Suppression of growth of highly-metastatic human breast cancer cells by norcantharidin and its mechanisms of action

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Abstract The effects of norcantharidin (NCTD) on the growth of highly-metastatic human breast cancer cells were investigated by in vitro and ex vivo assays. Our results indicated that norcantharidin inhibited the in vitro growth of human breast cancer MDA-MB-231 cell line in dose- and time-dependent manners after the cancer cells were treated with norcantharidin at the concentrations of 6, 30 and 60 $\mu\text{mol/L}$ for 24, 48 and 72 h. Moreover, the sera from the NCTD-treated rabbits after intravenous injection of NCTD at 15 and 30 min significantly suppressed the growth of the cancer cells ex vivo. The analyses by Hoechst 33258 staining and flow cytometry showed that the typical apoptotic morphological changes appeared and cell cycles arrested at G2/M phase in MDA-MB-231 cells after the cells were treated for 48 h with NCTD. In addition, NCTD down-regulated the expressions of anti-apoptotic protein Bcl-2 and up-regulated the expressions of pro-apoptotic protein

Bax, eventually leading to the reduction of Bcl-2/Bax ratio in MDA-MB-231 cells. Furthermore, NCTD at concentrations of 6, 30 and 60 $\mu\text{mol/L}$ dose-dependently reduced the phosphorylation of Akt and NF- κB expression in the breast cancer cell line. Induction of apoptosis and cell cycle arrest as well as reduction of Bcl-2/Bax ratio by NCTD may be the important mechanisms of action of NCTD suppressing the growth of MDA-MB-231 cells, which are associated with inhibition of the Akt and NF- κB signaling. Our findings suggest that norcantharidin may have a wide therapeutic and/or adjuvant therapeutic application in the treatment of human breast cancer.

Keywords Norcantharidin · Human breast cancer · Growth · Apoptosis · Cell cycle arrest · Bcl-2/Bax ratio · Akt signaling · NF- κB

Introduction

Breast cancer becomes the second leading cause of cancer related deaths among females worldwide (Jemal et al. 2008; Park et al. 2008), and its rate in China and other Asian countries is also increasing rapidly (Park et al. 2008; Ziegler et al. 2008). To find novel natural compounds with low toxicity and high selectivity of killing cancer cells is an important area in cancer research. To date, chemotherapy has been the most frequently used treatment for breast cancer

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and other cancers. However, some normal cells are destroyed as well by this method of treatment. Due to their wide range of biological activities and low toxicity in animal models, some natural products have been used as alternative treatments for cancers including breast cancer. Norcantharidin (NCTD) is the demethylated analog of cantharidin isolated from natural blister beetles. In China, NCTD as an anticancer drug is currently used to treat breast cancer, hepatoma, leukemia, colon cancer, etc. The chemical structure of NCTD is shown in Fig. 1A. NCTD induced apoptosis in hepatoma cells, which is involved in a caspase activation pathway (Chen et al. 2002). NCTD has tumor chemopreventive effect on *in vivo* models. There have been studies showing that NCTD significantly inhibited tumor cell growth, invasion, and metastasis in nude mice bearing human gallbladder carcinoma (Fan et al. 2006); NCTD suppressed the invasion and metastasis in CT26 colorectal adenocarcinoma cells on *in vitro* and *in vivo* models (Chen et al. 2005). It also induced apoptosis in human leukemic Jurkat cells without affecting the viability of normal mononuclear cells (Liao et al. 2007). However, whether or not NCTD has inhibitory activities against highly-metastatic human breast cancer cells remains unclear. In this study, we investigated the *in vitro* and *ex vivo* effects of NCTD on the growth of highly-metastatic human breast cancer cell line MDA-MB-231. We focused on disclosing the molecular mechanisms of action of NCTD against the Akt signaling and its down-stream targets. We show that suppression of the phosphorylation of Akt and NF- κ B p-65 expressions, and reduction of the ratio of Bcl-2/Bax proteins by NCTD play major roles in inhibiting the growth of MDA-MB-231 cells.

Materials and methods

Materials

Enhanced chemiluminescence Western blotting detection reagents were purchased from GE Healthcare Life Sciences (Piscataway, NJ). The primary antibodies to human Bcl-2, Bax, nuclear factor (NF- κ B p-65), Akt, p-Akt (Ser473), and β -actin were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Acrylamide and the protein assay kit were obtained from

A Chemical structure of NCTD

B

C

Fig. 1 *In vitro* and *ex vivo* effects of norcantharidin (NCTD) on the growth of highly-metastatic human breast cancer cell line MDA-MB-231. **A** The chemical structure of NCTD. **B** *In vitro* effects of NCTD on the growth of MDA-MB-231 cells. The cells were cultured for 24, 48, and 72 h in DMEM medium containing NCTD at the concentrations indicated in figure. **C** *Ex vivo* effects of NCTD on the growth of MDA-MB-231 cells. The cells were cultured for 24, 48, and 72 h in DMEM medium containing each rabbit serum obtained 0, 15, 30 min and 1 h after intravenous injection of NCTD, respectively. The effects on the cell growth were determined by MTT assay as described in the “Materials and methods” section. Each point and vertical bar represents the mean and SD for 6 wells. Statistical analysis was carried out using the ANOVA and Bonferroni test (**B** and **C**). * $P < 0.05$. All statistical tests were two-sided. This figure is the representative of three similar experiments

Bio-Rad (Hercules, CA). Norcantharidin (NCTD), DMEM, penicillin, streptomycin, fetal bovine serum, and trypsin/EDTA, 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258, propidium iodide, and all other chemicals employed in

this study were purchased from Sigma Chemical Co. (St. Louis, MO).

Animal experimentation and preparation of sera from NCTD-treated rabbits

These were done according to our published methods with slight modifications (Zhang et al. 2001). In brief, New Zealand White female rabbits (3.5–4 kg; from Luye Pharmaceutical Company, Yantai, China) were treated in accordance with guidelines established by the Animal Care and Use Committee at Yantai University. NCTD was given via the intravenous injection to the rabbits once daily at a dose of $10 \text{ mg mL}^{-1} 100 \text{ g}^{-1}$ body weight for 3 days. On the third day, the blood was then collected at 0, 15, 30 min, and 1 h from the rabbits (fasted for 16 h) after intravenous injection of NCTD. The collected blood was left to clot for 2 h at room temperature and centrifuged twice at $3000 \times g$ at 4°C for 20 min. The sera were sterilized by filtration and then heated at 56°C for 30 min. The prepared sera were aliquoted, and stored at -80°C until ex vivo cell growth assay.

Cell culture and in vitro and ex vivo cell growth assays

The highly-metastatic human breast cancer cell line MDA-MB-231 was obtained from the American Type Culture Collection. The cell line was cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Beijing, China), glutamine (2 mmol/L), penicillin (100 U/mL) and streptomycin (100 $\mu\text{g/mL}$) at 37°C in a humidified incubator with 95% air/5% CO_2 atmosphere. The in vitro and ex vivo assays were done according to our published methods (Zhang et al. 1999, 2001). In brief, cells were cultured in DMEM supplemented with 10% FBS (in the case of in vitro assay) containing NCTD at concentrations of 0–60 $\mu\text{mol/L}$, or 10% rabbit sera (in the case of ex vivo assay) obtained 0, 15, 30 min, and 1 h after intravenous injection of NCTD, respectively. Control groups received DMSO vehicle in vitro assay (0.1%, final concentration). The effects of NCTD on in vitro and ex vivo growth in MDA-MB-231 cells were measured 24, 48, and 72 h after the treatments using MTT assay kit following the manufacturer's instruction. Each experiment was repeated three times.

Apoptosis assays and cell cycle analysis

To examine the effects of NCTD on apoptosis and cell cycle distribution in MDA-MB-231 cells, we used our published methods (Zhang et al. 2000). The cells at 70% confluence were treated for 48 h with NCTD at different concentrations. The treated cells were fixed with 1% glutaraldehyde and stained with Hoechst 33258. The morphological changes in the nuclear chromatin were observed under a fluorescent microscope (Nikon, TE2000-U, Japan), using $40\times$ lens. For flow cytometry for cell cycle analysis and apoptosis, the treated cells were labeled with propidium iodide solution containing RNase A. The DNA content was analyzed by flow cytometry (Becton Dickinson FACS Vantage SE, San Jose, CA). The cell cycle distribution and apoptosis were determined based on DNA content and the sub- G_1 cell population, respectively.

Western blot analysis

To detect the effects of NCTD on protein expressions, we used the Western blot analysis as described in the method of Chen et al (2001). MDA-MB-231 cells were treated with NCTD at the concentrations of 0 (0.1% DMSO vehicle as control), 6, 30 and 60 $\mu\text{mol/L}$, and collected at 48 h. For detection of p-Akt, the cells were treated for 45 min with NCTD. The treated cells were harvested and lysed in lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4 , protease inhibitor cocktail, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride] for 20 min on ice. Cellular protein was loaded and separated on sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane by the standard electric transfer protocol. The membrane was blocked and probed with primary antibodies, then incubated with horseradish peroxidase-labeled second antibody. Immunoblots were developed using the enhanced chemiluminescence detection system.

Statistical analysis

The data were expressed as mean $\pm$ standard deviation (SD) and analyzed by the SPSS 13.0 software to evaluate the statistical difference. Statistical analysis

Fig. 2 Effects of NCTD on induction of apoptosis and cell cycle arrest in MDA-MB-231 cells. **A** Induction of apoptosis in MDA-MB-231 cells by treatment for 48 h with NCTD at concentrations of 0 (**A1**: 0.1% DMSO vehicle as the control), 6 $\mu\text{mol/L}$ (**A2**), 30 $\mu\text{mol/L}$ (**A3**) and 60 $\mu\text{mol/L}$ (**A4**). The treated cells were fixed with 1% glutaraldehyde and then stained with Hoechst 33258. Apoptotic morphological changes in the nuclear chromatin were observed under a fluorescent

microscope as described in the “Materials and methods” section. **B** Induction of apoptosis and cell cycle arrest in MDA-MB-231 cells by treatment for 48 h with NCTD at the concentrations indicated in figure. The treated cells were analyzed for DNA content by flow cytometry. Compared with the control (0.1% DMSO vehicle), NCTD at concentrations of 6, 30 and 60 $\mu\text{mol/L}$ dose-dependently induced apoptosis (cells in sub-G₁ phase) and G₂/M phase arrest in the cancer cells

was done using the ANOVA and Bonferroni test. Values between different treatment groups were compared. Results were considered significant at a *P* value of <0.05. All statistical tests were two-sided.

Results and discussion

NCTD suppressed in vitro and ex vivo growth of MDA-MB-231 cells

We first confirmed that NCTD inhibited the growth of highly-metastatic human breast cancer cell line

MDA-MB-231 in dose- and time-dependent manners after the cells were treated with NCTD at the concentrations of 6–60 $\mu\text{mol/L}$ for 24, 48 and 72 h, respectively (Fig. 1B). The ex vivo assay showed that the rabbit sera obtained 15 and 30 min after intravenous injection of NCTD significantly suppressed the growth of MDA-MB-231 cells after the cells were treated with these sera for 24, 48 and 72 h, while the 1 h rabbit sera did not show the significant inhibitory effect (Fig. 1C). This result suggests that NCTD has a certain bioavailability by intravenous injection of NCTD and the peak inhibition of the breast cancer cell growth is at

0.5 h after intravenous injection of NCTD in rabbits. There have been reports showing that the blood concentration of NCTD can be detected after intragastric administration of NCTD in mice (Wei et al. 2007); NCTD also appeared in human serum after oral administration of NCTD in human volunteers (Wei et al. 2008). These results partially explain why NCTD has its therapeutic and/or adjuvant therapeutic effects on treatment for some cancer patients.

NCTD induced apoptosis by reducing the Bcl-2/Bax protein ratio in MDA-MB-231 cells

To understand the mechanisms of action of NCTD against the growth in human breast cancer cells, we investigated the effects of NCTD on apoptosis and cell cycle arrest in the cancer cells as well as the expressions of related proteins. Hoechst 33258 staining indicated that the typical morphological changes, such as formation of apoptotic bodies appeared in MDA-MB-231 cells after the cells were treated for 48 h with NCTD at concentrations of 30 and

60 $\mu\text{mol/L}$, whereas the control cells without NCTD treatment did not show the evident apoptotic morphological changes (Fig. 2A). Flow cytometric analysis confirmed that NCTD at concentrations of 6, 30 and 60 $\mu\text{mol/L}$ dose-dependently induced apoptosis and cell cycle arrest at the G2/M phase in MDA-MB-231 cells after the cells were treated for 48 h with NCTD (Fig. 2B). Furthermore, the analyses of Western blotting demonstrated that NCTD down-regulated the expression of anti-apoptotic protein Bcl-2 and up-regulated the level of pro-apoptotic protein bax, eventually leading the reduction of ratios of Bcl-2 and Bax protein levels (Fig. 3) in MDA-MB-231 cells. There have been studies showing that Bcl-2 and its dominant inhibitor Bax are key regulators of cell proliferation and apoptosis. Overexpression of Bcl-2 enhances cell survival by suppressing apoptosis, but overexpression of Bax accelerates cell death (Oltvai et al. 1993). Induction of apoptosis and cell cycle arrest and reduction of Bcl-2/Bax protein ratio by NCTD may be one of the important mechanisms of action of NCTD against the breast cancer cell growth.

Fig. 3 Effects of NCTD on protein expressions of Bcl-2 and Bax in MDA-MB-231 cells. The expressions of Bcl-2 and Bax proteins were analyzed by Western blotting as described in “Material and methods” section. The cells were treated for 48 h with NCTD at the indicated concentrations; β -Actin was used in Western blot analyses as a sample loading control. The

ratio of Bcl-2 and Bax (the ratio of relative density of each band normalized to β -actin), shown as mean $\pm$ SD (Bar) is relative to that of zero (vehicle) as the control (designated as 1.0). For one experiment, three assays were carried out and only one set of gels is shown. Statistical analysis was carried out using the ANOVA and Bonferroni test. * $P < 0.05$

Fig. 4 Effects of NCTD on the phosphorylation of Akt (A) and NF- κ B (B) expressions in MDA-MB-231 cells. The cells were treated for 45 min (for p-Akt detection) and 48 h (for NF- κ B detection), respectively, with NCTD at the indicated concentration. Equal amounts of protein from total cell lysates were separated by SDS-PAGE, and Western blot analysis of p-Akt (Ser 473) and NF- κ B p-65 was done as described in the “Materials and methods” section. The density of the band (normalized to Akt) in the (A) or (normalized to β -actin) in the (B) shown as mean $\pm$ SD is relative to that of 0 (0.1% DMSO vehicle) as the control (designated as 100%). For one experiment, three assays were carried out and only one set of gels is shown. Statistical analysis was carried out using the ANOVA and Bonferroni test. * $P < 0.05$

NCTD reduced the phosphorylation of Akt and expressions of NF- κ B in MDA-MB-231 cells

To further understand the mechanisms of action of NCTD against the growth of human breast cancer cells, we investigated the effects of NCTD on Akt signaling in MDA-MB-231 cells. Our results confirmed that NCTD at concentrations of 6 μ mol/L to 60 μ mol/L significantly suppressed the phosphorylation of Akt (Fig. 4A) and NF- κ B p-65 expression (Fig. 4B) in MDA-MB-231 cells. There have been studies reporting that NF- κ B is a nuclear transcription regulator with a specific motif for bcl-2 transcription (Viatour et al. 2003; Marsden et al. 2002). Activation of p-Akt and the NF- κ B/bcl-2 pathway leads to inhibition of chemotherapy-induced apoptosis, which

results in treatment resistance (Wang et al. 1996). The NF- κ B family of transcriptional activators, p65, p50, p52, c-rel, and RelB, shares a rel homology domain. NF- κ B in its inactive state exists in the cytosol bound to inhibitory I κ B molecules. Activation of the pathway leads to phosphorylation and degradation of I κ B with subsequent nuclear localization of NF- κ B. On nuclear import, homodimeric or heterodimeric NF- κ B transactivators bind to decameric DNA consensus motifs to activate transcription (Aggarwal 2004; Hayden and Ghosh 2004). NF- κ B controls expression of numerous genes involved in inflammation and immune response processes, including proliferation, invasion and adhesion, angiogenesis, and apoptosis. It has been reported that inhibition of NF- κ B potentiates the anticancer effect

of chemotherapeutic agents. Anticancer drugs docetaxel or cisplatin treatment, like NF- κ B cDNA transfection, increased the expression of NF- κ B p65 and significantly induced NF- κ B DNA-binding activity in cancer cells, suggesting that the activation of NF- κ B by these chemotherapeutic agents may contribute to the resistance of cancer cells to these agents. In vitro and in vivo animal experiments demonstrated that the anticancer agent genistein and NF- κ B p65 siRNA exerted inhibitory effects on NF- κ B p65 expression and NF- κ B DNA-binding activity to a similar degree in MDA-MB-231 cells; both of them also abrogated the activation of NF- κ B stimulated by chemotherapeutic agents, suggesting that genistein may reduce cancer cell resistance to chemotherapeutic agents through inactivation of NF- κ B p65 expression and NF- κ B DNA-binding activity (Li et al. 2005). Our present results suggest that the suppression of NF- κ B p65 expression by NCTD may be one of mechanisms of action for inhibiting the growth in MDA-MB-231 cells although the further study is needed to clarify the effect of NCTD on the NF- κ B DNA-binding activity in the cancer cells. The Bcl-2/Bax, p-Akt, and NF- κ B have become the important targets of action by anticancer agents (Wang et al. 1996; Price et al. 1999; Helbig et al. 2003; Emi et al. 2005; Gupta et al. 2002). In the present study, we have demonstrated that NCTD down-regulates the levels of Bcl-2 protein and up-regulates the levels of Bax protein in MDA-MB-231 cells, leading the reduction of ratios of Bcl-2 and Bax proteins. Furthermore, we have confirmed that NCTD inhibits the phosphorylation of Akt in the human breast cancer cells. Such effects may be the important mechanisms of action of NCTD inducing apoptosis and suppressing the growth of the breast cancer cells. In addition, induction of cell cycle arrest at G2/M phase in MDA-MB-231 cells by NCTD may be partially responsible for inhibition of the growth of the breast cancer cells although the mechanisms of action of NCTD arresting the cell cycles require further investigation.

In summary, we have demonstrated that NCTD significantly suppressed the growth of highly-metastatic human breast cancer cell line MDA-MB-231 in vitro and ex vivo. Moreover, NCTD induced the apoptosis and cell cycle arrest at G2/M phase in MDA-MB-231 cells. In addition, NCTD reduced the Bcl-2/Bax protein ratio and inhibited the Akt and

NF- κ B signaling in MDA-MB-231 cells. All these findings suggest that NCTD may have the wide therapeutic and/or adjuvant therapeutic application in the treatment of human breast cancer.

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