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Original article

Anticancer activities of harmine by inducing a pro-death autophagy and apoptosis in human gastric cancer cells

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ABSTRACT

Background: Harmine, a β-carboline alkaloid from Peganum harmala, has multiple anti-tumor activities, especially for its folk therapy for digestive system neoplasm. However, the underlying mechanism of harmine on gastric cancer remains unclear.

Purpose: To illuminate the potential anti-tumor activity and mechanism of harmine against gastric cancer cells.

Methods/Study designs: The anti-proliferative activity of harmine in vitro was evaluated by MTT assay. The autophagic activity induced by harmine was assessed using GFP-LC3 transfection. FITC/PI double staining was applied for the apoptosis inspection. The mitochondrial membrane potential was detected by JC-1 fluorescence probe. The potential mechanisms for proteins level in autophagy and apoptosis were analyzed by Western blot.

Results: Harmine exhibited potent effects on both autophagy and apoptosis. Treatment with harmine could enhance dots of GFP-LC3 in cells. Meanwhile, the process had connection with Beclin-1, LC3-II, and p62 by the inhibition of Akt/mTOR/p70S6K signaling. However, high concentration of harmine led to apoptosis characterized by the propidium/Annexin V-positive cell pollution, cell shrunk and the collapse of mitochondrial membrane potential. The regulation of Bcl-2, Bax and the gathering of cleaved-PARP, cleaved-caspase 3 and cleaved-caspase 9 contributed to the induction of apoptosis. In addition, 10 μM LY294002 (a specific inhibitor of PI3K/Akt) combination with 40 μM harmine significantly increased the cytotoxicity to the gastric cancer cells and up-regulated both the apoptosis-related protein (cleaved-PARP, cleaved-caspase-3) and autophagy-related protein (Beclin-1, LC3-II, and p62). Adding the inhibitor of autophagy, 3-MA or BafA1, increased the viability of harmine-exposured gastric cancer cells, which confirmed the role of autophagy played in the gastric cancer cell death induced by harmine.

Conclusion: Harmine might be a potent inducer of apoptosis and autophagy, which offered evidences to therapy of harmine in gastric carcinoma in the folk medicine.

Introduction

Harmine (HM, Fig. 1) is a natural β-carboline alkaloid, isolated from the seeds of *Peganum harmala* L. (Zygophyllaceae family). In China, *P. harmala* mainly distributes in Xinjiang, Inner Mongolia and other places in Northwest desert areas, and it has been used extensively for digestive system neoplasm in traditional Chinese medicine and Uygur medicine (Frost et al., 2011). Previous research showed that HM presented remarkable therapeutic effect on various diseases, which include apoplexia, asthma, jaundice and lumbago (Wang et al., 2011). Besides, HM possesses anti-tumor, anti-Alzheimer, anti-inflammatory, antimycotic and anti-viral activities regulated by several signaling pathways such as mitochondrial-mediated signaling pathways, PI3K/Akt pathway and kinase (Wang et al., 2015; Hamsa and Kuttan, 2010; Y.H. Wang et al., 2015).

In the perspective of human cancer, autophagy (type II cell death) may be a potential method apart from apoptosis (the type I programmed cell death) for interventions in pharmacology. Autophagy is a crucial mechanism of cell death in the condition of cellular growth factor's absence, lower nutrient and other

Abbreviations: Akt, protein kinase B; AMPK, adenosine 5′-monophosphate-activated protein kinase; BafA1, bafilomycin A1; FBS, fetal bovine serum; HM, harmine; LC3, the light chain 3; 3-MA, 3-methyladenine; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; mTOR, mammalian target of rapamycin; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; PI, propidium iodide; PI3K, phosphatidylinositol 3-kinase; PARP, poly-ADP-ribose polymerase; cccp, carbonyl cyanide 3-chlorophenylhydrazone.

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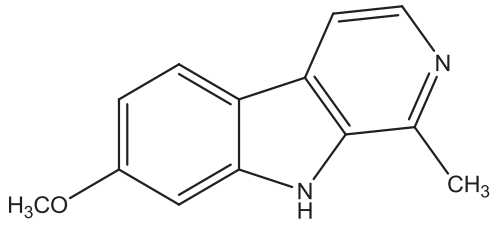

Fig. 1. Structure of harmine (HM).

analogous, which is highly conserved in mammalian, characterized the turnover of cellular with endolysosome, where autolysosome recycles breakdown contents (for instance, macromolecules and long-lived proteins) using the hydrolases and discarded organelles (Codogno and Meijer, 2005; Kroemer and Jäättelä, 2005; Levine and Klionsky, 2004). Autophagy is a stepwise of digesting its own intracellular contents depended on fusion. Intriguingly, the "self-digestion" manner plays not merely a protective role for cell survival but also a pro-apoptotic role for cell death in response to extrinsic stress (Hara et al., 2006). It is initiated with a biosynthesis for double-membrane structure regulated by autophagy related protein, such as Beclin-1, LC3. Several signaling pathways make crucial effects on autophagy process. mTOR (mammalian target of rapamycin) is an extremely important mediated factor in the regulation of autophagy. PI3K/Akt pathway plays crucial role in regulating cell cycle, cell growth, translation, apoptosis, autophagy, DNA repair. Studies showed that the mitogen-activated protein kinase (MAPK) signaling pathway and adenosine 5′-monophosphate-activated protein kinase (AMPK) signaling pathway were significant for up-regulation of autophagy, apoptosis, cell growth and development. Autophagy prevented DNA damage caused by ROS (Bester et al., 2011). Autophagy also could maintain tumor development and survival by recycling nutrient during extrinsic damage and cancer.

Type I programmed cell death, apoptosis, is characterized as a condensation in nuclear chromatin, DNA fragmentation, cell shrinkage, apoptotic body formation proceeds in a high level as a response (Maiuri et al., 2007). The classical pathway involved in apoptosis could be subdivided into extrinsic way and intrinsic way. The extrinsic way refers to the death receptor-mediated way. Moreover, the mitochondrial-regulated apoptotic way is the intrinsic way. Both of the two ways' execution depends on the cascade reaction of caspases (Soldatenkov and Smulson, 2000).

In recent years, reports have revealed the alkaloid from *P. harmala*, harmol and the derivatives of HM are a potent multi-target medicine, could intervene in both apoptosis and autophagy (Li et al., 2015). Therefore, the alkaloids from *P. harmala* have fair chance to exert an antitumor action in multiple tumor cells.

The current study tried to clarify the antiproliferative action of HM in gastric cancer cell (MGC-803 and SGC-7901), and elucidate the underlying molecular mechanisms. Our team innovated searching in the present study for autophagy (Akt/mTOR/p70S6K signaling pathway and AMPK pathway), apoptotic (Akt/mTOR/p70S6K pathway and mitochondrial-mediated signaling pathway) traits in HM-exposed gastric cancer cells.

Materials and methods

Experimental materials

MGC-803 and SGC-7901 cells were acquired from KeyGen Biotech (Nanjing, China). RPMI 1640 medium, fetal bovine serum (FBS), and Lipofectamine 2000 were products of Thermo Scientific (Waltham, MA USA). 3-MA, BafA1 and protease inhibitor cocktail were obtained from Sigma-Aldrich (St. Louis, MO, USA). LY294002 was purchased from Selleck (Houston, USA). LC3-II, Beclin-1, p62 (SQSTM1), mTOR, p-mTOR, Akt, p-Akt, p70S6K, p-p70S6K, caspase 3, caspase 9, PARP-1, Bax, Bcl-2 primary antibody were obtained from Cell Signaling Technology (Danvers, MA, USA). β-Actin was obtained from Proteintech Group, Inc. (Chicago, IL, USA). FITC-Annexin V apoptosis detection kit I was product of BD biosciences (Franklin Lakes, NJ, USA). RIPA cell lysis buffer and mitochondrial membrane potential assay kit with JC-1 were obtained from Beyotime biotechnology (Shanghai, China). Hyperfilm ECL was product of GE Healthcare Life Sciences (Uppsala, Sweden). Harmine was isolated and purified from the seeds of *P. harmala* in our lab. The purity is above 98% by HPLC analysis and its structure was identified through MS, IR and NMR spectra.

Cell culture and HM treatment

Human gastric cancer cell lines (MGC-803 and SGC-7901) were cultured in RPMI-1640 medium with 10% FBS and 1% 100 U/ml penicillin, 100 mg/ml streptomycin sulfate at 37 °C in humidified atmosphere containing 5% CO_2 and 95% air. HM was dissolved in dimethyl sulfoxide (DMSO) to be the stock solution (20 mM) and further diluted to final concentrations with culture medium. For the time-dosage experiments, the cells were treated with HM of 10, 20, 40, 80 μM for 24, 48, and 72 h.

Cell viability (MTT assay)

The cell viability was evaluated with 3-(4,5-dimethyl-2-thiazolyl)−2,5-diphenyl- 2H-tetrazolium bromide (MTT) assay. The cells were seeded in 96 well-plates with the density of 5×10^4 cells/ml at 37 °C in the incubator. Incubated for 12 h, the cells were treated with HM of 10, 20, 40, 80 μM for 24, 48 and 72 h. For the inhibition experiments, cells were treated with 2.5 mM 3-MA, 100 nM BafA1 or 10 μM LY294002 for 1 h before HM added. Thereafter, 5 mg/ml MTT solution was added to each well for 4 h in the incubator. Subsequently, 150 μl DMSO dissolves the formazan crystal. The absorbance was determined at 492 nm with a microplate reader. Cell viability was presented based on proportion of the untreated cells.

Western blot analysis

After treated with HM, cells were extracted with Radio Immunoprecipitation Assay containing 1% (v/v) protease inhibitor cocktail (Sigma, USA), and the concentration of the protein was measured using a BCA protein assay kit. Subsequently, the equal proteins (20 μg) were separated by sodium dodecyl sulfate-polyacrylamide Gel electrophoresis (Bio-Rad, Hercules, CA, USA) and electrotransferred to a polyvinylidene fluoride membrane (0.45 μm). After the transferring, the nonspecific binding blots were blocked in 5% no-fat milk dissolved in TBST including 0.1% v/v Tween-20 for 1 h at room temperature with a gently shaking. Then, the specific primary antibodies were incubated for overnight at 4 °C followed by incubation for 1 h with the respective second antibody at room temperature. The protein signals were visualized using ECL chemiluminescence detection reagents. β-Actin was performed as a loading control.

Plasmid transfection

The GFP-LC3 plasmid was transfected to the MGC-803 and SGC-7901 cells with Lipofectamine 2000 according to the manufacture's protocol. Briefly, 1×10^6 cells/well were seeded onto 6 cm dish for overnight at 37 °C, and transfected with the GFP-LC3 plasmid in