Cantharidin inhibits cell proliferation and induces apoptosis through G2/M phase cell cycle arrest in hepatocellular carcinoma stem cells

AI-PING LE,  LUN-LI ZHANG,  WEI LIU and  YU-FEI SHI

Departments of Blood Transfusion and Infectious Diseases, First Affiliated Hospital of Nanchang University, Nanchang, Jiangxi 330006, P.R. China

Received December 6, 2015; Accepted December 30, 2015

DOI: 10.3892/or.2016.4684

Abstract. The present study was designed to investigate the effect of cantharidin on cell proliferation, ability of self-renewal, cell cycle arrest and induction of apoptosis in HepG2 hepatocellular carcinoma stem cells (HCSCs). It was observed that cantharidin treatment exhibited dose- and time-dependent inhibitory effect on the viability of HCSCs. The inhibition of cell viability by cantharidin in HepG2 CD133+ and parental cells was significant at the concentration 5 and 15 µM, respectively after 48 h. Cantharidin treatment inhibited the self-renewal ability of the HCSCs and the expression of β-catenin and cyclin D1. Flow cytometry revealed that cantharidin treatment at 5 µM concentration significantly increased the cell population in G2/M phase and decreased the population in the G1 phase. Cantharidin treatment in the HCSCs for 48 h increased expression of histone H2AX, Myt1, cyclin A2, cyclin B1, p53 and cdc2 (Tyr15) phosphorylation significantly compared to the parental cells. Exposure of the HCSCs to cantharidin for 48 h at a concentration of 5 µM caused a significant increase in the proportion of apoptotic cells. Therefore, cantharidin is a promising agent for the hepatocellular carcinoma treatment.

Introduction

Hepatocellular carcinoma (HCC) is one of the major causes of cancer deaths throughout the globe and every year around 600,000 new cases are detected (1,2). The rate of prognosis in HCC patients is very poor and depends on the stage of diagnosis. The commonly used treatment strategies for HCC at present include, chemotherapy, radiation therapy and surgery, however, none of the strategies are sufficiently efficient (3). Metastasis of HCC to other organs including lungs, lymph nodes, kidneys and brain is commonly observed in the patients with liver cancer (4). Thus, the discovery of new molecules for the treatment of hepatocellular carcinoma is required. A small population of cancer cells bestowed with the ability to initiate, promote and sustain tumor growth is known as cancer stem cells (CSCs) (5,6). These cells are capable of undergoing self-renewal and proliferation at an enormous rate (7). CSCs are resistant to radiotherapy as well as chemotherapy and facilitate the metastasis of cancer cells to various other organs. Thus, it is believed that targeting the CSCs can be a promising strategy for the treatment of cancer.

Natural products isolated from plant and animal sources have been the source of drugs for various diseases (8). Cantharidin is obtained by the phytochemical analysis of Blister Beetles and belongs to the class of terpenoids (8). Cantharidin has a long traditional medicinal importance in Chinese system of medicine (9). The mechanism of action of cantharidin has been found to involve arrest of cell cycle and induction of apoptosis in the cancer cells (10,11). Cantharidin treatment inhibits cell proliferation and induces expression of COX2, PGE2 and causes cell cycle arrest in G2/M phase (13). However, the effect of cantharidin on cell proliferation and self-renewal, cell cycle arrest and induction of apoptosis in HCSCs have not been reported. Therefore, the effect of cantharidin on the hepatocellular carcinoma stem cells was investigated. It was observed that cantharidin treatment inhibited cell viability and self-renewal capacity, arrested the cell cycle and induced apoptosis in HepG2 CD133+ HCSCs.

Materials and methods

Chemical and reagents. Cantharidin and dimethyl sulphoxide (DMSO) were purchased from Sigma-Chemical Co. (St. Louis, MO, USA). Trypsin, MTT reagent and lithium chloride were purchased from Gibco-BRL (Grand Island, NY, USA). The antibodies for human β-catenin, cyclin D1 and β-actin were obtained from the Health Science Research Resources Bank (Osaka, Japan).
Cell lines and culture. The HepG2 hepatocellular carcinoma cell line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were maintained in RPMI-1640 medium (RPMI:ECM=4:1) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

Cell separation and culture of spheres. HepG2 hepatocellular carcinoma cells were sorted using magnetic activated cell sorting (MACS) separation column (BD Biosciences, Mountain View, CA, USA) based on the presence of surface marker, CD133⁺. Briefly, the cells after phosphate-buffered saline (PBS) washing were treated with 0.5% bovine serum albumin (BSA). To the 200 µl of anti-CD133 antibody were added 200 µl of CD133-conjugated MicroBeads, 2x10⁷ cells and the sample. After incubation for 1 h, the cells were rinsed in PBS and then CD133⁺ and CD133⁻ cells were separated. The CD133⁺ and parental cells were rinsed twice in PBS followed by incubation in serum-free RPMI-1640 containing antibiotics, penicillin and streptomycin. The cells at a density of 2x10⁵ cells/ml were distributed onto 6-well ultralow attachment plates (BD Biosciences) in stem cell media. Following incubation for 5 days the cultures were passaged to determine the number of colonies with more than 50 cells per colony using a microscope (IX71; Olympus, Tokyo, Japan). All the calculations were performed in triplicate.

Analysis of colony formation. The effect of cantharidin treatment on formation of colonies in HepG2 CD133⁺ cells was also determined. Briefly, the cells at a density of 2x10⁵ cells/ml were seeded onto 96-well plates. The cells were incubated with various concentrations of cantharidin dissolved in DMSO or with DMSO alone as negative control. Following incubation for a period of 5 days, the ability of the cells to form colonies was determined by counting the number of colonies using an Olympus CX22 microscope (Olympus).

The sphere forming ability of the cells was analyzed after 2.5x10⁵ cells/ml were seeded onto 6-well ultralow attachment plates. In another experiment, 1x10⁷ cells/ml were distributed on to the 6-well ultralow attachment plates and then treated with different doses of cantharidin. HepG2 CD133⁺ single cell suspensions were exposed to cantharidin (5 µM), lithium chloride (2 µM), casticin-LiCl (5 or 5 µM) or control to DMSO, respectively. After 24 h, the effect of cantharidin on the ability of cells to form a carcinoma mass was analyzed.

In vivo tumorigenicity assay. Twenty pathogen-free male Balb/c nu mice (56 weeks of age) were purchased from the Animal Institute of the Chinese Academy of Medical Science. The animal studies were performed in accordance with the standard protocols approved by the Ethics Committee of Hunan National University and the Committee of Experimental Animal Feeding and Management (Changsha, China). The study was approved by the Ethics Committee of Hunan National University and the Committee of Experimental Animal Feeding and Management (Changsha, China) under the reference number 007/2013-HNU. The mice were randomly divided into five groups (n=4 per group) and maintained under standard conditions, according to typical protocols. The cells were suspended in a serum-free DMEM/Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) mixture (1:1 volume). The mice were inoculated with different quantities of CD133⁺ SFCs (5x10², 1x10³, 5x10³, 1x10⁴ and 5x10⁴ cells) in one flank, and unsorted MHCC97 cells (5x10⁴, 1x10⁵, 2x10⁵, 5x10⁵ and 1x10⁶ cells) in the other. Tumorigenicity experiments were terminated two months after cell inoculation. Tumor size was measured using a caliper and the volume was calculated as follows: V (mm³) = L x W² x 0.5, where L denotes length and W the width. The harvested tumors were photographed and weighed immediately. Specimens from tumor tissue samples were fixed in 10% neutral buffered formalin, processed in paraffin blocks and sectioned. The sections were stained with hematoxylin and eosin (H&E) and examined under an inverted microscope (IX71; Olympus).

**MTT assay.** On to the 96-well tissue culture plates 2.5x10⁵ CD133⁺ or parental HepG2 cells were dispersed and cultured in RPMI-1640 supplemented with 10% fetal bovine serum along with 2 mM L-glutamine. The cells after attachment for 12 h were treated with various concentrations of cantharidin for 36 h. To each well of the plate, 150 µl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyloxazole bromide (MTT) and incubated for 4 h. DMSO (150 µl) was added to each well of the plate to dissolve the formed formazan crystals. A microplate reader (SpectraMax Plus; Molecular Devices) was used to measure absorbance for each well at 465 nm three times independently. All the experiments were performed three times.

**Analysis is apoptosis.** CD133⁺ or parental HepG2 cells were seeded at a density of 3x10⁵ cells into the 10-cm culture dishes (T75 flask; Nunc A/S). The flasks were supplemented with RPMI-1640 medium containing 2% FBS and incubated for 24 h with various doses of cantharidin. Following incubation, the cells were rinsed in PBS buffer and fixed in 70% ethanol overnight at 40°C. Cell contents were subjected to centrifugation at 15000 x g for half an hour and then treated with 200 µl PBS supplemented with 1 mM RNase A (Calbiochem, San Diego, CA, USA) and the fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit (BD Biosciences, San Diego, CA, USA) were used to analyze the percentage of apoptotic cells according to the manufacturer's instruction.

**Cell cycle analysis.** CD133⁺ or parental HepG2 cells were seeded at a density of 3x10⁶ cells into the tissue culture flasks (T75 flask; Nunc A/S). The flasks were supplemented with RPMI-1640 medium containing 2% FBS and incubated for 24 h with various doses of cantharidin. Following incubation, the cells were rinsed in PBS buffer and fixed in 70% ethanol overnight at 40°C. Cell contents were subjected to centrifugation at 15000 x g for half an hour and then treated with 200 µl PBS supplemented with 1 mM RNase A (Calbiochem, San Diego, CA, USA) for 40 min. Propidium iodide (Sigma-Aldrich) at the concentration of 50 µg/ml was added to the cell cultures and incubation was continued for half an hour. DNA content of the cells was examined using FACSVantage SE flow cytometry system and CellQuest program (BD Biosciences).

**Western blot analysis.** The cells deprived of serum were treated with cantharidin or only DMSO as control in 6-well plates for 24 h. Following incubation, the cells were washed with PBS and then treated with lysis buffer (50 µM Tris-HCl pH 7.4, 10% glycerol, 137 µM NaCl, 1 µM PMSF, 100 µM sodium vanadate, 10 mg/ml leupeptin, 10 mg/ml aprotinin,
1% NP-40 and 5 µM cocktail). Concentration of proteins in the cell lysates was determined by bicinchoninic acid assay (BCA) method. The proteins were separated on 10% polyacrylamide gel and then transferred to a PVDF membrane. The membrane was blocked overnight using 5% non-fat dry milk followed by TBST washing. The membrane was then incubated for 12 h with primary antibodies for β-catenin or cyclin D1, washed with TBST followed by incubation with secondary antibodies for 1 h. The X-ray autoradiography was performed and the gray scale images were analysed.

Statistical analysis. The data expressed are the mean ± SD. The differences between the groups were analyzed using Student’s t-test and SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA). The differences at P<0.05 were considered statistically significant.

Results

Separation of hepatocellular carcinoma stem cells from HepG2 cell line. The CD133+ cells were separated from HepG2 cell cultures by examination using FCM and then cultured to obtain stem cell rich cultures, and at 5 days, the spheroids formed by CD133+ and parental cells were collected. It was observed that compared to CD133+ cells, the parental cells formed larger sized and more number of tumorspheres (Fig. 1A). Analysis of the capacity of self-renewal revealed that CD133+ cells formed spheroidal mass of undifferentiated cells.
LE et al: INHIBITION OF HEPATOCELLLAR CARCINOMA BY CANTHARIDIN

2973

CD133+ cells within 5 days (Fig. 1B). Thus, CD133+ cells possess the capacity of self-renewal.

Inhibition of proliferation and selfrenewal of HCSCs from the HepG2 cell line by cantharidin. The cells were exposed to various concentrations of cantharidin from 0 to 20 µM for various time periods. The results revealed a concentration-dependent inhibition of cell viability of CD133+ HCSCs by cantharidin after 48-h treatment. The reduction in cell viability of CD133+ HCSCs was significant at 5 µM concentration of cantharidin. However, in parental cells the inhibition in viability was significant at 20 µM concentration after 48 h (Fig. 2A and B). Cantharidin treatment at a concentration of 5 µM significantly inhibited the tendency of colony formation in HCSCs after 48 h. Compared to the primary tumorspheres of HCSCs of untreated group, the number of primary as well as secondary tumorspheres in the cantharidin treated cultures were significantly reduced (Fig. 2C and D).

Inhibition of selfrenewal in HCSCs by cantharidin involves alteration in β-catenin expression. Effect of cantharidin treatment on the expression of β-catenin and cyclin D1 in CD133+ HCSCs and parental cells was analyzed by western blot analysis. Results showed a significantly higher expression of β-catenin and cyclin D1 in the CD133+ HCSCs compared to the parental cells. However, cantharidin treatment induced a concentration-dependent inhibition of β-catenin and cyclin D1 expression. The reduction in expression of β-catenin and cyclin D1 was significant at the concentration of 5 µM of cantharidin after 48 h in HCSCs (Fig. 3).

Treatment of the HCSCs with lithium chloride, a factor known for activation of Wnt/β-catenin pathway led to the enhancement in expression of β-catenin and cyclin D1. When the lithium chloride pretreated HCSCs were exposed to cantharidin the inhibition in expression of β-catenin and cyclin D1 caused by cantharidin was suppressed (Fig. 4). Since β-catenin pathway plays an important role in the self-renewal

---

Figure 3. Inhibition of β-catenin and cyclin D1 expression in HCSCs obtained from HepG2 cell line by treatment with cantharidin. The expression of β-catenin and cyclin D1 was higher in CD133+ HCSCs compared to the PCs. Cantharidin inhibited the expression of β-catenin and cyclin D1 in CD133+ HCSCs in concentration-dependent manner. PC, parental cell.

Figure 4. Treatment of lithium chloride pretreated HCSCs WITH cantharidin prevented the inhibition of β-catenin and cyclin D1. LiCl, lithium chloride.

Figure 5. Cantharidin treatment caused cell cycle arrest in HCSCs in G2/M phase. The cells after incubation with cantharidin for 24 h were fixed and then stained with propidium iodide to analyze the DNA content.
of cancer stem cells, cantharidin treatment in HCSCs inhibits self-renewal by inhibiting the expression of β-catenin and cyclin D1.

**Cantharidin treatment arrests cell cycle in G2/M phase in the HCSCs.** Flow cytometry was used to analyze the effect of cantharidin on cell cycle in the HCSCs. It was observed that cantharidin treatment at 5 µM concentration significantly enhanced the cell population in G2/M phase and decreased the population in the G1 phase. The population of CD133+ HCSCs in the G2/M phase increased from 19.5±1.8 to 73.4±4.2% with the increase in treatment time from 24 to 48 h. In G1 phase the population of cells decreased from 17±2.0 to 9.6±1.8% with the increase in treatment time from 24 to 48 h. However, in parental cells the proportion of cells in G2/M phase were 7.5±1.2 and 11.2±2.1%, respectively after 24 and 48 h (Fig. 5).

**Alteration in cell cycle regulatory proteins by cantharidin treatment in the HCSCs.** Cantharidin treatment in the HCSCs for 48 h induced a significant increase in histone H2AX expression compared to the parental cells. It also promoted the Myt1 protein expression and cdc2 (Tyr15) phosphorylation in HCSCs (Fig. 6). Analysis of cyclin A2, cyclin B1 and p53 revealed a significant increase in the expression of all the three proteins in the cantharidin treated HCSCs. However, the histone H3 expression was inhibited significantly in HCSCs on treatment with cantharidin for 48 h.

**Induction of apoptosis in HCSCs by cantharidin treatment.** Exposure of the HCSCs to cantharidin for 48 h at a concentration of 5 µM caused a significant increase in the proportion of apoptotic cells. Annexin V/FITC staining showed that cantharidin treatment significantly increased...
the Annexin V/FITC-stained HCSCs cells compared to the untreated cells (Fig. 7A). HCSCs cells treated with cantharidin for 48 h showed significant morphological alterations by immunofluorescence (Fig. 7B).

### Discussion

Carcinoma stem cells (CSCs) play a vital role in the progression of cancer and its resistance to chemotherapeutic agents. Therefore, inhibition of CSC proliferation by arresting cell cycle and induction of apoptosis is of promising importance for the treatment of cancer (16). It is reported that CSC proliferation can be inhibited by targeting a number of factors including hedgehog, Wnt/β-catenin, Notch and EGFR pathways (17,18).

The present study demonstrates the effect of cantharidin on inhibition of cell proliferation, self-renewal, cell cycle arrest and induction of apoptosis in CD133+ hepatocellular carcinoma cell line, derived from HepG2 cell line. It was observed that cantharidin treatment inhibited cell viability and tendency of self-renewal, arrested cell cycle in G2/M phase and induced apoptosis in CD133+ HCSCs. CSCs are present in various types of cancers and are identified on the basis of presence of CD133, surface marker (19). In the present study, CD133+ HCSCs were separated from HepG2 cell lines and cultured in media conditioned for stem cell to form tumorspheres. It was observed that CD133+ HCSCs formed larger sized and greater number of tumorspheres compared to the parent cells. Exposure of the HCSCs to cantharidin significantly inhibited the cell proliferation and tendency of self-renewal after 48 h of treatment compared to the parental cells. Cantharidin treatment also led to inhibition of the tendency to form spheres in HCSCs.

Wnt/β-catenin signaling pathway plays an important role in the self-renewal of the cancer stem cells and hence progression and invasion of carcinoma (20). Therefore, the effect of cantharidin on the β-catenin and cyclin D1 expression was also investigated. It was observed that cantharidin treatment significantly inhibited the β-catenin and cyclin D1 expression in CD133+ HCSCs compared to the parental cells. However, lithium chloride, a factor known for activation of Wnt/β-catenin pathway exposure to cantharidin pretreated cells prevented the cantharidin-induced inhibition of β-catenin and cyclin D1 expression. These findings suggest that inhibition of self-renewal in CD133+ HCSCs by cantharidin treatment is associated with β-catenin and cyclin D1 expression.

Progression of cell cycle is regulated by various factors including, cyclins and cyclin-dependent kinases and activated cdc2-linked cyclin A and cyclin B regulate the progression of G2/M phase. Activation of cdc2 Tyr15 and Thr14 by Myt1 kinases results in inactivation of the cdc2/cyclin B complex (21-23). In the present study cantharidin treatment significantly increased the expression of Myt1 protein in CD133+ HCSCs. The phospho-cdc2 (Tyr15) and cyclin D1 protein expression was also promoted in CD133+ HCSCs compared to the parental cells. The phosphorylation of histone H2AX proteins was increased and the cell cycle was arrested in the G2/M phase. The process of apoptosis involving the removal of damaged cell is inhibited in the carcinoma cells (24). In the present study cantharidin treatment induced apoptosis in CD133+ HCSCs significantly compared to the parental cells after 48 h.

In conclusion, cantharidin treatment inhibits proliferation and self-renewal, arrests cell cycle in G2/M phase and induces apoptosis in CD133+ HCSCs derived from HepG2 cell line. Therefore, cantharidin is a potent agent for the inhibition of hepatocellular carcinoma.

### Acknowledgements

The study was supported by the Jiangxi Major Technology Project (no. 2014BBG70001).

### References


