Phytomedicine 21 (2014) 348–355

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.de/phymed

Harmine induces apoptosis and inhibits tumor cell proliferation, migration and invasion through down-regulation of cyclooxygenase-2 expression in gastric cancer

Hao Zhang[a,1], Kun Sun[a,1], Jing Ding[b], Huae Xu[c], Lingjun Zhu[d], Kai Zhang[a], Xiaolin Li[a,*], Weihao Sun[a,*]

[a] Department of Geriatric Gastroenterology, the First Affiliated Hospital to Nanjing Medical University, Nanjing 210029, PR China
[b] Department of Respiratory Medicine, the Affiliated Nanjing Children Hospital to Nanjing Medical University, Nanjing 210029, PR China
[c] Department of Pharmacy, the First Affiliated Hospital to Nanjing Medical University, Nanjing 210029, PR China
[d] Department of Oncology, the First Affiliated Hospital to Nanjing Medical University, Nanjing 210029, PR China

ARTICLE INFO

Article history:
Received 16 April 2013
Received in revised form 28 July 2013
Accepted 19 September 2013

Keywords:
Harmine alcaloid
Gastric cancer
Cyclooxygenase-2
Proliferation
Apoptosis
Invasion

ABSTRACT

Cyclooxygenase-2 (COX-2) plays an important role in the carcinogenesis and progression of gastric cancer. Harmine is reported as a promising drug candidate for cancer therapy; however, effects and action mechanism of harmine on the human gastric cancer cells remain unclear. This study evaluated the anti-tumor effects of harmine on human gastric cancer both *in vitro* and *in vivo*. The cell proliferation was determined using MTT colorimetric assay. Apoptosis was measured by DAPI staining and flow cytometry analysis. The wound healing and transwell invasion assays were performed to evaluate the effects of harmine on the migration and invasion of gastric cancer cells. The expression of COX-2, proliferating cell nuclear antigen (PCNA), Bcl-2, Bax and matrix metalloproteinase-2 (MMP-2) was detected by Western blot analysis. Our results showed that harmine significantly inhibited cellular proliferation, migration, invasion and induced apoptosis *in vitro*, as well as inhibited tumor growth *in vivo*. In addition, harmine significantly inhibited the expression of COX-2, PCNA, Bcl-2 and MMP-2 as well as increased Bax expression in gastric cancer cells. These results collectively indicate that harmine induces apoptosis and inhibits proliferation, migration and invasion of human gastric cancer cells, which may be mediated by down-regulation of COX-2 expression.

Introduction

Gastric cancer is the fourth most prevalent cancer and the second most common cause of cancer-related deaths throughout the world (Sang, 2012; Schwarz and Smith, 2007; Jemal et al., 2011). At present, the management of gastric cancer includes surgery, radiotherapy, conventional chemotherapy, molecular targeted therapy and biological therapy, but the curative effects of existing chemotherapeutic drugs are not good enough and they have numerous side-effects, including myelosuppression, hepatotoxicity, and immunosuppression (Leite et al., 2012; Ali et al., 2013). Therefore, it has become a focus to search for new drugs from natural sources, which are capable of preventing and treating gastric cancer. One possible way to increase the efficacy of anticancer drugs and decrease toxicity or side effects is to develop traditional medicines, especially from medicinal plants (Ramsewak et al., 2000; El Gendy et al., 2010; Astulla et al., 2008).

Harmine (Fig. 1), originally isolated from the seeds of *Peganum harmala*, is a tricyclic compound belonging to the β-carboline alkaloids (Frost et al., 2011). These alkaloids possess a broad range of pharmacological activities, such as anti-inflammatory, anxiolytic and antinociceptive effects (Zhao et al., 2012). Recent studies have shown that harmine possessed significant anti-tumor potential both *in vitro* and *in vivo* (Abe and Yamada, 2009; Cao et al., 2011; Dai et al., 2012; Hamsa and Kuttan, 2010, 2011). However, effects and action mechanism of harmine on human gastric cancer remain unclear.

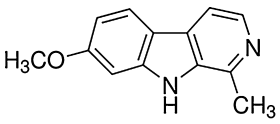
Cyclooxygenase (COX), a key enzyme in the conversion of arachidonic acid to prostaglandins (PGs) and other eicosanoids, exists as two isoforms: constitutive COX-1 and mitogen-inducible

Abbreviations: COX-2, cyclooxygenase-2; PGs, prostaglandins; MMPs, matrix metalloproteinases; ECM, extracellular matrix; PCNA, proliferating cell nuclear antigen; DMSO, dimethylsulfoxide; DAPI, 4′,6-diamidine-2′-phenylindole dihydrochloride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylformazan; FBS, fetal bovine serum; PBS, phosphate buffer saline; PI, propidium iodide; SDS, sodium dodecyl sulfate; SPF, specific pathogen-free; SD, standard deviation; ANOVA, analysis of variance analysis.

* Corresponding authors. Tel.: +86 25 68135151; fax: +86 25 83780170.
E-mail addresses: lxl@njmu.edu.cn (X. Li), swh@njmu.edu.cn, weihaosun@hotmail.com (W. Sun).

[1] These authors contributed equally to this work.

0944-7113/$ – see front matter © 2013 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.phymed.2013.09.007



$C_{13}H_{12}N_2O$
Molecular Weight: 212.25 g/mol

Fig. 1. Chemical structure, molecular formula and 212.25 molecular weight of harmine.

COX-2. COX-2 is also constitutively expressed in gastric cancer and is related to cell proliferation and apoptosis, tumor invasiveness and metastasis (van Rees et al., 2002; Sun et al., 2005; Almeida et al., 2012). Previous studies have shown that COX-2 inhibition by selective COX-2 inhibitors or small interfering RNA (siRNA) suppresses cell proliferation and induces apoptosis in human gastric cancer cells (Sun et al., 2008; Chan et al., 2007).

Matrix metalloproteinases (MMPs), a family of closely related enzymes that degrade the extracellular matrix (ECM), are considered to be important in facilitating tumor invasion and metastasis (Curran and Murray, 1999). In gastric cancer, MMP-9 and MMP-2 were reported to be overexpressed and associated with tumor aggressiveness (Sun et al., 2005; Noh et al., 2011). In addition, our previous study showed that MMP-9 expression significantly correlated with COX-2 expression in human gastric cancer (Sun et al., 2005).

Recently, we have found that ursolic acid, a natural pentacyclic triterpenoid compound, induces apoptosis of gastric cancer cells by down-regulation of COX-2 expression (Zhang et al., 2013). However, the effects of harmine on the expression of COX-2 in gastric cancer cells have not been examined thus far. In the present study, two human gastric cancer cell lines BGC-823 and SGC-7901, in which COX-2 were found to be expressed (He et al., 2012), were applied to investigate the anti-tumor effects of harmine on human gastric cancer *in vitro* and *in vivo*. In addition, the expression of COX-2, proliferating cell nuclear antigen (PCNA), MMP-2 and apoptosis-related proteins were detected to further elucidate the possible mechanism underlying the anti-tumor effects of harmine against gastric cancer.

Materials and methods

Reagents

Harmine (≥98% purity), dimethylsulfoxide (DMSO), 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Stock solutions of harmine were made in DMSO at a final concentration of DMSO (≤0.1%) and sterilized by passage through a 0.22 μm pore size filter (Immobilon, Millipore Corp., Bedford, MA, USA), diluted with culture media before use. RPMI-1640 medium, fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). All other chemicals were of analytical grade and used without further purification.

Cell lines and culture conditions

Human poorly differentiated BGC-823 and moderately differentiated SGC-7901 gastric cancer cell lines were obtained from Shanghai Institute of Cell Biology (Shanghai, China). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS, 100 units/ml penicillin G, and 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

MTT assay

BGC-823 and SGC-7901 cells were seeded into 96-well microplate at a density of 5×10^3 cells per well and incubated overnight in 10% FBS medium. The cells were then treated with different concentrations of harmine in serum-free conditions. Untreated cells in serum-free medium were used as controls. After incubation for 24, 48 and 72 h at 37 °C, the cell proliferation was determined by the MTT assay as described in our previous study (Sun et al., 2008, 2009).

DAPI staining

BGC-823 and SGC-7901 cells were treated with harmine at the final concentrations of 0, 4, 8 and 16 μg/ml for 24 h, and then washed once in phosphate buffer saline (PBS) followed by fixation in cold methanol:acetone (1:1) for 5 min. After washing twice in PBS for 5 min, these cells were stained with 4 μg/ml DAPI for 10 min at room temperature and subsequently examined by fluorescence microscopy (Eclipse E-800; Nikon, Tokyo, Japan). Apoptotic cells were identified by chromatin condensation and nuclear fragmentation.

Flow cytometry analysis

To further quantitative analysis of apoptosis, the cells were washed with PBS, stained with annexinV-FITC and propidium iodide (PI) using the AnnexinV-FITC kit (Bender Medsystem, Vienna, Austria). The cells were then subjected to flow cytometry according to manufacturer's instructions and the stained cells were analyzed by FACScan flow cytometer (Becton Dickinson, CA, USA).

Western blot analysis

The extraction of proteins from cells and western blot analyses were performed as described in our previous reports (Sun et al., 2008, 2009; He et al., 2012). Primary antibodies used include rabbit anti-COX-2, anti-Bcl-2, anti-Bax, anti-MMP-2 (Cell Signaling Technology, Inc., Beverly, MA, USA); anti-PCNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-GAPDH (Sigma–Aldrich). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was purchased from Vector Laboratories Inc. (Burlingame, CA, USA). Bands were quantified using densitometric image analysis software (Quantity One, Bio-Rad, Hercules, CA, USA). The relative expressions of COX-2, PCNA, Bcl-2, Bax and MMP-2 were normalized to that of GAPDH.

In vitro wound healing assay

Cell migration was assayed using *in vitro* wound healing assay. BGC-823 and SGC-7901 cells were plated into the 6-well plates and cultured in incubator until confluent monolayers were formed. The cells were serum starved for 12 h and then a 'wound' (clear space) was made using a sterile pipette tip. At this time point ($t = 0$ h) wound margins were observed using phase contrast microscopy and photographed. Then, serum-free medium containing 0 or 8 μg/ml harmine was added to the plates and the cells were incubated for up to 36 h at 37 °C. The same fields of the wound margin were photographed at 24 and 36 h. Pictures were superposed using Photoshop (Adobe) and areas were measured using Scion Image Analysis Software (Scion Corporation, Frederick, MD, USA). The wound healing rate was calculated according to the following formula: (the average area of wound in 0 h – the average area of wound in 24 or 36 h)/the average area of wound in 0 h. Experiments were repeated at least three times.