Pharmacological Reports 68 (2016) 874–880

Contents lists available at ScienceDirect

Pharmacological Reports

journal homepage: www.elsevier.com/locate/pharep

Original article

Norcantharidin induces apoptosis in human prostate cancer cells through both intrinsic and extrinsic pathways

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ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form 12 April 2016
Accepted 15 April 2016
Available online 29 April 2016

Keywords:
Norcantharidin
Prostate cancer
Apoptosis
Bcl-2 family
Inhibitor of apoptosis (IAP)

ABSTRACT

Background: Norcantharidin, a modified pure compound from blister beetles, was previously demonstrated to induce apoptosis of cancer cells. This study investigated its anti-cancer activity in prostate cancer cells and the mechanisms involved.
Methods: Two human prostate cancer cell lines, 22Rv1 and Du145, were treated with norcantharidin at concentrations ranging from 3 to 30 μg/ml. Cytotoxic effect of norcantharidin was determined by use of the 3-(4,5-dimethylthiazol-yl)-diphenyl tetrazoliumbromide (MTT) assay. The effects of apoptosis were evaluated by cell death assay, Caspase-3, -8, -9 activity and cytochrome *c* release. The apoptotic related protein expressions (Bcl-2 family and inhibitor of apoptosis proteins) were determined using western blotting.
Results: An MTT assay revealed that norcantharidin induced cytotoxicity against both prostate cancer cells in dose- and time-dependent manners. Treatment with norcantharidin at 3 μg/ml or higher significantly increased oligonucleosomal formation with concomitant appearance of PARP cleavage, implicating the induction of apoptosis. Norcantharidin intrinsically elevated cytosolic cytochrome *c* levels and activated caspase-3, -8, and -9. Extrinsically, it upregulated the expression of not only the death receptors Fas and DR5 in 22Rv1 cells, but also of RIP and TRADD adaptor proteins in Du145 cells. Mechanistically, norcantharidin increased ratios of pro-/anti-apoptotic proteins and decreased expression of IAP family member proteins, including cIAP1 and survivin, regardless of the distinct status of androgen receptor expression in both cells.
Conclusions: Norcantharidin exhibited cytotoxicity against 22Rv1 and Du145 prostate cancer cells by inducing both intrinsic and extrinsic apoptotic pathways and could thus potentially be a remedy for prostate cancer.

Introduction

Prostate cancer (PCa) is the most common malignant tumor in men in the United States. In 2014, approximately 233,000 new cases and 29,480 deaths were estimated in USA [1]. Currently, the major strategies for treating PCa encompass surgery, radiation and hormonal therapy. Hormonal therapy is frequently used in combination with radiotherapy and postoperative cancer recurrence, but it often leads to severe complications that affect life quality of patients, and eventually develops to castration-resistant prostate cancer (CRPC). Accordingly, chemotherapy becomes the sole treatment modality for CRPC. Since 2004, docetaxel has become the treatment of choice in metastatic CRPC, but it presents severe limitations including drug resistance and disease relapse [2].

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http://dx.doi.org/10.1016/j.pharep.2016.04.010
1734-1140/

Norcantharidin, a synthetic demethylated analog of cantharidin isolated from blister bettles (*Mylabris phalerata* Pall.). It has the advantages of easy synthesis, lower intrinsic toxicity and retaining anticancer activity [3]. Norcantharidin has been shown to inhibit the growth of numerous cancer cell lines via apoptosis, autophagy and cell cycle arrest, including oral cancer [4], hepatoma [5–8], leukemia [9], colorectal adenocarcinoma [10–12], melanoma [13,14], glioblastoma [15], gallbladder carcinoma [16] and PCa [17–19]. *In vivo* studies using animals bearing tumor xenografts have demonstrated that norcantharidin is able to prolong host survival rate. It inhibits angiogenesis, reduces pulmonary metastatic capacity, inhibit tumor growth by down-regulating NF-κB p65 expression and decrease plasma VEGF level, but without renal and liver toxicity [5,16,20–22]. The molecular mechanisms underlying norcantharidin-elicited cytotoxicity involve inhibition of cell adhesion, caspases activation, inhibition of invasion and metastasis, activation of the MAPK and protein kinase C (PKC) pathways, regulation of Bcl-2 family proteins and overcoming multidrug resistance [6,10,12,13,23–26].

Our previous *in vitro* studies have demonstrated that norcantharidin can induce apoptosis in human colorectal and breast cancer cell lines [12,25]. To extend its applicability, the current study aimed to investigate whether norcantharidin can inhibit PCa cell growth. And to elucidate the mechanisms involved. The cytotoxic effect of norcantharidin was tested in two human PCa cell lines with opposite status of androgen dependency (i.e., androgen-dependent 22Rv1 and androgen-independent Du145 cells). The norcantharidin-interrupted expression of apoptotic mediators in both intrinsic and extrinsic pathways was also analyzed.

Materials and methods

Chemical reagents

Norcantharidin was purchased from Sigma (Sigma, St. Louis, MO, USA) and prepared by serial dilutions in culture medium. Primary antibodies against Bax, Bak, Bad, Bid, Bcl-2, Bcl-xL, Mcl-1, Fas, RIP, TRADD, DR5, XIAP, survivin, cIAP1, cIAP2, poly (ADP-ribose) polymerase (PARP) and goat-anti-rabbit secondary antibody were obtained from Cell Signaling Technology (Beverly, MA, USA). Primary antibodies against actin and was from Millipore Biotechnology (Billerica, MA, USA). The goat-anti-mouse secondary antibody was from PerkinElmer (Wellesley, MA, USA).

Cell culture

Human PCa cell lines, 22Rv1 and Du145, were purchased from the National Health Research Institute Cell Bank, Taiwan. 22Rv1 cells were grown in RPMI 1640 medium with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Du145 cells were grown in 90% Minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate. The media of both cell lines contained 50 μg/ml gentamicin and 10% fetal bovine serum. The cell cultures were maintained in a humid chamber at 37 °C under 95% humidified air/5% CO_2 atmosphere.

Evaluation of antitumor activity

Norcantharidin antitumor activity was evaluated using a microculture tetrazolium test (MTT) (Sigma). Briefly, PCa cells (about 5000 cells in 100 μl complete medium per well) were seeded into a 96-well plate (Nunc, Roskilde, Denmark). After incubation at 37 °C for 24 h, 100 μl of culture medium with or without norcantharidin was added to each well in triplicate for consecutive 24, 48, and 72 h of incubation at 37 °C. Then 50 μl MTT solution (Sigma) was added to each well. Following incubation for an additional 4 h at 37 °C, supernatants were removed and 100 μl DMSO was added to dissolve the MTT-formazan product. Absorbance was read using a microplate reader (Labsystems, Helsinki, Finland) at 550 nm. Cell growth inhibition at each norcantharidin concentration was measured and the IC_{50} values for all incubation periods were calculated and normalized by setting the untreated control group as 100%.

Cell death determination

Human PCa cells were seeded into 96-well plates and cultured for 24 h. Cells were treated with or without norcantharidin for another 24 h. After treatment, DNA fragmentation was determined by examining the cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, cell lysates from untreated and norcantharidin-treated cells were transferred to a streptavidin-coated microplate. A mixture of biotinylated anti-histone- and horseradish peroxidase (HRP)-conjugated anti-DNA antibodies were added to cell lysates and incubated for 2 h. The complex was then conjugated to form an immune complex on the plate, which then was read for optical density at 405 nm. The enrichment of mono-oligonucleosomes in cell lysates was calculated as absorbance of norcantharidin-treated cells/absorbance of untreated controls.

Caspase activity assay

PCa cells treated with or without norcantharidin were subjected to caspase activity assay according to the manufacturer's protocol (R&D systems, Minneapolis, MN, USA). Briefly, cell lysates (100 μg total protein) were added to the reaction mixtures (final volume of 50 μl) containing colorimetric substrate peptides specific for caspase-3 (DEVD-pNA), caspase-8 (IETD-pNA), or caspase-9 (LEHD-pNA). The reaction was performed at 37 °C for 2 h. Absorbance at 405 nm was determined with a microplate reader.

Cytochrome c *assay*

The cytochrome *c* ELISA kit (Assay Designs, Ann Arbor, MI, USA) was used to determine whether cytochrome *c* was released during norcantharidin-induced apoptosis. Briefly, after treatment with norcantharidin, cells were collected and washed with PBS, resuspended in Digitonin cell permeabilization buffer, and incubated for 5 min on ice. After centrifugation, supernatants were collected for the determination of cytochrome *c* in the cytosol. All samples were diluted and added into the cytochrome *c* 96-well plate. After incubation for 1 h at 500 rpm, the cytochrome *c* conjugate was added into each well and incubated for another 30 min at 500 rpm. After the substrate solution was added to each well, the plate was incubated for 45 min at room temperature. The cytochrome *c* level was determined after adding the stop solution by reading absorbance at 405 nm with a microplate reader.

Western blot analysis

PCa cells treated with or without norcantharidin for 24 h were lysed with RIPA protein lysis buffer and protein concentration was determined using the BCA protein assay kit (Novagen, Madison, WI, USA). Equal amounts of protein lysates were separated by 10% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto a nitrocellulose membrane. After blocking

membrane with 5% non-fat milk in TBST buffer (50 mmol/L Tris–base, pH 7.4; 150 mmol/L NaCl; 0.05% Tween-20) for 1 h at room temperature, the blots were incubated overnight at 4 °C with the diluted primary antibodies. After washes, the membrane was incubated with appropriate secondary antibodies (HRP-conjugated goat-anti-mouse or goat-anti-rabbit IgG). Immunoreactive signal was visualized using an enhanced chemiluminescence detection kit (PerkinElmer). The anti-β-Actin antibody was used as an internal control.

Statistical analysis

All statistical analyses were performed using Student's *t*-test and ANOVA; statistical significance was $p < 0.05$. All *p* values were two-tailed.

Results

Norcantharidin exhibited cytotoxicity against PCa cells

To observe the suppressive effect of norcantharidin on PCa cell growth, two immortalized PCa cell lines, 22Rv1 and Du145, were consecutively treated with norcantharidin at indicated concentrations either for 24, 48 or 72 h. The dose- and time-responsive survival curves were determined by using an MTT-based cytotoxicity assay (Fig. 1). As shown in Fig. 1, after treatment with norcantharidin, both of cells were markedly inhibited by norcantharidin in dose- and time dependent manners. The IC_{50} values for treatment duration were calculated and shown in Table 1.

Norcantharidin induced apoptosis in PCa cells

For norcantharidin-induced apoptosis has been evidenced in many types of tumor cell lines, we examined whether norcantharidin induced apoptotic cell death in PCa cells. Both 22Rv1 and Du145 cells were treated with norcantharidin for 24 h and the lysates were used to detect fragmentation of chromosomal DNA into oligonucleosomes, which is one of major characteristics of apoptosis. The data clearly indicated that norcantharidin dose-dependently induced oligonucleosomal DNA fragmentation in both PCa cell lines (Fig. 2A). The induction folds relative to negative control were up to 15.3 and 20.1 folds in 22Rv1 and Du145 cells, respectively, with 30 μg/ml norcantharidin treatment. Besides, Western blotting detection indicated that exposure of 22Rv1 and Du145 cells to norcantharidin at 10 μg/ml or higher concentrations resulted in appearance of proteolytic cleavage of PARP (Fig. 2B), supporting that norcantharidin induced apoptosis of PCa cells.

Table 1
The IC_{50} values of 22Rv1 and Du145 prostate cancer cells with norcantharidin treatment.

Treatment duration (h)	IC_{50} values (μg/ml) in prostate cancer cells	
	22Rv1	Du145
24	23.5 ± 2.6	20.2 ± 0.5
48	12.8 ± 1.4	14.2 ± 1.2
72	7.1 ± 0.6	6.7 ± 0.6

Viability of the cells exposed to various concentrations of norcantharidin for 24, 48 and 72 h, respectively, was detected by an MTT assay.

Norcantharidin increased cytochrome c release and caspase activation in PCa cells

Since release of cytochrome *c* from mitochondria to cytoplasm is a key event in intrinsic pathway of apoptogenesis. Cytochrome *c* level in cytosolic fraction in both cells was determined after 24 h of norcantharidin treatment. It was clearly noted that norcantharidin significantly increased cytosolic cytochrome *c* levels in both PCa cells in a dose-dependent manner (Fig. 3A). Next, to determine the involvement of caspase activation in norcantharidin-induced PCa cell death, lysates of norcantharidin-treated cells were subjected to a kit for screening caspase activity, including caspase-3, -8 and -9. The results showed that norcantharidin significantly elevated the activities of caspase-3, -8 and -9 activities in PCa cells in a dose-dependent manner (Fig. 3B). As an important mediator of intrinsic mitochondrial pathway, the caspase-9 activity showed 2.20- and 2.21-fold induction in 22Rv1 and Du145 cells, respectively, when exposed to 30 μg/ml of norcantharidin. The two activities of downstream executive caspase-3 increased by about 5.4 and 2.8-fold in 22Rv-1 and Du145 cells, respectively. Moreover, higher than 2-fold increases of caspase-8 levels were noted in both cells, strongly suggesting that extrinsic pathway activation was also involved in the norcantharidin-induced PCa cell apoptosis.

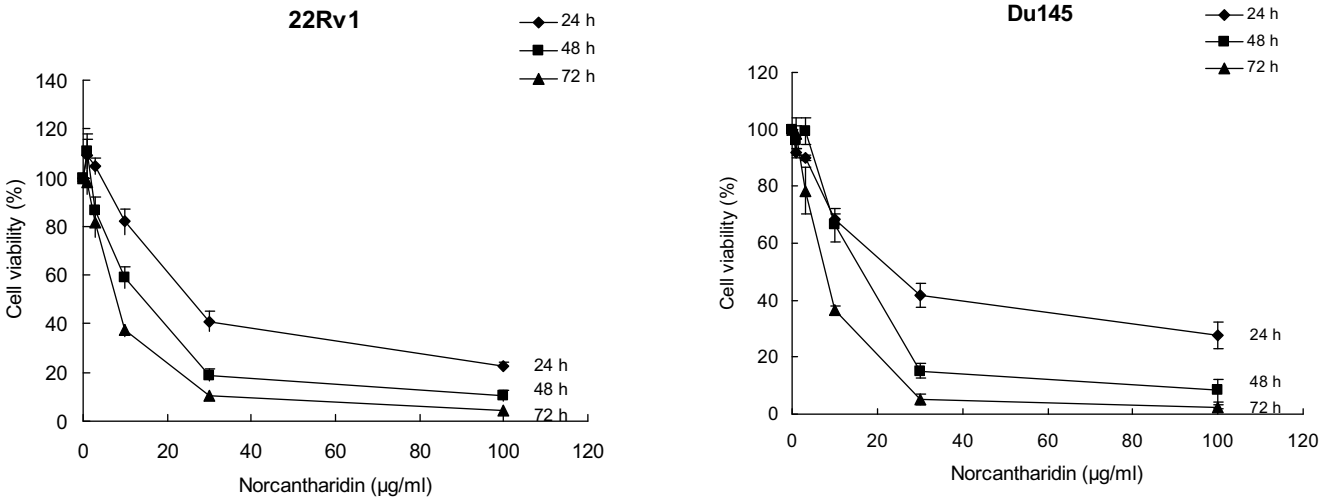

Fig. 1. Cytotoxic effect of norcantharidin on 22Rv1 and Du145 prostate cancer cells. Both cells were treated with 0, 3, 10, 30 and 100 μg/ml of norcantharidin for 24, 48 and 72 h and subjected to an MTT-based cytotoxicity assay. Data are presented as means ± SD from three independent experiments.

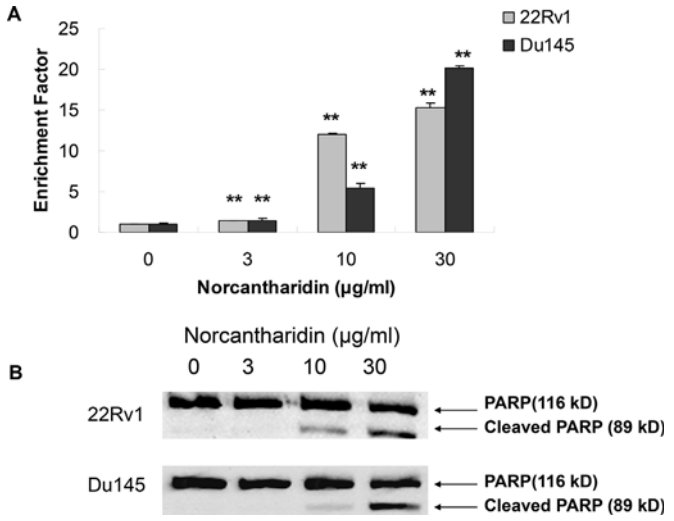

Fig. 2. Apoptotic induction of 22Rv1 and Du145 prostate cancer cells by norcantharidin. (A) Dose-dependent induction of apoptotic oligonucleosome formation by norcantharidin. Cells were treated with norcantharidin at indicated concentrations for 24 h and lysates were subjected to an oligonucleosome ELISA analysis. Data are presented as means ± SD from three independent experiments, ** $p < 0.01$ compared to control group. (B) Western blotting demonstrated that the cleavage of poly(ADP-ribose) polymerase (PARP) emerged in norcantharidin-treated 22Rv1 and Du145 cells.

Norcantharidin modulated extrinsic death receptor and adaptor protein expression in PCa cells

To clarify the involvement of extrinsic pathway in norcantharidin-induced apoptosis of PCa cells, we examined whether norcantharidin affects expression of membrane death receptors and adaptor proteins in the PCa cells. Western blotting results indicated that norcantharidin treatment for 24 h upregulated expression of Fas and DR5 receptors in 22Rv1 cells, whereas TNF receptor complex associated adaptor proteins such as RIP and TRADD remained unchanged (Fig. 4). In Du145 cells, norcantharidin did not alter Fas and DR5 expression levels, but remarkably upregulated RIP and TRADD protein levels. These findings suggested that the extrinsic death receptor signaling may participate in the norcantharidin-driven apoptogenesis in PCa cells.

Norcantharidin disrupted equilibrium between Bcl-2 and Bax family proteins in PCa cells

For the balance between anti- and pro-apoptotic family members determines whether or not a cell will undergo apoptosis, we next delineated the expression profiles of anti- and pro-apoptotic regulatory proteins in norcantharidin-treated cells. The results of Western blot showed that 24 h of incubation with norcantharidin upregulated expression of Bax and Bak proteins in 22Rv1 cells, while it additionally increased that of Bad and Bid in Du145 cells (Fig. 5). On the other hand, norcantharidin induced downregulation of Bcl-2, Bcl-xL and Mcl-1 expressions in 22Rv1 cells, while it reduced expression of only Bcl-xL and Mcl-1 in Du145 cells. These results suggest that the disequilibrium between pro-apoptotic and anti-apoptotic family proteins may contribute to norcantharidin-induced PCa apoptogenesis.

Involvement of inhibitors of apoptosis protein (IAP) family proteins in norcantharidin-induced PCa cell apoptosis

Since IAP family proteins play an inhibitory role against caspase activation during apoptogenesis, we next sought to answer whether norcantharidin also altered expression of IAP family

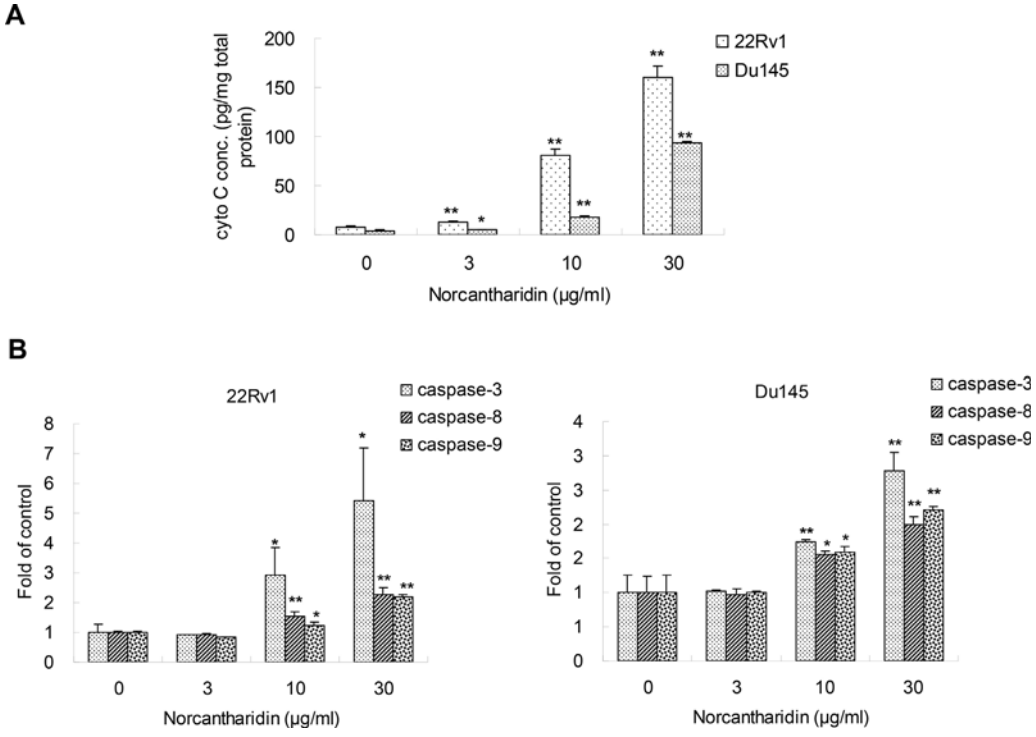

Fig. 3. Induction of cytochrome *c* (Cyt *c*) release and caspase activation in 22Rv1 and Du145 prostate cancer cells by norcantharidin. Cells with 24 h of norcantharidin treatment were subjected to Cyt *c* detection and caspase activity assays. (A) Norcantharidin dose-dependently induced elevation of intrinsic cytosolic Cyt *c* levels in both 22Rv1 and Du145 cells. (B) Elevation of intracellular levels of caspases (caspase-3, -8, and -9) in 22Rv1 and Du145 prostate cancer cells by norcantharidin. Data are presented as means ± SD from three independent experiments, * $p < 0.05$ and ** $p < 0.01$ (respectively) compared to control group.

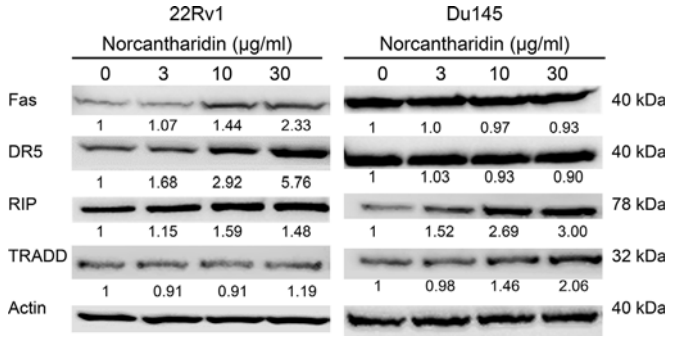

Fig. 4. Effects of norcantharidin on expression of death receptors and adaptor proteins in 22Rv1 and Du145 prostate cancer cells. Cells were treated with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were subjected to western blot detection for extrinsic death receptors (Fas and DR5) and their adaptor proteins (RIP and TRADD). β-Actin was used as an internal loading control. Representative images from three independent experiments are shown. The relative ratios of the band density of target versus actin, taking the ratio of untreated control as 1.0, are shown below each band.

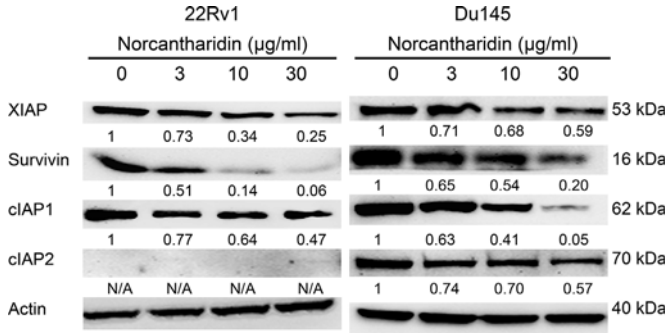

Fig. 6. Suppressive effect of norcantharidin on expression of IAP family proteins in 22Rv1 and Du145 prostate cancer cells. Cells were treated with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were resolved by western blot analysis and probed with antibodies specific for XIAP, survivin, cIAP1 and cIAP2. β-Actin was used as an internal loading control. Representative images from three independent experiments. The relative ratios of the band density of target versus actin, taking the ratio of untreated control as 1.0, are shown below each band. N/A: Not accessible.

members including XIAP, survivin, cIAP1 and cIAP2 in both PCa cells, thereby interrupting caspase activation. Western blot analysis revealed that treatment with norcantharidin for 24 h significantly downregulated expression of XIAP, survivin, cIAP1 and cIAP2 proteins in both PCa cells in a dose-dependent manner, in spite of undetectable cIAP2 protein in 22Rv1 cells (Fig. 6). These findings demonstrated that expressions of IAP family proteins were indeed suppressed in PCa cells during the progression of norcantharidin-induced apoptogenesis.

Discussion

PCa is the second leading cause of cancer death in men. Although the growth of PCa cells is largely androgen-dependent and can be inhibited by androgen ablation, androgen ablation therapeutic response is limited and eventual tumor recurrence to a CRPC and then become to advanced disease [27]. As existing

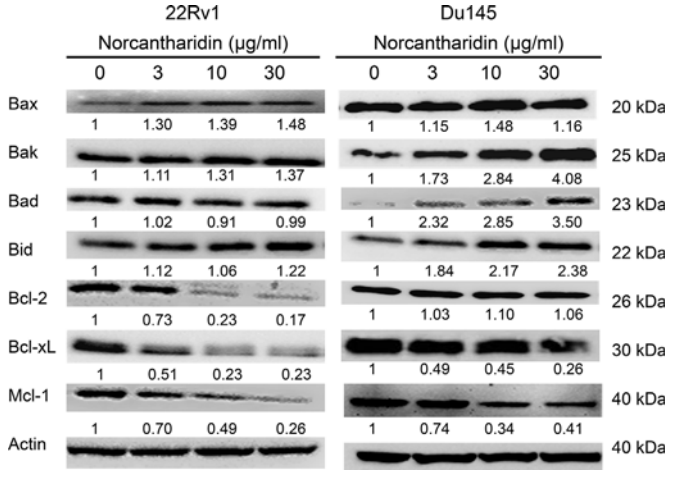

Fig. 5. Downregulation of Bcl-2 and upregulation of Bax family members in 22Rv1 and Du145 prostate cancer cells by norcantharidin. 22Rv1 and Du145 cells were treated with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were subjected to western blot analysis and probed with antibodies against either pro-apoptosis regulators including Bax, Bak, Bid, and Bad, or anti-apoptosis regulators including Bcl-2, Bcl-xL and Mcl-1. β-Actin was used as an internal loading control. Representative images from three independent experiments are shown. The relative ratios of the band density of target versus actin, taking the ratio of untreated control as 1.0, are shown below each band.

chemotherapeutic agents for PCa, such as docetaxel, have limitation in the improvement of morbidity and mortality, chemotherapy has provided significant survival benefit in the treatment of PCa. Due to the fact that most anticancer agents induced serious side effects, it is important to identify anticancer agents that are low toxic to normal cells and highly effective to induce apoptosis of both androgen-dependent and -independent PCa cells. Recently, the potential contributions of so-called complementary and alternative medicines in experimental cancer research have been recognized. Some herbal medicines have been reported to be effective in suppressing the growth of PCa cells and may have potential in treating this disease [28,29]. In this respect, norcantharidin is a modified cantharidin analog that has been previously shown to inhibit growth of various types of tumor cells, but has lower toxicity in normal cells [4,10,12,30]. In this study, two cell lines with distinct androgen-dependent status were tested. Norcantharidin was found to inhibit growth of both PCa cell lines in dose- and time-dependent manners (Fig. 1). Consistent with our findings, the cytotoxicity of norcantharidin in Du145 cells has been previously demonstrated [17–19]. Most importantly, our result implies that norcantharidin may provide a wide spectrum of application in killing PCa cells irrespective of androgen responsiveness.

Apoptosis underlies the molecular basis for new targets that induce cancer cell death through two major pathways. In this context, our previous studies also showed that norcantharidin induced apoptosis in colorectal and breast cancer cells through both extrinsic and intrinsic pathways [12,25]. As intrinsic pathway mediators, elevation of caspase-3 and -9 activities has been identified in norcantharidin-treated melanoma and hepatoma cells [6,13,31]. Similar to this study, Shen et al. reported that norcantharidin induced Du145 cells apoptosis by mitochondria membrane potential change and DNA fragmentation [18]. The present study further demonstrated that norcantharidin induced apoptotic oligonucleosome formation in a dose-dependent manner (Fig. 2A) and markedly increased release of cytochrome *c*, an important mediator in the intrinsic apoptosis pathway (Fig. 3A). As a consequence, the upregulated caspase-3, -8, and -9 activities (Fig. 3B) led to proteolytic cleavage of downstream target PARP (Fig. 2B). In agreement with our findings, the norcantharidin-triggered caspase activation was also seen in cervical cancer, colorectal cancer, breast cancer, urinary bladder carcinoma and lymphoma cells [12,23,25,32,33]. Our findings suggest that norcantharidin caused apoptosis through both extrinsic and intrinsic pathways in both androgen-dependent and -independent PCa cells.

In the context of extrinsic pathway, we intriguingly observed that norcantharidin upregulated Fas and DR5 expression in 22Rv1 but increased RIP and TRADD in Du145 cells (Fig. 4), strongly suggesting the involvement of extrinsic pathways in the norcantharidin-induced apoptosis of PCa cells. In fact, the apoptosis-inducing effect of norcantharidin in glioblastoma and colorectal tumor cells depends on expression of wild-type p53 [12,15]. Besides, p53 was found to activate extrinsic pathway via induction of Fas, DR5 and PERP mRNA expression [34]. Sun et al. previously reported that a retinoid chemical induces Fas and DR5 expression in wild-type p53 human lung cancer cell lines, but not in mutant p53 cell lines [35–37]. Our unpublished data also indicated that norcantharidin induced p53 upregulation in wild-type p53-expressing 22Rv1 cells, but did not affect cellular content in Du145 cells with mutant p53 (data now shown), supporting a p53-dependent regulation in Fas and DR5 expression in PCa cells. Taken together with the similar IC_{50} values observed in both cell lines (Table 1), this p53-independent cytotoxicity of norcantharidin is consistent with the apoptotic induction in oral cancers [38], again highlighting its applicability to the broad spectrum of tumor types.

With regard to the regulatory role of Bcl-2 and Bax family members in norcantharidin-induced apoptosis, our data clearly indicated that norcantharidin upregulated pro-apoptotic proteins, but downregulated anti-apoptotic protein expression in both PCa cells (Fig. 5). The results suggest that norcantharidin induces apoptosis by altering Bcl-2 family protein expression in PCa cells. In line with our findings, Shen and Chen et al. have reported that norcantharidin induces Du145 apoptosis through mitochondria membrane potential change, ROS induction, depletion of ATP, AMPK activation, cytochrome *c* release and cdc6 degradation [17,18]. Other lines of evidence also support the notion that norcantharidin enhances Bax expression in SAS cells and down-regulates Bcl-2 expression in Ca9-22 and Bcl-xL in SAS cells [38]. Moreover, decreased ratios of Bcl-2/Bax are evidenced in A375-S2 and MDA-MB-231 cells [13,39]. However, a contradiction was reported that norcantharidin increases phosphorylation of Bcl-2 and Bcl-xL in Hep-G2 cells [6]. This issue deserves to be further clarified in the norcantharidin-treated PCa cells.

Escape of cancer cells from cell death pathway is a typical feature and causes resistance to treatment. IAP proteins are a family of anti-apoptotic proteins that promote cell survival by blocking the activations of caspases. Over-expression of IAP proteins frequently observed in various human cancers is involved with tumor progression, recurrence, and metastasis [40,41]. Inhibition of IAP proteins is accordingly considered as a therapeutic target. The previous studies have shown that norcantharidin downregulates expression of survivin, XIAP and cIAP1 in gallbladder carcinoma and Mantle cell lymphoma [16,32,42]. Similar to previous reports, this study demonstrated that norcantharidin decreased survivin, XIAP, cIAP1 and cIAP2 levels in Du145 cells and reduced only survivin, XIAP, and cIAP1 expression in 22Rv1 cells (Fig. 6). Besides, it is worth to mention that the constitutive expression of cIAP2 in 22Rv1 cell was too low to be detected, implicating a role for cIAP2 to play in norcantharidin-induced apoptogenesis. In this regard, Conte et al. reported that the macrophages derived from cIAP2-deficient mice were hypersensitive to Fas-mediated apoptosis by lipopolysaccharide treatment [43]. This may provide an underlying rationale for the norcantharidin-induced Fas upregulation in 22Rv1 cells, but not in Du145 cells (Fig. 4). Together with the results of apoptotic regulators such as Bcl-2 and Bax family members, we suggest that norcantharidin differentially regulates the pro-apoptogenic machineries in androgen-dependent and androgen-independent PCa cells.

Although androgen-deprivation therapy and radiotherapy are currently available to treat early stage PCa, it is still problematic that approximate one-third of patients eventually progress to CRPC or metastasis within 10 years. Docetaxel (Taxotere) not only improves survival for CRPC patients, but also has many side effects including neutropenia, hypersensitivity reaction, stomatitis, peripheral neuropathy, and fluid retention [2,44]. In this regard, numerous studies have shown that norcantharidin increases the intracellular accumulation of doxorubicin and exerts strongly synergistic cytotoxic effects. A pharmacokinetics study of mice indicated that norcantharidin can be rapidly absorbed from the digestive tract and excreted by the urine when given orally or intravenously, whereas it is mainly distributed in the liver, bile, kidney, stomach, and tumor cells in mice. More importantly, norcantharidin shows no depressive effect on white and red blood cells on long-term administration [3]. Moreover, pre-clinical studies demonstrated that norcantharidin can prolong survival time, inhibit metastasis and angiogenesis [5,16,20–22], whereas a clinical trial on primary hepatoma indicates that it prolongs survival time from 4.7 to 11.1 months and increases l-year survival rate from 17% to 30% compared with the group using current chemotherapeutic drugs [3]. Mechanistically, norcantharidin has benefits in eradicating cancer stem cells, reversing multidrug resistance, and preventing leukopenia, but has fewer side-effects with combined docetaxel or other chemotherapeutic agents [3,16,17,22,26,45]. Taken together with the findings of our and other groups, norcantharidin is reasonably regarded as a potential complementary and alternative medicine in the treatment of both androgen-dependent and -independent PCa.

Conclusion

Norcantharidin treatment induces apoptosis of PCa cells via activating both extrinsic and intrinsic pathways. The former includes upregulation of death receptor, while the latter includes release of cytochrome *c* from mitochondria, disturbed equilibrium between pro-apoptotic and anti-apoptotic Bcl-2 family proteins, suppression of IAP expression. Convergence of both pathway signals eventually results in activation of caspases. Accordingly, norcantharidin may be used as a chemotherapeutic agent for treating PCa.

Conflict of interest

There are no conflicts of interest.

Acknowledgment

This study is financially supported by the grant from Show Chwan Memorial Hospital, Taiwan, ROC (Grant No. MI99003 and RD103017).

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