Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), a type II transmembrane protein, is a part of the TNF superfamily of cytokines. Cantharidin, a type of terpenoid, is extracted from the blister beetles (Mylabris genus) used in Traditional Chinese Medicine. Cantharidin elicits antibiotic, antiviral and antitumor effects, and can affect the immune response. The present study demonstrated that a cantharidin and TRAIL combination treatment regimen elicited a synergistic outcome in TRAIL-resistant DU145 cells. Notably, it was also identified that cantharidin treatment initiated the downregulation of cellular FLICE-like inhibitory protein (c-FLIP) and upregulation of death receptor 5 (DR-5), and sensitized cells to TRAIL-mediated apoptosis by initiating autophagy flux. In addition, cantharidin treatment increased lipid-modified microtubule-associated proteins 1A/1B light chain 3B expression and significantly attenuated sequestosome 1 expression. Attenuation of autophagy flux by a specific inhibitor such as chloroquine and genetic modification using ATG5 small interfering RNA abrogated the cantharidin-mediated TRAIL-induced apoptosis. Overall, the results of the present study revealed that cantharidin effectively sensitized cells to TRAIL-mediated apoptosis and its effects are likely to be mediated by autophagy, the downregulation of c-FLIP and the upregulation of DR-5. They also suggested that the combination of cantharidin and TRAIL may be a successful therapeutic strategy for TRAIL-resistant prostate cancer.

Introduction

Prostate cancer (PCa) is one of the major types of malignancy in men, with a high incidence rate (1). Currently, the primary treatment strategies for these tumors are surgical castration combined with androgen deprivation therapy or antiandrogens, radiotherapy, chemotherapy, immunotherapy and other combined treatments (2,3). In patients with PCa, the aim of these treatments is initiation of apoptosis in tumor cells, thereby inhibiting cancer growth and spread.

Cantharidin, a type of terpenoid, is extracted from the blister beetles (Mylabris genus) and is used in Traditional Chinese Medicine (4,5). Cantharidin possesses antibiotic, antiviral and antitumor qualities, and can affect immune responses (4,6). Cantharidin has been used as a medicinal agent for >2,000 years and has a number of applications, including treatment of edema and warts (4). Cantharidin treatment initiates cell cycle arrest and attenuates apoptosis in various types of carcinomas including those of the bladder (7), breast (8), liver (9,10), and colon (11), buccal mucosa and leukemia (12,13).

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is the only identified protein that can specifically initiate apoptosis of tumor cells without affecting noncancerous cells (3). TRAIL, a type II transmembrane protein, is a part of the TNF superfamily of cytokines identified in 1995 by Wiley et al (14). TRAIL is able to bind the death receptors (DR)-4 and DR-5, promoting the death-inducing signaling complexes activity of caspase-3 and caspase-8, thereby initiating tumor cell death (15). However, downregulation of the death receptors and pro-apoptotic proteins, and upregulation of the anti-apoptotic proteins such as cellular FLICE-like inhibitory protein (c-FLIP), leads to TRAIL-mediated apoptosis resistance (16-19). Therefore, identification of TRAIL sensitizers is may help to overcome the barrier of TRAIL resistance.
Autophagy is a multi-step cellular process in which cytoplasmic constituents are targeted to lysosomes for degradation, and is associated with several diseases including cancer (20). Recent studies have revealed that autophagy has a dual mechanism, having either pro-survival or pro-death characteristics depending on the cell type and stimulus strength (21-23). Autophagy acts in a pro-survival manner through cytoprotective events that favor cell survival and decrease the incidence of cell death. Conversely, autophagy can augment a pro-death signaling system and ultimately result in cell death in cancer (24). The complete autophagic flux engages microtubule-associated proteins 1A/1B light chain 3B (LC3), which serves an important role in cargo elimination, phagophore elongation and autophagosome composition, and the sequestosome 1 (p62)-cargo system is selectively attached to autophagosomes through the interaction of p62 and LC3 (20).

In the present study, the effectiveness of cantharidin in sensitization of PCa cells to TRAIL was examined. We aimed to elucidate the molecular pathways underlying the effect of cantharidin and synergistic effect of cantharidin and TRAIL in DU145 cells. It was identified that cantharidin treatment sensitizes DU145 tumor cells to TRAIL-mediated apoptosis via autophagy flux. The combined treatment of cantharidin and TRAIL may represent a viable therapeutic strategy for certain cases of TRAIL-resistant cancer.

Materials and methods

Cell culture. Human prostate DU145 and LNCaP cell lines were maintained in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. During the experiments, the culture medium was changed to RPMI-1640 containing 1% FBS. In the present study, TRAIL treatment intervals of 1 and 2 h were used for the western blot analysis and the cell viability assay, respectively. It was identified that a 2 h treatment period for the combination of TRAIL and cantharidin induced a high level of cell death that affected the detection of certain proteins. The cells were maintained at 37°C and 5% CO₂ in a humidified incubator.

Reagents. Cantharidin was acquired from Sigma-Aldrich; Merck KGaA, while TRAIL was attained from AbFrontier Co., Ltd.

Cell viability assay. The DU145 and LNCaP cells were plated 2x10⁵ cells/well in 12-well plates and treated with cantharidin (0, 0.25, 0.50 and 1 µM) for 18 h and were further exposed to TRAIL (200 ng/ml) for an additional 2 h. For autophagy inhibition, cells were treated 10 µM chloroquine (CQ) 1 h prior to cantharidin or TRAIL treatment at 37°C. Cell morphology was assessed under a light microscope (Nikon corporation) at magnification, x400. Untreated cells were used as control, and the cell viability was compared to the control. Each treatment was performed in triplicate.

Immunofluorescence staining. DU145 cells were cultured on poly-L-lysine-coated coverslips. Following cantharidin treatment with or without CQ or ATG5 siRNA, the cells were fixed with 4% paraformaldehyde at 4°C for 10 min and permeabilized with 0.1% Triton X-100. The cells were then incubated for 1 h at room temperature in a blocking solution (5% FBS in TBS) followed by overnight incubation at 4°C with anti p62 antibody (1:250; cat. no. PAS-20839; Invitrogen; Thermo Fisher Scientific, Inc.). Following washing with PBS, the cells were incubated with the Alexa Fluor® 488-conjugated donkey polyclonal anti-rabbit secondary antibody (1:500; cat. no. A-21206; Thermo Fisher Scientific, Inc.) for 2 h in the dark. Finally, immunostaining was visualized under a fluorescence microscope at magnification, x200.

Western blot analysis. Western blot analysis was performed as described previously (26). Immunoprecipitation assay buffer (Qiagen, Inc.) was used for total protein extraction. The supernatant was collected by centrifugation at 11,200 x g and 4°C for 10 min. The protein concentration was determined using a BCA protein assay kit (Thermo Fisher Scientific; Inc.). Protein (30 µg) were separated on 10% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membranes were blocked with 5% non-fat dried milk at 25°C for 1 h. Then membranes were incubated with primary antibodies for 1 h at 25°C, the following antibodies were used: DR-5 (1:1,000; cat. no. ab181846; Abcam); β-actin (1:1,000; cat. no. A5441; Sigma-Aldrich; Merck KGaA); c-FLIP (1:1,000; cat. no. ADI-AAP-440; Enzo life sciences, USA); LC3A/B (1:1,000; cat. no. A-21206; Cell Signaling Technology, Inc.); p62 (1:250; cat. no. PAS-20839; Invitrogen; Thermo Fisher Scientific, Inc.); ATG5 (1:1,000; cat. no. c.s12994s; Cell Signaling Technology, Inc.); and cleaved caspase-8 (1:1,000; cat. no. 551242; BD Pharmingen; BD Biosciences). Then membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat. nos. ADI-SAB-100 and ADI-SAB-300; Enzo Life Science, Inc.) at 25°C for 1 h. The immune reactive protein bands were visualized using enhanced chemiluminescence detection system (GE Healthcare Life Sciences) and detected with chemiluminescence imaging system (Fusion FX7; Viber Lourmat). The intensities of the protein bands were determined using Image J Java 1.8.0 software (National Institutes of Health).

Transmission electron microscopy (TEM) analysis. Following the fixation of cells in 2% glutaraldehyde (Electron Microscopy Sciences) and 2% paraformaldehyde (Electron Microscopy Sciences) in 0.05 M sodium cacodylate (pH 7.2; Electron Microscopy Sciences) for 2 h at 4°C, the specimens were fixed in 1% osmium tetroxide (Electron Microscopy Sciences) for 1 h at 4°C, dehydrated in increasing ethanol concentration (25, 50, 70, 90 and 100%) for 5 min each, and embedded in...
Embed 812 epoxy resin (Electron Microscopy Sciences) for 48 h at 60˚C according to the manufacturers' protocol. Ultrathin sections (60 nm) were prepared using a LKBIII ultratome (Leica Microsystems GmbH) and stained with 0.5% uranyl acetate (Electron Microscopy Sciences) for 20 min and 0.1% lead citrate (Electron Microscopy Sciences) for 7 min at room temperature. Images were captured using a Hitachi H7650 electron microscope (Hitachi, Ltd.) at magnification, x10,000, installed at the Center for University-Wide Research Facilities (CURF), Jeonbuk National University.

Figure 1. Effect of Can on TRAIL-mediated apoptosis in prostate cancer cells. DU145 cells were treated with Can (0, 0.25, 0.50 and 1 µM) for 18 h and then with TRAIL (200 ng/ml) for an additional 2 h. (A) The morphology of DU145 cells was evaluated by interference light microscopy (scale bar=100 µm; magnification, x100). (B) Cellular viability was assessed with crystal violet staining in DU145 cells. (C) Quantification of the average density of crystal violet staining in DU145 cells. (D) Cellular viability was evaluated with trypan blue dye exclusion assays in DU145. (E) Morphology, (F and G) crystal violet cellular viability and (H) trypan blue staining in LNCaP cells. *P<0.05, **P<0.01 and ***P<0.0001. Can, cantharidin; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
Small interfering RNA transfection. DU145 cells were seeded 1x10^5 cells/well in 24-well plates for 24 h and then transfected with Silencer Select small interfering RNA (ATG5 siRNA; oligo ID HSS114103; Sequence GGUUUGGACGAAUUC CAACUGUUU; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Concomitantly, non-targeting siRNA was transfected as a negative control. The cells were incubated with ATG5 siRNA or negative control siRNA for 6 h and the medium was then changed to RPMI-1640 with 10% FBS for 24 h. The cells were then treated with neferine, or neferine in combination with TRAIL.

Statistical analysis. Statistical analyses were performed using GraphPad v.5.01 software (GraphPad Software Inc.). All experiments were performed in triplicate, and the data are expressed as the mean ± standard error of the mean. Significant differences between control and treated samples were analyzed by one-way analysis of variance followed by Tukey's post hoc test.

Results

Effect of cantharidin on TRAIL-mediated apoptosis in PCa cells. To observe the potential suppressive effect of cantharidin on PCa cell growth, DU145 and LNCaP cells were treated consecutively with cantharidin at different concentrations for 18 h and then TRAIL for an additional 2 h. As shown in Fig. 1, TRAIL or cantharidin alone only marginally induced cell death, and no morphological changes were observed. In
addition, the combined regimen of cantharidin and TRAIL significantly attenuated cell viability, whereas treatment with cantharidin or TRAIL alone had no effect on apoptosis (Fig. 1A-H). The results suggested that cantharidin effectively sensitized human PCA cells to TRAIL-mediated apoptosis.

**Cantharidin induced autophagy and enhanced cells to TRAIL-mediated apoptosis.** In response to cantharidin treatment, DU145 cells exhibited significantly upregulated LC3-II expression levels and attenuated p62 expression levels (Figs. 2A, and S1A and B). The protein levels assayed by immunofluorescence staining were consistent with the results of western blot analysis (Figs. 2B and S1C). TEM demonstrated that both autophagic and vacuolar vacuoles were secreted in cantharidin-treated cells (Fig. 2C). Cells treated with cantharidin and TRAIL in combination exhibited increased expression of activated caspase (Ac-cas)-3 and Ac-cas8, respectively (Fig. 2D). These data suggest that cantharidin can initiate autophagy in DU145 cells.

Autophagy inhibition rescues TRAIL-mediated apoptosis sensitization through cantharidin. Autophagy control was further confirmed by examining the autophagy flux following treatment with CQ. CQ promoted the accumulation of membrane-bound LC3-II and led to an increase in p62 levels (Figs. 3A, and S2A and B). Immunofluorescence staining confirmed the increase in p62 expression (Figs. 3B and S2C). However, co-treatment with cantharidin, TRAIL and CQ attenuated the upregulation of Ac-cas3 and Ac-cas8 (Fig. 3C). The effects of cantharidin on the viability of the DU145 cells in response to CQ were also investigated. Cell morphology analysis confirmed that CQ treatment more effectively attenuated the cell death compared with the combined regimen of cantharidin and TRAIL (Fig. 4A). Co-treatment with cantharidin, TRAIL and CQ markedly increased the viability of DU145 cells (Fig. 4B-D). This suggests that cantharidin sensitized TRAIL-mediated apoptosis may be attenuated through CQ by blocking autophagy flux.
Figure 4. Can-mediated sensitization of TRAIL-mediated apoptosis is rescued by autophagy attenuation. DU145 cells were pretreated with CQ (10 µM) for 1 h prior to their exposure to Can (1 µM) for 18 h and then to TRAIL for an additional 2 h. (A) The morphology of the cells was evaluated by interference light microscopy (scale bar=100 µm; magnification, x100). (B) Cellular viability was assessed by crystal violet staining. (C) Bar chart demonstrating the average density of crystal violet staining. (D) Cellular viability was evaluated with trypan blue dye exclusion assays. *P<0.05 and **P<0.0001 vs. control and each treatment group, respectively. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CQ, chloroquine; Can, cantharidin.

Figure 5. Genetic attenuation of autophagy rescues Can-sensitized TRAIL-mediated apoptosis through the activation of autophagy flux. DU145 cells were pretreated with ATG5 siRNA or negative control siRNA for 24 h prior to their exposure to Can (1 µM) for 18 h. (A) The levels of LC3-II, p62 and ATG5 were assessed by western blot analysis. (B) Cells were immunostained with p62 antibody (green) and evaluated for fluorescence (scale bar=50 µm; magnification, x200). DU145 cells were pretreated with ATG5 siRNA or negative control siRNA for 24 h prior to their exposure to Can (1 µM) for 18 h and then to TRAIL for an additional 1 h. (C) The levels of Ac-cas3 and Ac-cas8 were assessed by western blot analysis. TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Can, cantharidin; ATG5, autophagy-related 5; siRNA, small interfering RNA; LC3-II, lipid-modified microtubule-associated proteins 1A/1B light chain 3B; p62, sequestosome 1; NC, negative control.
Genetic attenuation of autophagy rescues cantharidin-sensitized TRAIL-mediated apoptosis. To investigate the inhibition of autophagic flux, the autophagy-related 5 (ATG5) gene was knocked down. ATG5 knockdown decreased cantharidin-initiated expression of LC3-II and markedly increased p62 protein levels (Figs. 5A and S3A). The protein levels of p62, determined by immunofluorescence staining, were consistent with those determined by western blot analysis (Figs. 5B and S3B). However, co-treatment with cantharidin, ATG5 siRNA and TRAIL attenuated the increase in Ac-cas3 and Ac-cas8...
expression (Fig. 5C). Cell morphology analysis confirmed that the ATG5 siRNA regimen regulated cell death more effectively compared with combined treatment of cantharidin and TRAIL (Fig. 6A). Co-treatment with cantharidin, TRAIL and ATG5 siRNA markedly increased the viability of DU145 cells and significantly attenuated cell death (Fig. 6B-D). These data indicate that cantharidin-sensitized prolongation of TRAIL-mediated apoptosis can be facilitated by genetic attenuation of autophagy and induction of autophagy flux.

**Discussion**

The present study investigated the mechanism of cantharidin-mediated TRAIL sensitization. Cantharidin treatment induced autophagy flux, decreased c-FLIP expression and increased DR-5 expression. It also enhanced the cleavage of caspase-8 and caspase-3 leading to the induction of apoptosis. Furthermore, cantharidin treatment sensitized TRAIL-mediated apoptosis in multiple cancer cells. Therefore, the results suggested that cantharidin treatment may be used for suppression of TRAIL-resistance.

**Downregulation of c-FLIP and upregulation of DR-5 by cantharidin rescues TRAIL resistance.** The results from the western blot analysis revealed that cantharidin attenuated the expression of c-FLIP and upregulated the expression of DR-5 (Figs. 7A and S4A and B). The combined regimen of cantharidin and TRAIL decreased c-FLIP and increased DR-5 levels (Figs. 7B and S4C and D). c-FLIP expression was increased and DR-5 upregulation was partially inhibited by combined treatment with CQ and cantharidin (Fig. 7C). Furthermore, a combined regimen of ATG5 siRNA and cantharidin increased c-FLIP levels while DR-5 upregulation was partially inhibited (Fig. 7D). These data suggest that cantharidin down-regulated c-FLIP expression and upregulated DR-5 expression and thereby sensitized TRAIL-mediated apoptosis through the initiation of autophagy flux.

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**Availability of data and materials**

All data generated or analyzed during the present study are included in this published article or are available from the corresponding author upon reasonable request.

**Authors’ contributions**

UMN, HY and SYP designed, executed the study and analyzed the data. UMN and HY wrote the manuscript. All authors have read and approved the final manuscript.

**Ethics approval and consent to participate**

Not applicable.

**Patient consent for publication**

Not applicable.
Competing interests

The authors declare that they have no competing interests.

References


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