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Norcantharidin induces apoptosis of breast cancer cells: Involvement of activities of mitogen activated protein kinases and signal transducers and activators of transcription

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ABSTRACT

Involvement of activities of mitogen-activated protein kinases (MAPKs) and signal transducers and activators of transcription (STATs) remains unsolved in norcantharidin-associated breast cancer cell apoptosis. This study investigated the anti-cancer effect of norcantharidin and its underlying mechanism in two human breast cancer cell lines, estrogen receptor (ER)− HS-578T and ER+ MCF-7 cells. Norcantharidin induced potent cytotoxicity and arrested cell growth through increasing phosphorylation of Chk1, Chk2 and total $p21^{Waf1/Cip1}$ and reducing cyclin B and cdc25c expression. It also induced apoptosis through extrinsic death receptor and intrinsic mitochondrial pathways by cytochrome *c* release, caspase activation, oligonucleosome appearance, PARP cleavage, and aberration of Bcl-2 family protein expression and phosphorylation. Although norcantharidin did not affect STAT1, STAT3, and STAT5 protein expression, it suppressed STAT3 and STAT5 phosphorylation in HS-578T cells, whereas it up-regulated STAT1 phosphorylation and down-regulated STAT5 phosphorylation in MCF-7 cells. Moreover, norcantharidin activated MAPK family member proteins, extracellular signal-regulated kinase (ERK), $p38^{MAPK}$ and c-Jun N-terminal kinase (JNK), were all phosphorylated by treatment. Pretreatment with selective kinase inhibitors significantly attenuated the norcantharidin-induced cytotoxicity in breast cancer cells. These findings suggest the potential involvement of MAPK and STAT pathways in norcantharidin-induced apoptogenesis. Norcantharidin may be an effective anti-cancer drug against breast cancer.

1. Introduction

Breast cancer (BCa) is the second most common cancer worldwide after lung cancer and the fifth most common cause of cancer-related death. Notably, morbidity of BCa in females is roughly 100 times higher than males, survival rates for both sexes are equal (Jemal et al., 2009). Unfortunately, severe morbidity of BCa has not been improved by surgery, radiotherapy, biological therapy, chemo- or hormonotherapies, as BCa is highly resistant to chemotherapy and no effective cure exists for patients with advanced disease stages (Andre et al., 2004; Orlando et al., 2007). Several biomarkers have been identified that predict response to chemotherapy to variable extent. Since estrogen is well known for its ability to directly modulate expression of growth factor receptor pathways and cell cycle regulatory genes (Dickson and Russo, 2000), status of estrogen receptor (ER) in a tumor significantly correlates with the likelihood of achieving pathologic complete response (Tewari et al., 2008). Patients who bear operable and ER-negative (ER−) tumors are more likely to achieve higher chemosensitivity than those ER+ cases (Guarneri et al., 2006; Ring et al., 2004).

Norcantharidin is a demethylated analog of cantharidin purified from the dried body of the Chinese blister beetle Mylabris, (*Mylabris phalerata* Pallas), which is a traditional Chinese medicine long been used for treating malignant tumors (Wang, 1989). In comparison with cantharidin, the major bioactive compound in Mylabris,

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norcantharidin has the advantage of easy synthesis and reduced intrinsic toxicity while retaining its anti-cancer activity. The anti-cancer mechanisms of norcantharidin have been illuminated by the evidence showing its anti-proliferation, pro-apoptotic and anti-migratory effects on many cancer cells. Norcantharidin inhibits the growth of numerous cancer cell lines via apoptosis, including oral cancer (Kok et al., 2003), hepatoma (Chen et al., 2002), leukemia (Liao et al., 2007), colorectal adenocarcinoma (Peng et al., 2002), melanoma (An et al., 2004b), glioblastoma (Hong et al., 2000) and gallbladder carcinoma (Fan et al., 2007). Besides, animal studies demonstrated that norcantharidin reduced pulmonary metastatic capacity of colorectal adenocarcinoma cells (Chen et al., 2005, 2009b) and prolonged survival of HepG2 tumor-bearing nude mice (Yang et al., 1997).

The well-studied mechanism for the anti-cancer activity of norcantharidin is its ability to induce tumor cell apoptosis, the process of which encompasses interruption of cell cycle progression of tumors at mitotic phase, suppression of cell adhesion and motility, expressional modulation of Bcl-2 superfamily member proteins and of caspase activity, and induction of eventual apoptotic cell death. In fact, the apoptotic induction by norcantharidin has been evidenced in many types of tumor cells and, more recently, in human breast cancer cells (Huang et al., 2009). In addition, the activities of mitogen-activated protein kinase (MAPK) family members, such as extracellular signal-regulated kinase (ERK), $p38^{MAPK}$, and c-Jun N-terminal kinase (JNK), have been mechanistically implicated in the apoptogenesis of norcantharidin-treated tumor cells (An et al., 2004a; Chen et al., 2002, 2003, 2005; Hong et al., 2000; Peng et al., 2002). Although Huang et al. (2009) has recently demonstrated that norcantharidin interferes with Akt phosphorylation in a highly metastatic human BCa cell line, however, not enough is known about the role that MAPKs play in the norcantharidin-elicited apoptosis of breast tumor cells. Moreover, aberrant activation of signal transducers and activators of transcription (STATs), mainly STAT1, STAT3, and STAT5 proteins, is involved in the pathogenesis of cancer (Bromberg, 2001). Overall, STAT1 functionally promotes apoptosis and tumor suppression, while STAT3 and STAT5 modulate cell growth and inhibit apoptosis (Battle and Frank, 2002; Bromberg, 2001). To date, the role of STATs in the apoptotic induction of BCa cells by norcantharidin remains unclear.

Due to lack of knowledge for the above-mentioned viewpoints on the anti-cancer activity of norcantharidin in BCa cells, this study thus aimed at validating the norcantharidin-driven cytotoxicity in two BCa cell lines with opposite ER status (i.e., ER− HS-578T and ER+ MCF-7 cells), delineating the responsive profiles of STATs and MAPKs activities to drug treatment therein, as well as elucidating the pharmacological mechanism underlying the drug action.

2. Materials and methods

2.1. Chemical reagents

Norcantharidin was purchased from Sigma (Sigma, St. Louis, MO, USA) and prepared by serial dilutions in culture medium. Primary antibodies against Bax, Bid, Bad, Bak, Bim, Bcl-2, Bcl-xL, Mcl-1, phosphor-Bcl-2 (Ser70), c-Jun N-terminal kinase (JNK), phosphor-JNK (Thr183/Tyr185), ERK 1/2, phosphor ERK 1/2 (Thr202/Tyr204), $p38^{MAPK}$, phosphor-$p38^{MAPK}$ (Thr180/Tyr182), $p21^{Waf1/Cip1}$, phosphor-p53 (Ser15), $p27^{Kip1}$, phosphor-Chk1 (Ser345), phosphor-Chk2 (Thr68), STAT1, phosphor-STAT1 (Tyr701), STAT3, phosphor-STAT3 (Tyr705), STAT5, phosphor-STAT5 (Tyr694) and goat-anti-rabbit secondary antibody were obtained from Cell Signaling Technology (MA, USA). Primary antibodies against cyclin B, cdk1/cdc2, cdc25c, and goat-anti-mouse secondary antibody were from Millipore (NY, USA). Primary antibodies against poly (ADP-ribose) polymerase (PARP), Fas, Fas-associated death domain protein (FADD), TNFR1-associated death domain protein (TRADD), and receptor interacting protein (RIP) were from BD Biosciences (USA). Selective inhibitors, U0126 for MAPK/ERK kinase (MEK), SP600125 for JNK, SB203580 for $p38^{MAPK}$, were from Sigma (St. Louis, MO) and all dissolved in DMSO for stock preparation.

2.2. Cell culture

Both BCa cell lines, HS-578T and MCF-7, were obtained from the Cell Bank of the National Health Research Institute, Taiwan. HS-578T cells were grown in DMEM with 10 μg/ml bovine insulin and 10% FBS. MCF-7 cells were grown in MEM-Alpha containing 0.1 mM non-essential amino acids and 10% FBS. The media for both cell lines contained 50 μg/ml gentamicin. All cultured cells were maintained in a humid chamber at 37 °C under 95% humidified air/5% CO_2 atmosphere.

2.3. Evaluation of antitumor activity by cytotoxicity assay

Antitumor activity of norcantharidin was evaluated using a microculture tetrazolium test (MTT) as previously described (Phillips et al., 1990; Yang et al., 2010). Briefly, HS-578T and MCF-7 tumor cells seeded into a 96-well plate (5000 cells per well) were treated with or without norcantharidin in triplicate for 48 h of consecutive 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. The plate was read using a microplate reader (Labsystems, Helsinki, Finland) at 550 nm. Cell inhibition at each concentration was converted to percentage of control levels set at 100% and the IC_{50} value for norcantharidin was calculated.

2.4. Apoptotic cell death determination

Apoptosis-associated DNA fragmentation was determined by measuring the cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) using a Cell Death Detection ELISA Plus kit (Roche, Mannheim, Germany) as previously described (Yang et al., 2010). Briefly, HS-578T and MCF-7 cells seeded into 96-well plates were treated with or without norcantharidin for 24 h. Then cell lysates were collected and transferred to a streptavidin-coated plate supplied by the manufacturer. A mixture of anti-histone–biotine and anti-DNA-POD 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 by a microplate reader (Labsystems). The contents of mono-oligonucleosomes in lysates were calculated as absorbance of norcantharidin-treated cells/absorbance of untreated controls.

2.5. Cytochrome c assay

The cytochrome *c* ELISA kit (Assay Designs, Ann Arbor, USA) was used to determine the cytochrome *c* release from mitochondria in norcantharidin-treated cells, as previously described (Yang et al., 2010). 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 room temperature, the cytochrome *c* conjugate was added into each well and incubated for another 30 min. After the substrate solution was added to each well, the plate was incubated for

45 min at room temperature. The cytochrome *c* level was read at 405 nm by a microplate reader (Labsystems) after adding the stop solution.

2.6. Caspase activity assay

Caspase activities in the HS-578T and MCF-7 cells treated with or without norcantharidin were determined 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 (Labsystems).

2.7. Western blot analysis

The HS-578T and MCF-7 cells were cultivated with norcantharidin for 24 h and then lysed in RIPA protein lysis buffer. Protein concentration was determined using the BCA Protein Assay kit (Novagen, USA). Western blotting was performed as previously described (Yang et al., 2010). Equal amounts of sample lysate were resolved by 10% sodium dodecyl polyacrylamide gel electrophoresis (SDS–PAGE), and transferred onto a nitrocellulose membrane. After blocking the membrane with 5% non-fat milk in TBST buffer (50 mM Tris–base, pH 7.4; 150 mM 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 washing, the membrane was incubated with the appropriate secondary antibody (horseradish peroxidase conjugated goat-anti-mouse or goat-anti-rabbit IgG). Antibody binding was detected using an enhanced chemiluminescence detection kit (Perkin-Elmer, USA). β-Actin antibody was used as an internal control.

2.8. Statistical analysis

All statistical analyses were performed using the Student's *t*-test and ANOVA, with $P < 0.05$ considered as statistically significant. All *P* values were determined using two-tailed tests.

3. Results

3.1. Cytotoxicity of norcantharidin in BCa cells

To examine the cytotoxic effects of norcantharidin on established BCa cell lines, ER− HS-578T and ER+ MCF-7 BCa tumor cells were selected for 48 h of treatment with norcantharidin. The dose–responsive survival curves were determined by MTT cytotoxicity assay (Fig. 1). The results indicate that norcantharidin significantly reduced viability of both lines of BCa tumor cells in a dose-dependent manner. The calculated IC_{50} values for HS-578T and MCF-7 cells exposed to norcantharidin were 13.06 ± 1.27 and 18.27 ± 0.77 μg/ml, respectively. The distinction between the IC_{50} values of these two cells clearly indicates that ER− HS-578T cells possess higher chemosensitivity to norcantharidin treatment than ER+ MCF-7 cells.

3.2. Biomodulatory effects of norcantharidin on cell cycle regulators in BCa cells

Although norcantharidin is known to induce cytostaticity in MDA-MB-231 BCa cells by G2/M arrest (Huang et al., 2009), not enough is known about the molecular mechanism by which it elicits G2/M growth arrest in BCa tumor cells. Since our preliminary data had confirmed that norcantharidin also induced G2/M arrest of both HS-578T and MCF-7 BCa cells (data not shown), this study continued to delineate the expression profiles of cell cycle regulatory proteins in both cell lines exposed to norcantharidin (Fig. 2). Western blotting was used to measure intracellular contents of cell cycle regulation proteins (cyclin B, cdc2 and cdc25c), phosphorylation status of cell cycle checkpoint proteins (p53, Chk1 and Chk2) as well as levels of cyclin-dependent kinase inhibitors ($p21^{Waf1/Cip1}$ and $p27^{Kip1}$). In HS-578T cells, norcantharidin treatment for 24 h increased phosphorylated levels of p53, Chk1, and Chk2 as well as total protein level of $p21^{Waf1/Cip1}$, accompanied with down-regulation of cyclin B, cdc2, and cdc25c; no marked change was noted in $p27^{Kip1}$ protein expression. In MCF-7 cells, norcantharidin up-regulated phosphorylation of both Chk1 and Chk2, elevated $p21^{Waf1/Cip1}$ protein contents, whereas it down-regulated cyclin B and cdc25c levels. Besides, p53 phosphorylation as well as cdc2 and $p27^{Kip1}$ protein expression remained constant. These

Fig. 1. Induction of cytotoxicity in BCa cell lines by norcantharidin. HS-578T and MCF-7 BCa cells were treated with norcantharidin at indicated concentrations for 48 h. The drug effect on cell viability was measured using an MTT assay. Data are representative results in three independent experiments.

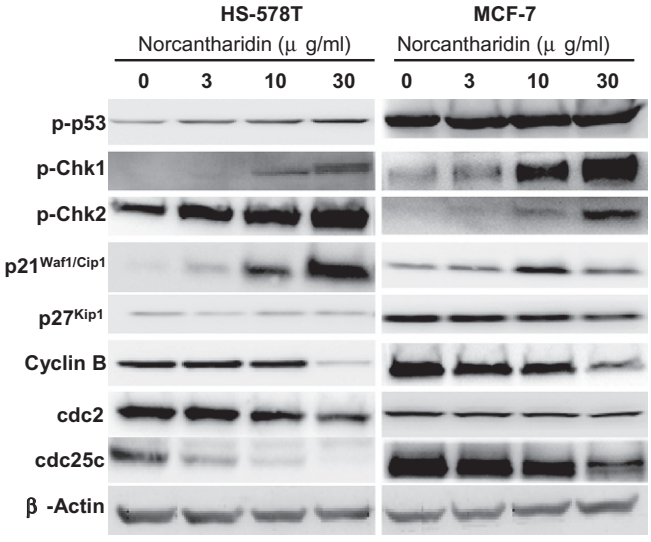

Fig. 2. Expression of cell cycle regulatory molecules in norcantharidin-treated BCa cell lines. HS-578T and MCF-7 BCa cells were cultured with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were resolved by Western blot analysis and probed with antibodies specific for either cell cycle checkpoint proteins (phosphor-p53, phosphor-Chk1, and phosphor-Chk2), cyclin-dependent kinase inhibitors ($p27^{Kip1}$ and $p21^{Cip1/Waf1}$), or cell cycle regulatory proteins (cyclin B, cdc2, and cdc25c). Meanwhile, β-Actin was used as an internal loading control. Representative images from three independent experiments are shown.

findings strongly suggest that cyclin B and cdc25c play a pivotal role in norcantharidin-induced BCa cell cycle arrest.

3.3. Apoptotic induction in BCa cells by norcantharidin treatment

Norcantharidin-induced apoptosis has been evidenced in many types of tumor cells and, more recently, in MDA-MB-231 BCa cells (Huang et al., 2009). To better characterize the mechanism underlying the norcantharidin-induced apoptogenesis, HS-578T and MCF-7 BCa cells were treated with norcantharidin for 24 h and the lysates were used for detection of various apoptosis-associated parameters. Fragmentation of chromosomal DNA into oligonucleosomes, which is one of characteristics of apoptosis, was dose-dependently induced by norcantharidin in both BCa cell lines (Fig. 3A). The normalized data indicated that 30 μg/ml of norcantharidin increased oligonucleosomal formation up to 14.73- and 3.63-folds in HS-578T and MCF-7 cells, respectively. Exposure of HS-578T and MCF-7 cells to various norcantharidin concentrations resulted in appearance of proteolytic cleavage of PARP, as revealed by Western blotting detection (Fig. 3B). Again, caspase activity assay clearly indicated that norcantharidin resulted in dose-dependent increases in the activities of pro-apoptotic caspases, such as caspase-3, -8, and -9 in HS-578T (Fig. 3C) as well as caspase-8 and -9 in MCF-7 cells (Fig. 3D). Intriguingly, the undetectable caspase-3 activity in MCF-7 cells is consistent to previous report, mainly due to an exon 3 deletion in *CASP-3* gene transcript and its functional deficiency (Jänicke et al., 1998). All evidence supports that norcantharidin potently induces apoptosis in BCa cells.

3.4. Involvement of both extrinsic and intrinsic pathways in norcantharidin-induced BCa cell apoptosis

To clarify the pharmacologic action of norcantharidin on the apoptogenesis of BCa cells, we first explored the role of the extrinsic membrane death receptor-mediated pathway in norcantharidin-induced BCa cell apoptosis. Western blotting results indicated that norcantharidin treatment for 24 h up-regulated Fas in HS-578T cells, although expression of FADD, RIP and TRADD is slightly reduced by 30 μg/ml of norcantharidin (Fig. 4). In comparison with the expression patterns of death receptor-associated proteins in HS-578T cells, norcantharidin did not alter Fas expression, slightly suppressed FADD content, whereas up-regulated RIP and TRADD levels in MCF-7 cells. These findings suggest that the death-receptor signaling may participate in norcantharidin-induced apoptogenesis, or at least be affected by drug treatment.

Next, to determine whether intrinsic signaling activity is involved in norcantharidin-induced BCa cell apoptosis, the cytosolic level of cytochrome *c*, a key initiator of the intrinsic apoptotic pathway, was assessed. The results indicated that norcantharidin significantly increased the cytosolic cytochrome *c* level in both HS-578T and MCF-7 cells in a dose-dependent manner (Table 1). Since the intrinsic pathway hinges on the balance of activities between pro- and anti-apoptotic members of the Bcl-2 superfamily proteins, Western blotting analysis was utilized to acquire the expression profiles of Bcl-2 superfamily members in norcantharidin-treated cells (Fig. 5). The results showed that 24 h of incubation with norcantharidin up-regulated the expression of Bax, Bak, and Bad proteins in HS-578T cells, while it only increased that of Bax, Bad, and

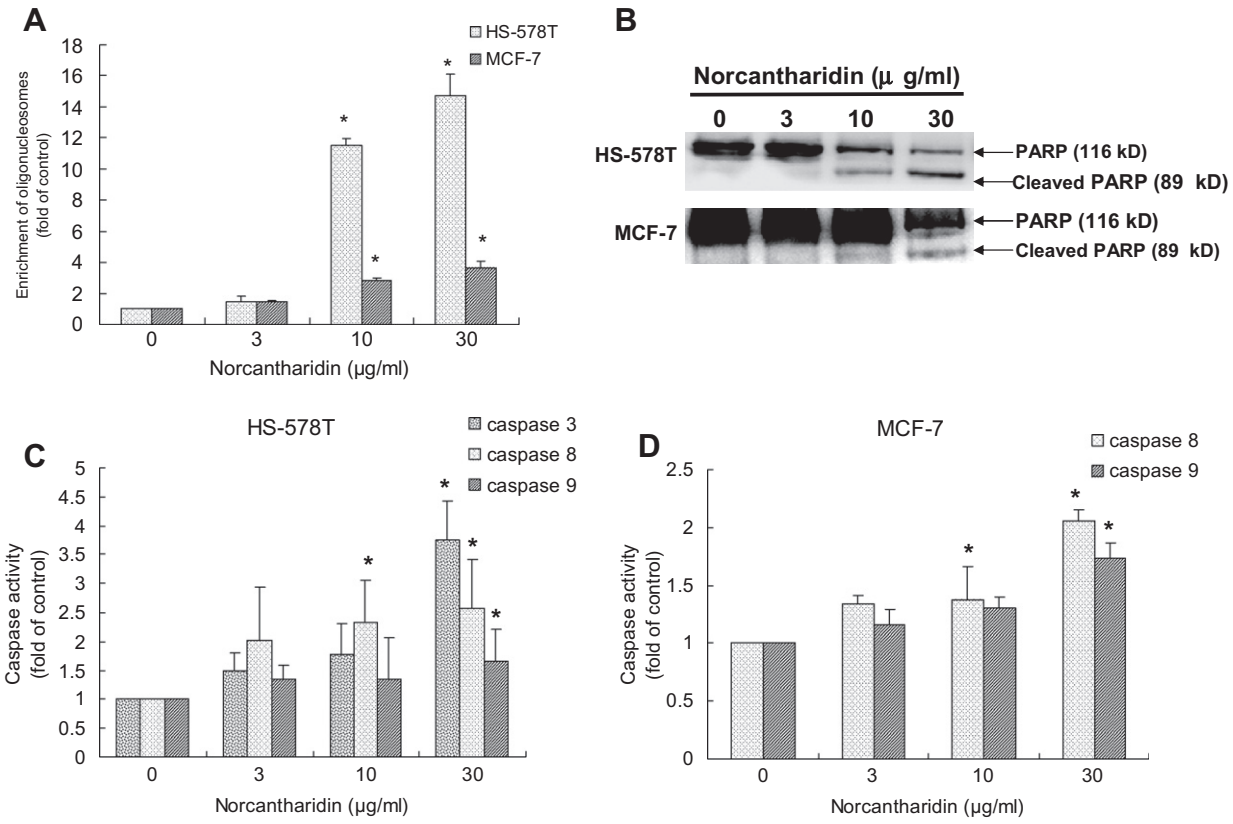

Fig. 3. Induction of apoptosis of BCa cell lines by norcantharidin. HS-578T and MCF-7 BCa cell lines were treated with norcantharidin at indicated concentrations for 24 h. Cellular lysates were collected for measuring apoptosis-associated parameters. (A) Dose-dependent induction of apoptotic oligonucleosome formation by norcantharidin was evidenced by using a commercially available ELISA kit. (B) Western blotting detection demonstrated that the cleavage of poly(ADP-ribose) polymerase (PARP) emerged in norcantharidin-treated HS-578T and MCF-7 cells. (C) and (D) Elevation of intracellular levels of caspases (caspase-3, -8, and -9) by norcantharidin was seen in HS-578T and MCF-7 cells, except undetectable caspase-3 activity in MCF-7. Data are presented as means ± SD from three independent experiments. ∗ indicates $P < 0.05$ compared with corresponding control levels.

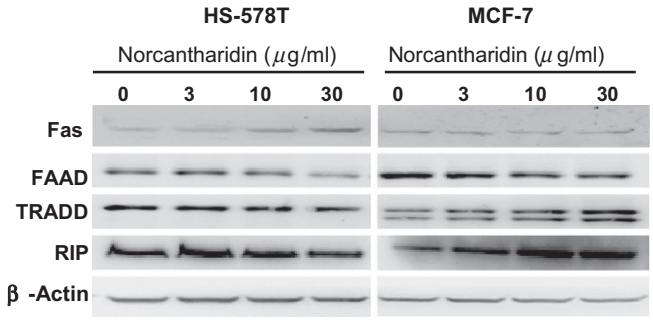

Fig. 4. Biomodulatory effect of norcantharidin on expression of death receptor-mediated extrinsic apoptotic pathway. HS-578T and MCF-7 BCa cells were treated with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were resolved by Western blot analysis and probed with antibodies specific for Fas, Fas-associated death domain (FADD), TNFR1-associated death domain (TRADD) and receptor-interacting protein (RIP). β-Actin was used as an internal loading control. Representative images from three independent experiments are shown.

Table 1
The norcantharidin-induced dose-dependent alteration in cytosolic cytochrome *c* (Cyt *c*) levels in HS-578T and MCF-7 cells.

Dose (μg/ml)	HS-578T		MCF-7	
	Cyt *c* (μg/10^6 cells)	Ratios to control	Cyt *c* (μg/10^6 cells)	Ratios to control
0	27.51	1	34.23	1
3	53.27	1.94	33.46	0.98
10	116.93	4.25	114.85	3.35
30	395.80	14.39	125.00	3.65

Cyt *c* in cytosolic fraction was determined in both cell lines treated with norcantharidin for 24 h.

p-Bcl-2 in MCF-7 cells (Fig. 5A). Conversely, norcantharidin induced down-regulation of Bcl-xL and Mcl-1 expressions in HS-578T cells as well as that of only Bcl-xL in MCF-7 cells (Fig. 5B). Of note, Bcl-2 expression in HS-578T was constitutively much lower than that in MCF-7 cells, which was intriguingly up-regulated by norcantharidin.

3.5. Biomodulatory effect of norcantharidin on STATs in BCa cells

To determine the dose-responsive effect of norcantharidin on STAT-mediated signaling activity, both total and phosphorylated protein levels of STATs, including STAT1, STAT3, and STAT5, in HS-578T and MCF-7 BCa cells with 24 h of treatment were examined by Western blot (Fig. 6). The results clearly showed that norcantharidin did not affect total STAT1, STAT3, and STAT5 levels in both HS-578T and MCF-7 cells. Moreover, STAT1 phosphorylation level was constitutively low in HS-578T cells, whereas the constitutive STAT3 and STAT5 phosphorylation were prominently down-regulated by norcantharidin. By contrast, MCF-7 cells possessed higher constitutive levels of phosphorylated STAT5 proteins, which could be further suppressed by norcantharidin. Conversely, the low constitutive level of STAT1 phosphorylation in MCF-7 cells was abruptly up-regulated by norcantharidin treatment.

3.6. Involvement of MAPK activities in norcantharidin-induced BCa cytotoxicity

Since the activities of MAPKs, including $p38^{MAPK}$, ERK1/2, and JNK, are known to mediate the norcantharidin-elicited apoptogenesis in cancer cells (An et al., 2004a, 2005; Chen et al., 2003, 2008), their roles in the drug-induced apoptosis of breast tumor cells remain unclear. Therefore, the expression of total and phosphorylated protein levels of MAPK family member proteins was monitored in both cell lines under exposure to norcantharidin. Norcantharidin at concentrations higher than 10 μg/ml elevated phosphorylation levels of $p38^{MAPK}$ and JNK in both HS-578T and MCF-7 cells, while ERK1/2 phosphorylation was more sensitive and inducible at 3 μg/ml therein (Fig. 7A and B). In addition, norcantharidin slightly reduced total JNK and $p38^{MAPK}$ protein content in MCF-7 cells. These results suggest that ERK1/2, JNK, and $p38^{MAPK}$ may participate in norcantharidin-induced BCa cell apoptosis.

To further determine whether phosphorylation of MAPK family members is involved in the norcantharidin-induced BCa cytotoxicity, a blockade of kinase activity kinase-specific inhibitors prior to drug treatment was performed to scrutinize the respective contribution of MAPK kinase to the norcantharidin-elicited BCa cytotoxicity. The data showed that pretreatment with U0126 (MEK1/2 inhibitor), SP600125 (JNK inhibitor) as well as SB203580 ($p38^{MAPK}$ inhibitor), although partially but significantly, attenuated the norcantharidin-induced cell death of HS-578T and MCF-7 cells (Fig. 8). This finding confirms and strongly suggests that the activity of MAPK signaling pathway, indeed, plays an important role in governing the drug-induced apoptogenesis in BCa cells.

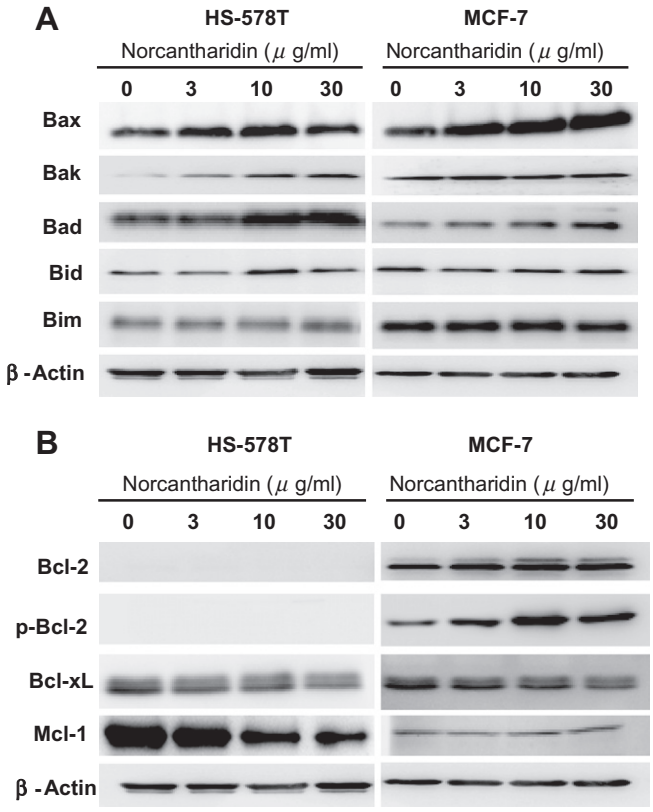

Fig. 5. Expression profiles of Bcl-2 superfamily members in norcantharidin-treated BCa cell lines. HS-578T and MCF-7 BCa cells were treated with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were subjected to Western blot analysis and probed with antibodies specific for pro-apoptosis member proteins (Bax, Bak, Bad, Bid, and Bim) (A) as well as with those against anti-apoptosis member proteins (Bcl-2, p-Bcl-2, Bcl-xL, and Mcl-1) (B). β-Actin was used as an internal loading control. Representative images from three independent experiments are shown.

4. Discussion

As current chemotherapeutic agents, such as anti-estrogens, taxanes and aromatase inhibitors, have limited effectiveness and many side effects in BCa patients (Marsh and McLeod, 2007), iden-

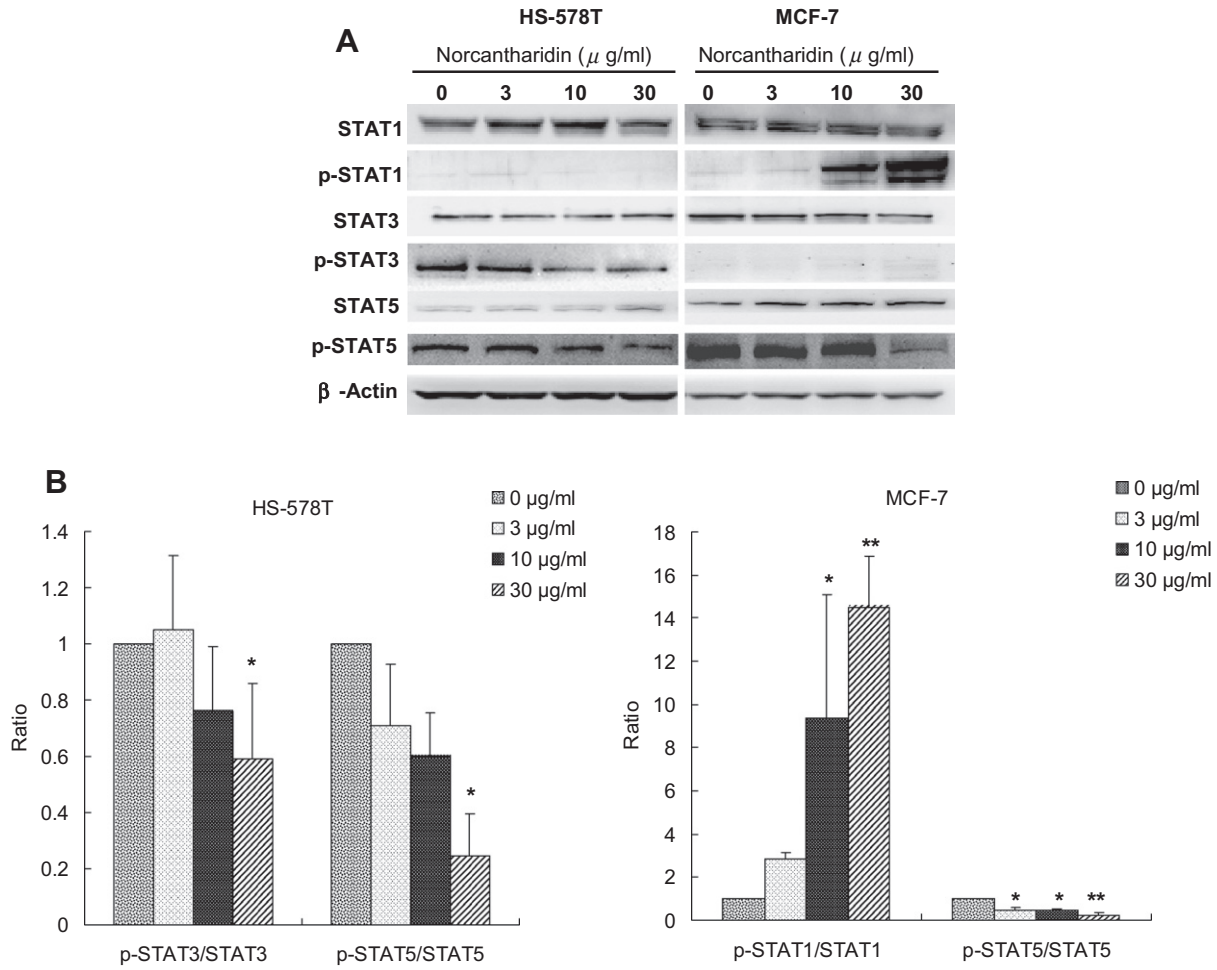

Fig. 6. Regulation of STATs in norcantharidin-treated BCa cell lines. HS-578T and MCF-7 BCa cells were cultured with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were resolved by Western blot analysis and probed with antibodies raised against either total or phosphorylated type of STAT1, STAT3, and STAT5. β-Actin was used as an internal loading control. Representative images (A) and densitometrical analysis data (B) from three independent experiments are shown.

tifying new agents effective for treating BCa with both ER status is important. This study evaluated the *in vitro* chemosensitivity of two immortalized BCa cell lines with opposing ER status after treatment with an anti-cancer drug, norcantharidin. The significantly different IC_{50} values between ER+ and ER− tumor cells in response to norcantharidin treatment confirmed that norcantharidin is more potent in inhibiting ER− than that ER+ BCa cells. In fact, other line of evidence derived from primarily isolated BCa cells also supports that the *ex vivo* chemosensitivity of ER– breast tumor cells was apparently higher than those ER+ cells (unpublished data), supporting again the crucial contribution of ER pathway to the resistance of cancer cells to chemotherapy.

The biomodulatory effect of norcantharidin on cell cycle progression in BCa cells has recently been reported (Huang et al., 2009). Norcantharidin was demonstrated to arrest BCa cell growth at G2/M phase in a dose-dependent manner. This growth-arresting effect may be associated with the upstream regulator, ataxia-telangiectasia mutated (ATM), in human leukemia Jurkat cells (Liao et al., 2007). Despite no alteration in Cdk1 and $p27^{Kip1}$ expressions in human hepatoma cell lines, the enhancement of cdc25c and $p21^{Waf1/Cip1}$ phosphorylation, the reduction of cyclin B level and kinase activity, as well as the decrease in p53 protein expression were all involved in norcantharidin-regulated M phase arrest (Chen et al., 2002). In human gallbladder carcinoma cells, norcantharidin retarded cell growth through down-regulation of proliferation-related genes, PCNA, Ki-67, and cyclin-D1, and through up-regulation of $p27^{Kip1}$ (Fan et al., 2007). In this study, we additionally found that norcantharidin decreased the expressions of cyclin B and cdc25c, increased the phosphorylated contents of Chk1, Chk2 and total $p21^{Waf1/Cip}$, whereas did not affect $p27^{Kip1}$ expression in norcantharidin-treated MCF-7 and HS-578T cells (Fig. 2). Notably, norcantharidin decreased cdc2 abundance in ER− HS-578T, but not in ER+ MCF-7 cells. Since cdc2 is one of key kinase mediators for G2/M checkpoint (Taylor and Stark, 2001), the differential responsiveness of cdc2 down-regulation between ER− and ER+ BCa cells may explain the higher chemosensitivity in ER− cells. Taken together, norcantharidin pharmacologically may be an effective agent for inducing G2/M growth arrest in BCa cells.

Two major pathways that lead to apoptosis have been identified, namely, the extrinsic death receptor pathway leading to caspase-8 activation and the intrinsic pathway that involves the release of cytochrome *c* from mitochondria and activation of caspase-9 (Hengartner, 2000). From the viewpoint of extrinsic pathway, we observed that norcantharidin up-regulated Fas/APO-1 expression in HS-578T cells and increased TRADD and RIP in MCF-7 cells (Fig. 4). Supportively, norcantharidin induces human colorectal cancer cell apoptosis by activating the CD95 receptor system and caspase-8 (Peng et al., 2002). In the light of intrinsic pathway, elevation of caspase-3 and -9 activities has been identified in norcantharidin-treated melanoma and hepatoma cells (An et al., 2004a; Chen et al., 2002, 2003). The activities of all three caspases were similarly up-regulated in norcantharidin-treated

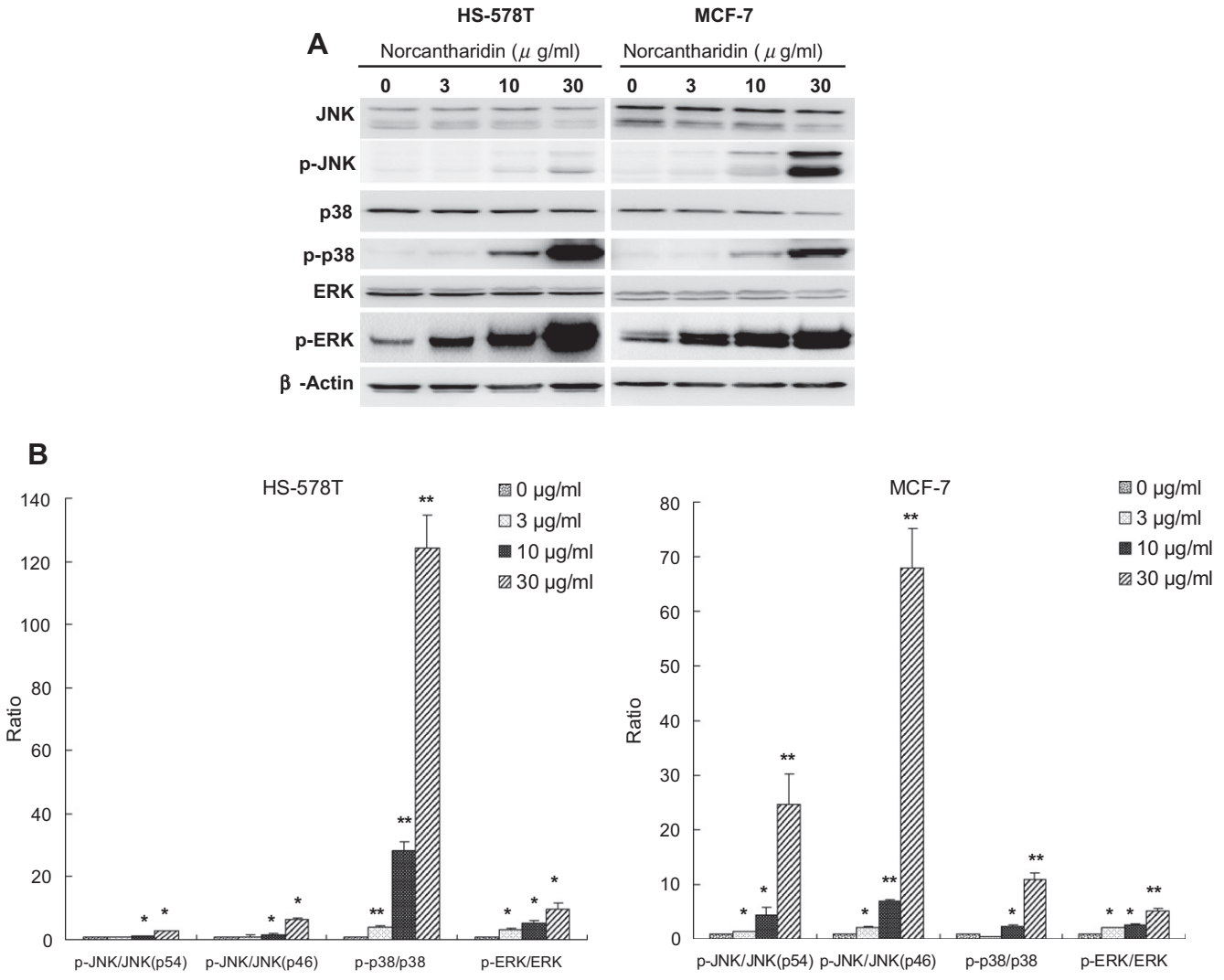

Fig. 7. Involvement of MAPK activation in norcantharidin-induced BCa cytotoxicity. HS-578T and MCF-7 cells were incubated with norcantharidin at indicated concentrations for 24 h. Cellular protein extracts were resolved by Western blot analysis and probed with antibodies raised against either total or phosphorylated type of JNK, $p38^{MAPK}$, and ERKs. β-Actin was used as an internal loading control. Representative images (A) and densitometrical analysis data (B) from three independent experiments are shown.

HeLa cells (An et al., 2004a). In this study, induction of BCa cell apoptosis by norcantharidin was evidenced with dose-responsive cytochrome *c* release (Table 1) and up-regulation of caspase activities in both HS-578T and MCF-7 cells (Fig. 3C and D), thereby leading to proteolytic cleavage of downstream target PARP (Fig. 3B). The results strongly suggest that both extrinsic and intrinsic pathways are involved in the norcantharidin-induce apoptosis.

With regard to the regulatory role of Bcl-2 superfamily members in norcantharidin-driven apoptogenesis, phosphorylation of Bcl-2 and Bcl-xL could be increased, while Bax or Bad expression were not affected in Hep-G2 cells (Chen et al., 2002). Similar to our results, the enhancement of Bax expression and the down-regulation of Bcl-xL by norcantharidin have been noted in SAS cells (Kok et al., 2005). The pro-apoptogenic propensity of norcantharidin in terms of decreased ratios of Bcl-2/Bax in A375-S2 (An et al., 2004b) and MDA-MB-231 cells (Huang et al., 2009) has also been documented. To support this, our data addressed further evidence showing that norcantharidin induced up-regulation of pro-apoptotic Bad, Bak, or Bax proteins, but down-regulation of anti-apoptotic Bcl-xL and Mcl-1 expressions in HS-578T and MCF-7 cells (Fig. 5). Consistent with previous findings (An et al., 2004b; Kok et al., 2005; Huang et al., 2009), although norcantharidin up-regulated Bcl-2 expression in MCF-7 cells, it decreased the ratio of anti-apoptotic to pro-apoptotic Bcl-2 family protein in HS-578T and MCF-7 cells. Interestingly, the lower constitutive level of Bcl-2 in ER-negative HS-578T cells may account for the higher chemosensitivity of tumor cells to norcantharidin treatment, as Bcl-2-targeted therapy increases the apoptotic response to chemotherapy (Emi et al., 2005; Yin et al., 2004). Conversely, ER+ MCF-7 cells possess not only higher constitutive level of Bcl-2, but also an integral responsiveness to the insult from anti-cancer drugs. The regulatory role of ER-mediated pathway in the constitutive Bcl-2 expression and relevant chemosensitivity in BCa cells is suggested by various reports (Tabuchi et al., 2009; LaPennsee and Ben-Jonathan, 2010). Estrogens affected both increasing cell growth and reducing cell death through activation of PI3K/Akt survival signals and Bcl-2 protein in ER+ BCa cells (Rodrik et al., 2005). Moreover, ER affected resistance to chemotherapeutic drugs by regulating Bcl-2 expression. In addition, post-translational modification on Bcl-2, such as multi-site phosphorylation, has been known to alter cellular chemosensitivity and regulates apoptosis. Since the Bcl-2 phosphorylation at serine 70 has been demonstrated to result in a loss of the binding ability of Bcl-2 to Bax and subsequent cell death (Shitashige et al., 2001), it may, at least in part, mechanisti-

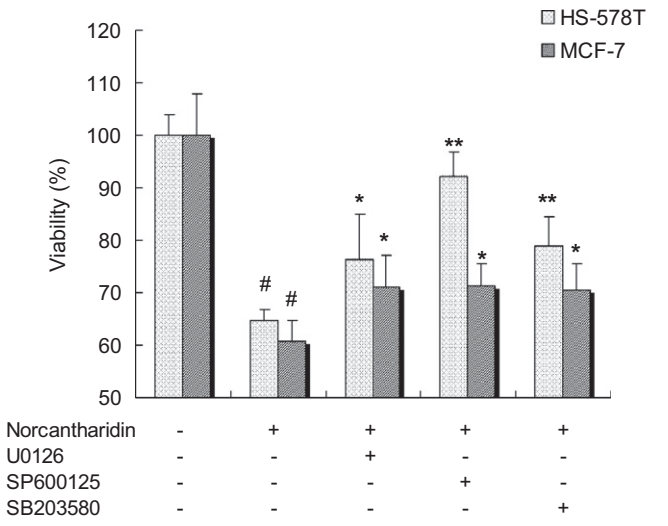

Fig. 8. Attenuation of norcantharidin-induced BCa cytotoxicity by blockage of kinase activity. HS-578T cells cultivated in a 96-well plate were treated with either 0.1% DMSO as solvent control or the selective kinase inhibitors, including 20 μM U0126 for MAPK/ERK kinase (ERK), 20 μM SP600125 for c-Jun N-terminal kinase (JNK), or 20 μM SB203580 for $p38^{MAPK}$, at 2 h before adding 20 and 30 μg/ml norcantharidin in HS-578T and MCF-7 cells, respectively. After being treated with norcantharidin 24 h, the drug-driven cytotoxicity was evaluated by MTT assay. Data are presented as mean ± SD. $^{\#}P < 0.01$ compared to negative control cells; $^{*}P < 0.05$ and $^{**}P < 0.01$, compared to the cells treated with norcantharidin at the same dose but without an inhibitor. The statistical differences were analyzed using Student's *t*-test.

cally explain the norcantharidin-triggered up-regulation of Bcl-2 protein expression and its phosphorylation at serine 70. Taken together, our findings support that norcantharidin induces apoptosis mainly through modulating the expression and the interaction among Bcl-2-related proteins in BCa cells.

The STAT proteins are a family of transcription factors that regulate cell proliferation, differentiation, survival, and apoptosis (Battle and Frank, 2002; Bromberg, 2001). Accumulating clinical evidence has pointed out the aberrant activities of STAT1, STAT3, and STAT5 in a diversity of tumors, including breast cancers. Among the STAT family members, STAT1 is generally thought to promote apoptosis and tumor suppression (Kim and Lee, 2007), while STAT3 and STAT5 modulate cell growth and inhibit apoptosis (Silva, 2004). Accordingly, STAT3 and STAT5 play important and distinct roles in mammary development and are activated in breast cancer in a manner of reciprocal regulation. Analysis of the activation state of both STAT3 and STAT5 has been thought to provide important diagnostic and prognostic information in breast cancer (Walker et al., 2009). Conversely, targeting constitutive STAT3 signaling is thus an attractive therapeutic approach for cancers (Kunigal et al., 2009). In terms of the role that STAT1 plays in norcantharidin-associated apoptogenesis, it was found that STAT1 protein directly interacts with TNFR1 and TRADD in HeLa cells (Wang et al., 2000) and that norcantharidin induces STAT1 nuclear translocation, thereby initiating Jurkat and CT26 cell apoptosis (Chen et al., 2009a; Liao et al., 2007). The norcantharidin-elicited STAT1 phosphorylation (Fig. 6) and TRADD protein up-regulation (Fig. 4) seen in MCF-7 cells may fortify the concept that norcantharidin possesses anti-cancer potency through activating extrinsic apoptotic pathway in tumor cells. Furthermore, Kunigal et al. (2009) demonstrated that siRNA-mediated STAT3 knockdown reduced expression of Bcl-xL and survivin in MDA-MB-231 cells, and induced the Fas-mediated extrinsic apoptotic pathway by activating caspases-3, -8, and -9 and PARP1 cleavage. The norcantharidin increased Fas protein level (Fig. 4) and the attenuated STAT3 phosphorylation (Fig. 6) found in HS-578T cells, again, demonstrated the involvement of extrinsic pathway. However, the mechanisms through which STATs mediate BCa cell apoptosis remain further elucidation.

The importance of MAPK-associated signaling activity in norcantharidin-induced apoptosis of various cancer cells has been widely addressed (An et al., 2004a, 2005; Chen et al., 2003, 2008). Although inhibition of Akt and NF-κB signaling has recently been implicated in the norcantharidin-induced apoptosis of human BCa cells (Huang et al., 2009), to our knowledge this is the first evidence showing that activities of ERK, JNK and $p38^{MAPK}$ contribute to norcantharidin-induced death of HS-578T and MCF-7 cells (Figs. 7 and 8). Given that the STAT1 phosphorylation has been suggested to be mediated by MAPKs (Kim and Lee, 2007; Wen et al., 1995), the coincidental dose-responsive profiles of MAPKs and STATs raised the possibility that the cross-talking effect between MAPK and STAT signaling pathways may be responsible for the norcantharidin-induced BCa cell apoptosis. Nevertheless, it remains to be determined whether $p38^{MAPK}$ phosphorylation induced by norcantharidin treatment contributes to STAT1 phosphorylation and/or STAT1-mediated pro-apoptotic effect in BCa cells.

In conclusion, norcantharidin may disturb cell cycle distribution of BCa cells through p53- and Chk-related pathways. Alternatively, it may induce BCa cell apoptosis via both extrinsic and intrinsic pathways. The MAPK- and STAT-mediated signaling pathways are mechanistically involved in the apoptogenesis of BCa cells triggered by norcantharidin. Therefore, norcantharidin is suggested as a chemotherapeutic agent for treating BCa.

5. Conflict of interest statement

None declared.

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