and MEK/ERK signalling pathway

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Summary

Purpose: *Berbamine is a plant-derived alkaloid with amazing and wide diversity of pharmacological properties which range from antimicrobial and anticancer. Nonetheless, the anticancer properties of Berbamine have not been thoroughly evaluated against colon cancer cells. This study was undertaken to evaluate the anticancer effects of Berbamine against the human colon cancer cell line HT-29.*

Methods: *CCK-8 assay was used to determine the cell viability. DAPI and propidium iodide (PI) staining assays were used for the detection of apoptosis. Electron microscopy was used for the determination of autophagy. Wound healing assay was used to monitor cell migration. Protein expression was determined by western blotting.*

Results: *The results showed that Berbamine caused a remarkable decrease in the HT-29 cell viability with an IC_{50} of 14 μM, while the high IC_{50} of Berbamine against the normal CDD-18Co cells indicated low toxicity of this molecule against the normal cells. DAPI and PI staining assays showed nuclear fragmentation, indicative of apoptosis in HT-29 cells. Berbamine also caused activation of caspase-3 and 9 and increased the Bax/Bcl-2 ratio. Electron microscopic analysis showed that Berbamine triggered the development of autophagic vesicles in the HT-29 cells which was concomitant with the increase in protein levels of LC3B-I, ATG-5, ATG-12 and Beclin-1. Wound healing assay showed that Berbamine decreased the migration potential of the HT-29 and also blocked the MEK/ERK signalling pathway in colon cancer cells.*

Conclusion: *Berbamine may prove an efficient lead molecule for the development of more potent anticancer agents through semi-synthetic approaches.*

Key words: *colon cancer, berbamine, autophagy, apoptosis, migration*

Introduction

Natural products have played a remarkable role in drug discovery paradigms [1]. A number of drugs have been isolated from natural sources owing to their great chemical diversity. This great chemical diversity present in living organisms is the outcome of millions of years of evolution to combat stresses, both biotic and abiotic [2]. Plants have served as a rich source of drugs in the past and will continue to provide more novel chemical scaffolds for alleviation of human diseases [3].

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Plant-derived alkaloids have shown remarkable anticancer properties and the pursuit to identify novel alkaloids that can serve as drugs or lead molecules for the semi-synthesis of anticancer drugs is a continuous search [4]. Berbamine is a potent bioactive alkaloid prevalently found in the plant species belonging to family *Berberidaceae* [5]. Berbamine has shown a number of bioactivities such as antimicrobial and anticancer [6]. It has been reported to induce Fas-triggered apoptotic cell death in hepatocellular cancer cells [7]. In hepatoma cells, Berbamine activates caspases and causes apoptosis [8]. The proliferation and migration of lung cancer cells is also suppressed by Berbamine [9]. Berbamine has also been shown to halt the growth of metastatic breast carcinoma cells [10]. Anti-leukemic activity of Berbamine has also been reported [11]. Nonetheless, the anticancer effects of Berbamine have not been thoroughly evaluated against the human colon cancer cells. Colon cancer is the fourth principal cause of cancer-related mortality and ranks as the third common type of cancer incidence and around 1.4 million new cases of colon cancer are reported annually [12]. In 2013, around 0.7 million deaths were reported to be due to colon cancer around the world [13]. Although the incidence of this disease has declined to some extent, it is believed that its incidence will increase by 60% till 2030 [14]. Late diagnosis and lack of potent and safe chemotherapeutic drugs and therapeutic targets form a considerable obstacle in the treatment of this malignancy [15].

This study was therefore undertaken to investigate the anticancer effects of Berbamine against human colon cancer cells. The underlying mechanism for the anticancer effects of Berbamine together with its effects on the MEK/ERK signalling pathway were also investigated.

Methods

Cell viability determination

The CCK-8 assay was used for the determination of the cell viability of the colon cancer cells. In brief, the transfected HT-29 colon cancer cells were seeded in 96-well plates and subjected to treatment with varied concentrations of Berbamine at 37°C for 24 h. Thereafter, 10 μL of CCK-8 solution were added to the cell culture and incubated for 2 h at 37°C in a humidifier (5% CO_2, 95% O_2). Optical density $(OD)_{450}$ was taken with the help of a microplate reader to determine the cell viability.

DAPI and PI staining assay

The colon HT-29 cells (0.6×10^6) were seeded in 6-well plates and subjected to incubation with varied concentration of Berbamine for 24 h at 37°C. As the cells were cast off, 10 μl cell cultures were put onto glass slides and stained with DAPI or PI. The slides were cover-slipped and examined under a fluorescent microscope.

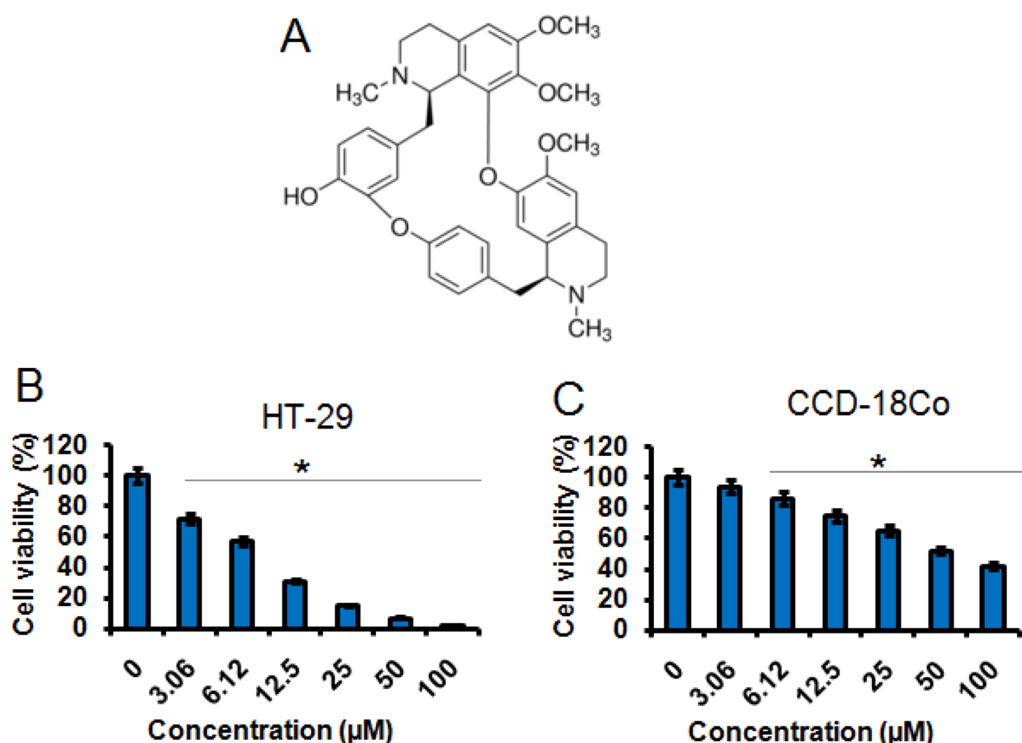

Figure 1. A: Chemical structure of Berbamine. **B:** Effects of Berbamine on the viability of HT-29 colon cancer cells and **C:** on normal CCD-18Co cells. The Figure shows that Berbamine decreases the viability of the HT-29 colon cancer cells concentration-dependently. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Transmission electron microscopy (TEM)

The HT-29 cells were treated 0, 7, 14 and 28 μM concentrations of Berbamine. The cells were then fixed in a solution of 4% glutaraldehyde 0.05 M sodium cacodylate, post-fixed in 1.5% OsO4, and dehydrated in alcohol. They were then prepared for flat embedding in Epon 812 and observed using Zeiss CEM 902 electron microscope.

Wound healing assay

After 24 h of treatment, the Dulbecco's Modified Eagle Medium (DMEM) was removed and cells were washed with phosphate buffered saline (PBS). A sterile pipette tip was employed to scratch a wound in each well and cells were washed again with PBS and a picture was taken. The cell plates were cultured for 24 h and a picture was taken again under an inverted microscope.

Western blot analysis

The determination of the protein expression was carried out by western blotting. The Berbamine-treated HT-29 cells were harvested with centrifugation and were then lysed in lysis buffer containing protease inhibitor. Afterwards, using SDS-PAGE (10 %) uniform protein amounts (45 μg) were separated and loaded over PVDF membranes bought from Millipore, Billerica, MA, USA. Next, skimmed milk was used to block the membrane at room temperature for 1 h. Afterwards, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the protein bands were detected by Odyssey Infrared Imaging System (LI-COR, USA). Actin was used as control for normalization.

Statistics

All the data are shown as mean±standard deviation (SD). One-Way Analysis of Variance (ANOVA) was used for determination of statistical significance. Multiple comparisons were performed with Tukey's test. Individual experiments were repeated thrice. A p value<0.05 indicated statistical significance.

Results

Effect of Berbamine on the viability of colon cancer cells

The CCK-8 assay was used to unveil the effects of Berbamine (Figure 1A) on the viability of the HT-29 colon cancer and CDD-18Co normal cells. Berbamine caused a significant reduction in the viability of the HT-29 cells. The effects of Berbamine on the viability of the HT-29 cells were dose-dependent and IC_{50} of 14 μM was observed for Berbamine against the HT-29 cells (Figure 1B). Interestingly, the effects of Berbamine on the normal CDD-18Co cells were lower and an IC_{50} of 50 μM was found for Berbamine against these normal cells (Figure 1C).

Apoptotic effects of Berbamine on colon cancer cells

To ascertain the underlying mechanism for the growth inhibitory effect of Berbamine, the HT-29 cells were treated with different doses of the compound and then stained with DAPI and PI separately. The results of DAPI staining showed that Berbamine caused nuclear fragmentation of the HT-29 cells, characteristic of apoptosis

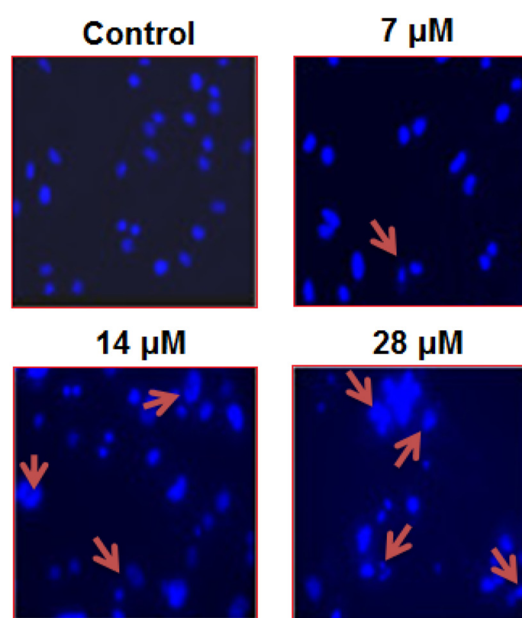

Figure 2. DAPI staining of the Berbamine-treated HT-29 colon cancer cells showing the induction of concentration-dependent apoptosis. Arrows depict the apoptotic cells. The experiments were performed in triplicate and expressed as mean ± SD.

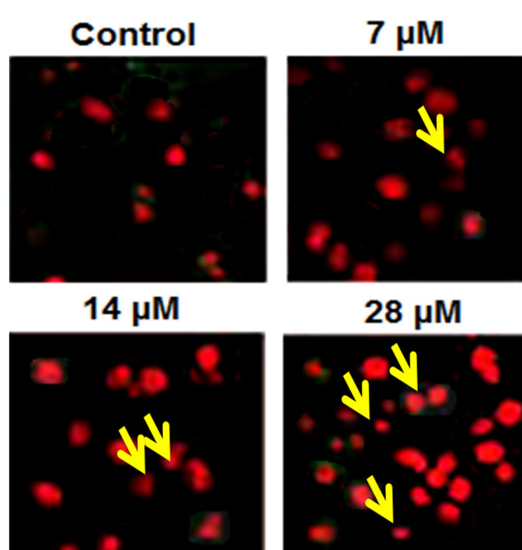

Figure 3. Propidium iodide (PI) staining of the Berbamine-treated HT-29 colon cancer cells, showing concentration-dependent increase in PI-positive cells indicative of apoptosis. Arrows depict the apoptotic cells. The experiments were performed in triplicate and expressed as mean ± SD.

(Figure 2). PI staining also showed increase in the PI-positive cells with increase in the concentration of Berbamine again, indicating apoptosis (Figure 3). Western blot analysis showed that Berbamine caused activation of Caspases 3 and 9 as well as increase in the Bax/Bcl-2 ratio, confirming the apoptotic cell death in the HT-29 cells (Figure 4).

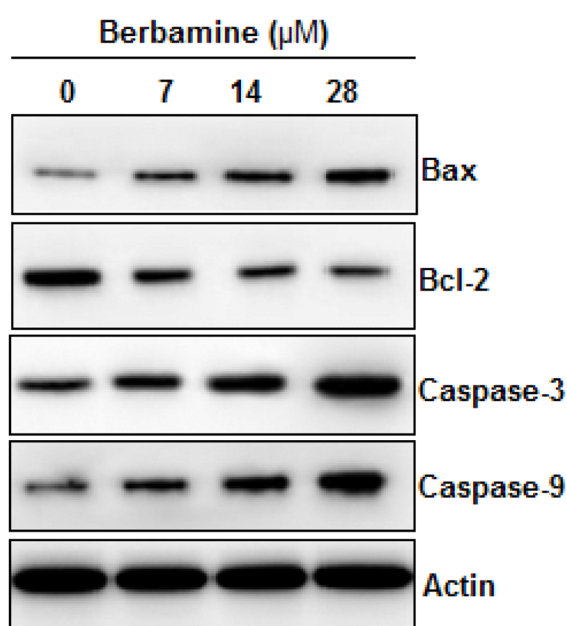

Figure 4. Western blot analysis showing Berbamine effects on the expression of apoptosis-related proteins. The expression of Bax, Caspase-3 and Caspase-9 increased while Bcl-2 decreased upon Berbamine treatment. The experiments were performed in triplicate.

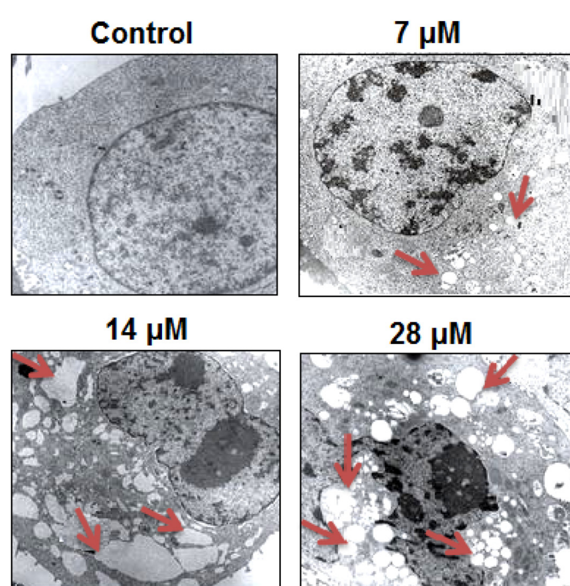

Figure 5. Electron microscopy showing the induction of autophagy in the Berbamine-treated HT-29 colon cancer cells in a concentration-dependent manner. Arrows depict the autophagosomes. The experiments were performed in triplicate.

Autophagy inducing effects of Berbamine on the colon cancer cells

Next, TEM analysis of the Berbamine-treated HT-29 cells was performed and it was observed that this molecule caused development of autophagic vesicles or autophagosomes in the HT-29 cells, which are the hallmarks of autophagy (Figure 5). Moreover, Berbamine also caused increase in the protein levels of LC3B-II, ATG-5 and 12 as well as in Beclin-1, indicative of autophagy. Nonetheless, no apparent effects were observed on the protein expression level of LC3B-I (Figure 6).

Effect of Berbamine on the colon cancer cell migration

The effects Berbamine of the migration potential of the HT-29 colon cancer cells was assessed

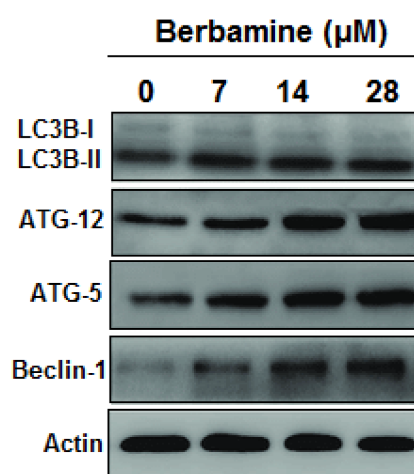

Figure 6. Western blot analysis showing that Berbamine effects the expression of autophagy-related proteins. The expression of LC3B-II, ATG-12, ATG-5 and Beclin-1 was increased upon Berbamine treatment. The experiments were performed in triplicate.

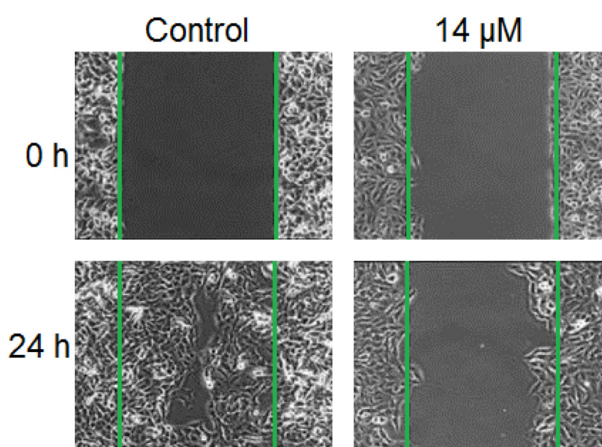

Figure 7. Wound healing assay showing that Berbamine inhibits the migration of the HT-29 colon cancer cells at 14 μM concentration after 24-h incubation. The experiments were performed in triplicate.

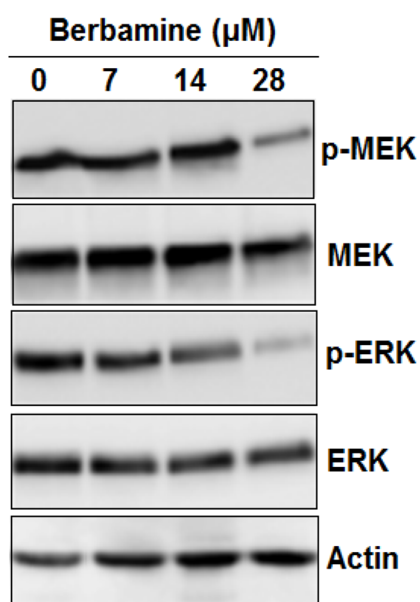

Figure 8. Berbamine blocks the MEK/ERK signalling pathway in HT-29 cells as depicted by western blot analysis. The Figure shows that Berbamine inhibits the phosphorylation of MEK and ERK in a concentration-dependent manner. The experiments were performed in triplicate.

by wound healing assay. The results showed that the treatment of HT-29 cells with Berbamine for 24 h caused reduction in the migration of these cells (Figure 7).

Effect of Berbamine on the MEK/ERK pathway in colon cancer cells

The effects of Berbamine were also assessed on the therapeutically important MEK/ERK signalling pathway. The results showed that Berbamine caused decline the phosphorylation of MEK and ERK proteins (Figure 8). Nonetheless, no apparent effects were observed on the total MEK and ERK protein levels.

Discussion

Colon cancer is a highly dangerous malignancy. The clinical outcome of colon cancer is unsatisfactory and treatment strategies have a number of flaws. The currently available chemotherapeutic agents have adverse effects and therapeutic targets are lacking [16]. This study was carried out to evaluate the anticancer effects of a bioactive alkaloid Berbamine against the human colon cancer cells. Interestingly, the outcomes of the CCK-8 cell viability assay showed that Berbamine decreased the viability of HT-29 colon cancer cells. However, the growth inhibitory effects were found to be very low against the normal cells, suggesting that Berbamine has cancer cell-specific activity. Previous studies have also shown remarkable anticancer effects of Berbamine. It has been reported that Berbamine significantly decreased the proliferation of glioblastoma cells [17]. Moreover, the semi-synthetic derivatives of Berbamine have also been shown to decrease the viability of osteosarcoma and melanoma cells [18,19]. Studies carried out earlier have proved that this compound halts cancer cell growth via apoptotic cell death. Investigations have also shown the selective apoptotic effects of Berbamine on human promyeloleukemic cells [20]. Given these studies, we sought to know whether Berbamine causes apoptosis of the colon HT-29 cancer cells and the DAPI and PI staining showed that this molecule induces apoptosis in HT-29 cells. This was also associated with the activation of caspases 3 and 9 as well as increase in the Bax/Bcl-2 ratio. Electron microscopy analysis of the Berbamine-treated cells was also performed which showed that Berbamine led to the development of autophagic vesicles in the HT-29 cells. The increased expression of LC3B-II, ATG-5 and 12 and Beclin-1 in the Berbamine-treated HT-29 cells further confirmed the involvement of the autophagic cell death, in addition to apoptosis. These observations are further validated by a previous study wherein Berbamine has been shown to trigger autophagy in the human liver cancer cells [21]. To investigate the anti-metastatic effects of Berbamine, the wound heal assay was performed which showed that Berbamine reduces the migration of the colon cancer cells. Owing to therapeutic implications of MEK/ERK signalling pathway in the treatment of human malignancies [22], researches are being directed at identifying molecules that block this pathway. Herein, we found that Berbamine blocked this pathway in the HT-29 cells in a concentration-dependent manner, indicating that this compound may prove a vital lead molecule for the development of systemic therapy in colon cancer.

Conclusion

The results of this study indicate that Berbamine selectively halts the growth of colon cancer cells via induction of apoptosis and autophagy. In addition to the inhibition of cell migration, Berbamine also blocks the MEK/ERK pathway in colon cancer cells. Further *in vivo* evaluation of Berbamine will help establish it as a lead molecule for the treatment of colon cancer.

Conflict of interests

The authors declare no conflict of interests.

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