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ORIGINAL ARTICLE

Bismahanine exerts anticancer effects on human cervical cancer cells by inhibition of growth, migration and invasion via suppression of NF-kB signalling pathway

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Summary

Purpose: *Bismahanine, a carbazole alkaloid, has been shown to exhibit tremendous pharmacological potential. In this study, the effect Bismahanine was examined against human cervical cancer cells.*

Methods: *The HeLa human cervical cancer cells were treated with various concentrations of Bismahanine from 0-320 μM for 24 h. The anticancer effects of Bismahanine were measured by WST-1 cell viability assay. DAPI and annexin V/propidium iodide (PI) assays were employed to examine the induction of apoptosis. Transwell assays were performed to examine the cell migration and invasion. The expression of the proteins was examined by western blot analysis.*

Results: *Bismahanine decreased the viability of HeLa cells and exhibited an IC_{50} of 20 μM due to induction of apoptosis as indicated by DAPI staining. Additionally, the annexin V/PI staining revealed that apoptotic cell percentage increased with increasing concentration of Bismahanine. The expression of Bcl-2 was decreased while that of Bax, Caspase 3 and 9 was increased. Bismahanine treatment also resulted in significant decrease of metalloproteinase (MMP) 2 , 3 and 9 expressions. Transwell assays showed that Bismahanine inhibited the migration and invasion of HeLa cells.*

Conclusion: *Bismahanine exhibits significant anti-proliferative effects on the cervical cancer cells and may prove essential in the development of chemotherapy for cervical cancer.*

Key words: *cervical cancer, bismahanine, apoptosis, migration, invasion*

Introduction

Cervical cancer being one of the common types of cancers in women, is ranked as the second most prevalent cancer world over [1]. Although cervical cancer is more frequent in underdeveloped countries, it still accounts for 10% of all the cancers in women [2]. Approximately 0.37 million new cervical cancer cases are detected throughout the globe annually [3]. Despite the development of biomarkers for early detection, the clinical outcomes are still unsatisfactory. The treatment for cervical cancer involves radical hysterectomy, chemotherapy and/or radiotherapy [4]. The currently available chemotherapeutic agents have adverse effects impacting the patient quality of life (QoL). Hence, the identification of novel, effective and safer anticancer agents is required to ameliorate the outcome of this disease. Bismahanine, a carbazole alkaloid from the medicinal herb *Murraya koenigii*, has been reported to exhibit antiproliferative effects on several types of cancers [5]. However, the anticancer effects of Bismahanine have not been examined against the cervical cancer cells. In this work, we

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aimed to study the suppressive effect of Bismahanine on HeLa cervical cancer cell line *in vitro* and to elucidate the mechanism underlying this suppressive effect. The NF-KB signalling pathway has been reported to be activated in cancer cells [6,7] and therefore the effects of Bismahanine were also examined on the NF-KB signalling pathway.

Methods

Cell culture conditions

The HeLa cervical cancer cell line and the normal cell line HCvEpC were obtained from the Cancer Research Institute of Beijing (Beijing, China) and maintained in Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Massachusetts, USA), supplemented with 10% fetal bovine serum (FBS) (Invitrogen Life Technologies, Massachusetts, USA),100 µg/ml streptomycin and 100 U/ml penicillin G (Himedia, Pennsylvania, USA) in an incubator at 37˚C with 5% CO_2.

Proliferation and colony formation assay

In this study, HeLa cells were seeded at a density of 2×10^5 in 100 µl culture medium in 48-well plates and were cultured for one day. Cells were then treated with various Bismahanine concentrations 0, 10, 20 and 40µM and incubated for 48 h. After the incubation period, WST reagent (25 µl) was added and further incubated for additional 2 h. Optical density (OD) was then measured at 450 nm with a reference wavelength of 695 nm. The colony formation assay was performed as described previously [8].

Apoptosis assay

The HeLa cervical cancer cells (0.6×10^6) were grown in 6-well plates. Following an incubation period of around 12 h, HeLa cells were treated with Bismahanine for 24 h at 37°C. Around 25 µl of cell culture were put onto a glass slide and stained with DAPI. The slides were cover-slipped and examined with a fluorescent microscope. Annexin V/PI staining was performed as described previously [9].

In brief, Bismahanine-treated HeLa cells (5×10^5 cells per well) were incubated for 24 h. This was followed by annexin V-FITC/PI staining. The percentage of apoptotic HeLa cells at each concentration was then determined by flow cytometry.

Cell migration and invasion assay

The migration and invasion abilities of the Bismahanine-treated HeLa cells were examined by transwell chamber assay. In brief, 1×10^4 HeLa cells were seeded in the upper chamber of the transwell (8 µm pore size polycarbonate filters). This was followed by the placement of the chambers into 24-well plates and incubation at 37°C for 48 h. However, in case of invasion assay, the inserts were coated with extracellular matrix gel (50 µl) (ECM, Sigma, USA). Swabbing was performed to remove the non-migrated and non-invaded cells from the upper surface. However, the migrated and the invaded cells on the lower surface were subjected to fixation with methanol for about 35 min which was followed by staining with crystal violet (0.5%) for about 50 min, subjected to washing with PBS and finally counted under light microscope (5 fields, 200x magnification).

Western blot analysis

The HeLa cells were lysed in lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were subjected to separation on 10% SDS-PAGE, followed by transferring 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

All the results are shown as mean ± standard deviation (SD) and p value <0.05 was considered as statistically significant. Student's t-test using GraphPad prism 7 software was used for statistical analyses.

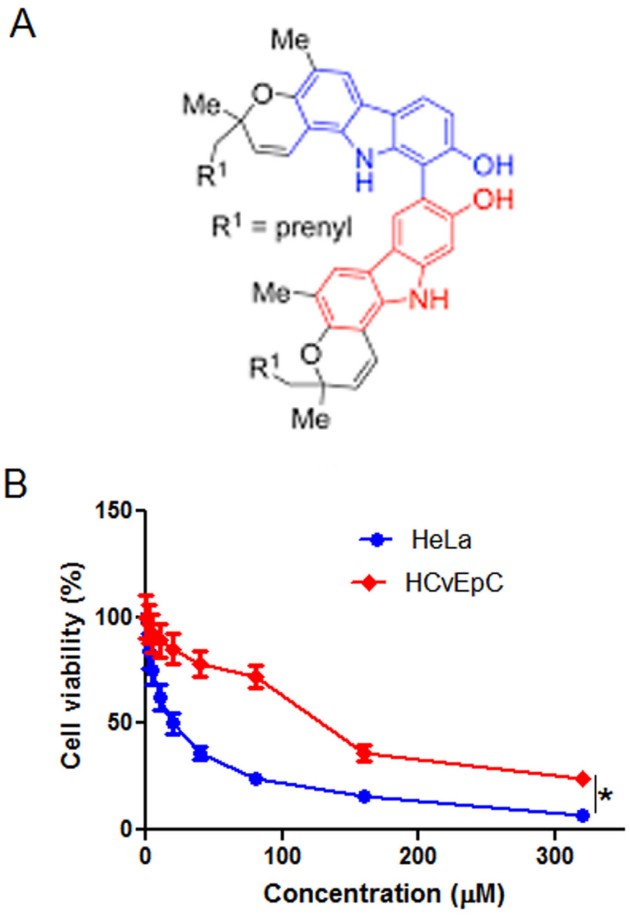

Figure 1. A: Chemical structure of bismahanine. **B:** Effect of Bismahanine on the viability of HeLa cells and HCvEpC non-cancer cells as determined by WST-1 assay. The values are mean of three experiments ± SD (*p< 0.05).

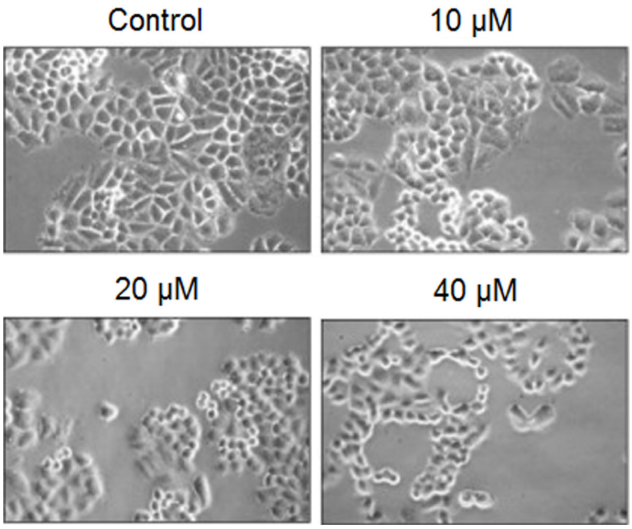

Figure 2. Effect of Bismahanine on the morphology of the HeLa cells at indicated concentrations. The figure shows that Bismahanine induces morphological changes in the HeLa cells concentration-dependently. The experiments were performed in triplicate.

Results

Bismahanine inhibits the proliferation and colony formation of HeLa cells

The antiproliferative effects of Bismahanine (Figure 1A) on the HeLa cervical cancer cell line were assessed by WST-1 assay. It was found that Bismahanine triggered anti-proliferative effects on the HeLa cells and exhibited an IC_{50} of 20 μM (Figure 1B). Additionally, it was found that the anticancer effects of Bismahanine on the HeLa cells were concentration-dependent. However, negligible toxicity was observed on the normal HCvEpC cells (IC_{50} 120 μM). Bismahanine also caused change in the morphology of the HeLa cells (Figure 2). The results of the colony formation assay showed that Bismahanine inhibited the colony development of the HeLa cells concentration-dependently (Figure 3).

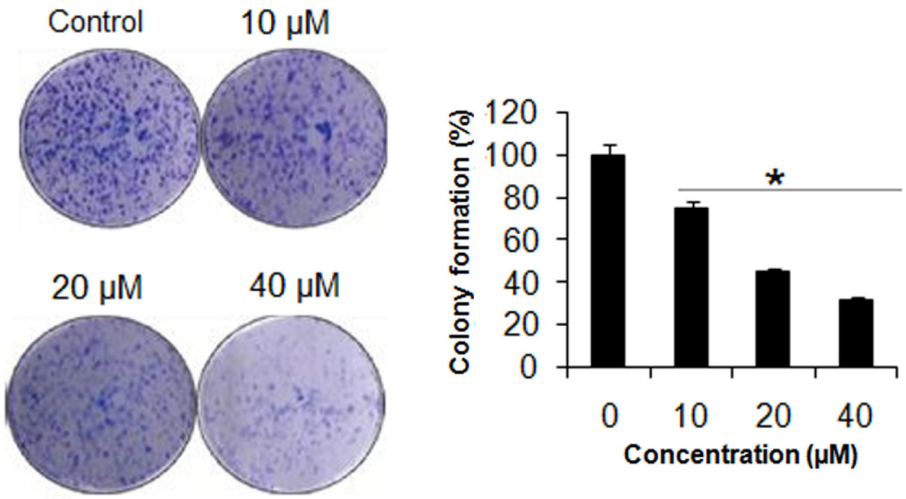

Figure 3. Effect of Bismahanine on the colony formation of the HeLa cells at indicated concentrations as determined by colony formation assay. The figure shows that Bismahanine inhibits colony formation of HeLa cells concentration dependently. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

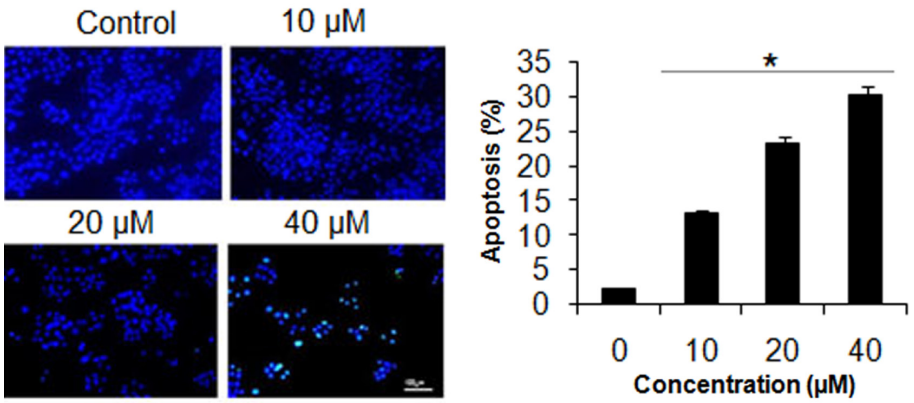

Figure 4. Bismahanine induced apoptosis in the HeLa cells as indicated by DAPI staining. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Bismahanine induces apoptosis in cervical cancer cells

To ascertain whether Bismahanine prompts apoptosis in HeLa cells, DAPI staining was performed which showed remarkable changes in the nuclear morphology and membrane blebbing of these cells (Figure 4). The percentage of the apoptotic HeLa cells was determined by Annexin V/PI staining which showed that the apoptotic cell percentage increased from 2.2 % in the control to 40.56% at 40 μM of Bismahanine (Figure 5). The apoptosis was further confirmed by the increased expression of Caspase 3, 9 and Bax and decreased expression of the Bcl-2 in HeLa cells (Figure 6).

Bismahanine inhibits the migration and invasion of HeLa cells

The effects of Bismahanine were also investigated on the migration and invasion of HeLa cells by transwell assay. It was found that Bismahanine could suppress the migration and invasion of the HeLa cells concentration-dependently (Figure 7).

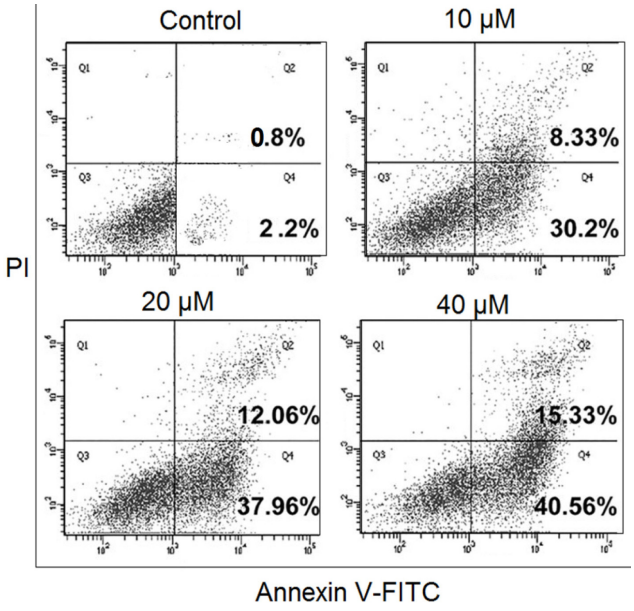

Figure 5. Determination of the percentage of the apoptotic cell populations as determined by Annexin V/PI staining . The figure shows that the percentage of apoptotic HeLa cells increases upon bismahanine treatment. The experiments were performed in triplicate.

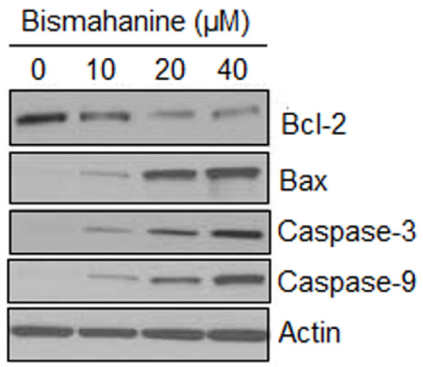

Figure 6. Effect of Bismahanine on the expression of Bax, bcl-2, Caspase-3 and 9 expression as indicated by western blot analysis. The figure shows that Bismahanine increases the expression of Bax,Caspace-3 and 9 and decreases the expression of Bcl-2 . The experiments were performed in triplicate.

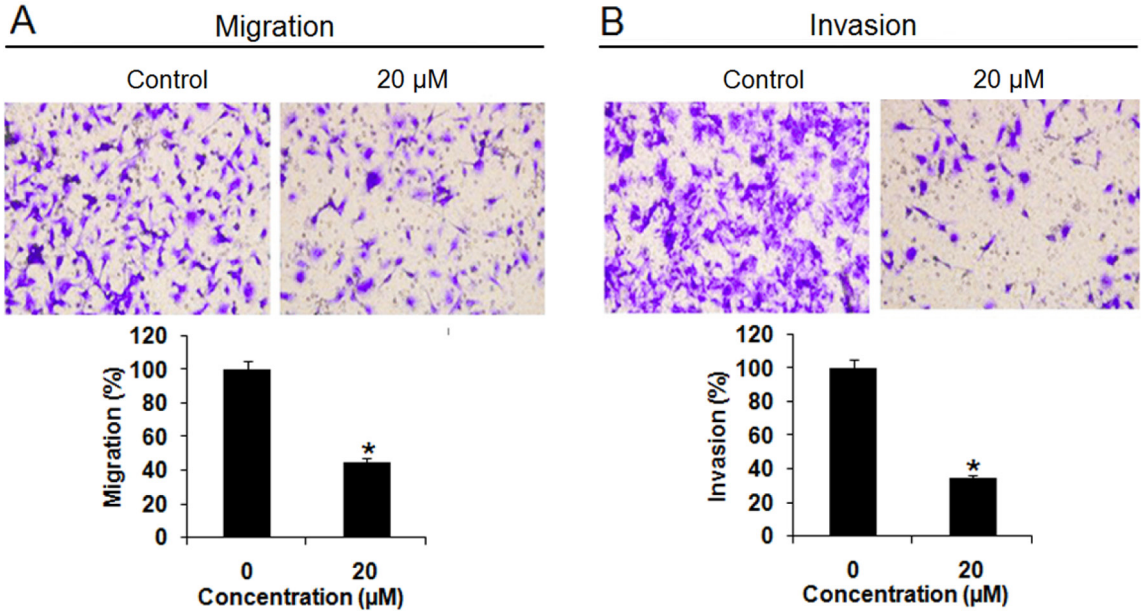

Figure 7. Inhibition of **A:** cell migration and **B:** invasion by Bismahanine at 20 μM concentrations as depicted by transwell assay . The Figure shows that Bismahanine inhibits the migration and invasion of HeLa cells. The values are mean of three experiments ± SD (*p<0.05).

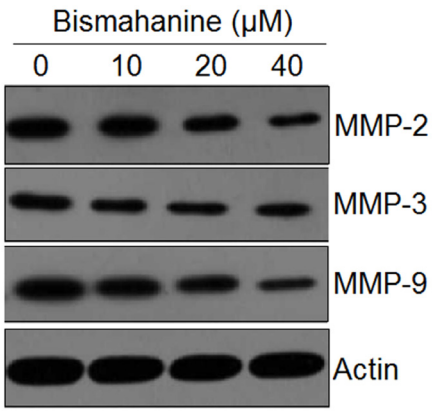

Figure 8. Effect of Bismahanine on the expression of MMP-2, 3 and 9 at indicated concentrations as depicted by western blot analysis. The figure shows that Bismahanine inhibits the expression of MMP-2, 3 and 9. The experiments were performed in triplicate.

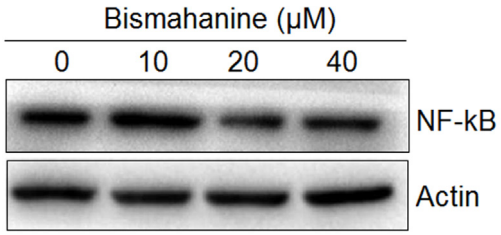

Figure 9. Western blots showing Inhibition of the phosphorylation of NF-kB by Bismahanine in HeLa cells at indicated concentrations as depicted by western blot analysis. The experiments were performed in triplicate.

Bismahanine inhibits the matrix metalloproteinase (MMP) expression and NF-kB signalling pathway

We also sought to know the effects of Bismahanine on the expression of matrix metalloproteinase (MMP) expression. It was found that Bismahanine inhibited the expression of MMP-2, 3 and 9 in a concentration-dependent manner (Figure 8). The effects of Bismahanine were also examined on the NF-kB signalling pathway which revealed that this molecule caused concentration-dependent decline in the expression of NF-kB in HeLa cells (Figure 9).

Discussion

Cervical cancer is considered as the second most common type of cancer in women across the world [10]. Since the clinical outcomes are far from descent and the treatment options have a number of side effects, the identification of novel anticancer molecules and subsequent development of efficient and safer treatment regimes for clinical cancer are required [1-3]. Herein, we examined the anticancer effects of Bismahanine on the HeLa cervical cancer cells. The results revealed that Bismahanine inhibits the growth of the cervical cancer cells in a concentration-dependent manner and also halted their ability to develop colonies. Previous studies have shown that Bismahanine-related compounds, such as Mahanine, can inhibit the growth of several types of malignancies such as leukemia [11,12]. Further studies have also shown that Mahanine, a compound similar in structure to Bismahanine, induces apoptosis in prostate cancer cells by blocking the AKT signaling pathway [13]. Similarly, Mahanine has been reported to induce apoptosis in myeloid leukemia cells [14]. Therefore, we investigated whether Bismahanine also induces apoptosis in HeLa cells. The results of DAPI and annexin V/PI showed that this molecule induced apoptosis in the HeLa cells and the percentage of the apoptotic cells increased as the concentration of Bismahanine was increased. Apoptosis is an important process that helps eliminate the harmful and cancer cells and several known anticancer drugs induce apoptosis in cancer cells [15]. The Bismahanine-induced apoptosis was also associated with concomitant increase in the expression of the cleaved caspase-3, 9 and Bax and downregulation of Bcl-2 which are the important markers for apoptosis [16]. Cervical cancer cells have the capacity to invade neighboring tissues and create metastasis [17] and hence we examined the effect of this molecule on the migration and invasion of HeLa cells. Interestingly, it was found that Bismahanine could suppress the migration and invasion of HeLa cells which was concomitant with downregulation of MMP-2, 3 and 9 expressions. NF-kB transduction pathway is an important pathway that has been reported to be dysregulated in cancer cells [7] and in this study we found that Bismahanine inhibited the phosphorylation of the HeLa cells, suggestive of the anticancer potential of this molecule.

Conclusion

It is concluded that Bismahanine exhibits significant anticancer effects on the cervical cancer cells via induction of apoptosis. In addition, Bismahanine also inhibited the migration and invasion of cervical cancer cells by modulating the expression of metalloproteinases. Therefore, Bismahanine may prove beneficial in the management of cervical cancer.

Conflict of interests

The authors declare no conflict of interests.

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