**Name of Journal:** ***World Journal of*** ***Gastroenterology***

**Manuscript NO: 38588**

**Manuscript Type:** **ORIGINAL ARTICLE**

***Basic Study***

Mitochondrial pathway mediated by reactive oxygen species involvement in α-hederin-induced apoptosis in hepatocellular carcinoma cells

Li J *et al*. The anti-hepatoma effects of α-hederin

Jiao Li, Dan-Dan Wu, Ji-Xiang Zhang, Jing Wang, Jing-Jing Ma, Xue Hu, Wei-Guo Dong

Jiao Li, Dan-Dan Wu, Ji-Xiang Zhang, Jing-Jing Ma, Xue Hu, Department of Gastroenterology, Renmin Hospital of Wuhan University, Central Laboratory of Renmin Hospital, Wuhan 430060, Hubei Province, China

Jing Wang, Department of Gastroenterology, Beijing Shijitan Hospital of Capital Medical University, Beijing 100038, China

Wei-Guo Dong, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

ORCID number: Jiao Li (0000-0002-4973-8255); Dan-Dan Wu (0000-0003-2626-2861); Ji-Xiang Zhang (0000-0002-8773-5020); Jing Wang (0000-0001-7718-7254); Jing-Jing Ma (0000-0002-5795-752X); Xue Hu (0000-0002-1918-2700); Wei-Guo Dong (0000-0002-4228-6508).

Author contributions: Li J, Wu DD, Zhang JX, Wang J and Dong WG designed the research; Li J, Wu DD and Ma JJ performed the research; Wang J and Zhang JX contributed new reagents/analytical tools; Hu X, Ma JJ and Li J analyzed the data; and Li J wrote the manuscript.

**Supported by** the National Natural Science Foundation of China, No. 81572426; and the Natural Science Foundation of Hubei Province, No. 2015CKB755.

Institutional animal care and use committee statement: All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Wuhan University.

Conflict-of-interest statement: The authors declare that there are no conflicts of interest regarding the publication of this paper.

**Data sharing statement:** No additional data are available.

**ARRIVE guidelines statement:** The ARRIVE guidelines have been adopted.

**Open-Access:** This article is an open-access article which was selected by an in-house editor and fully peer-reviewed by external reviewers. It is distributed in accordance with the Creative Commons Attribution Non Commercial (CC BY-NC 4.0) license, which permits others to distribute, remix, adapt, build upon this work non-commercially, and license their derivative works on different terms, provided the original work is properly cited and the use is non-commercial. See: http://creativecommons.org/licenses/by-nc/4.0/

**Manuscript source:** Unsolicited manuscript

Correspondence to: Wei-Guo Dong, MD, PhD, Department of Gastroenterology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuhan 430060, Hubei Province, China. [dongweiguo@whu.edu.cn](mailto:dongweiguo@whu.edu.cn)

Telephone: +86-13986167388

**Received:** March 1, 2018

**Peer-review started:** March 2, 2018

**First decision:** March 30, 2018

**Revised:** April 4, 2018

**Accepted:** April 9, 2018

**Article in press:**

**Published online:**

Abstract

***AIM***

To investigate the antitumor activity of α-hederin in hepatocellular carcinoma (HCC) cells and its underlying mechanisms *in vitro* and *in vivo*.

***METHODS***

SMMC-7721, HepG-2, and Huh-7 HCC cells were cultured *in vitro* and treated with α-hederin (0, 5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, 30 μmol/L, 35 μmol/L, 40 μmol/L, 45 μmol/L, 50 μmol/L, 55 μmol/L, or 60 μmol/L) for 12 h, 24 h, or 36 h, and cell viability was then detected by the Cell Counting Kit-8. SMMC-7721 cells were treated with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without DL-buthionine-*S*,*R*-sulfoximine (BSO) (2 mmol/L) or *N*-acetylcysteine (NAC) (5 mmol/L) pretreatment for 2 h, and additional assays were subsequently performed. Apoptosis was observed after Hoechst staining. Glutathione (GSH) and adenosine ATP levels were measured using GSH and ATP Assay Kits. Intracellular reactive oxygen species (ROS) levels were determined by measuring the oxidative conversion of 2’,7’-dichlorofluorescin diacetate. Disruption of the mitochondrial membrane potential was evaluated using JC-1 staining. The protein levels of Bax, Bcl-2, cleaved caspase-3, cleaved caspase-9, apoptosis-inducing factor (AIF), and cytochrome C (Cyt C) were detected by western blot. The antitumor efficacy of α-hederin *in vivo* was evaluated in a xenograft tumor model.

***RESULTS***

The α-hederin induced the apoptosis of HCC cells. The apoptosis rates in the control, low-dose α-hederin (5 μmol/L), mid-dose α-hederin (10 μmol/L), and high-dose α-hederin (20 μmol/L) groups were 0.90% ± 0.26%, 12% ± 2.0%, 21% ± 2.1%, and 37% ± 3.8%, respectively (*P* < 0.05). The α-hederin reduced intracellular GSH and ATP levels; induced ROS; disrupted the mitochondrial membrane potential; increased the protein levels of Bax, cleaved caspase-3, cleaved caspase-9, AIF and Cyt C; and decreased Bcl-2 expression. The α-hederin also inhibited xenograft tumor growth *in vivo*.

***CONCLUSION***

The α-hederin induces the apoptosis of HCC cells *via* the mitochondrial pathway mediated by increased intracellular ROS and may be an effective treatment for human HCC.

**Key words:** Hepatic carcinoma; α-hederin; Apoptosis; Reactive oxygen species; Mitochondria

**© The Author(s) 2018.** Published by Baishideng Publishing Group Inc. All rights reserved.

**Core tip:** Theα-hederin induces the apoptosis of hepatocellular carcinoma cells *in vitro* and *in vivo*. We found that reactive oxygen species and the mitochondrial pathway play a vital role in α-hederin-induced apoptosis.

Li J, Wu DD, Zhang JX, Wang J, Ma JJ, Hu X, Dong WG. Mitochondrial pathway mediated by reactive oxygen species involvement in α-hederin-induced apoptosis in hepatocellular carcinoma cells. *World J Gastroenterol 2018;* In press

INTRODUCTION

Hepatocellular carcinoma (HCC) is a highly prevalent disease worldwide, particularly in many Asian countries, with a very high incidence of over 20 cases/100000 individuals[1]. It is the fifth most common malignancy and the second most common cause of cancer-related death, and related deaths increased from 600000 in 2008 to 746000 in 2012[1,2]. And it is also recognized as the main cause of death in patients with cirrhosis[3]. HCC treatment mainly includes systemic chemotherapy, radiofrequency ablation, transarterial chemoembolization, ethanol or acetic acid injection, surgical resection, and, in rare cases, liver transplantation[4]. Although resection is the most common therapy, most patients are not eligible for this treatment because of tumor extent or poor hepatic condition[4,5]. Systemic chemotherapy is another possible treatment option, but it often has a low response rate and severe side effects. Multidrug resistance occurs frequently in patients treated with chemotherapy, leading to recurrence and poor survival[6]. The poor general prognosis is related to a low overall survival rate after 5 years, ranging from 24% to 41%[7]. Therefore, it is important to develop highly effective natural treatments with limited toxicity for HCC.

Triterpene saponins are natural amphiphilic compounds that have the potential to induce cancer cell death and increase the activity of chemotherapeutic agents or radiotherapy[8,9]. The α-hederin is a secondary saponin isolated from *Hedera* or *Nigella* species. It is the major active component of various traditional medicinal herbs and shows promising activity against colon and lung cancer. The α-hederin also has biological activity such as antioxidant activity, anti-inflammatory activity, and effects on smooth muscle contraction[10-14]. It is thought to promote cell apoptosis and/or membrane alterations[15], and excess reactive oxygen species (ROS) have been reported to be involved in these processes[16]. Excess ROS can cause oxidative damage to the mitochondrial membrane and trigger apoptosis through downstream signal transduction[17,18].

Reports on the anti-HCC activity of α-hederin are limited. In this study, we evaluated the effects of α-hederin on HCC cells both *in vitro* and *in vivo* and explored the underlying mechanisms.

MATERIALS AND METHODS

*Cell lines and culture*

The human SMMC-7721, HepG-2, and Huh-7 HCC cell lines were purchased from the Shanghai Cell Collection (Shanghai, China). HCC cells were cultured in DMEM (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, United States) and 1% penicillin/streptomycin. All cells were cultured in a 5% CO2 humidified incubator at 37°C. The α-hederin was purchased from Sigma-Aldrich (St. Louis, MO, United States), dissolved in 100% dimethyl sulfoxide, and stored at 5 °C.

*Cell proliferation assays*

Cells were seeded at a density of 5 × 103 cells per well in 96-well plates and then treated with 0, 5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, 30 μmol/L, 35 μmol/L, 40 μmol/L, 45 μmol/L, 50 μmol/L, 55 μmol/L, or 60 μmol/L α-hederin for 12 h, 24 h, or 36 h. Cell proliferation was assessed at different times using Cell Counting Kit-8 (Beyotime, Shanghai, China) according to the manufacturer's protocol. Ten microliters of CCK-8 solution was added to each well for 1 h, the absorbance was then measured at 450 nm with a microplate reader (Victor31420 Multilabel Counter, PerkinElmer, Waltham, MA, United States) to calculate the cell viability in different groups.

*Cell apoptosis assays*

Apoptotic cells were examined using the Hoechst 33258 staining kit (Beyotime, Shanghai, China). SMMC-7721 cells were treated with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without pretreatment with 2 mmol/L DL-buthionine-*S*,*R*-sulfoximine (BSO) (Sigma-Aldrich) or 5 mmol/L *N*-acetylcysteine (NAC) (Sigma-Aldrich) for 2 h and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min. After staining with 20 μmol/L Hoechst 33258 for 20 min, the cells were observed under a fluorescence microscope (Olympus, Tokyo, Japan), and apoptotic cells were identified by fragmented and condensed nuclei.

*Measurement of intracellular GSH and ATP*

Glutathione (GSH) and adenosine ATP levels were measured using a GSH Assay Kit (Beyotime, Shanghai, China) and an ATP Assay Kit (Beyotime, Shanghai, China). SMMC-7721 cells were treated with 0, 5 μmol/L, 10 μmol/L, or 20 μmol/L α-hederin for 24 h with or without pretreatment with BSO (2 mmol/L) or NAC (5 mmol/L) for 2 h, and the subsequent procedures were performed according to the manufacturers’ instructions. The experimental data were obtained with a microplate reader.

*ROS detection*

Intracellular ROS levels were determined by measuring the oxidative conversion of 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to the fluorescent compound dichlorofluorescin (DCF) using a ROS Assay Kit (Beyotime, Shanghai, China). After treatment with 0, 5, 10, or 20 μmol/L α-hederin for 24 h with or without pretreatment with BSO (2 mmol/L) or NAC (5 mmol/L) for 2 h, SMMC-7721 cells cultured in 6- and 96-well plates were incubated with 10 μmol/L DCF-DA for 20 min at 37 °C. Cells cultured in 6-well plates were observed under an upright fluorescence microscope, while cells in 96-well plates were evaluated with a microplate reader.

*Mitochondrial membrane potential*

Changes in the mitochondrial membrane potential (Δ*Ψ*m) were identified using JC-1 according to the manufacturer's specifications. SMMC-7721 cells were pretreated with BSO (2 mmol/L) or NAC (5 mmol/L) for 2 h, treated with 0 or 10 μmol/L α-hederin for 24 h, and then incubated with 1 mL of the JC-1 dye for 30 min in a 37 °C incubator. The cells were washed twice with PBS and then evaluated with a confocal laser scanning microscope (Olympus, Tokyo, Japan). JC-1 forms a red fluorescent aggregate at hyperpolarized membrane potentials, whereas it remains in the green fluorescent monomeric form at depolarized membrane potentials.

*Western blot analysis*

Total cellular protein was extracted on ice using RIPA lysis buffer containing protease inhibitors (Beyotime, Shanghai, China). Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 5% nonfat dry milk and incubated overnight with various primary antibodies at 4 °C. Next, anti-rabbit secondary antibodies were added for 1 h at room temperature. Band intensity was measured using the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, United States).

*Xenograft tumor model*

All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Wuhan University. The animal protocol was designed to minimize animal pain and discomfort. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark cycle, 50% humidity, and ad libitum access to food and water) for one week prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) after being fasted overnight, and tissues were collected.

The antitumor efficacy of α-hederin *in vivo* was evaluated using a xenograft tumor model. Male BALB/c-nu/nu nude mice (4-6 wk old) were purchased from HFK Experimental Animal Center (Beijing, China). HCC cells (5.0 × 106) suspended in 100 μL of PBS were subcutaneously inoculated into the right dorsal flank of nude mice. When the tumors reached 100-150 mm3, the mice were randomly divided into four groups (*n* = 6 per group): a control group, low-dose group (2.5 mg/kg), mid-dose group (5 mg/kg), and high-dose group (10 mg/kg). The α-hederin was administered *via* intraperitoneal injection every 3 d.

To create the tumor growth curve, the diameter of each xenograft tumor was measured with a caliper. We weighed the mice every 3 d. At the end of the experiment, xenotransplanted tumors, livers, lungs, and brains were harvested for additional analysis. Mouse blood was collected for hepatic and renal function tests.

*HE and* *TUNEL staining*

To further evaluate treatment efficiency, the tumors were dissected and fixed in 4% formaldehyde. Next, tumors were sectioned into slices and stained with hematoxylin and eosin (HE) for histological analysis. We performed TUNEL staining to detect apoptotic cells. Positive cells were identified, counted (eight random fields per slide), and analyzed by light microscopy (Olympus, Tokyo, Japan).

*Statistical analysis*

All data were collected from at least three independent experiments. One-way analysis of variance and *t*-tests were performed to analyze all the data (SPSS 20.0 software). *P <* 0.05 indicated statistical significance.

RESULTS

*α-hederin reduces HCC cell viability and induces the apoptosis of HCC cells via GSH depletion and ROS accumulation*

To investigate the effects of α-hederin on HCC cell growth, we treated HCC cells with different concentrations of α-hederin for 0, 12 h, 24 h and 36 h. As shown in Figure 1A, α-hederin significantly reduced HCC cell viability in a dose- and time-dependent manner, with IC50 values at 24 h for SMMC-7721, HepG-2, and Huh-7 cells were 13.880 μmol/L, 18.450 μmol/L, and 25.520 μmol/L, respectively. We further use the one-way ANOVA to analyze the IC50 values for each time period of SMMC-7721, HepG-2, and Huh-7 cells, there are statistical significance among the IC50 value of three time periods (*P* < 0.05). The Hoechst 33258 staining results are shown in Figure 1B; α-hederin induced the apoptosis of HCC cells in a dose-dependent manner. The apoptosis rates in the control, low-dose α-hederin (5 μmol/L), mid-dose α-hederin (10 μmol/L), and high-dose α-hederin (20 μmol/L) groups were 0.90% ± 0.26%, 12% ± 2.0%, 21% ± 2.1%, and 37% ± 3.8%, respectively (*P* < 0.05). To determine whether α-hederin affects intracellular ROS generation, SMMC cells were treated with α-hederin for 24 h. As shown in Figure 1C, the relative DCFH-DA fluorescence significantly increased in a dose-dependent manner. The α-hederin significantly reduced cellular GSH (Figure 1D) and ATP levels (Figure 1E) (*P* < 0.05). These results show that α-hederin may reduce HCC cell viability and induce the apoptosis of HCC cells *via* GSH depletion and ROS accumulation.

*BSO and NAC influence α-hederin-induced apoptosis of SMMC-7721 cells*   
To further determine whether α-hederin induces the apoptosis of HCC cells *via* GSH depletion and ROS accumulation, SMMC cells were treated with 10 μmol/L α-hederin for 24 h with or without BSO (2 mmol/L) or NAC (5 mmol/L) pretreatment for 2 h. As shown in Figure 2A, the apoptosis rate varied as expected: 0.94% ± 0.25% in the control group, 22% ± 2.4% in the α-hederin group, 27% ± 3.5% in the α-hederin and BSO group, and 13% ± 3.3% in the α-hederin and NAC group (*P* < 0.05). Intracellular ROS levels are shown in Figure 2B. Relative DCFH-DA fluorescence was significantly increased in the α-hederin (10 μmol/L) group compared to the control group (*P* < 0.05), and this increase was enhanced in the α-hederin and BSO group but reduced in the α-hederin and NAC group (*P* < 0.05). As shown in Figure 2C and Figure 2D, intracellular GSH and ATP levels were significantly decreased in the α-hederin (10 μmol/L) group compared to the control group (*P* < 0.05), and these decreases were enhanced in the α-hederin and BSO group but reduced in the α-hederin and NAC group (*P* < 0.05). This result suggested that, α-hederin inducing apoptosis of HCC cells in a indirect way which is closely related to GSH and ROS.

*α-hederin induces apoptosis through activation of the mitochondria-mediated pathway*

To investigate the underlying mechanism of apoptosis induced by α-hederin, we ascertained the effect of α-hederin on mitochondrial membrane depolarization (Δ*Ψ*m) using the JC-1 cationic dye. Compared to the control group, the ratio of aggregate-to-monomer fluorescence in the α-hederin (10 μmol/L) group was decreased (*P* < 0.05) as JC-1 fluorescence changed from red (aggregate) to green (monomer) (Figure 3A). Compared to that in the α-hederin group, the aggregate-to-monomer fluorescence ratio was decreased in the α-hederin and BSO group and increased in the α-hederin and NAC group (*P* < 0.05).

Then, we conducted western blotting to examine the effect of α-hederin on the levels of mitochondrial pathway-related proteins. As shown in Figure 3B, α-hederin increased the levels of Bax, cleaved caspase-3, and cleaved caspase-9 and decreased Bcl-2 expression levels. Meanwhile, the mitochondria-mediated apoptosis-related proteins apoptosis-inducing factor (AIF) and cytochrome C (Cyt C) in cytoplasm were increased by α-hederin, but AIF and Cyt C in mitochondria were decreased (Figure 3C). Pretreatment with BSO augmented the α-hederin-induced changes in protein levels, whereas pretreatment with NAC weakened these effects of α-hederin.

*α-hederin inhibits tumor growth in vivo*

The anticancer effects of α-hederin *in vivo* were analyzed in a human xenograft tumor model. As shown in Figure 4A, the transplanted tumor volume increased more slowly with increasing α-hederin concentration, and the final tumor weight was lower in the α-hederin-treated groups. At the end of the experiment, the tumor weights in the control and 2.5 mg/kg, 5 mg/kg, and 10 mg/kg α-hederin groups were 1217 mg ± 177 mg, 917 mg ± 84 mg, 778 mg ± 105 mg, and 539 mg ± 96 mg, respectively. Tumor growth was significantly suppressed in the α-hederin groups in a dose-dependent manner (*P* < 0.05). TUNEL staining of the tumors is shown in Figure 4B, and cells stained brown are apoptotic. Compared to the control group, the α-hederin groups showed a gradual increase in the proportion of apoptotic cells with increasing drug concentration (*P* < 0.05).

Liver, lung, and brain tissue from each group was stained with HE, and no tumor metastases were observed. We assayed the hepatic and renal functions of nude mice treated with control or α-hederin and found that alanine aminotransferase, aspartate aminotransferase, urea, and creatine levels were not significantly different.

DISCUSSION

The α-hederin has various biological activities, including anticancer activity in some cancer cells*.* However, its effects on HCC have not been clarified. In the present study, to investigate the effects of α-hederin on HCC cells, we performed cell proliferation and apoptosis assays; detected ROS, GSH, and ATP levels and the mitochondrial membrane potential; conducted western blotting analysis to examine related proteins; and generated a xenograft tumor model to evaluate the antitumor efficacy of α-hederin *in vivo*. Our results show that α-hederin induces the apoptosis of HCC cells *in vitro* and *in vivo* and suggest that the mechanism involves the mitochondrial pathway mediated by increased intracellular ROS.

In this study, we found that α-hederin significantly inhibited the proliferation of HCC cells and induced their apoptosis in a dose- and time-dependent manner. We also found that α-hederin decreased GSH and ATP levels and increased ROS levels in a concentration-dependent manner. These results are consistent with those of Swamy *et al*[16], who reported that α-hederin increased the apoptosis of murine P388 leukemia cells and increased the production of ROS in a dose- and time-dependent manner. It has been reported that cancer cells have increased ROS production compared to normal cells. ROS is generated through a variety of extracellular and intracellular actions. Severe accumulation of cellular ROS may induce lethal damage in cells. Glutathione (GSH) is one of the most common intracellular compounds that play a vital role in the cellular defense against ROS damage. GSH clears intracellular ROS by non-enzymatic and enzymatic catalysis. The non-enzymatic process is GSH acting directly. The enzyme catalyzed process is based on GSH as the substrate, and induces the clearance of ROS in cells under the catalysis of glutathione peroxidase (GSH-Px) or glutathione S transferase (GST)[19,20]. During intracellular GSH synthesis, two ATP-dependent enzyme catalysis are required: glutamate cysteine ligase (GCL) and glutathione synthetase (GS)[21]. Our study shows α-hederin significantly reduced cellular ATP levels. Therefore, a reduction in intracellular ATP contributes to a decrease in GSH, leading to ROS accumulation and cellular damage. To determine whether the apoptotic effect of α-hederin on HCC cells is associated with the generation of intracellular ROS, we pretreated SMMC-7721 cells with BSO or NAC, which improve/decrease the levels of intracellular GSH and ROS. The results showed that the apoptotic effect of α-hederin was greater after pretreatment with BSO but was ameliorated by NAC. These data indicate that the apoptosis-inducing potential of α-hederin is related to intracellular ROS production.

# Mitochondria play an important role in cancer cell survival[22], they are major sources of cellular bioenergetics and the target of ROS. ROS can induce oxidative damage that affects mitochondrial function, and a decrease in Δ*Ψ*m indicates damage to mitochondrial function. Cheng *et al*[23] reported the mitochondrial apoptotic activity of α-hederin in breast cancer cells. A previous study showed that ROS causes the mitochondrial permeability transition pore (mPTP) to open in HepG-2 cells[24]. We next evaluated whether ROS induce this mitochondria-mediated apoptotic mechanism in HCC cells treated with α-hederin. Similar to breast cancer cells, SMMC-7721 cells treated with α-hederin showed a clear decrease in Δ*Ψ*m compared to untreated cells. Additionally, the Δ*Ψ*m decrease was aggravated by BSO but relieved by NAC. To further investigate whether the ROS increase and Δ*Ψ*m loss induced by α-hederin led to HCC cell apoptosis, we detected the levels of related proteins. We found that α-hederin increased the protein levels of Bax, cleaved caspase-3, and cleaved caspase-9 but decreased Bcl-2 levels. Thus, the antiapoptotic/proapoptotic (Bcl-2/Bax) protein ratio decreased. AIF and Cyt C protein levels were increased by α-hederin. Although the α-hederin-induced changes in the above proteins were enhanced by pretreatment with BSO, they were weakened by NAC pretreatment. Bcl-2 family proteins are reported to be key factors in regulating the mitochondrial apoptosis pathway[25]. Disruption of the Bcl-2/Bax protein balance induces apoptosis. Bcl-2 family proteins are also components of the mPTP. A decrease in Bcl-2 levels alters the mPTP structure and the mitochondrial membrane potential, increasing mitochondrial membrane permeability[26]. Additionally, excess ROS can trigger opening of the mPTP[27]. As a result, AIF and Cyt C proteins are released to activate procaspase-9, which activates the caspase cascade that ultimately generates caspase-3 to induce apoptosis. On the other hand, AIF can mediate apoptosis directly in caspase-independent way[28]. These data indicate that the [mechanism](file:///C:\Users\Administrator\AppData\Local\youdao\dict\Application\7.2.0.0703\resultui\dict\?keyword=mechanism) by which α-hederin induces HCC cell apoptosis involves the mitochondrial pathway mediated by increased intracellular ROS.

In human xenograft tumor models in nude mice, α-hederin significantly inhibited tumor growth without causing liver and kidney damage, indicating the efficacy and safety of α-hederin for the treatment of HCC *in vivo*.

In conclusion, we show that α-hederin induces the apoptosis of HCC cells *via* the mitochondrial pathway mediated by increased intracellular ROS *in vitro* and *in vivo*. These findings identify α-hederin as a potential highly effective natural medicine with limited toxicity for HCC treatment. However, α-hederin has been reported to have other effects, such as membrane permeabilizing activity, which can directly induce cell death[29]. This study is not sufficient to clarify the antitumor effects of α-hederin. Further studies should focus on the detailed mechanism.

**ARTICLE HIGHLIGHTS**

***Research background***

Hepatocellular carcinoma (HCC) is a highly prevalent disease worldwide with poor general prognosis. To develop highly effective natural treatments with limited toxicity for HCC is important. The α-hederin is reported to have anti-tumor activity. However, the effect of α-hederin on HCC remains to be examined. We evaluated the effect and possible mechanism of α-hederin on HCC cells both in vitro and in vivo.

***Research motivation***

Developing new, effective and non-toxic chemotherapeutic drug contributes to the treatment and prognosis for HCC patients in clinic.

***Research objectives***

To investigate the antitumor activity of α-hederin in HCC cells and its underlying mechanisms in vitro and in vivo.

***Research methods***

Three HCC cells lines (SMMC-7721, HepG-2, and Huh-7 HCC cells) were used to detect the effect of α-hederin on HCC. Cell viability was detected by CCK-8 assay after cells were treated with α-hederin. NAC and BSO were used to interfere with the synthesis of Glutathione (GSH) in the SMMC-7721 cells, then, the effects of α-hederin on cell proliferation, cell apoptosis, adenosine triphosphate (ATP) and Reactive Oxygen Species (ROS) and mitochondrial membrane potential were detected. The protein levels of Bax, Bcl-2, cleaved caspase-3, cleaved caspase-9, apoptosis-inducing factor (AIF), and cytochrome C (Cyt C) were detected by western blot. The antitumor efficacy of α-hederin on HCC was also evaluated in nude mice with xenograft tumor. The apoptosis of cancer cells in xenograft tumor were examined by TUNEL staining In this research, as we used NAC and BSO to interfere with the synthesis of GSH, the mechanism we explored was more persuasive.

***Research results***

The α-hederin inhibited cell growth of the three cell lines in a dose- and time-dependent manner. The IC50 values at 24 h for SMMC-7721, HepG-2, and Huh-7 cells were 13.88, 18.45, and 25.52 μmol/L, respectively, so we used SMMC-7721 cells for the on-going experiments. The results showed that the apoptosis rates in the control, low-dose α-hederin (5 μmol/L), mid-dose α-hederin (10 μmol/L), and high-dose α-hederin (20 μmol/L) groups were 0.90% ± 0.26%, 12% ± 2.05, 21% ± 2.15, and 37% ± 3.8%, respectively. In comparison to the control, after treatment with α-hederin, ROS increased significantly, while the ATP levels decreased. When SMMC-7721 cells were pretreated with BSO (2 mmol/L), compared with the mid-dose α-hederin group, the apoptosis rate increased to 27% ± 3.5% (*P* < 0.05); what’s more, the increase of ROS and the decrease of ATP were both enhanced. However, NAC pretreatment had a protective effect on SMMC-7721 cells and could alleviate the change of ROS and ATP. The proteins involving in the mitochondria-mediated pathway were detected by western blot. The results showed α-hederin increased the levels of Bax, cleaved caspase-3, and cleaved caspase-9 and decreased Bcl-2 expression levels. Meanwhile, AIF and Cyt C in cytoplasm were up-regulated, but AIF and Cyt C in mitochondria were down-regulated. Subcutaneous xenografts were successfully constructed in 24 nude mice. After treatment with α-hederin for 3 wk, the weight of xenograft tumor was significantly reduced (*P* < 0.05). Compared to the control group, TUNEL staining showed a gradual increase in the proportion of apoptotic cells with the increase of α-hederin concentration (*P* < 0.05). There was no difference between the control mice and α-hederin-treated mic for the hepatic and renal functions. This research indicated that α-hederin could induce HCC cell apoptosis *via* mitochondria-mediated pathway by depleting GSH and accumulating ROS. But it didn’t explain how α-hederin changed the expression of GSH and ROS, and the effect of α-hederin on HCC cell invasion was not studied, either. In addition, apoptosis involves multiple factors and multiple links, it’s necessary to conduct an in-depth research to clarify specific mechanism.

***Research conclusions***

The α-hederin induces the apoptosis of HCC cells *via* the mitochondrial pathway mediated by increased intracellular ROS and may be an effective treatment for human HCC.

***Research perspectives***

It is of great value to discover the natural anticancer compounds which have high efficacy and low toxicity in the treatment of HCC. In our study, we show α-hederin could induce HCC cell apoptosis *via* mitochondria-mediated pathway by depleting GSH and accumulating ROS, which identifies α-hederin as a potential highly effective natural medicine with limited toxicity for HCC treatment. But some points remain unclear. How does α-hederin change the expression of ATP? The effect of α-hederin on HCC cell migration and invasion was not studied, either. In addition, apoptosis involves multiple factors and multiple links, it’s necessary to conduct an in-depth research to clarify specific mechanism. These results will facilitate the development of treatment for HCC.

**REFERENCES**

1 **Gomaa AI**, Khan SA, Toledano MB, Waked I, Taylor-Robinson SD. Hepatocellular carcinoma: epidemiology, risk factors and pathogenesis. *World J Gastroenterol* 2008; **14**: 4300-4308 [PMID: 18666317 DOI: 10.3748/wjg.14.4300]

2 **Torre LA**, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* 2015; **65**: 87-108 [PMID: 25651787 DOI: 10.3322/caac.21262]

3 **Sangiovanni A**, Prati GM, Fasani P, Ronchi G, Romeo R, Manini M, Del Ninno E, Morabito A, Colombo M. The natural history of compensated cirrhosis due to hepatitis C virus: A 17-year cohort study of 214 patients. *Hepatology* 2006; **43**: 1303-1310 [PMID: 16729298 DOI: 10.1002/hep.21176]

4 **Rahbari NN**, Mehrabi A, Mollberg NM, Müller SA, Koch M, Büchler MW, Weitz J. Hepatocellular carcinoma: current management and perspectives for the future. *Ann Surg* 2011; **253**: 453-469 [PMID: 21263310 DOI: 10.1097/SLA.0b013e31820d944f]

5 **Yu SJ**. A concise review of updated guidelines regarding the management of hepatocellular carcinoma around the world: 2010-2016. *Clin Mol Hepatol* 2016; **22**: 7-17 [PMID: 27044761 DOI: 10.3350/cmh.2016.22.1.7]

6 **Zhang X**, Ng HLH, Lu A, Lin C, Zhou L, Lin G, Zhang Y, Yang Z, Zhang H. Drug delivery system targeting advanced hepatocellular carcinoma: Current and future. *Nanomedicine* 2016; **12**: 853-869 [PMID: 26772424 DOI: 10.1016/j.nano.2015.12.381]

7 **Schmidt S**, Follmann M, Malek N, Manns MP, Greten TF. Critical appraisal of clinical practice guidelines for diagnosis and treatment of hepatocellular carcinoma. *J Gastroenterol Hepatol* 2011; **26**: 1779-1786 [PMID: 21875430 DOI: 10.1111/j.1440-1746.2011.06891.x]

8 **Lee SJ**, Sung JH, Lee SJ, Moon CK, Lee BH. Antitumor activity of a novel ginseng saponin metabolite in human pulmonary adenocarcinoma cells resistant to cisplatin. *Cancer Lett* 1999; **144**: 39-43 [PMID: 10503876 DOI: 10.1016/s0304-3835(99)00188-3]

9 **Jiang H**, Zhao P, Feng J, Su D, Ma S. Effect of Paris saponin I on radiosensitivity in a gefitinib-resistant lung adenocarcinoma cell line. *Oncol Lett* 2014; **7**: 2059-2064 [PMID: 24932289 DOI: 10.3892/ol.2014.2020]

10 **Park HJ**, Kwon SH, Lee JH, Lee KH, Miyamoto K, Lee KT. Kalopanaxsaponin A is a basic saponin structure for the anti-tumor activity of hederagenin monodesmosides. *Planta Med* 2001; **67**: 118-121 [PMID: 11301855 DOI: 10.1055/s-2001-11516]

11 **Gepdiremen A**, Mshvildadze V, Süleyman H, Elias R. Acute anti-inflammatory activity of four saponins isolated from ivy: alpha-hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside-F in carrageenan-induced rat paw edema. *Phytomedicine* 2005; **12**: 440-444 [PMID: 16008120 DOI: 10.1016/j.phymed.2004.04.005]

12 **Gülçin I**, Mshvildadze V, Gepdiremen A, Elias R. Antioxidant activity of saponins isolated from ivy: alpha-hederin, hederasaponin-C, hederacolchiside-E and hederacolchiside-F. *Planta Med* 2004; **70**: 561-563 [PMID: 15241892 DOI: 10.1055/s-2004-827158]

13 **Mendel M**, Chłopecka M, Dziekan N, Karlik W, Wiechetek M. Participation of cholinergic pathways in α-hederin-induced contraction of rat isolated stomach strips. *Phytomedicine* 2012; **19**: 591-595 [PMID: 22465216 DOI: 10.1016/j.phymed.2012.02.011]

14 **Wolf A**, Gosens R, Meurs H, Häberlein H. Pre-treatment with α-hederin increases β-adrenoceptor mediated relaxation of airway smooth muscle. *Phytomedicine* 2011; **18**: 214-218 [PMID: 20637581 DOI: 10.1016/j.phymed.2010.05.010]

15 **Rooney S**, Ryan MF. Modes of action of alpha-hederin and thymoquinone, active constituents of Nigella sativa, against HEp-2 cancer cells. *Anticancer Res* 2005; **25**: 4255-4259 [PMID: 16309225]

16 **Swamy SM**, Huat BT. Intracellular glutathione depletion and reactive oxygen species generation are important in alpha-hederin-induced apoptosis of P388 cells. *Mol Cell Biochem* 2003; **245**: 127-139 [PMID: 12708752 DOI: 10.1023/A:1022807207948]

17 **Lee HH**, Park C, Jeong JW, Kim MJ, Seo MJ, Kang BW, Park JU, Kim GY, Choi BT, Choi YH, Jeong YK. Apoptosis induction of human prostate carcinoma cells by cordycepin through reactive oxygen species‑mediated mitochondrial death pathway. *Int J Oncol* 2013; **42**: 1036-1044 [PMID: 23292300 DOI: 10.3892/ijo.2013.1762]

18 **Ryter SW**, Kim HP, Hoetzel A, Park JW, Nakahira K, Wang X, Choi AM. Mechanisms of cell death in oxidative stress. *Antioxid Redox Signal* 2007; **9**: 49-89 [PMID: 17115887 DOI: 10.1089/ars.2007.9.49]

19 **Circu ML**, Aw TY. Reactive oxygen species, cellular redox systems, and apoptosis. *Free Radic Biol Med* 2010; **48**: 749-762 [PMID: 20045723 DOI: 10.1016/j.freeradbiomed.2009.12.022]

20 **Gutteridge JM**, Halliwell B. Free radicals and antioxidants in the year 2000. A historical look to the future. *Ann N Y Acad Sci* 2000; **899**: 136-147 [PMID: 10863535 DOI: 10.1111/j.1749-6632.2000.tb06182.x]

21 **Jiang Y**, Tao R, Shen Z, Sun L, Zhu F, Yang S. Enzymatic Production of Glutathione by Bifunctional γ-Glutamylcysteine Synthetase/Glutathione Synthetase Coupled with In Vitro Acetate Kinase-Based ATP Generation. *Appl Biochem Biotechnol* 2016; **180**: 1446-1455 [PMID: 27380420 DOI: 10.1007/s12010-016-2178-5]

22 **Dias N**, Bailly C. Drugs targeting mitochondrial functions to control tumor cell growth. *Biochem Pharmacol* 2005; **70**: 1-12 [PMID: 15907809 DOI: 10.1016/j.bcp.2005.03.021]

23 **Cheng L**, Xia TS, Wang YF, Zhou W, Liang XQ, Xue JQ, Shi L, Wang Y, Ding Q, Wang M. The anticancer effect and mechanism of α-hederin on breast cancer cells. *Int J Oncol* 2014; **45**: 757-763 [PMID: 24842044 DOI: 10.3892/ijo.2014.2449]

24 **Zhang Y**, Han L, Qi W, Cheng D, Ma X, Hou L, Cao X, Wang C. Eicosapentaenoic acid (EPA) induced apoptosis in HepG2 cells through ROS-Ca(2+)-JNK mitochondrial pathways. *Biochem Biophys Res Commun* 2015; **456**: 926-932 [PMID: 25529445 DOI: 10.1016/j.bbrc.2014.12.036]

25 **Llambi F**, Green DR. Apoptosis and oncogenesis: give and take in the BCL-2 family. *Curr Opin Genet Dev* 2011; **21**: 12-20 [PMID: 21236661 DOI: 10.1016/j.gde.2010.12.001]

26 **Chen Q**, Lesnefsky EJ. Blockade of electron transport during ischemia preserves bcl-2 and inhibits opening of the mitochondrial permeability transition pore. *FEBS Lett* 2011; **585**: 921-926 [PMID: 21354418 DOI: 10.1016/j.febslet.2011.02.029]

27 **Voronina S**, Okeke E, Parker T, Tepikin A. How to win ATP and influence Ca(2+) signaling. *Cell Calcium* 2014; **55**: 131-138 [PMID: 24613709 DOI: 10.1016/j.ceca.2014.02.010]

28 **Delavallée L**, Cabon L, Galán-Malo P, Lorenzo HK, Susin SA. AIF-mediated caspase-independent necroptosis: a new chance for targeted therapeutics. *IUBMB Life* 2011; **63**: 221-232 [PMID: 21438113 DOI: 10.1002/iub.432]

29 **Lorent JH**, Léonard C, Abouzi M, Akabi F, Quetin-Leclercq J, Mingeot-Leclercq MP. α-Hederin Induces Apoptosis, Membrane Permeabilization and Morphologic Changes in Two Cancer Cell Lines Through a Cholesterol-Dependent Mechanism. *Planta Med* 2016; **82**: 1532-1539 [PMID: 27574896 DOI: 10.1055/s-0042-114780]

**P-Reviewer:** Cheng TH, Sun CK **S-Editor:** Wang XJ

**L-Editor:** **E-Editor:**

**Specialty type:** Gastroenterology and hepatology

**Country of origin:** China

**Peer-review report classification**

Grade A (Excellent): 0

Grade B (Very good): B

Grade C (Good): C

Grade D (Fair): 0

Grade E (Poor): 0

****

**Figure 1 α-hederin reduces hepatocellular carcinoma cell viability and induces the apoptosis of hepatocellular carcinoma cells through glutathione depletion and reactive oxygen species accumulation.** A: CCK-8 assays showed that α-hederin inhibits the viability of hepatocellular carcinoma cells (SMMC-7721, HepG-2, and Huh-7) in a dose- and time-dependent manner; B: SMMC-7721 cells were incubated with α-hederin (0, 5 μmol/L, 10 μmol/L, or 20 μmol/L) and stained with Hoechst 33258. Apoptotic cells were identified by fragmented and condensed nuclei under a fluorescence microscope. The percentage of apoptotic cells was calculated, *P* for trend < 0.01; C: SMMC-7721 cells were incubated with α-hederin (0, 5 μmol/L, 10 μmol/L, or 20 μmol/L), followed by incubation with DCFH-DA and observation under a fluorescence microscope or measurement using a microplate reader, *P* for trend < 0.01; D and E: SMMC-7721 cells were treated with α-hederin (0, 5 μmol/L, 10 μmol/L, or 20 μmol/L). GSH and ATP levels were measured using GSH and ATP Assay Kits and a microplate reader, *P* for trend < 0.01. a*P* < 0.05 *vs* control. Scale bars “-” represent 100 μm. GSH: Glutathione. ROS: Reactive oxygen species.

****

**Figure 2 DL-buthionine-*S*,*R*-sulfoximine and *N*-acetylcysteine influence the α-hederin-induced apoptosis of SMMC-7721 cells.** SMMC-7721 cells were incubated with α-hederin (10 μmol/L) with or without BSO (2 mmol/L) or NAC (5 mmol/L) pretreatment. A: Cell apoptosis was determined by Hoechst 33258 staining; B: ROS levels in SMMC-7721 cells; C and D: Effect of α-hederin on intracellular GSH and ATP levels. a*P* < 0.05 *vs* control; b*P* < 0.05 *vs* α-hederin (10 μmol/L). Scale bars “-” represent 100 μm. BSO: DL-buthionine-*S*,*R*-sulfoximine; NAC: *N*-acetylcysteine.

****

**Figure 3 α-hederin induces apoptosis through activation of the mitochondria-mediated pathway.** A: Mitochondrial membrane potential was detected with JC-1. JC-1 aggregates (red fluorescence) under conditions of a normal mitochondrial membrane and forms a monomer (green fluorescence) under depolarizing conditions. Fluorescence was detected by a confocal laser scanning microscope (400 ×). B and C: Western blots showing the expression of mitochondrial pathway-related proteins *in vitro*. SMMC-7721 cells were treated with α-hederin (0 or 10 μmol/L) with or without BSO (2 mmol/L) or NAC (5 mmol/L) pretreatment, and the protein levels of Bcl-2, Bax, caspase-9, caspase-3, AIF, and Cyt C in SMMC-7721 cells were then detected by western blotting. GAPDH expression was used as an internal control. The relative expression levels of these proteins in SMMC-7721 cells in different groups were compared. a*P* < 0.05 *vs* control; b*P* < 0.05 *vs* α-hederin (10 μmol/L). AIF: Apoptosis-inducing factor; Cyt C: Cytochrome C.

****

**Figure 4 α-hederin inhibits tumor growth *in vivo.*** Mice with xenograft tumors were divided into four groups (control and 2.5 mg/kg, 5 mg/kg, and 10 mg/kg α-hederin, *n* = 6 mice per group). A: Mean tumor volume at each time point and final tumor weight, *P* for trend < 0.05. B: TUNEL assays detected apoptotic cells in xenograft tumor tissue, as evidenced by the presence of nut-brown nuclei under a fluorescence microscope. The percentage of apoptotic cells was calculated, *P* for trend < 0.05. a*P* < 0.05 *vs* control. Scale bars “-” represent 100 μm.