

Cantharidin induces G₂/M arrest and triggers apoptosis in renal cell carcinoma

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Abstract. The present study aimed to investigate the effects of cantharidin on cell cycle distribution, the induction of apoptosis, and Notch1 and Jagged1 expression in ACHN and Caki-1 renal cancer cells. Cell viability assay, flow cytometry, cell cycle and western blot analyses were performed for ACHN and Caki-1 cells. Immunohistochemistry was used to analyze the expression of Notch1 and Jagged1 in RCC tissues. The results demonstrated that treatment with cantharidin exerted a dose- and time-dependent effect on cell viability, apoptosis induction and G₂/M phase cell cycle arrest. Exposure of ACHN and Caki-1 cells to 20 μ M cantharidin reduced cell viability to 26 and 32% respectively, after 48 h. In addition, treatment with cantharidin enhanced the number of ACHN and Caki-1 cells in G₂/M phase to 54.62 and 51.88% respectively, as compared with 17.16 and 16.53% in the control groups. In the ACHN and Caki-1 cells, treatment with cantharidin induced a marked increase in the proportion of apoptotic cells after 48 h. Furthermore, cantharidin enhanced the percentage ACHN and Caki-1 apoptotic cells to 57.23 and 62.34% respectively, as compared with 2.27 and 3.06% in the control groups. Detection of Notch1 and Jagged1 expression demonstrated that levels were significantly increased in carcinoma tissues. Conversely, cantharidin exhibited an inhibitory effect on Notch1 and Jagged1 expression after 48 h. Therefore, treatment with cantharidin may exert a promising effect on the inhibition of renal cancer, and may be of therapeutic importance for the treatment of renal cancer.

Introduction

Renal cell carcinoma (RCC) is one of the most commonly detected urological tumors, which comprises ~3% of detected human malignancies (1). During the last 30 years, a marked increase has been observed in the incidence and mortality rate of RCC (1). Histologically, there are several subtypes of RCC, and the associated genetic and biological features determine the success of the chosen treatment (2). In ~30% of patients RCC is detected at its metastatic stage, making treatment difficult (3). Despite advances in chemotherapy, which led to the development of multikinase inhibitors, the survival rate of patients with RCC remains low (4,5). Enhanced Jagged1 and Notch1 expression has been reported to be associated with a worse prognosis in patients with carcinoma (6).

Blister beetles constitute an important member of the Meloidae family of the Coleoptera order, and are of traditional medicinal importance (7). Phytochemical analysis of blister beetles has led to the isolation of several phytochemicals, including cantharidin, which is a terpenoid molecule. Cantharidin is used in Chinese medicine and has been shown to exert a wide range of biological activities (8). Treatment with cantharidin has been reported to induce cell cycle arrest and promote apoptosis in various types of carcinoma cell lines (9), including hepatoma (10), colon (11), bladder (12), breast (13), oral buccal, and leukemia cells (14). The present study aimed to investigate the effects of cantharidin on the inhibition of cell proliferation, cell cycle arrest and induction of apoptosis in RCC cell lines. Furthermore, its effects on Notch1 and Jagged1 expression in RCC tissues were investigated. The results of the present study revealed a significant reduction in cell viability, and an induction of cell cycle arrest at G₂/M phase and apoptosis in ACHN and Caki-1 RCC cells.

Materials and methods

Reagents and chemicals. Cantharidin, propidium iodide (PI) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany). RPMI-1640 medium, 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μ g/ml streptomycin were obtained from Merck Millipore.

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Cell lines and culture. ACHN and Caki-1 RCC cell lines were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium supplemented with 10% FBS and penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO₂ and 95% air.

Cell viability assay. The effects of cantharidin on the viability of RCC cells were determined using the CellTiter 96® Aqueous One Solution Cell Proliferation (MTS) assay (Promega Corporation, Madison, WI, USA). Briefly, the cells were dispersed at a density of 5x10⁵ cells/well onto 96-well microtiter plates in 100 µl culture medium and incubated for 48 h at 37°C. Next, the cells were treated with cantharidin (0, 5, 10, 15, 20, 25 µM) for 24 h, and the cells were incubated for an additional 24 h at 37°C or with 20 µM cantharidin for 0, 12, 24, 48 and 72 h at 37°C. The control cultures (received 0 µM cantharidin) were treated with DMSO only for 24 h. An EnVision Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) was used to measure absorbance at 465 nm. All experiments were carried out three times and the results are presented as the mean ± standard deviation.

Flow cytometric analysis. Apoptosis of RCC cells was determined by flow cytometry using Annexin V binding and PI staining. Following incubation with 20 µM cantharidin or DMSO (control) at 37°C for 48 h, the cells were washed twice with ice-cold phosphate-buffered saline (PBS). The cells were subsequently double-stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V (Trevigen, Inc., Gaithersburg, MD, USA) and PI (Sigma-Aldrich; Merck Millipore) for 30 min at room temperature in the dark. A 488-nm laser coupled to a cell sorter (FACSCalibur; BD Biosciences, San Jose, CA, USA) was used for flow cytometric analysis and microscopy.

Cell cycle analysis. ACHN and Caki-1 RCC cells were seeded at a density of 5x10⁵ cells/well onto 96-well cell culture plates (Corning Inc., New York, NY, USA). The cells were incubated with various concentrations of 20 µM cantharidin or DMSO (control) for 48 h at 37°C. After 48 h of incubation, the cells were trypsinized, washed with PBS and resuspended into single cell suspension. Then, the single cell suspension was fixed in 70% ethanol at -20°C for 24 h. The cells were washed with PBS and incubated in 100 µg/ml PI (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 200 µg/ml RNase (Sigma-Aldrich; Merck Millipore) for 30 min in the dark. Flow cytometry (BD FACSArray; BD Biosciences, Franklin Lakes, NJ, USA) was used to determine cell cycle distribution following incubation.

Tissue samples. The tumor renal tissue samples were obtained from patients with RCC during an operation at the Department of Urology, Ningbo Urology and Nephrology Hospital (Ningbo, China). The renal tissue samples were obtained from 10 patients between the age of 32-49 (6 males and 4 females). Immediately after extraction, the RCC tissue samples were frozen and stored in liquid nitrogen until further analysis. The present study was approved by the ethical committee of Ningbo Urology and Nephrology

Hospital. Written consent was obtained from all of the patients.

Western blot analysis. The non-cantharidin-treated neoplastic renal tissue and 20 µM cantharidin-treated renal cell carcinoma tissue grind broken fully in liquid nitrogen, and then treated with lysis buffer containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.9), 0.5% Triton X-100, 0.6% NP-40, and the following protease inhibitors: 1 mg/ml leupeptin, 1 mg/ml pepstatin A and 2 mg/ml aprotinin. Sonication of the lysates for 15 sec was followed by centrifugation at 12,000 x g for 45 min at 4°C. The detergent compatible protein assay kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used for determination of protein content. Subsequently, the proteins (50 µg) were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Inc.). The membranes were incubated with blocking buffer (PBS containing 7.5% non-fat dry milk, 2% bovine serum albumin and 0.1% Tween) for 2 h at room temperature, and were then incubated with anti-Notch1 (cat. no. 3680; 1:1,000; Cell Signaling Technology, Inc.), anti-GAPDH (cat. no. 2118; 1:1,000; Cell Signaling Technology, Inc.) and anti-Jagged1 (cat. no. ab109536; 1:1,000; Abcam, Cambridge, UK) at 4°C overnight followed by washing with PBS containing 0.1% Tween-20. Subsequently, the membranes were incubated with peroxidase-conjugated goat anti-rabbit immunoglobulin G (cat. no. BA1055; Boster Biological Technology, Ltd., Wuhan, China) at room temperature for 1 h. After washing with PBS, the membranes were developed using an enhanced chemiluminescence detection system (Amersham; GE Healthcare Life Sciences, Uppsala, Sweden).

Immunohistochemistry. Paraffin-embedded RCC tissues were sliced into 2 µm sections. The sections were deparaffinized in xylene, followed by rehydration in gradient alcohol, and were treated with hydrogen peroxide. Following boiling with citrate buffer (pH 6.0) for 20 min, the tissues were incubated for 1 h at room temperature with goat serum (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and were washed with PBS. The primary antibody was added for incubation at room temperature for 30 min and then maintained overnight at 4°C. The primary antibodies were anti-Notch1 (1:400), anti-GAPDH (1:800) and anti-Jagged1 (1:300) and the slices were incubated overnight at 4°C. The secondary antibody was goat anti-rabbit IgG H&L (cat. no. ab109536; 1:5,000; Abcam) and the slices were maintained at room temperature for 30 min. The slides were then stained with 3,3'-diaminobenzidine and were counterstained with hematoxylin & eosin after being washed with PBS. The stained slices were photographed using fluorescence inverted microscope (BSF-60, Shanghai Batuo Instrument Co., Ltd., Shanghai, China).

Statistical analysis. All experiments were performed in triplicate and the results are presented as the mean ± standard deviation. One-way analysis of variance was used for statistical analysis followed by Tukey's Honest Significant Difference test as a post-hoc comparison. SPSS version 18 (SPSS, Inc., Chicago, IL, USA) was used for analysis. P<0.05 was considered to indicate a statistically significant difference.

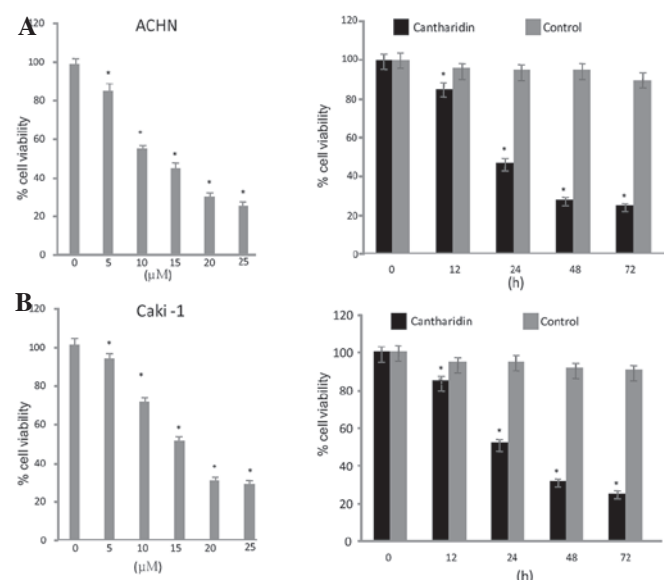


Figure 1. Effects of cantharidin on viability of (A) ACHN and (B) Caki-1 renal carcinoma cell lines. MTS assay revealed a dose- and time-dependent decrease in cell viability after 48 h. All experiments were performed in triplicate. Data are presented as the mean \pm standard deviation. *P<0.05 vs. control.

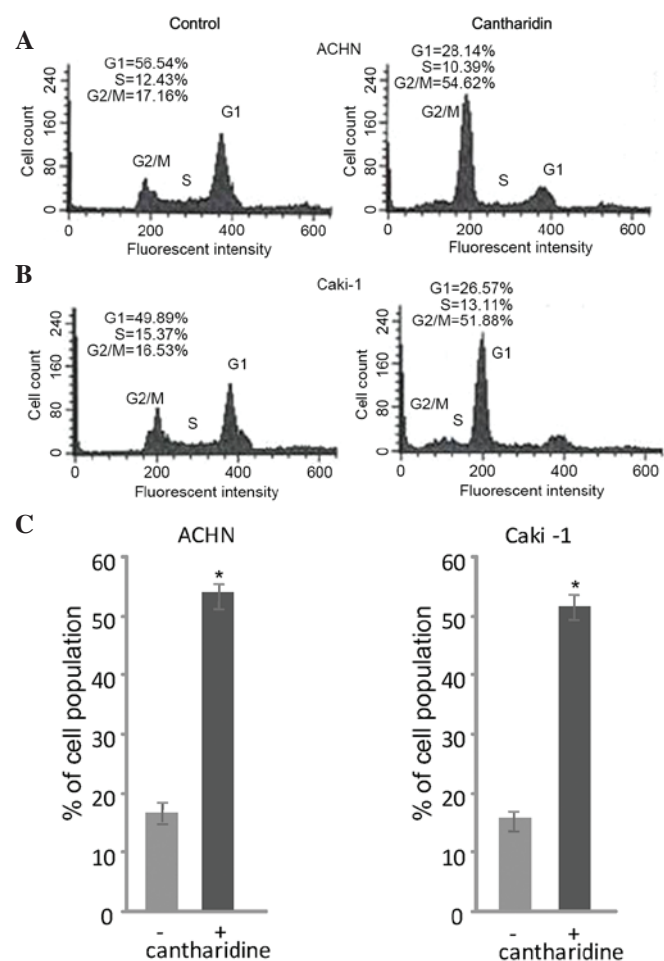


Figure 2. Effects of cantharidin on cell cycle distribution of human (A) ACHN and (B) Caki-1 renal carcinoma cells. Treatment with cantharidin induced a cell cycle arrest at G₂/M phase, as determined by flow cytometric analysis. (C) Proportion of cells in G₂/M phase with (+) or without (-) cantharidin treatment. Data are presented as the mean \pm standard error. *P<0.05 vs. control.

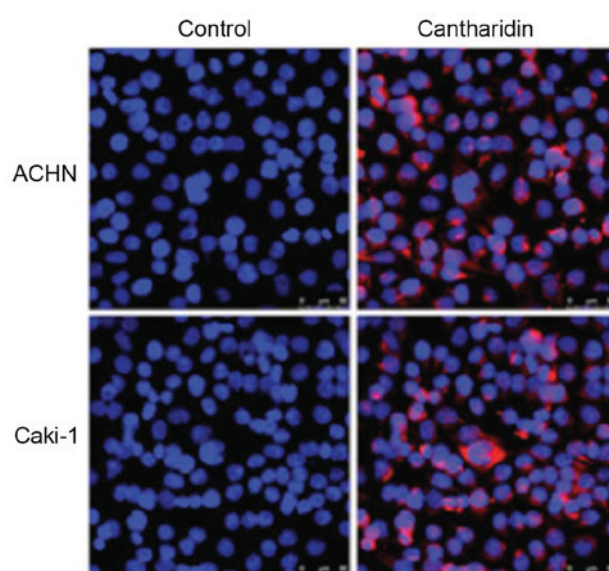


Figure 3. Representative images showing the effects of cantharidin on apoptosis induction of ACHN and Caki-1 renal cancer cells. Cells were incubated with 20 μ M cantharidin and were analyzed by flow cytometry following Annexin V-fluorescein isothiocyanate/propidium iodide double-staining.

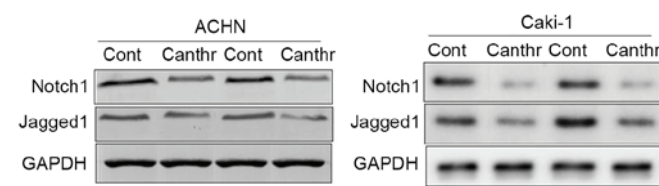


Figure 4. Notch1 and Jagged1 expression in non-neoplastic renal tissues and renal cell carcinoma tissues using western blot analysis. GAPDH, glyceraldehyde 3-phosphate dehydrogenase

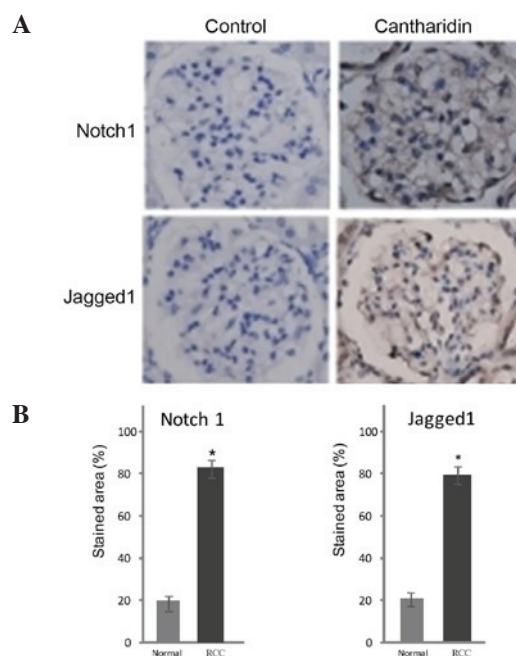


Figure 5. (A) Immunohistochemical examination of Notch1 and Jagged1 expression in non-cantharidin-treated neoplastic renal tissue and cantharidin-treated renal cell carcinoma tissue. Magnification, x250. (B) Expression of Notch 1 and Jagged 1 in normal and RCC tissue. Data are presented as the mean \pm standard error. *P<0.05 vs. control.

Results

Cantharidin inhibits growth of RCC cells. Exposure of the RCC cell lines to a range of concentrations of cantharidin (5-25 $\mu\text{g/ml}$) for various durations resulted in a dose- and time-dependent reduction in cell viability. ACHN and Caki-1 cells exhibited a significant reduction in viability after 48 h treatment with 20 μM cantharidin. Cell viability was reduced to 26 and 32% in ACHN and Caki-1 cells respectively, after 48 h (Fig. 1).

Cantharidin treatment induces a cell cycle arrest at G₂/M phase. Flow cytometry revealed that treatment of RCC cells with cantharidin induced a cell cycle arrest at G₂/M phase. Treatment of ACHN and Caki-1 RCC cells with 20 μM cantharidin induced a marked increase in the population of cells in G₂/M phase after 48 h. In cantharidin-treated ACHN cells the proportion of cells in G₂/M phase was 54.62%, as compared with 17.16% in the control group. Similarly, cantharidin treatment enhanced the proportion of Caki-1 cells in G₂/M phase to 51.887%, as compared with 16.53% in the control group (Fig. 2).

Effects of cantharidin on apoptosis. In order to investigate the effects of cantharidin (20 μM) on apoptosis of RCC cells, flow cytometry using Annexin V-FITC/PI double-staining was performed. Condensation of chromatin and fragmentation of nuclear material was detected in ACHN and Caki-1 cells following a 48 h exposure to cantharidin (Fig. 3). The percentage of apoptotic ACHN cells in the cantharidin-treated group was 57.23%, as compared with 2.27% in the control. The percentage of apoptotic cantharidin-treated Caki-1 cells was 62.34%, as compared with 3.06% in the control group.

Notch1 and Jagged1 expression in renal carcinoma tissues. Notch1 and Jagged1 expression was detected in RCC tissues, as determined by western blot analysis. The results demonstrated that Notch1 and Jagged1 expression was increased in all 12 tissues collected from patients with RCC (Fig. 4; representative blots from two patients are presented).

Immunohistochemical analysis. Notch1 and Jagged1 expression was detected in tissue samples from patients with RCC. Markedly lower levels of these proteins were detected in control tissues compared with in RCC tissues (Fig. 5). Treatment of RCC tissues with cantharidin resulted in a marked reduction in Notch1 and Jagged1 expression after 48 h. Notch1- and Jagged1-positive staining was 86 and 79% in the RCC tissues (control) and 18 and 21% respectively in the cantharidin-treated tissues (Fig. 5).

Discussion

Cantharidin is a terpenoid compound, which has been reported to exhibit promising biological activity (8). Cantharidin induces apoptosis in various types of cancer cells, including, hepatoma (10), colon (11), bladder (12), breast (13), oral buccal, and leukemia cells (14). In addition, treatment of bladder carcinoma cells with cantharidin induces cell cycle arrest (12). Therefore, the present study aimed to investigate the effects of

cantharidin on suppression of cell viability, cell cycle arrest and induction of apoptosis in RCC cell lines. In addition, the effects of cantharidin on Notch1 and Jagged1 expression were detected in RCC tissues. The results of the present study revealed that cantharidin resulted in a marked reduction in cell viability, and induced cell cycle arrest at G₂/M phase and apoptosis.

Apoptosis is a type of programmed cell death, which is responsible for the elimination of unwanted and damaged cells from the body. In the case of carcinoma, the process of apoptosis is disrupted resulting in uncontrolled cell growth. The results of the present study indicated that treatment of RCC cells with cantharidin markedly induced apoptosis compared with the control cells. The Notch pathway exhibits a dual role in the progression of carcinoma, either by promoting or inhibiting cell proliferation (15,16). Examination of RCC tissues in the present study revealed markedly increased Notch1 and Jagged1 expression; however, treatment of the RCC tissues with cantharidin led to a reduction in Notch1 and Jagged1 expression.

Notch1 expression promotes the progression of tumor angiogenesis and inhibits the expression of cyclin-dependent kinase, which is involved in cell cycle regulation (17-20). The results of the present study demonstrated that treatment with cantharidin induced cell cycle arrest at G₂/M phase; therefore, it may be suggested that cantharidin-induced cell cycle arrest, apoptosis induction and cell proliferation inhibition in ACHN and Caki-1 RCC cells is associated with inhibition of Notch signaling proteins. In conclusion, cantharidin exhibited an inhibitory effect on RCC, and may be considered of vital importance for its treatment. Nevertheless further investigations are required to identify the precise underlying mechanism. In conclusion, cantharidin treatment exhibits an inhibitory effect on renal cell carcinoma. Therefore, it may be of therapeutic importance for the treatment of renal cell carcinoma.

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