Magnesium cantharidate suppresses PP2A and ERK1/2 pathway to inhibit hepatocellular carcinoma cells

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Abstract
Background: Magnesium cantharidate (MC) is a protein phosphatase 2A (PP2A) inhibitor antitumor drug. However, its antitumor mechanism in hepatocellular carcinoma cell (HCC) remains unclear. Methods: PP2A lentiviral vector over expression strategy was utilized both in vivo and in vitro to explore the antitumor effect in MC and okadaic acid (OA). Tumor weight was detected in mice after MC and OA exposure. Cell proliferation, cell cycle, apoptosis rate, and western blotting were detected to explore the effects on MC and OA in human hepatocarcinoma SMMC-7721 cells. Results: In vivo results demonstrated that MC inhibited HCC progression while OA promoted tumor growth. In vitro results demonstrated that MC effectively inhibited the growth of SMMC-7721 cells by arresting the cell cycle at the G2/M phase with inhibiting Cdc25C and activating the phosphorylation of the Cdc2 protein. Flow cytometry results further showed that MC increased apoptosis. Furthermore, the expression of phosphorylated ERK1/2 was lower in the MC group but higher in the OA group. Molecular docking results showed that MC docked well with ERK1/2. Conclusions: MC inhibited HCC progression by suppressing the growth and activating the apoptosis of cancer cells and suppressing the expression of PP2A and ERK1/2.

Keywords: magnesium cantharidate; ERK1/2; HCC; PP2A

Background

Liver cancer is the leading cause of cancer-related deaths worldwide. It is the fifth most common form of cancer, and the number of cases is increasing each year in the United States [1, 2]. The standard treatment for hepatocellular carcinomas cell (HCC) is surgery, which can be supplemented with chemotherapy and immunotherapy. Approximately 90% of HCC patients undergo surgery [3]. However, chemotherapy often has the side effect of suppressing the proliferation of bone marrow cells, leading to leukopenia. This condition affects the patient's immune response to tumors and their overall quality of life [4]. To address these issues, natural products are being explored as potential alternatives for HCC treatment. These products have the potential to improve treatment outcomes, reduce systemic toxicity, and minimize side effects. Consequently, natural products can gradually become an integral part of complementary medicine in cancer therapy [5, 6].

*Mylabris*, a blister beetle in China, is a traditional natural product that has been used for cancer treatment for over 2,000 years. Mylabris specifically improves the functioning of leukocytes against tumors, including HCC, leukemia, colorectal carcinoma, bladder carcinoma, and breast cancer [7, 8]. Clinical reports have shown that Mylabris has synergistic effect on survival, liver function, immune function, and quality of life in patients with HCC. In clinical reports [9, 10]. And experiment studies also have shown that Mylabris effectively suppresses HCC growth [11–14]. Cantharidin (CTD) is the active constituent of *Mylabris*. Side effects, including nephrotoxicity and hepatotoxicity, limit the clinical applications of Mylabris [15–18]. Several chemical modifications have been performed to enhance the efficacy and reduce the toxicity of CTD [19, 20]. Interestingly, our previous study shows that magnesium cantharidate (MC) has better anti-tumor activity than other CTD analogs [21]. However, the anti-cancer mechanism of MC remains to be validated.

Mechanically, CTD is a classical inhibitor of PP2A, which plays a significant role in cell cycle regulation, growth control, and cell apoptosis [22–26]. CTD is known to inhibit PP2A activity, leading to an imbalance in cellular signaling pathways [27]. Proteomic analysis has revealed that CTD regulates the MAPK and ERK signaling pathways [25, 28]. These pathways are crucial for cell proliferation, survival, and differentiation. By affecting these pathways, CTD may exert its anti-tumor effects [29]. However, the specific mechanism by which MC acts as an anti-tumor agent in HCC is not fully understood. Therefore, this study aims to investigate the potential anti-tumor mechanism of MC against HCC using a lentiviral vector overexpressing PP2A (Figure 1). Additionally, the potential involvement of the ERK pathway in mediating the anti-tumor effects of MC should be explored.

Materials and methods

Reagents

CTD, okadaic acid (OA), CCK-8 solution, Cell Cycle Kit, and Annexin V-FITC Apoptosis Detection Kit were purchased from Sigma (Louis, MO, USA). DMEM, fetal bovine serum, penicillin, and streptomycin were purchased from Gibco (Carlsbad, CA, USA). ProFluor® Ser/Thr PPase Assay Kit was purchased from Promega (Madison, WI, USA). LA Taq enzyme and DNA marker were purchased from Takara (Tochigi, Japan). Anti-ERK1 and ERK2 antibody, anti-Erk1 (pT202/pY204) and Erk2 (pT185/pY187) antibody, anti-GAPDH antibody, anti-Cdc2 antibody, anti-Cdc2 (phospho Y15) antibody, and anti-Cdc2C5 antibody were purchased from Abcam (Cambridge, UK).

Preparation of MC

MC (90% purity) was prepared according to the Chinese patent (ZL201110149288.5). Briefly, magnesium hydroxide (0.2 g) was mixed with 30 mL of distilled water to prepare a suspension of magnesium hydroxide. The suspension was added to 1 g CTD in 30 mL chloroform solution and then heated at 60 °C for 24 h on a magnetic stirrer. The chloroform was then evaporated to obtain a white suspension. The white suspension was finally dried and recrystallized to obtain a white crystalline powder of MC.

Cell lines and cultures

Human liver cancer cell line SMMC-7721 was purchased from Shanghai Genechem Co., Ltd. (Shanghai, China) and was maintained at 37 °C. The cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum and 1% double antibody at 37 °C in a humidified atmosphere with 5% CO2. The cells were passaged upon reaching 90% confluence to maintain exponential growth.

Infection of SMMC-7721 cells with lentiviruses overexpressing PP2A

The CDS region of human PPP2CA (Protein phosphatase 2 catalytic subunit alpha) (NM_002715.3) was synthesized by adding XbaI and Not I cleavage sites in 5’ and 3’, respectively. The PPP2CA fragment and LV5Fe1a-GFP/Puro plasmid were double-digested and purified using the Gin Recovery Kit. The linearized LV5Fe1a-GFP/Puro vector was then ligated with PPP2CA using DNA ligase and sequenced for verification. The LV5Fe1a-GFP/Puro-PPP2CA, psPA2X, and pMD2G plasmids were extracted using the Plasmid Mini Extraction Kit. The lentivirus titer was determined by transfecting 293T cells with liposomes, including LV5Fe1a-GFP/Puro-PPP2CA 1,000 ng, psPA2X 100 ng, and pMD2G 900 ng, and filtering the supernatant to obtain the viral stock solution. Expression of green fluorescent protein in each well of PP2A recombinant lentivirus-infected cells was observed under inverted fluorescence microscopy. Subsequently, the SMMC-7721 cells were infected with the PPP2CA-and control lentiviruses using an appropriate amount of virus dilution in a fresh growth medium.

 Xenograft tumors in nude mice

SPF healthy male Balb/c nude mice (16–20 g, 4 weeks) were purchased from Tengxin Bioscience Co., Ltd. (Chongqing, China), (SCXK (Jing) 2016-0002) and maintained in SPF condition. After adaptive feeding, the 54 mice were randomly divided into nine groups comprising the control, negative control (NC), PP2A-over expression (OE), OA, MC, (MC + NC), (MC + PP2A-OE), (OA + PP2A-OE), and (OA + NC) groups. SMMC-7721 cells (2 × 10^6 cells) were subcutaneously injected on the right posterior side of the back of nude mice. Once the mean tumor volume reached 70 mm3, the mice were injected intratumorally with the lentivirus (1 × 10^7 IU/mL) once a week for three weeks. The MC and OA treatment groups were intratumorally injected at 1-day intervals with MC (120 μg/mL) and OA (6.4 ng/mL) for 11 times. The control group was intratumorally injected with normal saline. The mice were sacrificed a day after the last injection by cervical dislocation, and the tumor was extracted and weighed. The protocol for the animal experiments was approved by the animal ethics committee of Zunyi Medical University (2020)02-090 and was conducted in line with the Helsinki declaration.

Histological examination of tumor tissue in mice

Paraffin-embedded tumor tissues were stained with Hematoxylin/Eosin (H & E) according to standard procedures. The histological images were captured using a light microscope (OLYMPUS, RX43, Tokyo, Japan).

Measurement of the PP2A activity in SMMC-7721 cell

Cells were divided into control, NC, PP2A-OE, OA (0.059 nmol/L), MC (2.266 μmol/L), (MC + NC), (MC + PP2A-OE), (OA + PP2A-OE), and (OA + NC) groups. The activity of PP2A in each group was measured using the ProFluor® Ser/Thr PPase Assay Kit. Briefly, grins were rinsed three times with pre-cooled PBS and centrifuged at 1,000 g for 5 min. The cell lysate was resuspended and placed on ice for 40 min. The suspension was centrifuged at 13,000 g for 5 min, and the supernatant was passed through a pre-desalted Sphadex G-25 column.

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The collected protein was adjusted to a concentration of 0.5 μg/μL using a buffer. The protein concentration was quantified using the BCA method and detected with a fluorescence chemiluminescence analyzer. The fluorescence intensity of R110 was measured and recorded at an emission wavelength of 538 nm and an excitation wavelength of 485 nm.

CCK-8 assay
Cell proliferation was evaluated using the CCK-8 assay. CCK-8 solution was added to the control, NC, PP2A-OE, OA, MC, (MC + NC), (MC + PP2A-OE), (OA + PP2A-OE), and (OA + NC) groups, followed by incubation at 37 °C for 4 h. The absorbance of the mixture was measured at 450 nm using a microplate reader. The absorbance of each experimental group was recorded in three duplicate wells and calculated as follows: proliferation rate = Absorbance (Experimental group)/Absorbance (Control) × 100%. The calculation was performed using Graph Pad Prism 5 software (GraphPad Inc., USA).

Cell cycle analysis
The serum was collected and left at room temperature for 24 h to synchronize the cell cycle before treatment. MC and OA were then added to the serum at the same time. After 24 h, the cells were collected, and GENMED staining solution was added to the cells in centrifuge tubes according to the assay kit protocol. Cell cycle analysis was then performed using flow cytometry (Beckman, Brea, CA, USA).

Apoptosis analysis
Cell apoptosis rate was measured using Annexin V-FITC Apoptosis Detection Kit. Cell culture supernatant was collected and washed once with PBS. The supernatant was digested with trypsin and centrifuged at 1,000 g for 5 min. The supernatant was discarded, and Annexin V-FITC binding solution was added to gently resuspend the cells, which were re-incubated at room temperature for 10 min in darkness. The supernatant was discarded, and Annexin V-FITC-PI staining solution was added. The cells were finally analyzed using a cell cytometer. The apoptosis rate was analyzed using the CXP software (Beckman, Brea, CA, USA).

Western blot analysis
The total protein of tumor tissue was extracted using RIPA lysis buffer (RIPA: PMSF = 100:1) and quantified using the BCA kit. The protein extract was separated according to their molecular weight through SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The membranes were blocked by sealing fluid and incubated at 4 °C overnight with the following primary antibodies: anti-Cdc25, p-Cdc2, Cdc2, p-ERK1/2, ERK1/2 (1:1,000). The protein expression was determined using ImageJ2X. GAPDH was used as the internal control (1:10,000).

Molecular docking
To further observe the binding site of MC and ERK1/2 via molecular docking. Firstly, the ERK1/2 structures were obtained through the RCSB PDB database (http://www.rcsb.org), and the MC structure was constructed by Chemdraw 17.0 software. Then, the docking active site, binding energy, and root mean square deviation were calculated by AutoDock Vina software. Finally, the docking results were

Figure 1 Antitumor mechanism research strategy of magnesium cantharidate in HCC. HCC, hepatocellular carcinomas cell.
visualized and analyzed using Discovery Studio 4.5 software.

**Statistical analysis**
Each experiment was performed in triplicates. All data were expressed as the mean ± standard deviation (mean ± SD). Statistical analysis was performed using the SPSS software v29.0 (International Business Machines Corp., Chicago, IL, USA). The samples were tested for normality and variance homogeneity using the one-way analysis of variance. *P* < 0.05 was considered statistically significant.

**Results**

**Construction of a lentiviral vector overexpressing PP2A**
To evaluate the structure of lentiviral vector LV5EF1a-GFP/Puro-PPP2CA, the PCR product was subjected to Sanger sequencing and online sequence alignment. The sequence alignment score was 100%, indicating that overexpression of PP2A in the lentiviral vector was successfully achieved. SMMC-7721 cells were infected at a dilution rate of 10⁻³, 10⁻⁴, 10⁻⁵, and 10⁻⁶, and the virus titer of the constructed lentivirus was measured using inverted fluorescence microscopy. More than 30% of the cells at the dilution rate of 10⁻⁵, corresponding to a virus titer > 1 × 10⁹ IU/ml, still expressed GFP. The virus titer analysis showed that the lentivirus vector was successfully constructed.

**Determination of infection efficiency, mRNA transcription, and protein expression rate**
The infection efficiency of PP2A overexpressed lentivirus in SMMC-7721 was quantified after 24 h of infection using inverted fluorescence microscopy (Figure 2A) and was found to be 87%. The PCR results showed that the transcription of the PP2A mRNA was higher in the PP2A-OE group (Figure 2B) compared with the NC group. Western blot results revealed comparable findings (Figure 2C, 2D). The PP2A-OE lentivirus was therefore used in the subsequent experiments.

**Xenograft tumor construction in nude mice**
Tumors were observed at the right axillary subcutaneous inoculation site in nude mice one week after the injection (Figure 3A). H & E staining of the tumors (Figure 3B) showed that the tumor cells were arranged in nests and were diffusely distributed throughout the field with little interstitial space. Moreover, the tumor cells were significantly large, with high nuclear heterogeneity and poor differentiation, typical of HCC.

**Anti-tumor effect of MC**
The tumor masses of the nude mice in each group were peeled off and weighed (Figure 4). Results showed that compared to the control

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**Figure 2** PP2A-OE lentivirus infection in SMMC-7721 cells. (A) Infection efficiency of PP2A-OE lentivirus observed under inverted fluorescence microscope. (B) The relative expression of PP2A mRNA. (C) The expression of PP2A protein detected by Western blot. (D) The relative expression of PP2A protein (scale bar 100 μm, magnification ×100; mean ± SD, n = 3; **P** < 0.01, compared to the NC group). OA, okadaic acid; MC, magnesium cantharidate; NC, negative control.

**Figure 3** Xenograft tumor in nude mice of SMMC-7721 cells. (A) Nude mice model injection of SMMC-7721 tumor cells. (B) H & E staining of tumor tissue of nude mice model. (Scale bar 100/20 μm, magnification ×100, ×400).
group, the tumor weight in the MC group was significantly lower ($P < 0.01$), whereas that of the OA group was significantly higher ($P < 0.01$). Compared to the NC group, the tumor weight in the PP2A-OE group and (MC + NC) group was significantly lower ($P < 0.01$), whereas that in the (OA + NC) group was significantly higher ($P < 0.01$). Compared to the PP2A-OE group, the anti-tumor activity in (MC + PP2A-OE) was significantly lower ($P < 0.05$). Interestingly, there was no significant difference in the anti-tumor effect in (MC + PP2A-OE) and the MC group, indicating that MC could activate processes downstream of PP2A in HCC.

MC inhibits PP2A activity and cell proliferation

The inhibition effects of MC on PP2A activity in SMMC-7721 cells were evaluated using phosphatase assay. As shown in Figure 5A, MC and OA significantly inhibited PP2A activity ($P < 0.01$). On the other hand, the PP2A activity was significantly lower in the (OA + PP2A-OE) and (MC + PP2A-OE) groups ($P < 0.01$) than in the PP2A-OE group. CCK-8 assay was performed to investigate the effect of MC on cell proliferation. Compared to the control group, MC significantly modulated the proliferation of cancer cells ($P < 0.01$). However, compared to the control group, the proliferation of cancer cells was significantly lower in the PP2A-OE group and (MC + PP2A-OE) groups ($P < 0.001$), indicating that MC could activate processes downstream of PP2A in HCC.

Figure 4 The growth of xenograft tumor in nude mice and the tumor weight value (mean ± SD, n = 6). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$, compared to the Control, NC, and PP2A-OE group, respectively. OA, okadaic acid; MC, magnesium cantharidate; NC, negative control.

Figure 5 Detection of PP2A activity and cell proliferation. (A) The relative PP2A activity in each group detected by CCK-8 assay (mean ± SD, n = 3). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, compared to the Control, NC, and PP2A-OE group, respectively. OA, okadaic Acid; MC, magnesium cantharidate; NC, negative control.
higher in the OA group ($P < 0.01$) (Figure 5B), indicating that MC inhibited the proliferation of SMMC-7721 cells. Interestingly, although both MC and OA target PP2A, their effects on cell proliferation differ.  

**Cell cycle arrest**  
The inhibition effect of MC was further investigated using the flow cytometry assay and SMMC-7721 cells (Figure 6A). Results showed that there were more SMMC-7721 cells in the G2/M phase in the MC and PP2A-OE groups than in the OA group (Figure 6B). The expression of selected proteins was detected for further analysis (Figure 7A). Cyclin-dependent kinase 1 specifically regulates the cell cycle at the G2/M phase. Our results showed that p-Cdc2 was overexpressed in the MC, PP2A-OE, and (MC + PP2A-OE) groups and was inhibited in the OA group (Figure 7B). The expression of Cdc25C in MC, PP2A-OE, and (MC + PP2A-OE) groups was down-regulated but was upregulated in the OA group (Figure 7C). However, there was no difference in the expression of Cdc2 among the groups. Overall, MC modulated the proliferation of cancer cells by arresting the cell cycle, upregulating p-Cdc2 expression, and down-regulating Cdc25C expression.

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**Figure 6** Effect of MC to Cell cycle of SMMC-7721 cell by flow cytometry assay. (A) The cell cycle diagram in each group. (B) The cell number (%) in each cell cycle (mean ± SD, n = 3). OA, okadaic Acid; MC, magnesium cantharidate; NC, negative control.
MC-induced apoptosis of cancer cells

The apoptotic effect of MC in SMMC-7721 cells was quantified using flow cytometry. The percentage of cell populations at different stages of apoptosis stages is shown in Figure 8A. The apoptotic rate in control, MC, OA, and (PP2A-OE) groups was 13.21 ± 1.31, 26.46 ± 0.98, 7.18 ± 0.35 and 45.54 ± 2.13, respectively. Compared with the control group, the apoptotic rate in the MC group was significantly higher (P < 0.01), whereas that of the OA group was lower (P < 0.01) (Figure 8B), suggesting that MC induced apoptosis in SMMC-7721 cells.

MC inhibited the proliferation of HCC cells by activating the ERK1/2 pathway

This study showed that MC inhibits PP2A expression, also known as serine/threonine phosphatase. Inhibiting PP2A activity induced the phosphorylation and activation of several kinases. ERK1/2 is a key PP2A substrate that regulates cascades downstream of the MAPK signaling pathway. The expression level of ERK1/2 was quantified using western blotting. As shown in Figure 9A, there was no difference in the expression of ERK1/2 among the experimental groups. In addition, compared to the control group, NC, and PP2A-OE groups, ERK1/2 phosphorylation was down-regulated in the MC, PP2A-OE, and (MC + PP2A-OE) groups (Figure 9B). On the other hand, ERK1/2 phosphorylation was higher in the OA group than in the control group.

Molecular docking verification

The docking accuracy of protein ERK1/2 and MC was verified using the molecular docking method. The results showed that the binding energy of ERK1 was −6.02 kcal/mol, whereas that of ERK2 was −6.19 kcal/mol, which was both lower than −5.0 kcal/mol. These results indicate that MC binds strongly with ERK1/2. The best molecular docking pose of MC and ERK1/2 is shown in Figure 9C, suggesting that ERK1/2 plays a significant role in the effect of MC on HCC cells.

Discussion

HCC is one of the most prevalent primary liver cancers, with an age-adjusted global incidence of 10 cases per 100,000 person-years.
Figure 8 Cell apoptosis rate detection by flow cytometry. (A) The cell apoptosis diagram in each group. (B) The apoptosis rate (%) in each group (mean ± SD, n = 3). ****P < 0.0001, **P < 0.01, ***P < 0.001, compared to the Control, NC, and PP2A-OE group, respectively). OA, okadaic acid; MC, magnesium cantharidate; NC, negative control.
Figure 9 Western blotting assay. (A) The Western blotting band. (B) Relative protein expression of ERK1/2 and p-ERK1/2 protein in each group (mean ± SD, n = 3). "P < 0.01, ""P < 0.001, """"P < 0.0001, compared to the Control, NC, and PP2A-OE group, respectively. (C) The docking pose between MC and ERK1/2. OA, okadaic acid; MC, magnesium cantharidate; NC, negative control.

CTD is one of the most important traditional Chinese medicine (TCM) insect drugs widely used in cancer therapy, especially primary liver cancer [7, 8, 11]. MC is a self-synthesized CTD analog with higher efficacy and lower toxicity compared to CTD. However, the pharmacological mechanism of MC against HCC is still unclear. In this paper, the anti-tumor mechanism of MC was investigated in vivo and in vitro. The findings of this study provide a basis for drug development and expand the clinical application of MC and other CTD analogs.

First, an HCC xenograft model was developed in nude mice to assess the effect of MC and OA on the tumor. The xenograft tumor was developed by transfecting the SMMC-7721 cell line with a lentivirus overexpressing PP2A. We found that MC effectively inhibited tumor growth, but OA exerted the opposite effect. Notably, the tumor growth was also inhibited significantly in the PP2A-OE group after treatment with MC, suggesting that MC inhibits HCC progression through multiple pathways besides suppressing PP2A.

Second, the pharmacodynamics of MC in animals was further investigated using cell experiments. The anti-tumor effect of MC on HCC line SMMC-7721 cells was evaluated by overexpressing PP2A. The PP2A overexpression model was successfully constructed. The CCK-8 assay showed that overexpression of PP2A inhibited the proliferation of SMMC-7721 cells, indicating that the proliferation of the cancer cells was closely related to overexpression of PP2A. PP2A is a serine/threonine protein phosphatase that regulates the activity of at least 50 different protein kinases in several cellular signaling pathways, such as cell division, stress response, growth factor response, cell adhesion, and cell death [24]. Inhibiting PP2A activity arrests the cell cycle progression, induces abnormal cell proliferation, and promotes the growth of tumor cells [22, 31]. It is generally believed that PP2A inhibitors such as okadaic acid (OA) and microcystin could promote cancer development [23, 27, 31]. However, different from the classic PP2A inhibitor, CTD has excellent anti-tumor activity both in vivo and in vitro [23]. In this study, MC treatment inhibited PP2A activity and cell proliferation rates but prompted the apoptosis rate of SMMC-7721 cells, suggesting that MC suppresses HCC progression by inhibiting PP2A activity. Notably, MC inhibited the PP2A activity in the PP2A-OE group. However, MC treatment increased the cell proliferation rate and decreased the apoptosis rate of cancer cells in the PP2A-OE group, suggesting that PP2A plays a minimal role in cell death after MC treatment or that the inhibition was not enough to regulate the downstream process to regulate the cell growth. Therefore, MC may inhibit HCC progression via another pathway besides suppressing PP2A.

We investigated whether MC exerts its anti-tumor effect via the MAPK signaling pathway MAPK signaling pathway is a signal cascade involving PP2A and ERK1/2, and it plays an important role in regulating cell proliferation, growth, and apoptosis [28, 32, 33]. PP2A inhibitors such as OA promote tumor cell proliferation by activating the ERK pathway [28, 34]. Therefore, investigating the regulation mechanism of MC on ERK1/2 is key to unraveling the difference in the ant-tumor activity of MC and OA. We found that MC inhibited the phosphorylation of ERK1/2. On the other hand, OA increased the phosphorylation of ERK1/2. Interestingly, inhibiting PP2A and PPA overexpression suppressed the phosphorylation of ERK1/2, suggesting that MC directly regulated ERK1/2 independently of PP2A. ERK1/2 is a novel MC target in HCC. The binding between MC and ERK1/2 was evaluated using molecular docking. The results showed that MC
Advances couldn't SMMC-7721 is activated in protein in the target-multiple inducing mice PP2A protein in ERK1/2 activity G2/M expression and pathway. It both of contrast, been related MC pathway of the tumor and strongly promoted p-ERK1/2 that inhibited inhibiting PP2A 10 next mice. elucidated. low cells Therefore, the increased regulation functions, p-ERK1/2 of the is Our and ERK1/2 of the and G2/M 2024;6(1):4 pathway OA addition, and the and the phase. and the population arrest phosphorylation Cdc2 After the phase, observed OA the proliferation Cdc25C total an through fully not expression. MC the inhibited or pathway over proliferation activates of the induction area apoptosis Figure on independent promotes (Figure inhibiting 43 hand, and Cdc2 the cyclin-dependent investigated. contrast, the cells apoptosis ERK1/2. Finally, the key proteins with phosphorylation MC with promoted Cdc2 phosphorylation. The activity of overexpressed PP2A overexpression was partially abrogated by MC treatment. Conversely, OA promoted the expression of Cdc25c and inhibited the phosphorylation of Cdc2. Our results strongly suggested that inhibiting PP2A activity and ERK1/2 signal pathway arrests the cell cycle at the G2/M phase, inducing apoptosis of SMMC-7721 cells (Figure 10). In contrast, OA activates the ERK1/2 pathway and promotes the proliferation of tumor cells. However, the relationship between PP2A and ERK1/2 is still not fully elucidated. First, the PP2A subunit has several functions, and the total PP2A activity couldn’t completely reflect the specific PP2A inhibition. ERK1/2 is a novel MC target. MC inhibited HCC progression by directly suppressing ERK1/2 independent of PP2A inhibition. Despite our findings, further studies on the relationship between PP2A and ERK1/2 are needed.

Conclusions

In this study, MC significantly inhibited the tumor weight, while OA significantly increased the tumor weight both in WT mice and OE-PP2A mice. In addition, MC inhibited the SMMC-7721 cell proliferation rate, induced cell arrest and apoptosis through activating p-Cdc2 and inhibiting Cdc25, p-ERK1/2 protein expression. In contrast, OA increased the SMMC-7721 cell proliferation rate, inhibited cell arrest and apoptosis through inhibiting p-Cdc2 and stimulating Cdc25, p-ERK1/2 protein expression. After over expression of PP2A gene, MC also inhibited tumor weight while OA increased tumor weight, indicating that MC can also exert antitumor effect separated from PP2A target. However, it alleviated the effect of MC in the cell cycle and apoptosis in SMMC-7721 cells. Interestingly, both OA and MC activated the p-ERK1/2 protein expression in PP2A-OE SMMC-7721 cells. Overall, MC exerted an antitumor ability which was similar to PP2A-OE, ERK1/2 may be a novel therapeutic target for treating HCC of MC.

In conclusion, HCC was inhibited by MC through inhibiting PP2A and ERK1/2 with inhibition of cell proliferation or induction of apoptosis. Importantly, the antitumor effect of MC against HCC may be related to the "multiple target-multiple pathway" anti-tumor mechanism and the cross-talk relationship in PP2A-ERK1/2 of MC. Therefore, the PP2A-ERK1/2 pathway regulation of MC is an attractive research area that is not fully elucidated. In the next research, network pharmacology and multi-omics should be utilized to explore the comprehensive antitumor mechanism of MC. Moreover, gene knock down strategy also need to be utilized to elucidate the specific role in PP2A-ERK1/2 in HCC treatment, providing basis of antitumor mechanism of MC to expand CTD development and clinical use.

Figure 10 Brief molecular mechanism of MC against HCC. PP2A and ERK1/2 were suppressed by magnesium cantharidate, further inducing cell apoptosis and cell cycle arrest with down-regulated Cdc25c and up-regulated P-Cdc2 protein.
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