Cantharidin-induced cytotoxicity and cyclooxygenase 2 expression in human bladder carcinoma cell line

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Abstract
Mylabris is used in clinical therapy, but is always accompanied by cystitis. The toxic effects of mylabris on bladder are attributed to its active principle: cantharidin. In the present study, we explored how cantharidin induces cytotoxicity in the bladder. Human bladder carcinoma cell line T 24 cells were used as target cells, and human colon carcinoma HT 29 cells as native cells. Cantharidin exhibited acute cytotoxicity in the T 24 cells, and IC50 was 21.8, 11.2 and 4.6 μM after treatment for 6, 24 and 48 h, respectively. The cytotoxicity of cantharidin was not significantly enhanced when T 24 cells were treated for a longer time. Moreover, PARP proteins and pro-caspase 3, Bcl-2 were significantly inhibited after cantharidin treatment in T 24 cells. Pretreatment with the caspase 3 inhibitor markedly inhibited cantharidin-induced cell death. Therefore, we suggested that cantharidin could induce apoptosis via active caspase 3 in T 24 cells. When T 24 cells were treated with cantharidin at a low dose, the cell cycle was arrested in the G2/M phase. Furthermore, p21Cip1/Waf1 was enhanced, and cyclin A, B1 and cdk1 decreased. At a high dose (more 12.5 μM), cantharidin could stimulate T 24 cells to deplete a large number of ATP and induce secondary necrosis. In addition, cantharidin also stimulated COX 2 over-expression and PGE2 production in T 24 cells, in a dose-dependent manner. However, cantharidin also induced apoptosis and G2/M phase arrest in HT 29 cells, but did not induce COX 2 over-expression. Therefore, we suggest that cantharidin may induce cystitis through secondary necrosis and COX 2 over-expression.

Keywords: Chinese blister beetle (Mylabris phalerata Pallas); Cantharidin; Cytotoxicity; Inflammation; Cyclooxygenase 2; Cystitis

1. Introduction
The dried body of the Chinese blister beetle (Mylabris phalerata Pallas), known as mylabris, is one of the animal-derived Chinese medicines and is applied topically to treat malign sores and to relieve blood statis (Wang, 1989). Mylabris is a strong poison, and has been used to cure patients with hepatoma recently (Wang, 1989). The use of mylabris in traditional Chinese medicine is frequently accompanied with several side effects, such as a burning sensation of the digestive tract, vomiting, nephritis, cystitis, and so on (Wang, 1989; Nickolls and Teare, 1954; Karras et al., 1996; Oak et al., 1960). Mylabris contains 0.6–1.9% cantharidin (Fig. 1).
Cantharidin has been previously shown to be a strong protein phosphatase (PP1) and phosphatase 2A (PP2A) inhibitor (Graziano et al., 1988; Li et al., 1993), and to inhibit cAMP phosphodiesterase activity in hepatoma cells (Zhang and Chen, 1985). In our previous study, cantharidin can induce several types of tumor cell death, but not all through the apoptosis pathway (Efferth et al., 2005). In leukemia cells, cantharidin-induced apoptosis by a p53-dependent mechanism and p38 activation pathway (Efferth et al., 2005; Huh et al., 2004). The molecular pathway of cantharidin-induced cell death is still not clear, and this has produced complicated problems that have impeded the development of cantharidin as an anticancer drug. Therefore, we were interested in learning how cantharidin induces side effects, such as cytisitis.

When the bladder is stimulated with toxic compounds from metabolism, it will become inflamed, which will induce epithelial cell death, and then cystitis and hematuria. Moreover, cantharidin metabolizes through the kidney and liver (Oak et al., 1960), and undergoes specific binding to the brain, stomach, skin, heart, kidney, spleen and liver (Graziano et al., 1988). How cantharidin induces the cytotoxic effects in the bladder was explored in the present study. We used human bladder carcinoma cell line, T 24 cells as target cells to mimic the epithelial cells of bladder, and human colon carcinoma, HT 29 cells as a native control. The cytotoxic effects of cantharidin in T 24 and HT 29 cells were compared.

2. Materials and methods

2.1. Materials

Cantharidin was isolated from Chinese blister beetle (Mylabris phalerata), with a purity was 99.0%, as in a previous study (Wang et al., 2000). Dimethyl sulfoxide (DMSO), MTX (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide), and other chemicals were purchased from Sigma Industries (St. Louis, MO, USA). Dulbecco’s modified Eagle medium (DME), fetal bovine serum (FBS), antibiotics, glutamine, and trypsin-EDTA were purchased from Gibco (Grand Island, NY, USA). Western blot was performed by using antibody specific to human Bcl-2 (sc-783), poly(ADP-ribose)polymerase (PARP, sc-7150), caspase 3 (sc-7148), human cyclin A (sc-239), cyclin B1 (sc-7393), cdk1 (sc-163), P21s(rf)85(1) (ep-74), COX 2 (sc-1745), u-tubulin (sc-8035), anti-mouse IgG-AP (sc-2008), anti-rabbit IgG-AP (sc-2007) and anti-mouse IgG-AP (sc-2008), which were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). All other reagents and chemicals were of the highest purity grade available.

2.2. Cell cultures

The human bladder carcinoma cell line T 24 cells and human colon carcinoma HT 29 cells were obtained from American Type Cell Culture (ATCC) (Rockville, MD, USA). The two cell lines were maintained in DMEM (Gibco) supplemented with 10% FBS, 100.0 mg/l streptomycin, and 100 IU/ml penicillin (Gibco). The cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO₂.

2.3. Cytotoxicity assays

The stock solution of cantharidin (50 μM) was prepared by dissolving cantharidin in DMSO and then storing it at −20 °C until use. Serial dilutions of the stock solution were prepared in the culture medium in 96-well microplates. Cantharidin at the appropriate concentrations was added to cell cultures (1 × 10⁵ cells/well) for 1, 6, 24 and 48 h without renewal of the medium. The number of surviving cells was then counted by using the MTT assay (Bruggisser et al., 2002). Finally, the products were evaluated by measuring the optical density for each well at 600 nm, using an MRX microplate reader (Dynex Technologies, Guernsey, Channel Islands, Great Britain, UK).

The cytotoxicity index (CFI) was calculated according to the following equation:

$$\text{CFI} = \left[1 - \frac{T}{C}\right] \times 100\%$$

where T and C represent the mean optical density of the treated group and vehicle control group, respectively. In accordance with the CFI of the dose-response curve, the concentration of the test compound giving 50% of cell growth inhibition (IC₅₀ value) was estimated.
2.4. Flow cytometry analysis

After 24 h treatment, T 24 cells in six-well dishes (5 x 10^5 cells/well) were harvested by centrifugation and washed with PBS. The cells were fixed with ice-cold 80% ethanol for 30 min, washed with PBS, and then treated with 0.25 ml of 0.5% Triton X-100 solution containing 1.0 mg/ml RNase A at 37°C for 30 min. Finally, 0.25 ml of 50 μg/ml propidium iodide was added to the sample for 30 min in the dark (Wang et al., 2000). Samples were run through a FACScan (Becton Dickinson, San Jose, CA, USA). Results are presented as the number of cells versus the amount of DNA as indicated by the intensity of fluorescence.

2.5. Western blot analysis

T 24 cells in six-well dishes exposed to a serial concentration of cantharidin for 24 h were collected into tubes and then washed with PBS. Cell pellets were lysed with lysis buffer containing 40 mM Tris–HCl (pH 7.4), 10 mM EDTA, 120 mM NaCl, 1 mM dithiothreitol, 0.1% Nonidet P-40, and protease inhibitors. Total proteins (30 μg) were used for Western blot analysis. Western blot analysis was performed using 10% Tris–glycine–SDS-polyacrylamide gels, and the protein was transferred to a nitrocellulose membrane by electroblotting. The membranes were probed with anti-Bcl-2 (a rabbit polyclonal antibody), anti-PARP (a rabbit polyclonal antibody), anti-caspase 3 (a rabbit polyclonal antibody), anti-cyclin A (a mouse monoclonal antibody), anti-cyclin B1 (a mouse monoclonal antibody), anti-cdk 1 (a mouse monoclonal antibody), and visualized using a BCIP/NBT kit (BCIP/NBT, Gibco), according to the manufacturer’s instructions. As a loading control, we used anti-α-tubulin (a mouse monoclonal antibody).

2.6. Measurement of PGE2 production

T 24 cells were cultured with a serial concentration of cantharidin in 96-well microplates for 24 h. One hundred microliters of supernatant of culture medium was collected for the determination of PGE2 concentrations with an ELISA kit (Amersham Pharmacia Biotech, UK).

2.7. Measurement of ATP release

T 24 and HT 29 cells were cultured with a serial concentration of cantharidin in 96-well microplates for 24 h. The intracellular ATP content was measured with the luciferin–luciferase assay. After 24 h, the mammalian cell lysis solution was added to each well, and the white plates were shaken for 5 min at 750 rpm. Finally, each well was added into a substrate solution for 5 min at 700 rpm, and white-adapted plates for 10 min to reduce plate phosphorescence (Luminescence ATP Detection Assay by ATPliteTM kit, Perkin-Elmer®, USA). The resulting luminescence was monitored at 3-min intervals in a luminometer (Plate Chamelove® Multilabel Detection Platform, FIN-20750), and expressed as counts per second (CPS).

2.8. Statistical analysis

Each experiment was performed at least in triplicate. Results are expressed as the mean value ± standard deviation (S.D.). Statistical analysis was performed using an unpaired Student’s t-test. p Values <0.05 were considered significant.

3. Results

3.1. Cantharidin-induced apoptosis in T 24 and HT 29 cells

As shown in Table 1, the IC50 values of cantharidin were lower in HT 29 cells than T 24 cells for the over 24-h treatments. However, the cytotoxicity effects of cantharidin were stronger in T 24 than HT 29 cells for the 6-h treatment, and both exhibited dose- and time-dependence (Fig. 2). Cantharidin more quickly induced cytotoxicity in T 24 than in HT 29 cells.

To characterize the cell death of T 24 and HT 29 cells, we further measured PARP, pro-caspase 3 and Bcl 2 protein changes in cells, in detail, using a Western blotting assay. Caspase 3 is primarily responsible for the cleavage of PARP (116 kDa) during cell death, producing an 89-kDa fragment. When T 24 and HT 29 cells were treated with cantharidin for 24 h, 116-kDa band activity progressively diminished, while the 85-kDa signal increased dose-dependently, and pro-caspase 3 was reduced (Fig. 3). Pretreatment with the caspase 3 inhibitor markedly inhibited cantharidin-induced T 24 cell death (Fig. 4). Bcl 2 protein was also reduced after cantharidin treatment in HT 29 and T 24 cells, in a dose-dependent manner (Fig. 3). As the above results show, cantharidin could induce apoptosis in T 24 and HT 29 cells after treatment for 24 h.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>IC50 (μM)</th>
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<tbody>
<tr>
<td>T-24</td>
<td>HT-29</td>
</tr>
<tr>
<td>6</td>
<td>21.8</td>
</tr>
<tr>
<td>24</td>
<td>11.2</td>
</tr>
<tr>
<td>48</td>
<td>4.64</td>
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Table 1
The IC50 values of cantharidin in T 24 and HT 29 cells for various durations of treatments
Fig. 2. The cytotoxic effects of cantharidin in T 24 and HT 29 cells in a dose- and time-dependent manner. T 24 (A) and HT 29 (B) cells were treated with cantharidin at 1.57–25 μM for 1, 6, 24, and 48 h, and each experiment was repeated three times.

3.2. Cantharidin-induced G2/M phase cell cycle arrest in T 24 and HT 29 cells

Cell cycle analysis of the T 24 and HT 29 cells treated with cantharidin at 3.13, 6.25, 12.5, and 25.0 μM for 24 h showed alterations in the distribution of the DNA content, using flow cytometric analysis. The significant accumulation of T 24 and HT 29 cells in the G2/M phase is demonstrated in Fig. 5.

Cyclins are a family of proteins implicated in the induction and control of mitosis, while cyclins A and B1/cdk1 regulate the G2/M transition and p21 Cip1/Waf1 is a universal inhibitor of the cell cycle (Malumbres and Barbacid, 2001; Jackman et al., 2003; Xiong et al., 1993). As Fig. 5 shows, the levels of cyclins A and B1/cdk1 were significantly decreased and p21 Cip1/Waf1 enhanced in a concentration-dependent manner (Fig. 6). These results indicate that the G2/M transition of the T 24 and HT 29 cells was destroyed after treatment with cantharidin.

3.3. Cantharidin-induced COX 2 over-expression in T 24 cells

PGE2 level derived via COX protein expressed lead to the inflammatory reaction. When T 24 and HT 29 cells were treated with cantharidin, COX 2 protein was over-expressed in T 24 cells in a dose-dependent manner, but
Fig. 5. Effects of cantharidin on progression through the cell cycle. T24 cells were treated with various concentrations of 3.13–12.5 μM for 24 h (A) and HT 29 (B). C, solvent control (0.05% DMSO). Data are from three separate experiments, one of which is illustrated.

Fig. 6. The protein expression of p21, cdk1, cyclins A, and B1 in cantharidin-treated T24 and HT 29 cells. α-Tubulin was used as an internal control to identify equal amounts of proteins loaded in each lane. T24 cells were treated with various concentrations of 3.13–12.5 μM cantharidin for 24 h (A), and HT 29 (B). C, solvent control (0.05% DMSO). Data are from three separate experiments, one of which is illustrated.

not significantly changed in HT 29 cells (Fig. 7). Then the PGE2 level of the cantharidin-cultured medium in T24 cells was detected using the EIA kit. The results showed the PGE2 level was significantly increased in a dose-dependent manner (Fig. 8).

3.4. Cantharidin stimulated intracellular ATP depletion in T24 cells

Cells will lose a large amount of ATP when cell death via necrosis occurs (Eguchi et al., 1999). Data of Fig. 9 indicates that cantharidin, at the dose more than 12.5 μM, a large decrease in ATP level in T24 cells, but did not do so in HT 29 cells. Therefore, we suggest that cantharidin-induced secondary necrosis in T
Fig. 8. PGE\textsubscript{2} production from cantharidin-treated T\textsubscript{24} cells. Statistical analysis was done using the Student’s t-test. *\( p < 0.05; *** p < 0.005, \) significantly different from the 0.05% DMSO-treated group (without cantharidin).

Fig. 9. The intracellular ATP amount of cantharidin-treated T\textsubscript{24} and HT\textsubscript{29} cells. T\textsubscript{24} (A) and HT\textsubscript{29} (B) cells were treated with cantharidin at 1.57–25 M for 24 h, and each experiment was repeated three times.

4. Discussion

Previous studies have demonstrated that cantharidin could induce a p53-dependent apoptosis in leukemia cell lines (Efferth et al., 2005) and in HT\textsubscript{29} cells by p38 and JNK activation (Huh et al., 2004). On the other hand, cantharidin could also induce acute Hep\textsubscript{3B} cell death, but not via apoptosis at a high dose; the mechanism of cantharidin-induced cell death was the G\textsubscript{2}/M phase arrest of the cell cycle (Wang et al., 2000; Sakoff et al., 2002; Sakoff et al., 2004). One of the reasons may be that the protein phosphatases inhibitor can make an abnormal entry into the S phase of the cell cycle and disturb cyclin-dependent kinase activity (Taylor et al., 2000). Progression through G\textsubscript{1} involves the activation of cyclin D/cdk2, 4, 5 and 6; G\textsubscript{1}/S involves the activation of cyclin E/cdk2; S involves the activation of cyclin A/cdk2, while cyclins A and cyclin B/cdk1 regulate the G\textsubscript{2}/M transition (Malumbres and Barbacid, 2001; Jackman et al., 2003). The controlled activation of the kinase complexes at various intervals of the cell cycle is regulated by the availability of the...
Furthermore, the mitotic inhibitor, p21 Cip1/Waf1 of the cell line. In clinical studies of patients with non-small cell lung cancer, the PGE2 production and COX 2 expression of the tumor tissue increased more with cantharidin (Fig. 7A and 8) but not in HT 29 cells. We suggested that cantharidin-induced COX 2 over-expression in human mammary epithelial cells (Kotha et al., 2000). There were papers shown, a selective induction of COX 2 in with Ras mutation cell line, which results in enhanced chemotherapy resistance (Hoang et al., 2006).

Cantharidin is considered as an anticancer candidate, but its toxic effects are not acceptable. Therefore, several cantharidin analogues have been synthesized that decrease the toxic affects and enhance the anticancer effects (Sakoff et al., 2002; Walter, 1989). However, there has still not been any good anticancer drug development using cantharidin’s derivatives. Since the toxic mechanism of cantharidin is not clear, there is difficulty in developing a new anticancer drug from its components. In summary, we explored the difference in the effects of cantharidin between T 24 and HT 29 cells. According to the above results, cantharidin could significantly induce apoptosis and G2/M phase arrest in T 24 and HT 29 cells, but only induced COX 2 over-expression in T 24 cells. We suggest that cantharidin may induce its partial side effects through COX 2 over-expression in the bladder.

References


