Harmine induces apoptosis in HepG2 cells via mitochondrial signaling pathway

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BACKGROUND: Harmine has antitumor and antinociceptive effects, and inhibits human DNA topoisomerase. However, no detailed data are available on the mechanisms of action of harmine in hepatocellular carcinoma. This study aimed to investigate the effects of harmine on proliferation and apoptosis, and the underlying mechanisms in the human hepatocellular carcinoma cell line HepG2.

METHODS: The proliferation of HepG2 cells was determined by the cell counting kit-8 (CCK-8) assay and the clone formation test. The morphology of HepG2 cells was examined using fluorescence microscopy after Hoechst 33258 staining. Annexin V/propidium iodide (PI) was used to analyze apoptosis and PI to analyze the cell cycle. Western blotting was used to assess expression of the apoptosis-regulated genes Bcl-2, Bax, Bcl-xl, Mcl-1, caspase-3, and caspase-9. Mitochondrial transmembrane potential (Ψm) was determined using JC-1.

RESULTS: Harmine inhibited the proliferation of HepG2 cells in a dose-dependent manner. Hoechst 33258 staining revealed nuclear fragmentation and chromosomal condensation, cell shrinkage, and attachment loss in HepG2 cells treated with harmine. The percentage of the sub/G1 fraction was increased in a concentration-dependent manner, indicating apoptotic cell death. PI staining showed that harmine changed the cell cycle distribution, by decreasing the proportion of cells in G0/G1 and increasing the proportion in S and G2/M. Harmine induced apoptosis in a concentration-dependent manner, with rates of 20.0%, 32.7% and 64.9%, respectively. JC-1 revealed a decrease in Ψm. Apoptosis of HepG2 cells was associated with caspase-3 and caspase-9 activation, down-regulation of Bcl-2, Mcl-1, and Bcl-xl, and no change in Bax.

CONCLUSIONS: Harmine had an anti-proliferative effect in HepG2 cells by inducing apoptosis. Mitochondrial signal pathways were involved in the apoptosis. The cancer-specific selectivity shown in this study suggested that harmine is a promising novel drug for human hepatocellular carcinoma.

KEY WORDS: hepatocellular carcinoma; harmine; Bcl-2 protein; caspase-3; apoptosis

Introduction

Hepatocellular carcinoma (HCC) is a common and aggressive malignant tumor worldwide. Primary liver cancer, which consists predominantly of HCC, is the fifth most common cancer worldwide and the third most common cause of cancer mortality. In China, HCC accounts for 90% of primary liver cancer, which is the second most common cause of death.[1] Recent data indicate that the mortality of HCC in China is tending to increase, severely threatening the health and life of people. Currently, the treatments for HCC are mainly surgery and chemotherapy, but the curative effect of existing chemotherapeutic drugs is not good enough and they have numerous side-effects, including myelosuppression, neutropenia, and thrombocytopenia.[2, 3] Therefore, it has become a focus to search for drugs which are capable of preventing and treating HCC and other malignancies. 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.[4, 5] Herbal
medicines are an important source of novel agents with pharmaceutical potential. Harmine is a major component isolated from *Peganum harmala* L. (*Zygophyllaceae*) seed extract. The pharmacological characterization shows that harmine has antitumor and antinociceptive effects, and inhibits human DNA topoisomerase. However, no detailed data are available on the mechanisms of action of harmine in HCC. We investigated its effect on the growth of human HepG2 cells and the underlying intracellular signal transduction pathways involved in regulating apoptosis.

### Methods

#### Materials

Harmine was from Xi’an Feida Bio-Tech Co., Ltd. The following were all from Cell Signaling (Danvers, Massachusetts, USA): antibodies for immunoblots anti-caspase-3 (#9662), anti-caspase-9 (#9502), anti-Bcl-2 (#2772), and anti-Mcl-1 (#4572), anti-Bcl-xl (#2764), anti-Bax (#2772), and GAPDH (#3683); and cell lysis buffer.

#### Cell culture and treatment with harmine

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO₂. The harmine was dissolved in dimethyl sulfoxide (DMSO), and diluted to appropriate concentrations with culture medium. The final concentration of DMSO in the culture medium did not exceed 0.1%.

#### CCK-8 assay

The CCK-8 test was used to monitor cell proliferation. Cells were plated at a density of 5000 cells/well in 96-well plates. After 24 hours in culture, the cells were treated with harmine at the final concentrations 0, 0.625, 1.25, 2.5, 5, 10, or 20 μg/mL for 48 hours. Control cells were treated with DMSO. After addition of test compounds, 10 μL CCK-8 was added to each well. Absorbance was detected with an enzyme calibrator at 570 nm and then optical density (OD) values were measured. Inhibition of cell growth was computed by the percentage of viable cells compared with control. Percentage (%)=(ODC-ODT)/ODC×100%. ODT is the OD value of the treated sample, and ODC is the OD value of the control sample. Experiments were done in triplicate.

#### Clone formation assay

A clone formation assay was used to evaluate the effects of harmine on the proliferation of HepG2 cells. Cells were first cultured in 12-well microplates (300 cells/well) in 2.5 mL of complete RPMI-1640 for 24 hours. Then the cells were treated with the indicated concentrations harmine for 7 days. Finally, the cells were stained in crystal violet for 20 minutes. Images of the colonies were captured by a digital camera.

#### Fluorescence microscopy assay

Harmine-induced apoptosis in HepG2 cells was assessed by Hoechst 33258 staining. Treated with 5 μg/mL harmine for 48 hours, the cells were harvested and smeared on slides. The slides were air-dried, fixed in methanol-acetone (3/1, v/v), and stained with Hoechst 33258 (5 μg/mL) at 37 °C for 20 minutes. Nuclear morphology was examined under fluorescence microscopy (DFC480; Leica Microsystems, Wetzlar, Germany) to identify cells undergoing apoptosis.

#### Flow cytometry

HepG2 cells at a density of 20 000 cells/well were incubated in 6-well plates for 48 hours with DMSO, 0, 5, 10, or 20 μg/mL harmine. After incubation, the cells were collected and analyzed by flow cytometry. Then the cells were harvested, washed with phosphate buffer solution (PBS), and fixed in 70% ice-cold ethanol overnight. The fixed cells were incubated with 20 U/mL RNase I and 50 μg/mL propidium iodide (PI) for 30 minutes. The DNA content was determined by flow cytometry (Beckman Coulter, Fullerton, CA, USA). Apoptotic cells were identified by the sub-G1 phase in the cell-cycle distribution. For assessment of the apoptotic rate, Annexin V-FITC/PI (Becton Dickinson, USA) staining was performed according to the manufacturer’s protocol. The cells stained with Annexin V but not with PI were defined as apoptotic. The apoptotic rate was measured by flow cytometry (FCM, Becton Dickinson, USA) using CellQuest software.

#### Western blotting

Total proteins were extracted by incubation of cell pellets with lysis buffer. The protein concentration of the extracts was determined by bicinchoninic acid (Sigma) according to the manufacturer’s instructions. Cell lysates (50 μg/well) were electrophoresed in 12% SDS-PAGE and then transferred onto nitrocellulose membranes. After blotting in 5% non-fat dry milk in TBST buffer, the membranes were incubated with primary antibodies in 5% non-fat milk overnight at 4 °C, and then secondary antibodies conjugated with horseradish peroxidase for 1 hour at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence regents (Amersham, UK). To assess
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the presence of comparable amounts of proteins in each lane, the membranes were stripped to assess GAPDH.

Membrane potential of mitochondria (Ψm)

Changes of Ψm were monitored by determination of JC-1. HepG2 cells were treated with 5 μg/mL harmine for 48 hours, then the cells were harvested, washed with PBS, and fixed in JC-1 at 37 °C in the dark for 30 minutes, when they were harvested and smeared on slides. Changes in Ψm were measured after staining under fluorescence microscopy.

Statistical analysis

Data were analyzed by ANOVA and Student’s t test. These analyses were performed using SPSS 13.0 software. Differences with P<0.05 were considered significant.

Results

Harmine inhibits HepG2 cell proliferation

Cell viability was decreased remarkably by harmine treatment (Fig. 1A). Harmine inhibited the growth of HepG2 cells in a dose-dependent manner (P<0.05 vs control), with an IC50 of 9.80 μg/mL. To assess the effect of harmine on proliferation, the cells were treated with the indicated concentrations for 7 days. The result showed that harmine strongly inhibited their proliferation (Fig. 1B).

Harmine induces apoptosis in HepG2 cells

Apoptotic nuclear morphology was observed after Hoechst 33258 staining using fluorescence microscopy. After treatment with 5 μg/mL harmine for 48 hours, HepG2 cells began to exhibit apoptotic characteristics, such as cell shrinkage, nuclear condensation, and fragmentation. In the control group, the cells were regular in morphology and grew fully in patches and were confluent, rarely sloughing off (Fig. 2).

Apoptosis was also detected through fluorescein Annexin V-FITC/PI double labeling. The staining of cells with Annexin V-FITC and PI was used to

![Fig. 1. A: Effects of harmine on viability of HepG2 cells. The indicated concentrations of harmine were added, and the cells were incubated for 48 hours. Viability was measured by CCK-8 assay. Results represented 3 independent experiments. B: Clone formation assay was used to evaluate the effects of harmine on the proliferation of HepG2 cells. HepG2 cells were treated with 0, 0.625, 1.25, 2.5, or 5 μg/mL harmine for 7 days and stained in crystal violet for 20 minutes.](image)

![Fig. 2. Harmine-induced apoptosis in HepG2 cells was assessed by Hoechst 33258 staining. Morphology of HepG2 cells exposed to harmine at different concentrations photographed under a fluorescence microscope (original magnification ×200). A: DMSO; B: 0 μg/mL; C: 5 μg/mL.](image)
distinguish and quantitatively determine the percentage of apoptotic cells. HepG2 cells underwent apoptosis after exposure to harmine at 5, 10, and 20 μg/mL for 48 hours. The percentage of apoptotic cells stained by Annexin V-FITC is shown in Fig. 3A. PI staining and flow cytometry were used to investigate whether harmine inhibited the growth of HepG2 cells by initiating the apoptotic pathway. HepG2 cells underwent apoptosis after being exposed to harmine at 5, 10, and 20 μg/mL for 48 hours (Fig. 3B). The percentage of apoptotic cells in the sub-G1 phase of the cell cycle increased. The percentage of the sub-G1 fraction, which was indicative of apoptotic cell death in harmine-treated cells, increased in a dose-dependent manner.

**Harmine changes the cell cycle in HepG2 cells**

Flow cytometry with only PI staining showed that treatment of HepG2 cells with 5, 10, and 20 μg/mL harmine for 48 hours resulted in a higher number of cells in S phase and G2/M phase compared with the control group (Fig. 4). This increase was coupled with the decreased percentage of cells in G0/G1 phase.

**Effect of harmine on the protein expression level**

Loss of Ψm is a crucial step in the apoptotic process and is lethal to cells because it leads to the release of diverse pro-apoptotic factors from the mitochondria into the cytoplasm. In this study, we used the unique cationic dye JC-1 to determine the status of mitochondria. In non-apoptotic cells, JC-1 enters the negatively-charged mitochondria where it aggregates and turns red. However, in cells undergoing apoptosis, where Ψm has collapsed, JC-1 exists as monomers in the cytosol and turns green. Our results showed that harmine induced a depletion of Ψm in HepG2 cells (Fig. 5A).

The Bcl-2 family of proteins are key regulators of mitochondrial permeability. Therefore, we investigated whether the mitochondria-mediated apoptosis in HepG2 cells induced by harmine was modulated by Bcl-2 family members. Harmine suppressed the expression of Bcl-2, Mcl-1, and Bcl-xl, and did not change the expression of Bax (Fig. 5B). As a result of these changes, the ratios of Bcl-2/Bax, Mcl-1/Bax, and Bcl-xl/Bax were reduced significantly during apoptosis. To delineate the possible signaling pathways by which harmine induced HepG2 cell

![Fig. 3. Staining cells with Annexin V-FITC and PI was used to distinguish and quantitatively determine the percentage of apoptotic cells. The rate of apoptosis increased in a dose-dependent manner.](image)

![Fig. 4. PI staining showed characteristic features of apoptosis in harmine-exposed cells. The number of cells in S phase and G2/M phase increased and the percentage of cells in G0/G1 phase decreased compared with the control group.](image)
Although harmine shows effective antitumor activity, contemporary therapeutic strategies have shown remarkable anticancer ability, severe side-effects are unavoidable. The search for new antitumor agents that are more effective but less toxic has kindled great interest. Since apoptosis plays an important role in developmental processes, homeostasis, and the elimination of damaged cells, accumulating data indicate that many anticancer drugs cause the death of tumor cells through the induction of apoptosis.\[16, 17\] Although harmine shows effective antitumor activity, its effects on human hepatocytes are still unclear. Therefore, the purpose of the present study was to reveal the underlying molecular mechanism by which harmine induces apoptosis in the human HCC cell line HepG2.

We demonstrated that harmine dose-dependently inhibited the proliferation of HepG2 cells, and moreover, it also induced apoptosis and arrested the cell cycle. Morphological changes in apoptotic characteristics, such as cellular shrinkage, rounding, poor adherence, and round floating shapes in harmine-treated cells were also observed by fluorescence microscopy. Flow cytometry analysis revealed that harmine treatment resulted in an increase of apoptotic cells. These results suggested that the growth inhibition of HepG2 cells by harmine is due to its ability to induce apoptosis.

Mitochondria play an essential role in apoptosis, since both the intrinsic and extrinsic apoptosis pathways converge at the mitochondrial level and trigger mitochondrial membrane permeabilization.\[15\] Mitochondria-mediated apoptosis is precisely regulated by Bcl-2 family proteins, which can be divided into two subfamilies: one is anti-apoptotic such as Bcl-2 and Bcl-xl, the other is pro-apoptotic like Bax, Bad, and Bid.\[20\] The expression of Bcl-2 and Bax is important in the balance of pro-apoptotic and anti-apoptotic signals at the mitochondrial level.\[21\] In our experiments, harmine had no effect on expression of the pro-apoptotic Bax protein but decreased the expression levels of anti-apoptotic Bcl-2, Bcl-xl, and Mcl-1, leading to up-regulation of the Bax/Bcl-2, Bax/Bcl-xl, and Bax/Mcl-1 ratios. This might be responsible for the concomitant execution phase of apoptosis, which included disruption of $\Psi_{in}$. Caspase, a family of cysteine acid proteases, is an integral part of the apoptotic pathway. Caspase-9 is the apical caspase in the intrinsic or mitochondria-initiated apoptosis pathway and requires the release of cytochrome C from the mitochondria. Activation of caspase-3 correlates with activation of caspase-9. Caspase-3 in particular, when activated, has many cellular targets.\[22\] During apoptosis, caspase-3 is one of the key executioners of apoptosis in response to various stimuli.\[22\] In many studies, it has been determined that a variety of chemotherapeutic agents induce apoptosis through the activation of caspase.\[16\] Consistent with an increase in the ratio of Bax/Bcl-2 and disruption of $\Psi_{in}$, this study showed that harmine resulted in dose-dependent activation of caspase-9 and caspase-3.

In conclusion, we found that harmine-induced apoptosis was accompanied by modulation of the Bcl-2 family, mitochondrial dysfunction and activation of caspase in HepG2 cells, implying that harmine induced apoptosis via the mitochondrial death pathway. Therefore, we believe that harmine might be a promising molecule in cancer chemoprevention or chemotherapy and further efforts to explore this therapeutic strategy are needed.

**Discussion**

Although contemporary therapeutic strategies have shown remarkable anticancer ability, severe side-effects are unavoidable. The search for new antitumor agents that are more effective but less toxic has kindled great interest. Since apoptosis plays an important role in developmental processes, homeostasis, and the elimination of damaged cells, accumulating data indicate that many anticancer drugs cause the death of tumor cells through the induction of apoptosis.\[16, 17\] Although harmine shows effective antitumor activity, its effects on human hepatocytes are still unclear. Therefore, the purpose of the present study was to reveal the underlying molecular mechanism by which harmine induces apoptosis in the human HCC cell line HepG2.

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