Original Paper

Cantharidin Inhibits the Growth of Triple-Negative Breast Cancer Cells by Suppressing Autophagy and Inducing Apoptosis *in Vitro* and *in Vivo*

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Key Words

Cantharidin • Apoptosis • Autophagy • Triple-negative breast cancer

Abstract

Background/Aims: Cantharidin, a type of terpenoid secreted by the blister beetle *Mylabris phalerata* (Pallas), has attracted great attention in cancer therapy because of its potential anti-cancer activities. Here, we report the effects on apoptosis and autophagy in human triple-negative breast cancer (TNBC) cell lines after treatment with cantharidin and attempt to elucidate the underlying mechanisms. **Methods:** MDA-MB-231 and MDA-MB-468 cells were treated with cantharidin and cell proliferation was examined using CCK-8 and clone formation assays. The expression of apoptosis- and autophagy-associated proteins was detected by western blotting. Cells were infected with lentivirus carrying the Beclin-1 gene, and MDA-MB-231-beclin1 (MB231-Bec) and MDA-MB-468-beclin-1 (MB468-Bec) cells stably expressing Beclin-1 were established. Autophagic vacuoles in cells were observed with LC3 staining using fluorescence microscopy, and apoptotic cells were detected via flow cytometry. Tumor growth was assessed by subcutaneous inoculation of TNBC cells into BALB/c nude mice. **Results:** Cantharidin inhibited the proliferation of MDA-MB-231 and MDA-MB-468 cells, and induced cell apoptosis. Cantharidin additionally inhibited the conversion of LC3 I to LC3 II and autophagosome formation by suppressing the expression of Beclin-1. Furthermore, overexpression of Beclin-1 in TNBC cells attenuated the cytotoxicity of cantharidin. *In vivo*, cantharidin inhibited the growth of MDA-MB-231 and MDA-MB-468 xenografts in nude mice by suppressing autophagy and inducing apoptosis, and Beclin-1 overexpression in TNBC cells reduced the efficacy of cantharidin. **Conclusions:** Cantharidin inhibits autophagy by

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suppressing Beclin-1 expression and inducing apoptosis of TNBC cells *in vitro* and *in vivo*, thereby representing a potential strategy for the treatment of TNBC.

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Introduction

Breast cancer is the most frequently diagnosed cancer and the leading cause of mortality among females, accounting for 23% of the total cancer cases and 14% of cancer-related deaths, according to the global cancer statistics for 2011[1]. Cancers of the breast are classified into five distinct types based on molecular heterogeneity and gene expression: two ER (estrogen receptor)-positive luminal subtypes, HER2 (human epidermal growth factor receptor type 2)-overexpression subgroup, a normal breast gene expression subgroup, and a basal-like subgroup. The basal-like subgroup is characterized by the expression of basal-cell cytokeratins 5/6 and 17 and an absence or low levels of ER and HER2 expression [2]. TNBC (triple-negative breast cancer) was first defined in 2005[3] as comprising tumors lacking expression of ER, PgR (progesterone receptor), and HER2. TNBC accounts for approximately 15% of all types of breast cancer [4]. Early diagnosis of breast cancer significantly improves 5-year survival rates. There are studies focusing on identification of novel biomarkers such as glycoprotein for use in TNBC detection [3]. Still patients with TNBC exhibit relatively poor outcomes, and specific treatment guidelines are not available for this type of cancer. Moreover, these patients are resistant to existing endocrine therapies and HER2-targeted therapies, such as trastuzumab, as a result of specific molecular expression features of TNBC[5]. Therefore, targeted therapeutics is urgently needed.

Cantharidin, a type of terpenoid obtained from the blister beetle (*Mylabris phalerata* Pall. and *Mylabris cichorii* Linn.), is used as a traditional Chinese medicine with anticancer, antibiotic, antiviral, and immune-regulating activities [6, 7]. Recent studies have shown that cantharidin and its derivatives exert cytotoxic effects against cancer cells. In addition, these compounds have been shown to effectively inhibit cell proliferation in numerous cancer cell lines such as leukemic cells [8, 9], ENREF_7, as well as in bladder [10, 11], ENREF_8, colorectal [12], pancreatic [13], hepatic [14, 15], ENREF_11, oral [16], and breast cancers [17, 18], ENREF_16. Previous studies have reported that cantharidin and its derivatives exhibit strong antitumor activity through cell growth inhibition and cell apoptosis induction *in vitro* and *in vivo*. Furthermore, the cytotoxicity of these compounds was found to be independent of estrogen, progesterone and epidermal growth factor receptors. Cantharidin is therefore considered a highly promising candidate agent for treatment of TNBC.

The various types of PCD (programmed cell death), apoptosis, and autophagy are highly regulated by both the extrinsic death receptor (DR) pathway and intrinsic mitochondrial pathway [19]. Apoptosis ultimately leads to cell death, whereas autophagy plays either pro-survival or pro-death roles [20]. Bax belongs to the pro-apoptotic Bcl-2 family and Bcl-2 belongs to the anti-apoptotic Bcl-2 family [21-23], ENREF_21. Activation of PARP and caspase-3 triggers downstream pathways, resulting in apoptosis and cell death [24-27], ENREF_22, ENREF_22. The Beclin-1 pathway plays a role in pro-tumor autophagy [28, 29], and LC3 participates in autophagosome formation [30]. The interplay between apoptosis and autophagy is complex and poorly understood. In this study, we treated TNBC cells with cantharidin and detected cell apoptosis and autophagy after treatment to clarify the cytotoxicity in TNBC cells induced by cantharidin.

In the present study, cantharidin was firstly demonstrated to exert anti-tumor effects in human MDA-MB-231 and MDA-MB-468 triple-negative breast cancer cells by inducing apoptosis and inhibiting pro-survival autophagy by suppressing Beclin-1 expression both *in vitro* and *in vivo*. The present study strongly indicates that cantharidin represents a novel treatment agent and represents a potential strategy for the treatment of TNBC.

Materials and Methods

Reagents

Cantharidin was purchased from Sigma. Propidium iodide (PI) and DAPI (dihydrochloride) were purchased from Thermo Fisher Scientific. PARP, cleaved PARP, caspase 3, cleaved caspase 3, Bax, Bcl-2, LC3B I, LC3B II, p62, Beclin-1, and GAPDH antibodies were purchased from Cell Signaling Technology (MA, USA).

Cell lines and cell culture

The human MDA-MB-231 and MDA-MB-468 breast cancer cell lines were purchased from the American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 U/mL), and penicillin (100 U/mL) at 37°C in a humidified incubator with 5% CO₂.

Beclin-1 overexpressing cell line establishment

Lentivirus carrying Beclin-1 gene were produced by Shanghai Genechem Company. MDA-MB-231 and MDA-MB-468 cells were inoculated in 12-well plate and incubated with complete medium with lentivirus. 24h after seeding, medium contained were aspirated and replaced with 1ml fresh medium. In order to obtain a stable Beclin-1 overexpressing cell line, the lentivirus infected cells were screened by the culture medium containing 2ug/ml of puromycin for 2 weeks.

CCK-8 assay

The CCK-8 assay was used to determine cell viability following treatment with cantharidin. MDA-MB-231 and MDA-MB-468 cells were seeded at a density of 2,000 cells/well in 96-well plates and treated with cantharidin for 72 h. Cell viability was assessed using Cell Counting Kit-8 (CCK-8) (Dojindo) according to the manufacturer's instructions.

Colony formation assay

MDA-MB-231 and MDA-MB-468 cells were plated into a 6-well plate at a concentration of 500 cells/well and treated with 0, 1, and 5 µg/ml cantharidin. Colony formation after 10 days of culture was examined by staining with crystal violet at room temperature for 20 min.

Flow cytometric analysis of apoptosis

Annexin V-FITC Apoptosis Detection Kit (BD) was used to evaluate the percentage of apoptotic cells. MDA-MB-231 and MDA-MB-468 cells were treated with 0, 1, and 5 µg/ml cantharidin for 48 h. Then, 5×10^5 treated cells were centrifuged and re-suspended in cold 1 × binding buffer; after which 1.25 µl of Annexin V-FITC and 10 µl of propidium iodide (PI) were added and the suspension was analyzed by flow cytometry (FACScan).

Immunofluorescence analysis of autophagy

MDA-MB-231 and MDA-MB-468 cells were plated on glass-bottom cell culture dishes (Nest) at 50% confluency and then treated with 0, 1, and 5 µg/ml cantharidin for 48 h. The prepared cells were washed in PBS, fixed in 4% paraformaldehyde (PFA) at room temperature for 15 min, permeabilized in 0.2% Triton X-100 for 15 min, and blocked with 1% bovine serum albumin (BSA) in PBS for 1 h. Then, the cells were stained with the primary LC3 antibody at 4°C overnight and incubated with an Alexa 488-conjugated donkey anti-mouse secondary antibody (Jackson ImmunoResearch) at 37°C for 1 h.

Immunoblotting

Treated cells were collected and suspended in mammalian protein extraction reagent (Thermo) for at least 30 min on ice. Cell lysates were clarified by centrifugation at $12,000 \times g$ for 10 min at 4°C, and the supernatants were mixed with 5 × loading dye and boiled for 10 min. The protein content in the supernatant was measured using a Bio-Rad DC protein assay kit II (Bio-Rad). Proteins were separated by electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel and electrotransferred onto a PVDF membrane. The membrane was blocked in 5% nonfat skim milk and probed with primary antibodies for PARP, cleaved PARP, caspase 3, cleaved caspase 3, Bax, Bcl-2, LC3B I, LC3B II, p62, Beclin-1, and GAPDH; this was followed by

incubation with horseradish peroxidase-conjugated secondary antibodies. Protein expression was detected using an enhanced chemiluminescence system (Amersham Pharmacia).

Xenograft mouse models

All animal experiments were approved and conducted by the Institutional Animal Care and Use Committee. BALB/c nude mice (4-6 weeks-old) were subcutaneously injected with Beclin-1-overexpressing cells and their parent TNBC MDA-MB-231 and MDA-MB-468 cells, followed by treatment with cantharidin or vehicle. Mice were treated with either 10 mg/kg cantharidin or the vehicle through intravenous injection every 2 days. Tumor volumes were measured every three days until mice were sacrificed 3 weeks later.

TUNEL assay

TdT-mediated dUTP nick end-labeling assay (TUNEL) reaction mixture (Roche) was used to detect apoptotic xenograft tumor cells. After incubation with the reaction mixture, samples were analyzed by comparing relative fluorescence units.

Statistical analysis

All statistical analyses were performed using SPSS 14.0 software. Values are presented as the ratio $\pm$ SD of the control. Student's t-test and one-way ANOVA were used to analyze statistical significance. $p < 0.05$ was considered to indicate statistical significance. Each experiment was performed in triplicate.

Results

Growth was inhibited by cantharidin in human TNBC cells

It has been reported that cantharidin inhibits cancer cell proliferation. To evaluate the cytotoxicity of cantharidin against TNBC cells, MDA-MB-231 and MDA-MB-468 cells were treated with a cantharidin gradient for 48 h. As shown in Fig. 1 A and B, cell viability decreased following treatment with cantharidin in a concentration-dependent manner.

Fig. 1. Proliferation inhibition by cantharidin in TNBC cells. (A) Triple-negative breast cancer MDA-MB-231 and MDA-MB-468 cells were treated with 10^{-1} , 10^0 , 10^1 , 10^2 , 10^3 , and 10^4 $\mu\text{g/ml}$ cantharidin for 48 h. Then, cell viability was determined using a CCK-8 assay. The results are the mean $\pm$ SD of independent experiments performed in triplicate. (B) MDA-MB-231 and MDA-MB-468 cells were seeded on the plates and treated with 0, 1, and 5 $\mu\text{g/ml}$ cantharidin. Colony formation was assessed by crystal violet staining after 10 days of culture. The results are the mean $\pm$ SD of independent experiments performed in triplicate. $P < 0.05$ was considered statistically significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control.

In addition, the colony formation assay suggested that the number of developed colonies generated from cantharidin-treated MDA-MB-231 and MDA-MB-468 cells was lower than that among control cells (Fig. 1 C). It was concluded that cantharidin inhibits the growth of MDA-MB-231 and MDA-MB-468 cells in a concentration-dependent manner.

Apoptosis was induced by cantharidin in human TNBC cells

To determine whether cantharidin-induced cytotoxicity resulted from the induction of apoptotic cell death, we investigated the level of apoptosis in TNBC cells following treatment with cantharidin by flow cytometry with Annexin V and PI staining. As shown in Fig. 2A, the apoptotic index was significantly elevated in TNBC cells treated with cantharidin. We then examined the expression of apoptosis-associated proteins in MDA-MB-231 and MDA-

Fig. 2. Induction of apoptosis by cantharidin in TNBC cells. (A) MDA-MB-231 and MDA-MB-468 cells were treated with 0, 1, and 5 $\mu\text{g/ml}$ cantharidin for 48 h. Annexin V-FITC assay was performed to determine the apoptotic index of cells treated with cantharidin at various concentrations; the results were analyzed by flow cytometry. (B) Proteins expressed by treated TNBC cells were examined by immunoblotting. Apoptosis-associated proteins (active PARP, active caspase 3, Bax and Bcl2) were detected using the corresponding antibodies with GAPDH as the control. The results are the mean $\pm$ SD of independent experiments performed in triplicate. $P < 0.05$ was considered statistically significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control.

MB-468 cells. As shown in Fig. 2B, cantharidin treatment increased the expression of Bax, cleaved Caspase-3, and PARP, but had no effect on the expression of Bcl-2. Taken together, these findings show that cantharidin induces apoptosis of MDA-MB-231 and MDA-MB-468 cells.

Autophagy was inhibited by cantharidin in human TNBC cells

Furthermore, we explored whether cantharidin affects TNBC cell autophagy. Autophagy in MDA-MB-231 and MDA-MB-468 cells was detected via testing LC3 puncta by immunofluorescence staining with an LC3 antibody and the conversion of LC3 I to LC3 II by western blot, which are both classic indices for autophagy detection. Cantharidin treatment resulted in significant autophagy inhibition, as evidenced by a decrease in LC3 puncta (autophagosomes) (Fig. 3A). Consistently, western blot data showed that cantharidin decreased the conversion of LC3-I into LC3-II in MDA-MB-231 and MDA-MB-468 cells (Fig. 3B). Western blot results suggested that the expression of Beclin-1, a vital autophagy-induced protein, decreased after cantharidin treatment. Taken together, the data show that cantharidin inhibits autophagy in human TNBC cells by inhibiting expression of Beclin-1.

Beclin-1 overexpression attenuated the cytotoxicity of cantharidin in vitro

MDA-MB-231 and MDA-MB-468 cells were infected by lentivirus carrying the Beclin-1 gene, and MDA-MB-231-Beclin-1 (MB231-Bec) and MDA-MB-468-Beclin-1 (MB468-Bec)

Fig. 3. Inhibition of autophagy in TNBC cells by cantharidin. (A) MDA-MB-231 and MDA-MB-468 cells were seeded on plates and treated with 0, 1, and 5 µg/ml cantharidin for 48 h. Autophagosome formation was determined using immunofluorescence with an LC3 antibody. (B) Proteins expressed by treated TNBC cells were examined by immunoblotting. Autophagy-associated proteins (LC3 II, p62, and Beclin-1) were detected using the corresponding antibodies with GAPDH as the control.

Fig. 4. Pro-survival autophagy in cantharidin-treated TNBC cells, Beclin-1-overexpressing cells, and their parent TNBC MDA-MB-231 and MDA-MB-468 cells. Cells were treated with cantharidin at 5 µg/ml for 48 h. (A, B) Cell viability was then determined using the CCK-8 assay. (C) Annexin V-FITC assay was performed to examine Beclin-1 expression and the apoptotic index of cells following various treatments. Results were analyzed by flow cytometry. (D) Proteins expressed by cells were examined by immunoblotting. Autophagy-associated proteins (LC3 II, p62, and Beclin-1) and an apoptosis-associated protein (active caspase 3) were detected using the corresponding antibodies with GAPDH as the control. The results are the mean ± SD of independent experiments performed in triplicate. $P < 0.05$ was considered statistically significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus cantharidin in Beclin-1 overexpressing cells.

cells stably expressing Beclin-1 were established. The cells stably expressing Beclin-1 and their parent cells were treated with cantharidin at 5 µg/ml for 48 h, and cell viability was analyzed. As shown in Fig. 4A and 4B, Beclin-1 expression promoted TNBC cell proliferation.

Fig. 5. Effects of cantharidin and putative underlying mechanisms in BALB/c nude mice. (A, B) Beclin-1-overexpressing cells and their parent TNBC MDA-MB-231 and MDA-MB-468 cells were subcutaneously injected into the right flanks of nude mice in each group, and tumor volume was measured every three days. Mice were either treated with 10 mg/kg cantharidin or with control vehicle through intravenous injection every 2 days. (C, D) After the mice were sacrificed, cell apoptosis of each xenograft was determined by TUNEL assay. Green fluorescence represents apoptotic cell and blue fluorescence indicates cell nuclei. $P < 0.05$ was considered statistically significant, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ versus the control, # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ versus cantharidin in Beclin-1 overexpressing tumors.

In addition, the inhibition of cell growth by cantharidin was attenuated in Beclin-1-overexpressing cells. Furthermore, FACS results suggested that the rate of apoptosis decreased in Beclin-1 overexpressing cells (Fig. 4C). Western blot results were similar to the FACS results (Fig. 4D). Taken together, the data show that Beclin-1 overexpression attenuated the cytotoxicity of cantharidin *in vitro*.

Cantharidin inhibited the growth of TNBC xenografts in nude mice by inhibiting autophagy and inducing apoptosis in vivo.

To examine the effect of cantharidin *in vivo*, nude mice bearing MDA-MB-231 and MDA-MB-468 tumor xenografts and Beclin-1 overexpressing tumors were treated with vehicle and cantharidin at a dose of 10 mg/kg through intravenous injection every 2 days. Tumor volume was measured every 3 days and tumor weight was measured at the end of the study (Fig. 5A, 5B). The data suggest that cantharidin inhibited the growth of TNBC xenografts in nude mice, and that Beclin-1 overexpression in cells reduced the efficacy of cantharidin. TUNEL staining demonstrated a significant increase in apoptotic cells in the cantharidin-

treated group, and indicated decreased apoptosis in Beclin-1 overexpressing cells (Fig. 5C, 5D). Together, these data show that cantharidin inhibited the growth of TNBC xenografts in nude mice by inhibiting autophagy and inducing apoptosis *in vivo*.

Discussion

Cantharidin is reported to exert cytotoxic effects against various kinds of cancer cells, including breast cancers. In this study, cantharidin was shown to exert strong cytotoxicity against human MDA-MB-231 and MDA-MB-468 triple-negative breast cancer cells, inhibiting cell proliferation *in vitro* or *in vivo* by inducing apoptosis and inhibiting autophagy. Moreover, our results showed that cantharidin inhibited both the conversion of LC3 I to LC3 II and autophagosome formation by suppressing the expression of Beclin-1. However, Beclin-1 overexpression was found to attenuate the cytotoxicity of cantharidin, which suggests that cantharidin may inhibit pro-survival autophagy to attenuate TNBC cell proliferation. The *in vivo* assay also showed that cantharidin inhibited the growth of TNBC xenografts by suppressing autophagy and inducing apoptosis, and that Beclin-1 overexpression in TNBC cells decreased the efficacy of cantharidin. Therefore, cantharidin induced TNBC cell apoptosis and inhibited pro-survival autophagy by suppressing Beclin-1 both *in vitro* and *in vivo*. The findings suggest that cantharidin is a potential agent for the treatment of TNBC.

Cantharidin, an active compound obtained from blister beetles, is a known inhibitor of PP1 and PP2A (serine/threonine protein phosphatases types 1 and type 2A) [31]. It has been shown to play important roles in the regulation of cell growth [32, 33], cell cycle [34–36], and cell fate determination [37]. Cantharidin exhibits strong cytotoxicity and induces apoptosis in human leukemic cells by activation of the p38 and JNK kinase pathways associated with p53 and caspase-3 [9]. Cantharidin has additionally been confirmed to induce G2/M arrest and apoptosis through mitochondrial-dependent signaling pathways in bladder cancer cells [11]. Another study has shown that cantharidin induces G2/M phase arrest and apoptosis in human colorectal cancer cells through inhibition of CDK1 activity and caspase-dependent signaling pathways [12]. Therefore, the anti-tumor cytotoxicity of cantharidin has been demonstrated to be effective in various cancer cells; however, the underlying mechanisms are potentially complex and may differ according to the specific cancer. In the present study, cantharidin was found to be cytotoxic to TNBC cells, both *in vitro* and *in vivo*, and to induce cell apoptosis and inhibit cell autophagy. The attenuation of cantharidin-induced cytotoxicity by Beclin-1 suggests that the Beclin-1 pathway is involved in the inhibition of autophagy. Previous studies have indicated that the effects of cantharidin in cancer cells may involve DNA damage, cell cycle arrest, and mitochondria- or caspase-dependent apoptosis pathways and associated regulatory proteins. Therefore, more studies are needed to understand the mechanism beyond beside apoptosis and autophagy. The development of cantharidin-based therapies against TNBC requires further elucidation of these mechanisms.

The present immunofluorescence data show that cantharidin inhibits autophagosome formation. The immunoblotting results further indicate that cantharidin suppressed both the conversion of LC3 I to LC3 II and Beclin-1 expression. Beclin-1 overexpression attenuated cantharidin-induced inhibition of cell proliferation inhibition. Autophagy is a homeostatic process involving the degradation of cellular proteins and damaged, obsolete organelles [38]; these proteins and organelles are engulfed by autophagosomes, digested in lysosomes, and recycled to sustain protein homeostasis, cellular metabolism, and cell self-renewal [39]. Autophagy has dual roles in cancer as both a tumor suppressor and tumor promoter [40]. The inhibition of pro-survival autophagy has been shown to trigger apoptotic cell death and kill cancer cells [28, 41–43]; furthermore, in preclinical models, the administration of autophagy inhibitors in combination with chemotherapy induced cancer cell death and inhibited cell growth to a greater extent than chemotherapy alone [44]. Our study has confirmed that cantharidin, when administered alone, was able to inhibit autophagy in TNBC cells and cantharidin could be considered as a potential treatment agent for TNBC. While

the administration of cantharidin combined with autophagy inhibitors, such as chloroquine or hydroxychloroquine [41], may enhance anti-tumor cytotoxicity and exerts stronger cell proliferation-inhibiting effects, which needs further study.

TNBC is characterized by the lack of estrogen, progesterone, and epidermal growth factor receptors, which makes this cancer resistant to existing endocrine therapies and targeted therapies. Therefore, novel therapeutic strategies against TNBC are urgently needed. Cantharidin and its derivative norcantharidin have both been demonstrated to induce cell apoptosis and inhibit cell growth, adhesion, and migration in human breast cancer cells [18]. Cantharidin additionally exerts potent anti-tumor effects. We only treated the TNBC cells with cantharidin resulting in cancer cells proliferation inhibition. Several studies aimed at the development of synthetic cantharidin analogues with strong anti-tumor ability and fewer side effects have been reported [45]. In addition, the development of safer and more effective cantharidin derivatives may be considered as a strategy for the treatment of TNBC. Recent studies showed that thiostrepton [1] and bicalutamide [2] may also be potential therapeutic approach, which can be developed as promising combination therapeutic strategies for treating TNBC patients.

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Disclosure Statement

The authors declare that they have no conflict of interest. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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