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Magnesium Demethylcantharidate induces apoptosis in hepatocellular carcinoma cells via ER stress

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Abstract: Cantharidin (CTD) is a bioactive ingredient isolated from *Cantharis vesicatoria* (blister beetles), which has potential therapeutic value as an anticancer agent. Magnesium Demethylcantharidate (MDC) is a recently developed derivative of Cantharidin (CTD), and previous studies have illustrated its excellent anticancer activity on HCC cells. However, the effect and mechanism of MDC remains unclear and need to be further studied. In particular, whether MDC can cause ER stress in HCC is still unknown. In this study, we demonstrated that endoplasmic reticulum stress (ERS)-related proteins were changed in SMMC-7721 and Bel-7402 cells after being exposed to MDC. Moreover, we found that MDC could significantly inhibit the growth of xenograft tumor in nude mice. In summary, we confirmed that MDC could induce ERS in HCC cells and thus induce apoptosis.

Introduction

Being ranked as the seventh most common cancer and the third lethal cause in cancer all over the world according to GLOBOCAN 2020, there were approximately 905,677 new liver cancer cases and 830,180 deaths annually (Sung *et al.*, 2021). The main type of primary liver cancer was HCC, which comprises more than 80% of the cases (Yang *et al.*, 2019). Chronic Hepatitis B Virus (HBV), Hepatitis C Virus (HCV), alcohol abuse, diabetes and exposure to aflatoxin B1 are risk factors for HCC (Kulik and El-Serag, 2019). Besides, Surgical resection, liver transplantation, radio-embolization, radiofrequency ablation, chemotherapy, targeted therapy and immunotherapy are commonly used for HCC patients (Lee *et al.*, 2020). However, the 5-year survival rate of HCC is 18% globally and even as low as 12% in China (Craig *et al.*, 2020).

Endoplasmic reticulum (ER) is responsible for the synthesis, folding, processing, transportation of protein, the storage of Ca^{2+} (Oakes, 2020), and participate in lipid metabolism. The disorder of Ca^{2+} and accumulation of unfolded proteins in ER lumen, which is called ER stress (ERS), can be caused by reactive oxygen species (ROS), hypoxia and nutrient deprivation, as they could result in the disorder of cellular redox regulation (Kim and Kim, 2018). Unfolded protein response (UPR), mediated by inositol-requiring enzyme 1 (IRE1), protein kinase RNA-like ER kinase (PERK) and activating transcription factor 6 (ATF6), can alleviate ERS and maintain intracellular homeostasis (Marciniak, 2019). In addition, excessive and persistent ERS will lead to the binding of glucose-regulated protein 78 (GRP78) or binding protein (BIP) to the unfolded protein and promote the activation of IRE1, PERK and ATF6 pathways, thereby activating the pro-apoptotic pathway to induce cancer cell death (Chen and Cubillos-Ruiz, 2021; Eugene *et al.*, 2020; Zachariah *et al.*, 2021). Recently, a large number of studies have confirmed the role of ERS in nonalcoholic steatohepatitis, chronic liver disease and HCC (Hernández-Alvarez *et al.*, 2019; Lebeaupin *et al.*, 2018; Liu *et al.*, 2019; Wu *et al.*, 2021; Xia *et al.*, 2020). Therefore, it is necessary to find effective ERS inducers for the treatment of HCC.

In recent years, an increasing number of researchers have realized that some natural compounds can kill cancer cells, and exploring the molecular mechanisms would also be interesting by which we can find out how these compounds work against tumors. *Cantharis vesicatoria* (blister beetles)

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has been used as a traditional Chinese folk medicine for more than 2000 years (Puerto Galvis *et al.*, 2013). Cantharidin (CTD) is an anticancer active ingredient isolated from *Cantharis vesicatoria*. Current studies have reported that the anticancer mechanisms of cantharidin and its derivatives include inhibition of invasion and metastasis of tumor cells (Hsia *et al.*, 2016), induction of apoptosis (Wang *et al.*, 2018), regulation of cell cycle (Ye *et al.*, 2017), induction of autophagy (Sun *et al.*, 2017). Magnesium Demethylcantharidate (MDC) is a derivative of cantharidin with anti-HCC activity (Liu *et al.*, 2021). Because there are two methyl groups less than cantharidin, the toxicity of MDC is greatly reduced, and its LD_{50} is much higher than cantharidin (Li and Casida, 1992; Liu *et al.*, 2021). But the mechanism of MDC remains to be further studied. In particular, whether MDC can cause ER stress of HCC were still unknown. In this study, we elucidated the anticancer mechanism of MDC in two HCC cell lines through ERS signaling pathway. Our study suggests that MDC is a potential drug for the treatment of HCC.

Materials and Methods

Materials

Magnesium Demethylcantharidate (MDC) was synthesized according to the Chinese patent (ZL201410163711.0), and the purity was over 95%. The HCC cell lines SMMC-7721 and Bel-7402 were obtained from Genechem Co., Ltd. (Shanghai, China). The cell lines were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Biological Industries, Beit Haemek Ltd., Israel) and 1% penicillin/streptomycin (Solarbio, Beijing, China) and were maintained at 37°C in a humidified incubator containing 5% CO_2.

Cell viability assay

SRB colorimetry was performed to test cell viability. The cells (8×10^3) at logarithmic growth stage were seeded into 96-well plates. After culturing for 24 h, different concentrations of MDC (0, 6.25, 12.5, 25, 50 and 100 μM) were added for further culture for 24 or 48 h. Staining with SRB (Sigma-Aldrich, Saint Louis, MO, USA), then the SpectraMax i3X tablet reader (Molecular Devices, Silicon Valley, CA, USA) was used to measure the absorbance at 530 nm. The cell viability measurements were performed in three independent replicates.

Colony formation assay

Cells (500 cells/well) were seeded into 6-well plates and cultured for 24 h. Then, SMMC-7721 and Bel-7402 cells were treated with MDC (0, 9, 18 or 36 μM), respectively, and cultured for another 14 days without drugs. The clones were counted after staining with Giemsa solution (Solarbio, Beijing, China).

Fluorescent staining

The cells in 96-well were treated with MDC (0, 9, 18 or 36 μM) for 24 h, fixed with pre-cooled 4% paraformaldehyde for 15 min, then added 80 μL Hoechst33342 (Beyotime, Shanghai, China) staining reagent to each well for 25 min, and observed under fluorescence microscope (Olympus, Tokyo, Japan).

Western blot assay

Cells were harvested after treatment with various concentrations of MDC (0, 9, 18 or 36 μM) for 24 h. The BCA (Solarbio, Beijing, China) method detected the protein concentration of cells and the dissected tumors, lysed by RIPA lysis buffer (Solarbio, Beijing, China). 50 μg of proteins per well was sequentially electrophoretic, electrotransfer, blocked, incubated primary antibody, and incubated secondary antibody. Protein bands were finally incubated using Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA) and then detected by the BIO-RAD ChemiDoc Imaging System (Hercules, CA, USA).

Xenograft tumors in nude mice

4-week-old male BALB/c nude mice (Beijing HFK Bioscience Co., Ltd., Beijing, China) were maintained in specific pathogen free (SPF) condition. After one week of adaptation, SMMC-7721 cells (2×10^6) were inoculated subcutaneously on the right posterior side of the nude mouse's back. Once the mean tumor volume reached 70 mm^3, the drug treatment group ($n = 6$) was intraperitoneally injected with MDC (4.3 mg/kg) every other day, and the vehicle group ($n = 6$) was intraperitoneally injected with normal saline. The nude mice were weighed and tumor volume was measured.

Statistical analysis

All experiments were independently repeated for thrice. All data were expressed as means ± SD. GraphPad Prism 7.0 (Graphpad Software, San Diego, CA, USA) was used for statistical analysis of data. One-way ANOVA and Student's *t*-test were used for data difference analysis. A p value < 0.05 was indicated statistically significant.

Results

MDC effectively inhibits the proliferation of HCC cells

SRB assay was used to detect the effect of MDC on cell viability of SMMC-7721 and Bel-7402 cells after 24 and 48 h treatment. In this study, MDC significantly reduced SMMC-7721 and Bel-7402 cells viability in a dose- and time-dependent manner (Figs. 1B and 1C). The colony formation showed that the colony number of SMMC-7721 and Bel-7402 cells decreased significantly in a dose-dependent manner (Figs. 1D–1F). These results proved that MDC could inhibit the proliferation of the two HCC cell lines.

MDC induced apoptosis in HCC cells

The results of Hoechst staining showed that the chromatin of condensation, polynucleosomal fragmentation and nuclear contraction increased with the increase of MDC (Fig. 2A). Furthermore, Western blot was used to detect the expression of apoptosis-related proteins after MDC treatment. Western blot showed that cleaved caspase-3, cleaved caspase-9, and Bax/Bcl-2 expressions were increased in SMMC-7721 and Bel-7402 cells in a dose-dependent manner after MDC treatment (Fig. 2B). According to these results, it indicated that MDC treatment could induce apoptosis of HCC Cells.

MDC induced ERS in HCC cells

As shown in the Fig. 3, western blot analysis revealed that ERS occurred when SMMC-7721 and Bel-7402 cells were exposed

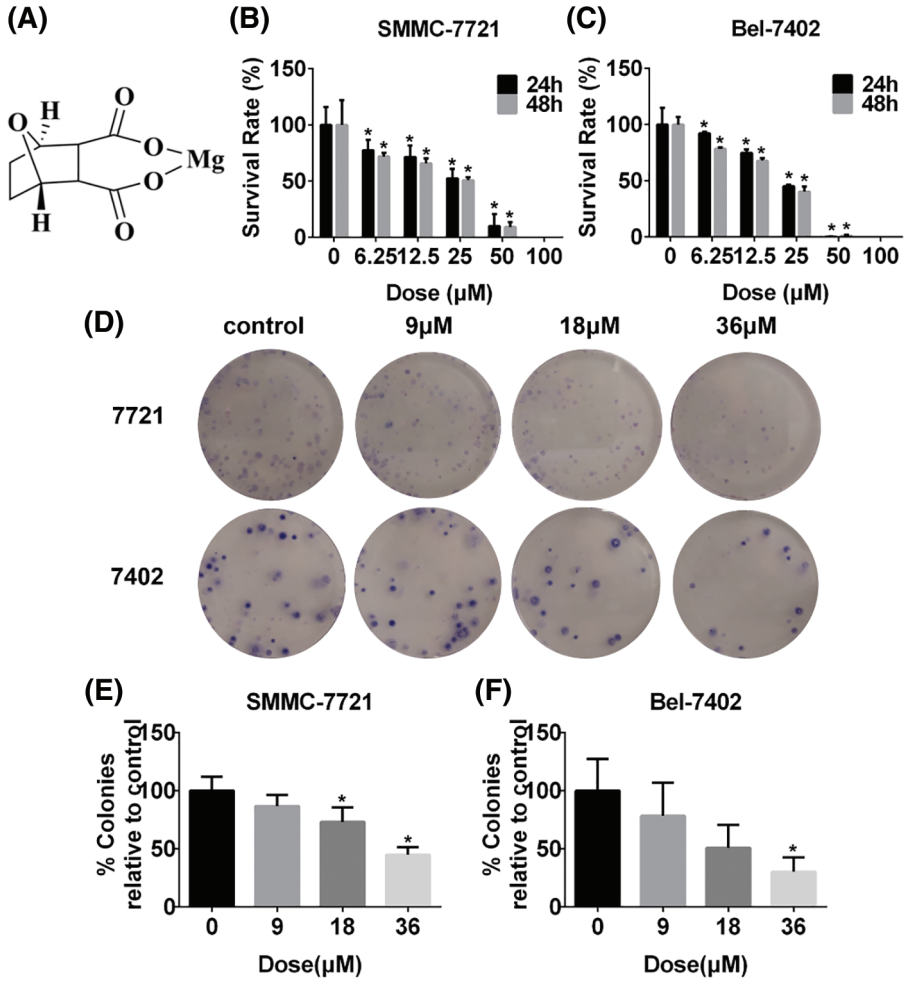

FIGURE 1. MDC effectively suppressed the proliferation of HCC cells. (A) The chemical structure of MDC. After treatment with various doses of MDC for 24 or 48 h, the SRB colorimetry was used to detect cell viability of SMMC-7721 and Bel-7402 cells (B and C). The colony formation assay was performed to detect proliferation, after treating SMMC-7721 and Bel-7402 cells with 0, 9, 18 or 36 μM of MDC for 24 h (D–F). The data of three independent replicates were expressed as the means ± SD ($^*p < 0.05$ *vs.* control).

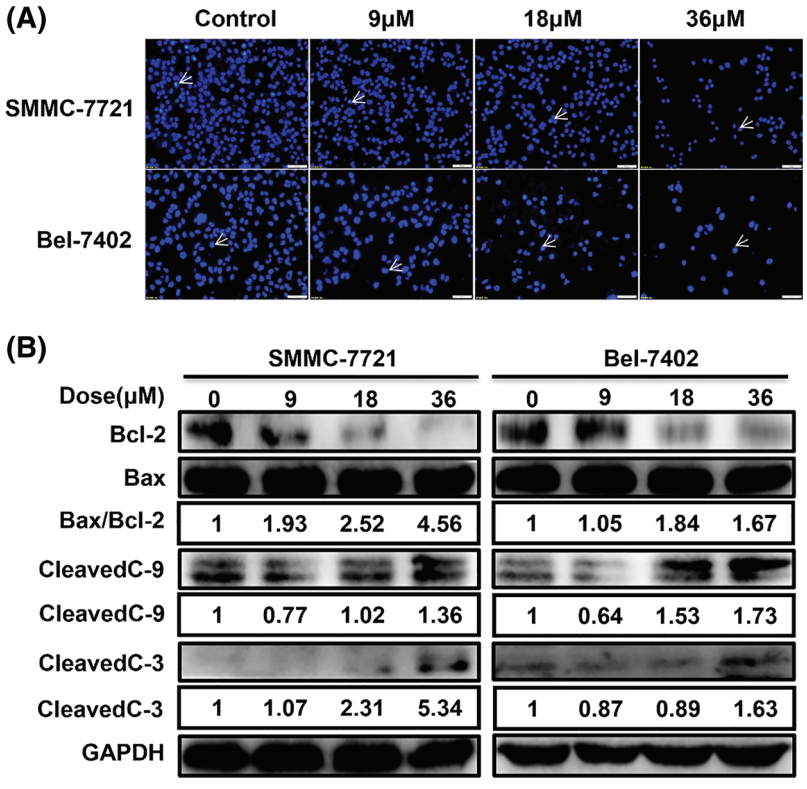

FIGURE 2. MDC induced apoptosis in HCC cells. (A) Hoechst staining showed that the chromatin of condensation, polynucleosomal fragmentation and nuclear contraction, after treating SMMC-7721 and Bel-7402 cells with 0, 9, 18 or 36 μM of MDC for 24 h. Hoechst staining ×200, bar: 50 μm. (B) Western blotting was used to analyze apoptotic proteins Bax, Bcl-2, cleaved caspase-3, and cleaved caspase-9.

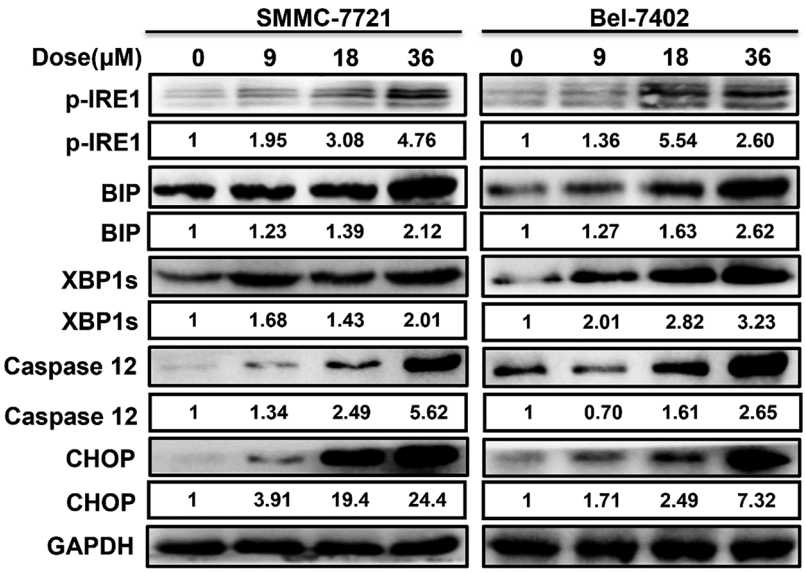

FIGURE 3. MDC induced ERS in HCC cells. Western blotting was used to analyze ERS related proteins, including p-IRE1, GRP78/BiP, XBP1s, Caspase 12 and CHOP.

to MDC, resulting in high expression of ERS-related proteins p-IRE1, GRP78/BiP, XBP1s, Caspase 12 and CHOP. It suggested that ERS induced by MDC must be an important feature of HCC apoptosis.

MDC significantly inhibited the growth of xenograft tumors in nude mice

SMMC-7721 cells were inoculated subcutaneously into the dorsal side of the right hind limb of nude mice to construct allograft tumor model. After the average tumor volume reached 70 mm^3, the nude mice were randomly assigned to control group and drug treatment group. The drug treatment group was intraperitoneally injected with MDC (4.3 mg/kg) every other day for 20 days. Compared with the control group, the weight of the nude mice treated with MDC did not change significantly, but the mass and volume of the allograft tumor were significantly reduced (Figs. 4A–4D). These data suggested that MDC could inhibit the growth of HCC *in vivo*.

Discussion

HCC is a serious threat to human health due to its high incidence and mortality (He *et al.*, 2021; Piñero *et al.*, 2020). In recent decades, many researchers found that certain natural products can kill HCC cells, and it would also be interesting to explore the anti-tumor molecular mechanisms. Cantharidin is one of natural products that can significantly inhibit the proliferation of HCC cells both *in vitro* and *in vivo* (Wang *et al.*, 2000; Zhu *et al.*, 2020). MDC is a novel agent derived from cantharidin, which can inhibit the invasion and metastasis of HCC cells by activating transcription factor FOXO1 (Liu *et al.*, 2021).

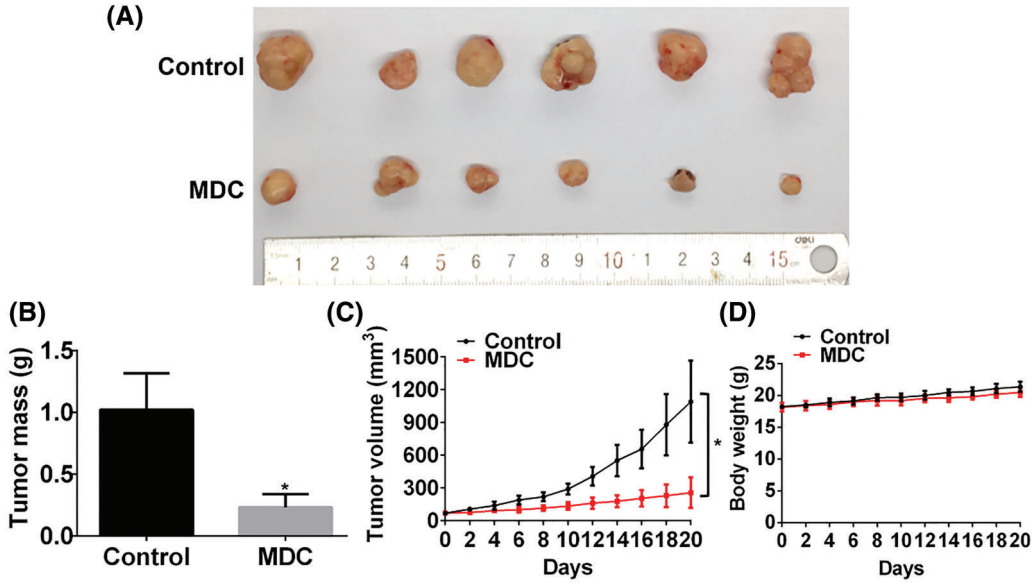

FIGURE 4. MDC significantly inhibited the growth of xenograft tumors in nude mice. (A) After treatment with MDC for 20 days, nude mice were sacrificed and tumor masses were exfoliated. (B) The exfoliated tumor mass of the two groups, six nude mice in each group. (C and D) Tumor volumes and body weight of nude mice were measured every other day. Data are represented as means ± SD. *$p < 0.05$ *vs.* control.

However, the mechanism of MDC needs further investigation. In particular, whether MDC can cause ER stress of HCC remains unknown.

It is known that apoptosis includes intrinsic and extrinsic pathways. The intrinsic pathway can be triggered by ERS, excess reactive oxygen species (Kesavardhana *et al.*, 2020). ERS is the adaptive response of cells to external adverse stimuli. In the early stage, ERS can inhibit cell apoptosis, which is beneficial to cell growth. However, when ERS is too intense and persistent, it can induce apoptosis and therefore promote cell death (Fernández *et al.*, 2015). In this study, we observed the elevation of Bax/Bcl-2 levels, and the caspase-9 and caspase-3 were cleaved after HCC cells were treated with MDC (Fig. 2B), which indicated that intrinsic apoptosis occurred in HCC cells. The increased expression of BIP was observed through western blot, leading to the phosphorylation of IRE1 and further proved that MDC induced apoptosis is generated through ERS signaling pathway (Fig. 3). Phosphorylated IRE1 can promote the synthesis of spliced X-box binding protein1 (XBP1s), and activate ASK1/JNK signal to activate C/EBP homologous protein (CHOP), Caspase12 and Bax (Liu *et al.*, 2020). When CHOP and Caspase12 elevated, the expression of Bcl-2 is inhibited, then, cleaved Caspase-9 was released, and cleaved Caspase-3 was further released, inducing apoptosis (Meng *et al.*, 2019).

Overall, we demonstrated that MDC could induce apoptosis of HCC cells through the ERS pathway. This finding suggest that MDC may be a potential anti-liver cancer drug.

Availability of Data and Materials: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author Contributions: Xinting Zhu, Yun Liu, Rong Yan and Xiaofei Li conceptualized and designed the study; Xinting Zhu, Meng Ye, Kelan Fang, Fang Liu, Jing Hui and Meichen Liu performed the experiments and the data analysis. Xinting Zhu and Meng Ye wrote the manuscript. All authors have read and approved the final manuscripts.

Ethics Approval: This study was approved by the Institutional Animal Ethics and Use Committee of Zunyi Medical University at 2016-3-15, and the ethical approval code was LS(2016)-2-065.

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Conflicts of Interest: The authors declare that they have no conflicts of interest to report regarding the present study.

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