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Brucine, an alkaloid from seeds of *Strychnos nux-vomica* Linn., represses hepatocellular carcinoma cell migration and metastasis: The role of hypoxia inducible factor 1 pathway

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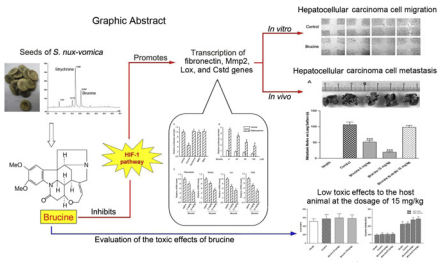
HIGHLIGHTS

- Brucine represses HCC cell migration *in vitro*.
- Brucine represses HCC metastasis *in vivo*.
- Brucine exhibits low toxic effect at the dosage of 15 mg/kg.
- Brucine suppresses HIF-1 dependent transcription.

GRAPHICAL ABSTRACT



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ABSTRACT

Brucine is an alkaloid derived from the seeds of *Strychnos nux-vomica* Linn. which have long been used as a traditional medicine for the treatment of hepatocellular carcinoma (HCC) in China. HCC prognosis can be greatly influenced by metastasis. There has thus far been little research into brucine as a source of anti-metastasis activity against HCC. In this study, we revealed that brucine dramatically repressed HepG2 and SMMC-7721 HCC cell migration with few cytotoxic effects. Hypoxia inducible factor 1 (HIF-1) is a key transcription factor mediating cell migration and invasion. Brucine suppressed HIF-1-dependent luciferase activity in HepG2 cells. The transcriptions of four known HIF-1 target genes involved in HCC metastasis, *i.e.*, fibronectin, matrix metallopeptidase 2, lysyl oxidase, and cathepsin D, were also attenuated after brucine treatment. Experiments *in vivo* showed that an intraperitoneal injection of 5 and 15 mg/kg of brucine resulted in dose-dependent decreases in the lung metastasis of H22 ascitic hepatoma cells. Moreover, a dosage of brucine at 15 mg/kg exhibited very low toxic effects to tumor-bearing mice. Consistently, brucine downregulated expression levels of HIF-1 responsive genes *in vivo*. Our current study demonstrated the capacity of brucine in suppressing HCC cell migration *in vitro* and lung metastasis *in vivo*. The inhibition of the HIF-1 pathway is implicated in the anti-metastasis activity of brucine.

Abbreviations: ALT, alanine aminotransferase; AST, aminotransferase; BUN, blood urea nitrogen; CRE, creatinine; Ctsd, cathepsin D; FACS, flow cytometry analysis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCC, hepatocellular carcinoma; Hb, hemoglobins; HIF-1, hypoxia inducible factor 1; Lox, lysyl oxidase; Mmp2, matrix metallopeptidase 2; MS, mass spectrum; MTT, methyl thiazolyl tetrazolium; NMR, nuclear magnetic resonance; OD, optical density; PCR, polymerase chain reaction; PHD, prolyl hydroxylase; PLT, platelets; RBC, red blood cells; *S. nux-blanda*, *Strychnos nux-blanda* Hill; *S. nux-vomica*, *Strychnos nux-vomica* Linn; SPF, specific pathogens free; TASNB, total alkaloids from *S. nux-blanda*; TASNV, total alkaloids from *S. nux-vomica*; UA, uric acid; WBC, white blood cells.

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1. Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and a leading cause of cancer-related mortality worldwide (Yang and Roberts, 2010). Despite much impressive advancement in surgical and radiotherapeutic technologies, the long-term prognosis of HCC still remains very poor due to high incidences of metastasis and recurrence (Tang et al., 2004). It is thus essential to search for new agents for controlling HCC metastasis as part of a wider strategy to treat HCC.

Hypoxia inducible factor 1 (HIF-1) is one of the key transcription factors mediating cancer cell metastasis and tumor vascularization, both of which play significant roles in tumor progression (Lu and Kang, 2010; Thirlwell et al., 2011). HIF-1 is known to respond to a hypoxia microenvironment and promote the expression of an array of adaptive genes. Structurally, it is a heterodimer consisting of one α (HIF-1α) and one β (HIF-1β) subunit. HIF-1β proteins constitutively exist in mammalian cells, while the protein levels of HIF-1α are regulated in an oxygen-dependent manner. Under normoxia, HIF-1α is hydroxylated by prolyl hydroxylases (PHDs) on one or both conserved proline residues (proline 402 and 564 in human HIF-1α). Oxygen is required as a substrate in this reaction (Aragones et al., 2009). Meanwhile, hydroxylated HIF-1α proteins are committed for 26S proteasomal degradation (Kaelin and Ratcliffe, 2008). Under hypoxia, HIF-1α is stabilized due to the inhibition of PHD-catalyzed hydroxylation. HIF-1α aggregates in the nucleus, dimerizes with HIF-1β, and activates the transcription of its target genes. A link between hepatic HIF-1 activity and the development and progress of HCC has been reported previously (Li et al., 2011). Similarly, protein levels of HIF-1α in HCC tissues positively correlate with HCC lymph node metastasis events (Xiang et al., 2011). These findings suggest that HIF-1 has great potential to be a drug target to inhibit HCC metastasis.

Traditional literatures on medical plants provide a new angle of threads for discovering potential therapeutic chemicals (El Gendy et al., 2012; Hazra et al., 2005). *Nux vocima* are the dry seeds of *Strychnos nux-vomica* Linn. (*S. nux-vomica*) and have long been used in traditional Chinese medicine for the treatment of HCC (Xu et al., 2003). Alkaloids are copiously found in *Nux vocima*, consisting mostly of brucine and strychnine (Behpour et al., 2012). These two compounds exhibit very strong toxic effects (Chen et al., 2012). According to the principles of traditional Chinese medicine, their toxicity can be reduced by processing *Nux vocima* in hot sands (220 °C for 3–4 min) before clinical practice, thus transforming part of brucine and strychnine into their *N*-oxidized derivatives (Deng et al., 2006b). Until now, the scientific basis underlying the anti-HCC activity of *Nux vocima* has not been fully understood. In our previous study, it was revealed that 0.5 mM brucine, but not strychnine or their processed products, induce remarkable apoptosis in cultured HCC cells *in vitro* (Deng et al., 2006a; Yin et al., 2007). Furthermore, its cytotoxic activity against cancer cells has been supported by a recent study demonstrating a brucine-induced G1 phase arrest and apoptosis in LoVo cells (Zheng et al., 2013). In addition, several investigations have reported the ability of brucine to attenuate angiogenesis both *in vitro* and *in vivo* (Agrawal et al., 2011; Saraswati and Agrawal, 2013). These observations indicate that brucine is the major active ingredients in *Nux vocima*. Prognosis of HCC is largely influenced by metastasis. However, it remains largely unknown if brucine provides any anti-metastasis activity against HCC. Moreover, since brucine is a highly toxic compound, it is important to systematically evaluate its anti-HCC activity and toxic effect to host animals *in vivo*.

Evolutionarily, *Strychnos nux-blanda* Hill (*S. nux-blanda*) is closely related to *S. nux-vomica*. In fact, the seeds of *S. nux-blanda* are also sometimes used as the herb "*Nux vocima*" in parts of China. In this study, we found that brucine, which exists only in *S. nux-vomica* but not in *S. nux-blanda*, significantly repressed HepG2 and SMMC-7721 HCC cell migration independent of inducing cell death or arresting cell proliferation. Since tight connections between HCC cell migration and metastasis have been demonstrated by previous investigations (Zheng et al., 2011; Kanno et al., 2012; Yeh et al., 2012), the *in vivo* anti-metastasis activity of brucine and its toxic effects to host animals were also examined. Our further investigations revealed that brucine suppressed HIF-1-dependent gene expression in HCC cells both *in vitro* and *in vivo*.

2. Materials and methods

2.1. S. nux-blanda and S. nux-vomica seeds preparation and analysis

The seeds of *S. nux-vomica* and *S. nux-blanda* were collected in Yunnan Province, and identified by Professor Dingrong Wan (College of Pharmacy, South-Central University for Nationalities, Wuhan, PR China). Voucher samples were then deposited at the Herbarium located in the College of Pharmacy South-Central University for Nationalities.

Air-dried seeds of the two species (500 g for each) were firstly ground into powder (20-mesh). The powder was then extracted with acidified 50% ethanol (pH 2.0–3.0) at room temperature for 3 times (24 h each). The ethanol extracts were combined, filtered and centrifuged. Then the supernatant was subsequently basified to pH 9.0 with ammonia water and extracted with chloroform. The chloroform fraction was evaporated to yield crude total alkaloids (15 g for each species). To obtain brucine and strychnine, crude total alkaloids from *S. nux-vomica* were further subjected to extensive chromatography on a silica gel column and eluted with petroleum ether-acetone or acetone-methanol gradient solvent system. To obtain brucine *N*-oxide and strychnine *N*-oxide, the seeds of *S. nux-vomica* were processed in hot sands (220 °C) for 3 min and then subjected to the aforementioned phytochemical extraction and isolation procedures. The chemical structures of these compounds were confirmed by spectrum methods including mass spectrum (MS), ^{1}H nuclear magnetic resonance (NMR) and ^{13}C NMR. Their purity was determined by high-performance liquid chromatography (HPLC) as described previously (Liu et al., 2007).

2.2. Cells cultures and treatment

Human HepG2 and SMMC-7721 HCC cells were cultured in the RPMI 1640 medium containing 10% fetal calf serum (FCS) purchased from Invitrogen (Carlsbad, CA, USA) containing 100 U/mL of penicillin and streptomycin. Cells were incubated at 37 °C in a humidified atmosphere supplied with 5% CO_2. For hypoxia treatment, cells were cultured in an atmosphere with 5% CO_2, 1% O_2 and 94% N_2. Murine H22 ascitic hepatoma cells were maintained by weekly transplanting them into the peritoneal cavities of Kunming mice as described previously (Zhao et al., 2012).

2.3. Cell viability assay

HepG2 cells were seeded on 24-well plates and allowed to adhere overnight. After treatment with different concentrations of chemicals, the medium was replaced by 250 μL of medium containing methyl thiazolyl tetrazolium (MTT, 0.5 mg/mL). This was followed by incubating the cells at standard culture conditions for 3 h. The mediums were then discarded and 500 μL of DMSO was added to each well. After thorough mixing, cell viabilities were indicated by the optical density (OD) values of the solutions at 570 nm.

2.4. Scratch wound healing assay

Scratch wound healing assays were performed as described previously (Shi et al., 2009). Briefly, cells were seeded into 12-well plates in the medium containing 10% FCS to near confluency. After generating a cross scratch in the monolayer with a sterile pipette tip, the medium was changed into 1640 containing 1% newborn calf serum. Photographs of the wound area were captured at the time points as indicated in the text. Data were quantified through analyzing the areas in the scratch not covered by cells.

2.5. Flow cytometry analysis (FACS)

FACS assay was performed as described previously (Zhang et al., 2009; Shu et al., 2011). After treating HepG2 cells as indicated, they were trypsinized and fixed with 70% ethanol for at least 2 h at 4 °C. The cells were then pelleted and washed with PBS with 20 mM EDTA. RNA was removed by incubating samples with 1 mg/mL RNaseA at 37 °C for over 1 h. Cells were then stained with 30 μg/mL propidium iodide and cell cycle distribution was analyzed by a flow cytometer (Becton Dickinson FACSCalibur, Franklin Lakes, NJ, USA).