RESEARCH ARTICLE

Berberine Hydrochloride Impact on Physiological Processes and Modulation of Twist Levels in Nasopharyngeal Carcinoma CNE-1 Cells

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Abstract

Objective: The main purpose of this work was to investigate the effect of berberine hydrochloride (BH) on the proliferation, apoptosis, migration, and invasion of CNE-1 nasopharyngeal carcinoma cells. Our results shed light on the functional components of traditional Chinese herbs for potential use in modern medicine. Methods: The CNE-1 cell line was treated with different concentrations of BH and effects on cell viability and proliferation were evaluated using the Cell Counting Kit-8 (CCK-8) assay. Anti-migratory and anti-invasive actions of BH were investigated using wound healing assays and the Millicell Hanging cell culture insert system, respectively. Expression of the epithelial-mesenchymal transition (EMT)-related gene twist (Twist) was analyzed by real-time PCR and Western blotting. Apoptosis was estimated with an annexin-V fluorescein (FITC) apoptosis detection kit, as well as with reference to levels of activated caspase-3 of CNE-1 cells before and after treatment with BH utilizing fluorescence spectroscopy. Results: BH was capable of reducing proliferation and viability of CNE-1 cells in a dose- and time-dependent manner, also demonstrating anti-migratory and anti-invasive capacities which correlated with reduction in expression of Twist. Finally, BH was able to induce significant amounts of apoptosis in CNE-1 cells, as demonstrated by an increase in the activity of caspase-3 and in annexin-V staining following treatment. Conclusion: BH extracted from rhizoma coptidis demonstrated an ability to block proliferation, induce apoptosis, and impair the migration and invasion of the CNE-1 cell line Considering these properties, our results suggest that BH could be an important compound for consideration in the treatment of nasopharyngeal carcinoma.

Keywords: Berberine hydrochloride - invasion - migration - apoptosis - cell proliferation - CNE-1

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Introduction

Nasopharyngeal carcinoma (NPC) is the most common epithelial malignancy of the nasopharynx and it is also an endemic disease, with a distinct racial and geographic distribution. High-incidence areas of NPC are found in Southeast Asia and North Africa, and low-incidence areas are in the USA, Europe, and Japan (Segawa et al., 2009). At this time, the pathogenesis of NPC is not clear, nor are there effective and low-toxicity therapeutics available for treatment. Thus, research and development of potential drug candidates for NPC is of utmost importance.

Berberine hydrochloride (BH) is a natural alkaloid abundantly present in Coptis chinensis Franch, but is also found in a variety of herbs of the Berberis species. It is known to have many pharmacological properties, including antibacterial, anti-inflammatory, anticholinergic, antihypertensive, and antioxidative activities (Tan et al., 2011). BH also possesses a repressive effect on the proliferation of various cancer types via the induction of cell cycle arrest and cellular apoptosis (Mantena et al., 2006; Lin et al., 2007; Kuo et al., 2011; Wen et al., 2013). Furthermore, the application of BH has attracted considerable attention for its use as an anti-metastatic agent, suggesting its use in a variety of advanced cancers (Kim et al., 2008; Ho et al., 2009; Kim et al., 2011; Singh et al., 2011). In this study, we investigated the effects of BH on the viability, migration, invasion, and apoptosis of a highly differentiated nasopharyngeal cancer cell line CNE-1 to determine BH's efficacy in blocking nasopharyngeal carcinoma (NPC) in vitro.

In an effort to further explore the use of BH as an antimetastatic agent, we studied the potential of BH in repressing the expression of Twist, an essential transcription factor for embryogenesis and epithelial-to-mesenchymal transitions (EMT). Twist has long been described as an oncogene due to its role as an antiapoptotic factor (Maestro et al., 1999), but has also been shown to play an essential role in metastasis of breast cancer cells

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by promoting EMT (Yang et al., 2004). A previous study found that Twist is a key factor responsible for metastasis of breast cancer in an in vivo system, with downregulation of Twist suppressing this metastatic ability (Yang et al., 2004). In addition, in primary melanoma patients, increased Twist is correlated with poor outcome and shorter survival (Hoek et al., 2004). These results implicate Twist as a novel oncogene that induces tumorigenesis in nonmalignant cells and promotes tumor progression in malignant cells.

Previously, we have found that Twist gene amplification is associated with the development of acquired resistance to an anticancer drug (taxol), and ectopic expression of Twist leads to resistance to microtubule-disrupting agents (Wang et al., 2004). These lines of evidence further suggest that Twist may be a positive factor in promoting resistance to anticancer drugs, a key characteristic of advanced cancers. The results from our study identify BH as a novel compound to treat NPC through its various antineoplastic activities, including its antimetastatic abilities via inhibition of Twist gene expression.

Materials and Methods

Materials

BH was purchased from China's food and drug administration (lot number: 110713-200911) and dissolved in Milli-Q water to a stock concentration of 1 mM, then stored at -70˚C until use. The cell counting kit-8 (c0038) and Annexin V-FITC apoptosis detection kit (c1063) were purchased from Beyotime Institute of Biotechnology. The millicell 8.0μm hanging PET inserts, preloaded in 24-well receiver plates, were purchased from Merck Millipore. THE BD BioCoat™ BD matrigelTM invasion chamber, 8.0 μm PET membrane 24-well cell culture inserts packaged ready-to-use in BD Falcon™ Companion plates, were purchased from BD Biosciences. The apoalert™ caspase-3 fluorescent assay kit (630215) was purchased from Clontech Trizol reagent was purchased from Invitrogen Corporation. PCR primers were synthesized by Sangon Biotech and the Realtime Q-PCR kits were purchased from Takara. The primary antibodies used for cleaved-Twist and ß-actin, as well as the horseradish peroxidase (HPR)-linked goat anti-rabbit IgG secondary antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture

CNE1 cells (a human nasopharyngeal carcinoma cell line) used in this study were obtained from the Institute of Biochemistry and Molecular Biology, Guangdong Medical College. Monolayer subcultures of the cells were cultured in DMEM (Gibco) medium supplemented with 10% fetal bovine serum (FBS) (Gibco) and 100μg/mL penicillin-streptomycin (Gibco), and maintained at 37˚C in a humidified atmosphere of 5% CO_2. An inverted microscope was utilized to observe cell growth and density, and the cells were regularly replated by digestion with trypsin EDTA solution (0.25% trypsinase and 0.02% EDTA). Cells were used in the logarithmic growth phase in all experiments performed in this study.

WST-8 conversion assay to measure the effect of BH on activety of CNE-1 cells

The effect of BH on cell viability/proliferation was determined using the WST-8 assay, which has higher detection sensitivity than other tetrazolium salt-based assays such as MTT, XTT, MTS, or WST-1. Briefly, CNE-1 cells were seeded into 96-well plates at a density of 5000 cells/well and incubated for 24 hours. The cells were then treated with different concentrations of BH (2.5, 5, 10, 20, 40 μg/mL) for 6, 12, 18, 24 and 48 hours. WST-8 conversion was then assessed using a one-step Cell Counting Kit-8 (CCK-8), according to the manufacture's instructions. The WST-8 method only generates a signal from the viable cells. All tests were carried out in triplicate. The absorbance was measured at 450 nm by the Synergy2 Multifunctional microplate reader (Bio Tek, USA) with a reference at 650 nm serving as blank. The cell viability of BH-treated cells was calculated as the percentage of cell viability compared to untreated cells, which were arbitrarily assigned 100% viability.

Wound healing assay

Briefly, CNE1 cells were seeded at a density of 8×10^4 per well in a 24-well plate and allowed to grow to confluency. A uniform wound was introduced on the monolayers by scraping with a sterile yellow pipette tip. After being washed thrice with PBS, scratches including the flanking front lines of cells were imaged (100-fold magnification). The culture medium was replaced with fresh medium containing a series of concentrations of BH (5, 10 μg/mL). The rate of wound closure in BH-treated and untreated cells was monitored by images captured with a phase-contrast microscope immediately after wound incision (0 hours) and after 24 and 48 hours. Experiments were independently performed thrice, and four to eight scratches were evaluated for each experimental condition.

Boyden Millipore chamber system

Cells ($2.5\text{x}10^4$/well) were seeded onto the membrane of the upper chamber insert and placed in a 24-well plate containing 600 μl of culture medium/well and treated with different concentrations (5, 10 μg/mL) of BH. After a 36 hour treatment with BH, the non-migrating cells remaining on the upper surface of the membrane were removed by cotton swabs. The cells that migrated across the upper membrane without collagen to the lower surface of the membrane were fixed by ice-cooled absolute ethanol and stained with 5% Giemsa solution for visualization of cells. The number of stained cells was quantified using a cell counter. Experiments were independently performed thrice.

Invasion

Transwell plates (BD BioCoat™ BD matrigelTM invasion chamber) were used to measure tumor invasion upon treating with BH. Cells (5×10^4/well) were seeded onto the Matrigel-coated upper chamber insert and placed in a 24-well plate containing 600 μL of culture medium/well and treated with different concentrations (5, 10 μg/mL) of BH. Plates were incubated at 37°, 5% CO_2 for 48 hours. After incubation, the non-invading cells and the Matrigel were removed from the upper

surface of the membrane using a cotton swab. Inserts were removed, washed twice with PBS, and then cells were fixed by ice-cooled absolute ethanol and stained with 5% Giemsa solution for visualization of cells. The membrane was transferred to a microscope slide, imaged at 200-fold magnification, and the number of stained cells was quantified using a cell counter. Experiments were independently performed thrice.

Reverse transcription-polymerase chain reaction and Real-time Q-PCR (RT-PCR)

After CNE- 1 cells treated with BH were harvested, the total cellular RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. The concentration and purity of RNA was assessed by NanoDrop® ND-1000 spectrophotometry (Thermo Scientific, Rockford, IL, USA). Reverse transcription-polymerase chain reaction was performed on samples from each group with the GeneAmp PCR System 9700 (Applied Biosystem, Foster City, CA, USA), using TaKaRa One step RT-PCR Kit (Takara Bio Inc., Dalian, China) according to the manufacturer's specifications. Real-time PCR was performed on the samples from each group with the ABI PRISM 7500 Sequence Detection System (Applied Biosystem, Foster City, CA, USA), using One Step SYBR® PrimeScript® RT-PCR Kit II (Takara Bio Inc., Dalian, China) according to the manufacturer's specifications.

Sequences for RT-PCR Primers were as follows: human Twist forward, 5'- GCCGGAGACCTAGATGTC AT -3', human Twist reverse, 5'-CCTAGAAGCATTTGC GGTGGA -3', the amplified fragment length is 351bp; human β-actin forward, 5- CCGGGACCTGACTGACT ACCTC -3', human β-actin reverse, 5'-TGCTGTCGCCTT CACCGTTC-3', the amplified fragment length is 580bp. Reaction conditions were as follows: 50°C for 30 min, 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 57°C for 30 sec and 72°C for 1 min, 72°C for 10 min. Each sample was performed in triplicate in a 25 μL reaction volume, and β-actin was used as reference gene. Sequences for real-time Q-PCR Primers were as follows: human twist forward, 5'-GCGTCTGCAGCTCCTCGTAAGACTG-3', human twist reverse, 5'-CCATCCTCCAGACCGAGAA GGCGT -3'; human β-actin forward, 5-TGGCACCCAGCACAATGAA -3', human β-actin reverse, 5'-CTAAGTCATAGTCCGCCTAGAAGCA -3'.

Reaction conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Each sample was performed in triplicate in a 25 μL reaction volume, and β-actin was used as reference gene. Relative quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method (Fendri et al., 2011) based on Ct values for both target and reference genes. Results of real-time PCR analysis are given as mean ± S.E.M. For each experiment, mRNA levels from untreated berberine samples were used for comparison. The mRNA levels were represented relative to untreated samples.

Western blotting

Cells were washed for three times with ice-cold PBS. Cell lysates were prepared with RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS 50 mM Tris-Cl, pH 7.4, and 20% (V/V) cocktail protease inhibitors (Sigma-Aldrich). After a brief sonication on ice, cell lysates were incubated on ice for 30 min, and centrifuged at 12, 000rpm for 30 min at 4°C to remove insoluble debris. Protein concentrations were determined using the Bradford method (Sigma (B6916), Shanghai, China). Whole cell lysates were treated by boiling in loading buffer containing SDS and resolved by SDS-PAGE, then electrophoretically transferred to membranes (Immobilon-P, Millipore). Membranes were pre-blotted over night in TBS-T buffer (3 g/L Tris-base, 8 g/L NaCl, 0.2 g/L KCl, 0.1%Tween-20, pH 7.4) containing 5% skim milk at 4°C. After that, the membranes were incubated with specific primary and secondary antibodies in TBS-T plus 5% skim milk for 1 hour each at room temperature. Membranes were developed using enhanced chemical luminescence (ECL), and the result was detected by Bio-Rad ChemiDoc XRS (Bio-Rad, USA). All antibodies were purchased from Santa Cruz Biotechnology, Inc.

Apoptosis detection by flow cytometry

The detection was performed utilizing the annexinV-FITC apoptosis detection kit (Roche, China). Briefly, cells were seeded in 6-well plates and incubated for 24 hours and then treated with BH (5, 10 μg/mL) for 6, 12, 18, 24 or 48 h. After treatment, approximately 1×10^6 cells were harvested, washed twice in PBS, and then stained with Annexin V-FITC and PI according to the manufacturer's instructions. The resulting fluorescence was detected by FACS canto II flow cytometer (BD Biosciences, USA) with Cellquest analysis software.

Fluorimetric assay for caspase-3 activity

Briefly, the harvested (adherent plus floating) cells were washed with ice-cold PBS, and resuspended (106 cells/100 μL) in the following lysis buffer: 20 mM Hepes-NaOH, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 0.2% NP-40, 1 mM EDTA, 5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Sigma-Aldrich, P8340). After 30 minutes on ice, cells were sonicated for 10 s (Vibracell Sonicator; amplitude 60, 25 W), centrifuged at 12, 000 rpm for 30 s, and the supernatant stored at −80°C until use. Protein concentrations were determined using the Bradford method (Sigma (B6916), Shanghai, China). The rest of the experiment was performed according to the manufacturer's specifications. The Synergy2 Multifunctional microplate reader (Bio Tek, USA) (excitation/emission wavelenghts: 400/505 nm) was used to measure the samples. Using the slope (ΔFU/ ΔμM AFC or AMC) of the curve, caspase-3 activity was calculated with the following formula: Caspase activity = (ΔFU/hr)×1/curve slope. ΔFU/hr= the difference in FU between an uninduced control and an induced sample.

Statistics

The results are presented as a mean ± SD, unless otherwise noted. The differences between the BH-treated groups and the controls at a specific time point (4 weeks)

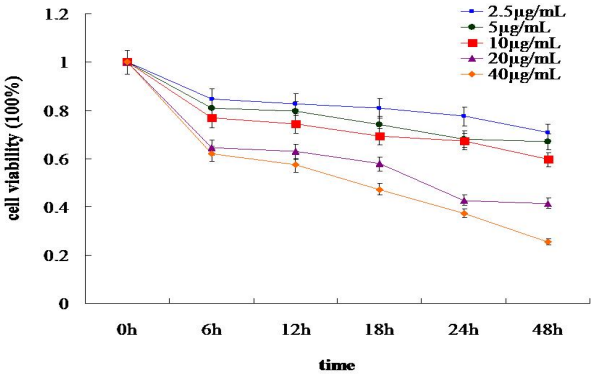

Figure 1. The Effects of Different Concentrations of BH at Different Times on CNE-1 Cell Proliferation (*P <0.01,P <0.001)**

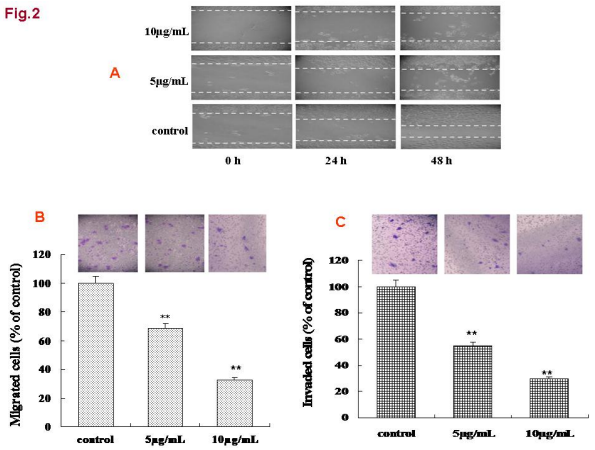

Figure 2. Suppression of Migration and Invasion of CNE-1 Cells at Low Doses of BH (P <0.001).** A. Wounds of similar size were introduced onto confluent monolayers, and different concentrations (5 and 10μg/mL) of BH were added to the culture medium. The rate of wound healing was monitored at the indicated time points (0, 24 and 48 hours; magnification=100X). B. Boyden chambers were used to confirm the inhibition of migration by BH. C. Boyden chambers coated with Matrigel were used to determine the ability of BH to inhibit invasion. In B and C, the number of stained cells was quantified using a cell counter, and five visual fields were evaluated for each experimental condition (magnification=200X). Results were averaged over the group and compared to results of the control group. (**P <0.001)

were analyzed using generalized linear models, which included 'group' and 'time after treatment' as predictors for each different dependent variable. Statistical analysis was performed using SPSS (version 13.0) for Windows. P-values less than 0.05 were considered statistically significant.

Results

BH inhibits proliferation of CNE-1 cells

After treatment with different concentrations of BH for various times, the viability of CNE-1 cells dropped markedly compared to the untreated group (P <0.01). As shown in Figure 1, proliferation of the BH-treated cells decreased even more with longer periods of treatment time or with increased concentrations of BH. At a concentration of 40 μg/mL of BH, 74.0% of the cells were lost after

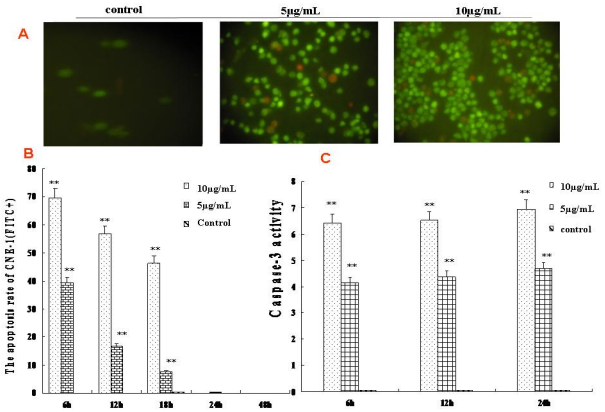

Figure 3. BH Induced CNE-1 Cells Apoptotis. A. Apoptosis was observed by Annexin V-FITC/PI staining of cells after 6 hours of BH-treament ((magnification=200X). B. Apoptosis was detected by flow cytometry of Annexin V-FITC/PI-stained cells (**P <0.001). C. Apoptosis was measured by a fluorometric assay for caspase-3 activity (**P <0.001)

treatment for 48 hours. These results indicate that BH (0-40 μg/mL) produces a dose- and time-dependent reduction in CNE-1 cell growth. The IC50 for 48 hours of berberine treatment in CNE-1 cells was 11.7 μg/mL. Based on these results, concentrations of 5 μg/mL and 10 μg/mL of BH were used for further experiments.

Suppression of migration and invasion of CNE-1 cells at low doses of BH

BH effectively inhibited cell migration at low doses (5 and 10 μg/mL) while remaining relatively non-toxic to cells. This is shown as an increased distance between the two leading edges of the cells, marked with white lines. When the treating the cells for 48 hours, the 10 μg/mL group showed more powerful inhibition of migration than the 5 μg/mL group (Figure 2A).

Boyden chambers coated with or without collagen were used to confirm the inhibition of CNE-1 cellular migration by BH. As shown in Figure 2B, the migration of cells decreased gradually with increasing concentrations of BH. The ability of CNE-1 cells to invade into Matrigel-coated transwell plates was also decreased with increasing concentrations of BH (Figure 2C), confirming that BH effectively inhibited cell migration and invasion (Figure 2B and 2C, P <0.001). Strikingly, most of the suppressed cell migration and invasion was observed at 10 μg/mL of BH.

BH induces apoptotic cell death

Annexin V-FITC staining was utilized to explore the level of apoptosis in CNE-1 cells following BH treatment. Propidium iodide (red fluorescent channel) was used as a co-stain to identify necrotic cells or terminal apoptotic cells that have lost membrane integrity. Morphological observation using fluorescent microscopy at 6 hours post-treatment (Figure 3A)indicates that the control group had only a few cells undergoing early apoptosis, whereas he BH-treated group had a large number of cells in early apoptosis.

Flow cytometric analysis (Figure 3B) confirmed that the number of AV- and AV/PI-positive cells significantly

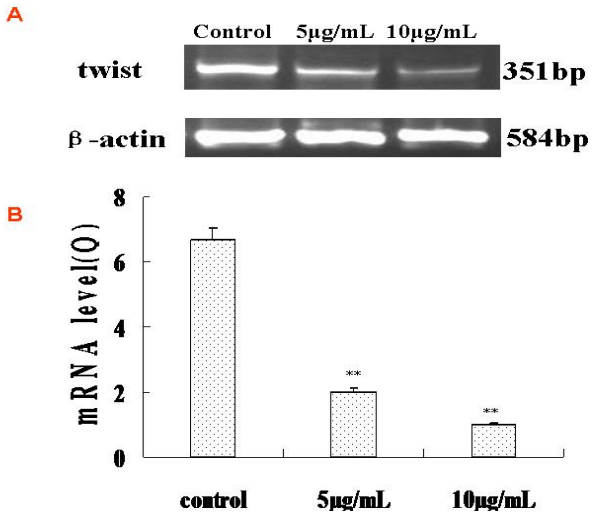

Figure 4. The Effect of BH Treatment of CNE-1 Cells on Twist Gene Expression. A. RT-PCR analysis of Twist expression in CNE-1 cells treated with 0, 5, or 10μg/mL of BH. B. qRealTime-PCR analysis of Twist expression in CNE-1 cells treated with 0, 5, or 10 μg/mL of BH. (**P <0.001)

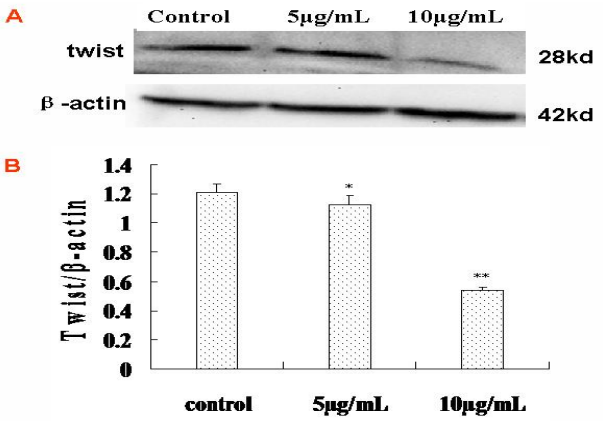

Figure 5. Western Blot Analysis of Twist Expression in CNE-1 Cells Treated for 12 Hours with 0, 5, or 10 μg/mL of BH (*P <0.05, **P <0.001)

increased in both the 5 μg/mL and 10 μg/mL BH-treated groups at 6, 12 and 18 hours post-treatment. The 10 μg/mL BH-treated cells at 6 hours had a higher apoptotic ratio (69.5%) compared to the 5 μg/mL group (39.2%) (**P <0.001). The rate of apoptosis gradually declined as treatment time elapsed (higher at 6 hours compared to 24 and 48 hours), and the apoptotic cells appeared to undergo cell death after BH treatment for 24 to 48 hours, as apoptotic cells could no longer be detected at this time. These results suggest that BH is capable of inducing apoptosis of a high proportion of CNE-1 cells within 48 hours of treatment.

To further substantiate BH's ability to induce CNE-1 cell apoptosis, caspase-3 activity was evaluated. Activation of caspase-3 by cleavage plays an extremely important role in apoptosis and is considered to be the terminal event preceding cell death (Snigdha et al., 2012). Treatment of CNE-1 cells with BH (5 and 10 μg/mL) for 6, 12 and 24 hours resulted in a dose-dependent increase slightly in the activation of caspase-3 when compared with cells that were not treated with BH (Figure 3C; P < 0.001). Thus, these data suggest that caspase-3 activation is involved in the BH-induced apoptosis of CNE-1 cells. Twist mRNA is downregulated during BH -induced apoptosis

For the previous study suggest that high levels of Twist may be able to functions as an anti- apoptotic factor or the metastatic nature of NPC. (Horikawa et al., 2007; Zhang et al., 2007). In an attempt to explore the mechanism of BH-induced apoptosis and reductions in proliferation migration and invasion, we turned out attention to a protein implicated in all four processes. To determine the expression pattern of Twist after BH treatment, RNA was isolated from CNE-1 cells following treatment with 0, 5, and 10 μg/mL of BH. RNA extraction and reverse transcription PCR reactions were performed as previously described (Tamboli et al., 2011). qRealTime-PCR was performed using One Step SYBR® PrimeScript® RT-PCR Kit in an Applied Biosystems 7900 Sequence Detector System, and quantified mRNA levels were expressed as relative fold change normalized to β-actin.

Results following semi-quantitative RT-PCR are shown in Figure 4A. With the increase in drug concentration, the intensity of the Twist mRNA electrophoretic band became weaker. In addition, relative fold changes of Twist mRNA in the control, 5, and 10 μg/mL BH-treated groups were determined by quantitative real-time PCR (Figure 4B). Similar to other assays, we found a dose-dependent decrease in the expression of Twist in CNE-1 cells treated with BH (**P <0.001). This suggests that the expression of Twist mRNA is inversely proportional to the concentration of BH.

Effect of BH on Twist protein levels

Decreased Twist mRNA levels compelled us to determine if Twist protein levels were also reduced by BH treatment. In order to do this, serial dilutions of total cellular protein samples (containing equal amounts of protein) from the CNE-1 control group or other groups treated with 5 and 10μg/mL BH for 12 hours were separated by 10% SDS-PAGE and subsequently analyzed by Western blot. The lower and upper panels of Figure 5 showed the chemiluminescent diagrams of the Western blot analysis. Immunobands of Twist were present in all untreated and BH-treated cells. These results showed Twist protein levels were also significantly decreased after treatment with 5 and 10 μg/mL BH for 12 hours, with the 10 μg/mL group having a stronger effect than the 5 μg/mL (Figure 5). Combined, these data support the fact that BH treatment reduces mRNA and protein levels of Twist, possibly resulting in the observed phenotypes.

Discussion

BH, an active ingredient extracted from the traditional medicinal herb Coptidis rhizoma, has been reported to have anticancer properties in multiple cancer cell lines through induction of apoptosis (Letasiova et al., 2006; Mantena et al., 2006). A recent study has also revealed that BH can impair the invasive properties of lung cancer cells by decreasing the production of urokinase-plasminogen activator and matrix metalloproteinase (Mitani et al.,

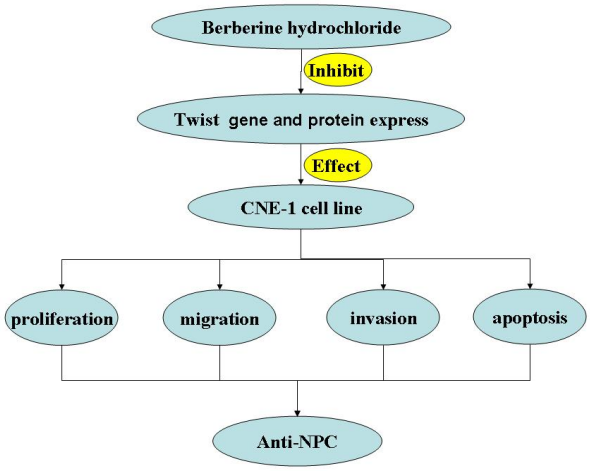

Figure 6. The Potential Mechanism of BH on NPC CNE-1 Cell Line

2001). The ability of BH to inhibit invasion of cancer cells may prevent cancer metastasis. NPC is a highly invasive head and neck cancer commonly associated with regional spread and systemic dissemination (Gao et al., 2012). Prognosis is poor when NPC cells metastasize to distant organs (Ong et al., 2003), and there are limited drugs available for the prevention of cancer metastasis. The present study aimed to examine the potential application of berberine in the treatment of NPC, particularly in the prevention of metastatic spread, using an established NPC cell line, CNE-1. The findings obtained in this study may also be applicable to other human cancer cells and support the possible potential of Coptidis rhizoma in the therapy of human cancers.

In our study, the cytotoxic dose of BH in NPC cells was analyzed first (Figure 1). Through the experimental studies, we found that BH inhibited CNE-1 cell proliferation with a dose and time-dependent tendency. The concentration of BH and time required to inhibit proliferation in CNE-1 cells were both lower than what has been previously reported (Lin et al., 2006; Jantova et al., 2007; Eom et al., 2008). Furthermore, BH under these conditions was shown to significantly inhibit CNE-1 migration and invasion in addition to inducing apoptosis (Figure 2, 3). Previous reports demonstrating tumor cell apoptosis induced by BH utilized higher concentrations of BH and required more time for induction than our studies (Hsu et al., 2007; Hyun et al., 2010). Despite this, lower concentrations of BH required to induce tumor cell apoptosis have been reported, though the time required for this was longer than our report and with a lower apoptosis ratio (Liu et al., 2009). This suggests that BH has different effects on cellular processes depending on the type of tissue under investigation.

The results of this study show that the effect of BH on the nasopharyngeal carcinoma cell line CNE-1 is very remarkable, considering that lower concentrations and shorter action time could suppress CNE-1 invasion and metastasis while inducing apoptosis. After prolonged periods of treatment, CNE-1 cells gradually appear more necrotic. Wang reported that paclitaxel combined with hyperthermia could inhibit CNE-1 proliferation and induce apoptosis (Wang et al., 2009). Our study demonstrates that the only low concentrations of BH are able to achieve the same effect, suggesting that BH has the potential to be a very significant and efficacious anti-nasopharyngeal cancer drug.

On the basis of the above research, we went on to investigate the relationship between BH and Twist expression to gain initial insights on the mechanism of BH actions on the CNE-1 cell line. Twist is known to play important roles in organizing the actin filaments involved in changes in cell shape, cell adhesion (Leptin 1991; Barnesm et al., 2009), cell polarity, cell migration (Yang et al., 2004; Yang et al., 2006) and apoptosis (Chen et al., 1995). To the best of our knowledge, this is the first study demonstrating the ability of BH to inhibit activation of Twist. Our experimental results revealed an interesting phenomenon that BH could suppress CNE-1 proliferation, migration, invasion and induce apoptosis, while at the same time inhibiting Twist gene and protein express, suggesting a correlation between Twist and all of these processes in CNE-1 cells that maybe a potential mechanism of BH on NPC CNE-1 cell line (Figure 6). The specific mechanism by which BH exerts its effects on the CNE-1 cell line requires further study. Considering all of our data, BH has strong potential for the prevention of cancer cell metastasis and induction of cancer cell apoptosis. Our data support further study of BH for its use as a treatment in nasopharyngeal and other types of cancer.

Acknowledgements

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