

Promotion of cytoplasmic vacuolation-mediated cell death of human prostate cancer PC-3 cells by oxidative stress induced by daucusol, a new guaiane-type sesquiterpenoid from *Daucus carota* L.

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Abstract: We investigated the antitumor activity of daucusol (DS) derived from *Daucus carota* L. in PC-3, A549 and HeLa cell lines by the MTT assay. Optical microscopy revealed that exposure of PC-3 cells to DS resulted in cytoplasmic vacuolation. Flow cytometry analysis of the phase of the cell cycle did not reveal a sub-G1 peak, and no caspase-dependent activation was observed after DS treatment. The levels of endoplasmic reticulum (ER) stress biomarkers, LC3B-II and ubiquitinated proteins were increased. It was also observed that oxidative stress played an important role in the activation of the cytoplasmic vacuolation-mediated cell-death pathway. *In vivo*, DS inhibited tumor growth in nude mice by 39.13% compared to the vehicle. Protein expression in the tumor tissue was consistent with their expression *in vitro*. Our findings indicate that DS induced cytoplasmic vacuolation-mediated death in PC-3 cells by triggering oxidative stress and suggest that targeting this pathway could serve as a novel therapeutic approach for prostate cancer.

Key words: daucusol; cytoplasmic vacuolation; oxidative stress; endoplasmic reticulum stress; prostate cancer

INTRODUCTION

Prostate cancer is the most common malignant tumor in men, causing the deaths of hundreds of thousands of men each year worldwide [1], making it the second highest cause of cancer-related deaths after lung cancer. Prostate cancer is heterogeneous, and treatment varies depending on the stage of the disease. While guidelines for the treatment of prostate cancer have been developed and have facilitated treatment decisions, its poor response to chemotherapy and frequent recurrence continue to present major obstacles in overcoming the disease. Recent studies showed that chemotherapy induces apoptosis or autophagy in prostate cancer cells [2,3]. However, resistance to chemotherapy in prostate cancer can arise via similar antiapoptotic or antiautophagic mechanisms [4,5]. Therefore, targeting a non-apoptotic cell death

pathway may be an effective strategy of avoiding drug resistance in prostate cancer treatment.

Cytoplasmic vacuolation-mediated cell death is a non-apoptotic cell death pathway characterized by extensive cytoplasmic vacuolation and endoplasmic reticulum (ER) dilation; it is not accompanied by caspase activation or DNA fragmentation [6]. Cytoplasmic vacuolation-mediated cell death induces upregulation of the autophagic biomarker LC3B-II, the ER stress markers GRP78, GADD153, and the accumulation of ubiquitinated proteins [7]. It was reported that the protein synthesis inhibitor cycloheximide (CHX) blocks cytoplasmic vacuole formation [8]. Although other mechanisms of cell death, like apoptosis and autophagy, have been well characterized, the mechanisms through which cytoplasmic vacuolation-mediated cell death occurs are not well understood.

In a previous study, we isolated the novel compound daucosol (DS) (Fig.1A) [9], a guaiane-type sesquiterpenoid from *Fructus carotae*, the fruit of *Daucus carota* L. (Umbelliferae) [10]. In this study, we tested the effects of DS on three human cancer cell lines: prostate cancer PC-3 cells, lung cancer A549 cells and cervical cancer HeLa cells. We found that DS inhibited growth of PC-3 cells via a novel pathway related to cytoplasmic vacuolation-mediated cell death. We next focused our attention on the resistance mechanisms of prostate cancer to exposure to DS *in vitro* and *in vivo*. Our findings revealed the therapeutic potential of DS for treating prostate cancer.

MATERIALS AND METHODS

Experimental animals

All animal research was conducted in accordance with the Declaration of Helsinki and with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the United National Institutes of Health. All experimental protocols were approved by the Review Committee for the Use of Animal Subjects of Changshu Realistic Technology Co. Ltd (Suzhou, China).

Reagents

Antibodies against the following proteins were used in this study: LC3B, ubiquitin, caspase-3, PARP, β -Actin (Cell Signaling Technology, Beverly, MA); GRP78, GADD153 (Santa Cruz Biotechnology, Santa Cruz, CA); calreticulin (Abcam, Cambridge, MA). The secondary antibodies goat anti-rabbit IgG, goat anti-mouse IgG (Biosharp Biological science and technology, Shanghai, China) and FITC goat anti-rabbit IgG (Huabio, Hangzhou, China) were used. DS was extracted and identified according to methods described in a previous study [9]. The chemicals cycloheximide (CHX, Sigma-Aldrich, St. Louis, MO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Solarbio, Beijing, China), N-acetylcysteine (NAC, Sigma-Aldrich, St. Louis, MO), TritonX-100 (Solarbio, Beijing, China) and dimethyl sulfoxide (DMSO, Gibco, Grand Island, New York, USA) were also used. Cell incubation medium was

purchased from Gino Biomedical Technology Co. Ltd (Hangzhou, China) and fetal bovine serum (FBS) was purchased from Gemini Bio Products (Gemini, CA, USA). The following kits were used in this study: DNA content quantitation assay kit (KeyGEN Nanjing, China), Reactive Oxygen Species (ROS) determination kit (Jiancheng, Nanjing, China), Giemsa Stain kit (Solarbio, Beijing, China), BCA protein concentration determination kit (Beyotime, Shanghai, China) and ECL system (Beyotime, Shanghai, China).

Cell lines and culture conditions

The human cancer cell lines PC-3, A549 and HeLa were obtained from the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle's medium (HeLa) or RPMI-1640 medium (PC-3, A549) supplemented with 10% FBS and 1% penicillin/streptomycin solution (100 IU/mL penicillin, 100 mg/mL streptomycin) at 37°C in 5% CO₂. Experiments were conducted after cells had been incubated for 24 h. DS was dissolved in DMSO and diluted into culture medium containing 5% FBS, with the final concentration of 0.05% DMSO in each well. The control group received the same amount of DMSO.

Cell viability assay

Cell viability was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in six replicates. Cells were seeded in 96-well plates at 3000 cells/well in a final volume of 100 μ L. After 24-h incubation at 37°C in 5% CO₂, cells were treated with DS (at final concentrations of 40, 60, 80, or 100 μ M for PC-3 and HeLa cells, and 50, 100, 150, or 200 μ M for A549 cells), or PC-3 cells were pre-treated with CHX (final concentration 5 μ g/mL) or NAC (final concentration 5 mM) for 1 h, and then various concentrations of DS (final concentrations 40, 60, 80 and 100 μ M) were added to the media. After incubation for 24 h, 48 h and 72 h, cells were treated with 20 μ L of the MTT stock solution (5 mg/mL) in each well for another 2 h. The medium was then carefully removed from each well and replaced with 200 μ L DMSO. Absorbance was measured at 570 nm on a plate reader.

Giemsa staining

PC-3 cells were seeded into 12-well plates at 1×10^5 cells/well in a final volume of 1 mL. After 24 h, cells were treated with DS (final concentrations 60 and 100 μM) in 2 mL medium or pretreated with CHX (final concentration 5 $\mu\text{g}/\text{mL}$) or NAC (final concentration 5 mM). All treated cells were incubated at 37°C in 5% CO_2 for another 48 h. The medium was carefully removed from each well and cells were washed three times with PBS, fixed for 3 min with methanol (chilled at -20°C), then stained with Giemsa for 30 min. After washing with water, 1 mL PBS was added to each well before optical microscopy.

Flow cytometry cell cycle analysis

For cell cycle analysis, the DNA content quantitation assay kit was used. Briefly, 48 h after drug treatment, cells were harvested in ice-cold PBS (3×10^5 cells in 300 μL) and 700 μL 100% ethanol was added for fixation. After incubation at 4°C overnight, fixed cells were re-suspended in 100 μL RNase A and incubated at 37°C for 30 min, followed by incubation in 400 μL propidium iodide (PI) for 30 min in darkness. The distribution of cells in phases of the cell cycle was determined using a flow cytometer (Cytomics FC 500, Beckman Coulter, USA) and analyzed using Multicycle AV for Windows advanced DNA Cell Cycle Analysis software.

Reactive oxygen species (ROS) assay

Cells were seeded into 6-well plates at 5×10^5 cells/well and treated with a range of concentrations of DS for 24 h, or with 20 μM H_2O_2 for 1 h to serve as a positive control. The medium was removed and replaced with 1 mL of PBS containing 1 μM CM- H_2DCFDA , and cells were incubated in the CO_2 incubator at 37°C for 30 min in the dark. The cells were collected into 15-mL centrifuge tubes. After washing with PBS three times, the ROS level of each concentration was determined using a flow cytometer (Cytomics FC 500, Beckman Coulter, USA) and analyzed using FlowJO 7.6 software.

Immunofluorescence analysis

Cells treated with drugs were washed with cold PBS, fixed with methanol (chilled at -20°C) for 5 min at

room temperature, permeabilized with 0.5% Triton X-100 in PBS for 10 min, and blocked with 1% BSA in PBS for 30 min. After washing with PBS, the cells were incubated overnight with anti-calreticulin antibody in 1% BSA at 4°C . The cells were incubated with FITC goat anti-rabbit IgG in 1% BSA for 2 h at room temperature. Nuclei were stained with DAPI in Vectashield mounting medium (Vector Laboratories). Confocal images were obtained using the 63X oil immersion lens in a confocal microscope (LSM 780, Carl Zeiss Jena, Germany).

Western blotting

Cells were treated with DS or pretreated with CHX (final concentration 5 $\mu\text{g}/\text{mL}$) or NAC (final concentration 5 mM) for 1 h, and then with different concentrations of DS for 48 h. Cells were collected from 6-cm dishes by scraping and centrifugation. The cells were washed once with ice-cold PBS and subsequently incubated in 75 μL lysis buffer for 30 min. Lysates were centrifuged for 15 min at 4°C at 13200 rpm. Protein concentrations in the supernatants were determined using a BCA protein concentration determination kit. Equal amounts of proteins were separated in a 7.5% SDS polyacrylamide gel and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk in 0.1% Tween-20 in TBS (TBST) for 2 h and incubated in primary antibody (1:500-1:1000) at 4°C overnight. After washing three times with TBST, the membranes were incubated for 2 h at room temperature with the appropriate secondary antibody (1:5000). The immunoblots were visualized with an enhanced chemiluminescence (ECL) system.

Xenografts in BALB /C male mice

We established a xenograft tumor model by transplanting PC-3 cells into nude mice as described previously [11]. BALB/C male mice (5-7 weeks of age, NO.11401300028261) were purchased from HuaFu-Kang Biotechnology Co. Ltd (Beijing, China). Briefly, xenograft tumors were established by subcutaneous injection of 5×10^6 PC-3 cells in a total volume of 0.1 mL of serum-free medium. Once tumors were measurable (100-200 mm^3), the animals were randomized into three groups ($n=5$) and intraperitoneally ad-

ministered the vehicle (10% DMSO, 20% polyethylene glycol 400, and 5% Tween 80 in sterile H₂O), the chemotherapeutic cyclophosphamide (30 mg/kg, as a positive control), or DS (200 mg/kg in 10% DMSO, 20% polyethylene glycol 400, and 5% Tween 80 in sterile H₂O) daily for 3 weeks. Mouse body weight was measured daily. Tumor volumes were calculated from caliper measurements using a standard formula: $V = 0.5 \times (\text{width}^2 \times \text{length})$ [12,13]. The mice were killed and the tumors were harvested, snap-frozen in liquid nitrogen and stored at -80°C until further use.

Statistical analysis

The data are presented as means ± standard deviation (SD). One-way analysis of variance (ANOVA) and least significant difference (LSD) tests were used for all experiments. Differences were considered significant at $p < 0.05$. Statistical analyses were performed using SPSS Statistics 17.0 software.

RESULTS AND DISCUSSION

DS inhibition of cells growth *in vitro*

The MTT assay was used to evaluate the antitumor activity of DS in the cervical cancer cell line, the PC-3 prostate cancer cell line and the A549 human lung cancer cell line. DS induced dose- and time-dependent reductions in cell viability in all three cell lines. These effects were most marked in the HeLa and PC-3 cell lines (Fig. 1B-D). The IC₅₀ values for DS ranged between 46 μM and 78 μM at 72 h.

DS induction of vacuolization and death signals in PC-3 cells

Several forms of non-apoptotic cell death have been described, including oncosis [14], autophagy [15], entosis [16] and necroptosis [17]. Giemsa staining revealed a dramatic change in cell morphology in

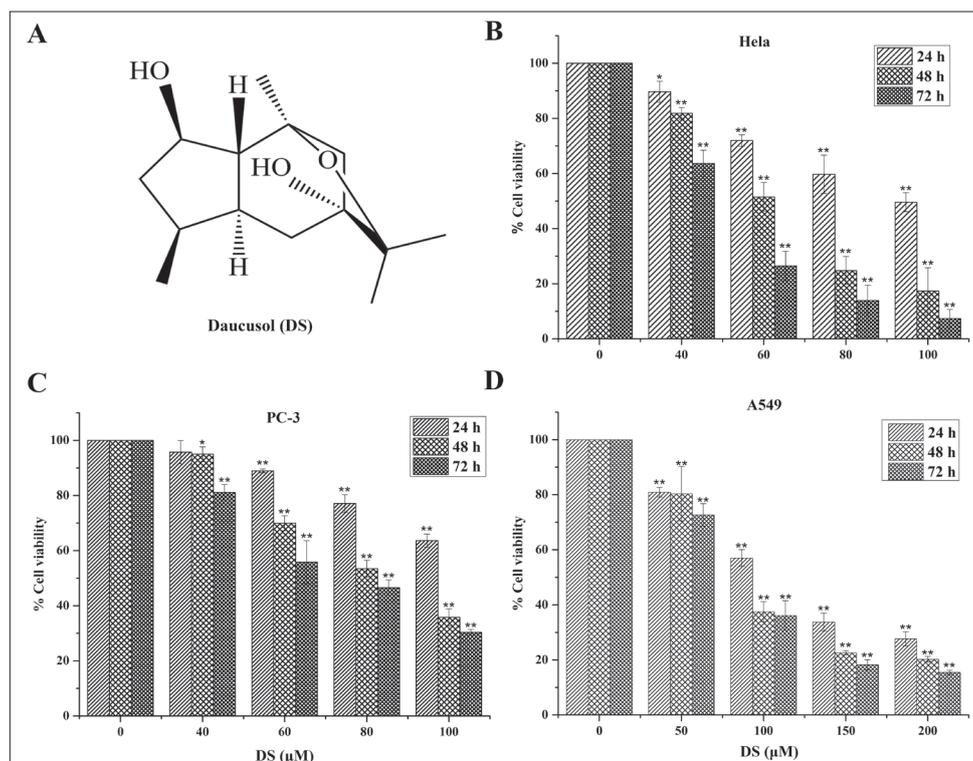


Fig. 1. The effect of DS on cell proliferation. **A** – Structure of DS. **B, C, D** – Concentration-dependent effects of DS on cell viability of cervical, prostate and lung cancer cell lines, HeLa, PC-3 and A549, respectively, after incubation with DS for 24-72 h. Cell viability was determined by the MTT assay. Bar, SD (N=6), * $p < 0.05$ and ** $p < 0.01$ vs. control group. IC₅₀ values: 46 μM (HeLa), 71 μM (PC-3) and 78 μM (A549).

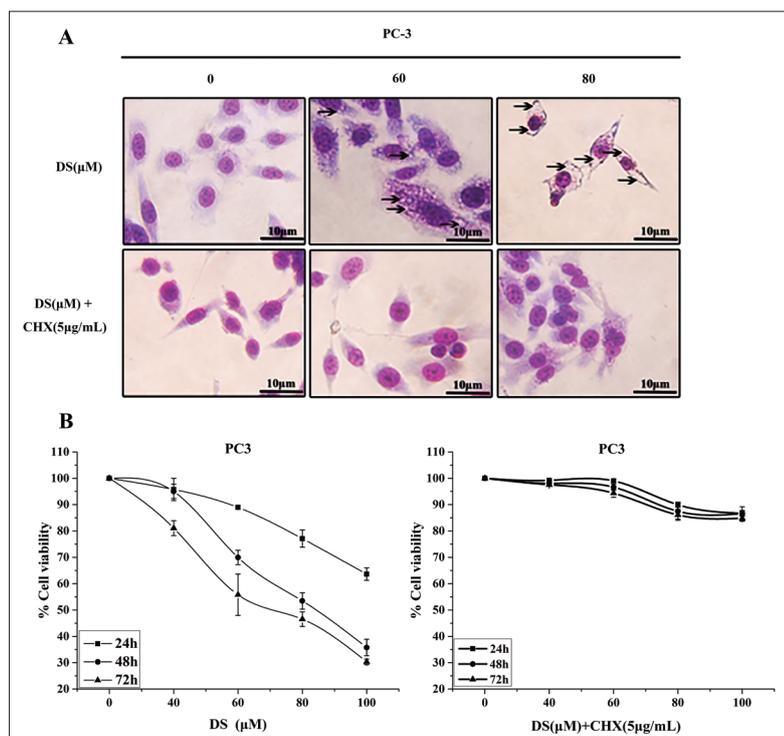


Fig. 2. DS induced cytoplasmic vacuolation in the PC-3 cell line blocked by cycloheximide (CHX). **A** – Cells were treated with DS (60 μM and 100 μM) or with DS and CHX (5 μg/mL). After staining with Giemsa, the cells were observed by optical microscopy (magnification $\times 600$). **B** – Cell viability of the PC-3 cell line was measured by the MTT assay after treatment with CHX (5 μg/mL) and different concentrations of DS treatment for 24–72 h (right panel). Points, mean; bar, SD (N=6).

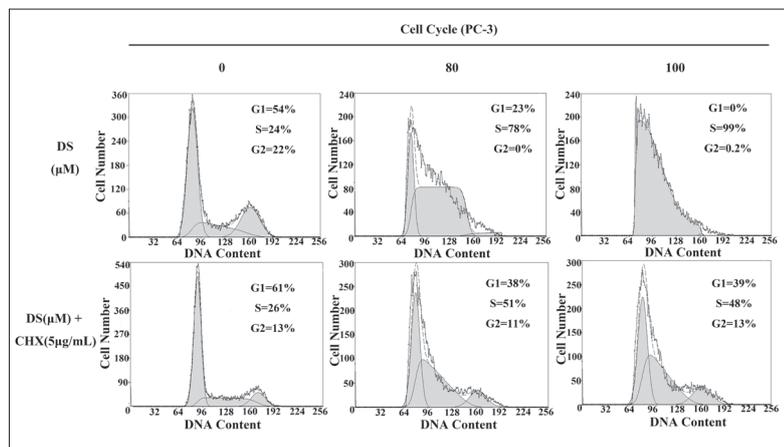


Fig. 3. DS induced S phase retardation but no sub-G1 peak as determined by flow cytometry. Cells were treated with either DS (0, 80, 100 μM) (left side) or with DS (0, 80, 100 μM) and CHX (5 μg/mL) (right side) for 48 h.

response to the DS treatment in PC-3 cells. DS induced a dose-dependent formation of cytoplasmic vacuolation. Vacuoles could be clearly visualized by their lack of cytoplasmic materials (Fig. 2A). The presence of vacuolation suggests that the mechanism by which DS induces cell death in PC-3 cells may be a novel non-apoptotic pathway.

DS-induced cytoplasmic vacuolization was blocked by CHX

CHX was used to determine its effect on vacuolation in DS-treated cells. Pretreatment with CHX diminished the ability of DS to induce vacuolation (Fig. 2A) and cell death (Fig. 2B). This result was consistent with a previous report [8] and suggests that DS-induced cell death is mediated primarily through cytoplasmic vacuolation. Based on this finding, we hypothesized that ER stress, which is related to the formation of cytoplasmic vacuoles, plays an essential role in DS-induced PC-3 cell death.

Flow cytometry analysis of the cell cycle

To further confirm that DS induced a non-apoptotic form of cell death, we analyzed cell cycle progression by flow cytometry. No sub-G1 peak (Fig. 3), a marker of apoptosis, was observed in the DNA profile. Instead, DS induced S phase retardation, an effect that was decreased by pretreatment with CHX, a general inhibitor of protein synthesis [18]. These results provide evidence that DS-induced non-apoptotic cell death is mediated by cytoplasmic vacuolation.

Effect of DS on ER stress, upregulation of LC3B-II and ubiquitinated proteins in the PC-3 cell line

Considering the above findings, we examined the effects of DS on ER stress and autophagy in PC-3 cells by Western blotting. Treatment with DS induced a dose-dependent increase in the expression of the ER biomarkers 78 kDa glucose-regulated protein (GRP78) and GADD153 (Fig. 4) as compared to the control. A previous report demonstrated that knockdown of LC3 conferred significant protection against cytoplasmic vacuolation and cell death, suggesting a novel role of LC3 in a death process other than autophagy [7], and increased protein levels of the autophagy-related protein LC3B-II were also induced by DS as reported herein (Fig. 4). In contrast, treatment with DS did not give rise to detectable levels of caspase-9 activation or PARP-1 cleavage (Fig. 4).

Additional evidence for the occurrence of DS-induced cell death by a cytoplasmic vacuolation-mediated pathway was provided by the increase in ubiquitinated proteins. Mitophagy, the specific autophagic elimination of mitochondria, plays an important role in protein ubiquitination [19]. When mitochondria are damaged, PINK1, a key regulator of mitophagy, is rapidly activated in the mitochondria, resulting in

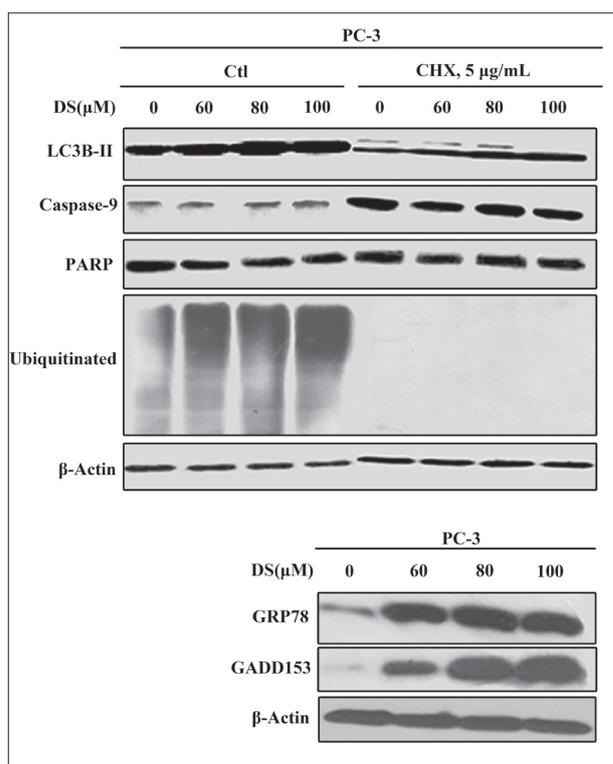


Fig. 4. Effect of DS on ER stress (right) and on the expression of LC3B-II and ubiquitinated proteins (left) in the PC-3 cell line. Western blotting analysis of DS-treated cells and the effect of CHX on the expression of proteins related to DS-induced cytoplasmic vacuolation-mediated cell death.

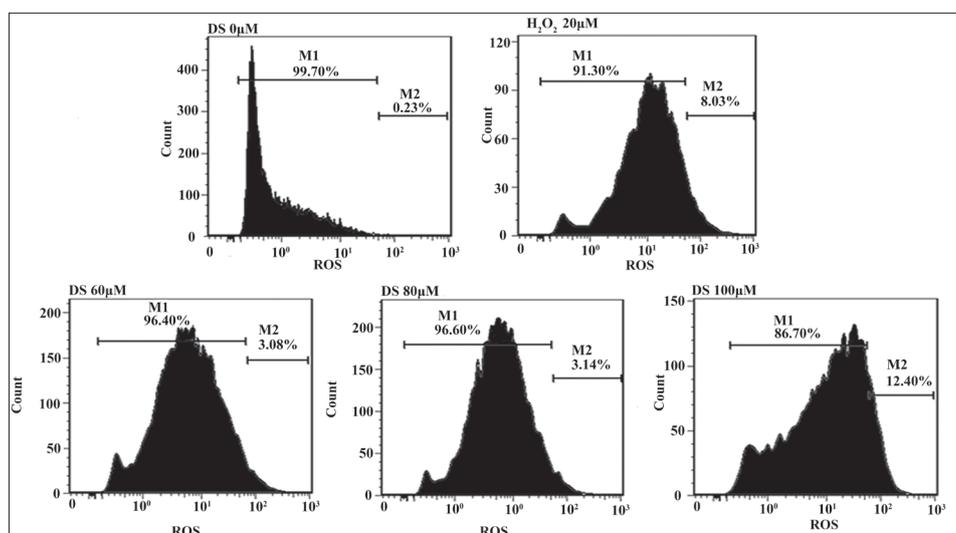


Fig. 5. Induction of ROS production in PC-3 cells by DS. H₂O₂ (20 μM) was used as a positive control.

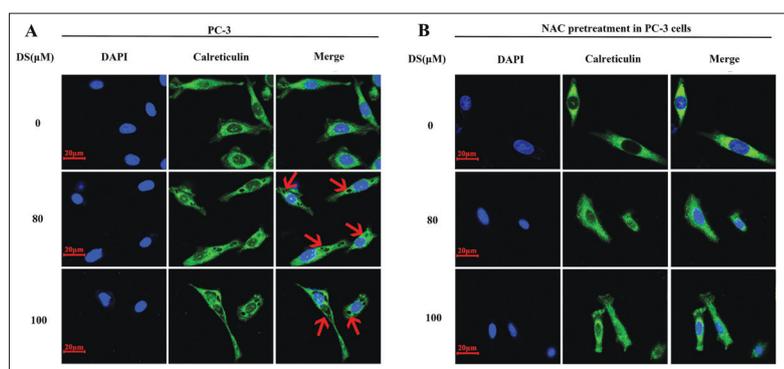


Fig. 6. DS-induced vacuoles were derived from the ER and blocked by NAC. **A** – Immunofluorescent images of prostate cancer PC-3 cells following a 48-h incubation in 0-100 μ M DS or in cells pretreated with NAC (5 mM) (**B**). Confocal images were obtained using a 63X oil immersion lens in a confocal microscope. Green – calreticulin (1:500). Blue – DAPI. Arrows – visualized vacuolation.

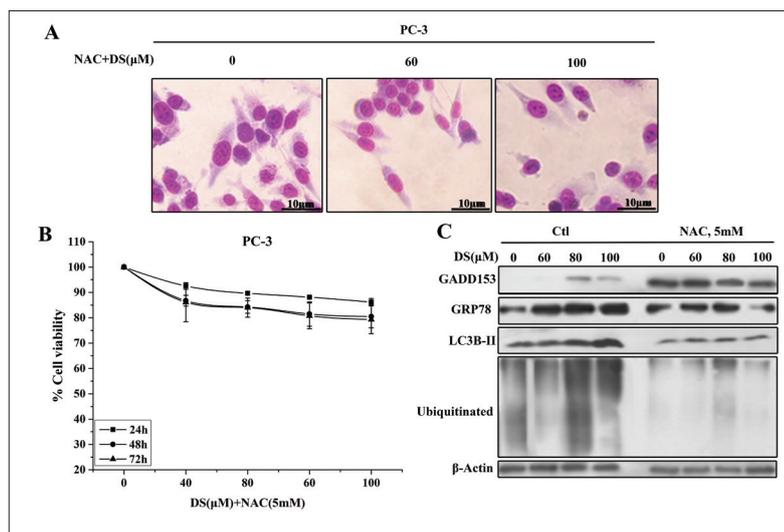


Fig. 7. ROS plays an important role in DS-induced ER stress and cytoplasmic vacuolation. **A** – Cells pretreated with NAC (5 mM) for 1 h, followed by treatment with DS (60 μ M and 100 μ M) and NAC (5 mM) for another 48 h. After staining with Giemsa, the cells were observed by optical microscopy (magnification \times 600). **B** – Cell viability of the PC-3 cell line was measured by the MTT assay after treatment with NAC (5 mM) for 24 h to 72 h. Points – mean; bar – SD (N=6). **C** Western blot analysis of the effect of NAC on ER stress and vacuolation-mediated cell death.

the recruitment of the E3 ubiquitin ligase parkin to facilitate mitochondrial protein ubiquitination [20]. These processes can be blocked by CHX, i.e. by inhibition of protein synthesis. Pretreatment with CHX abolished the ability of DS to induce ubiquitin accumulation (Fig. 4). Therefore, it can be assumed that DS upregulated protein ubiquitination by activating the mitophagy pathway, however, further research remains to be conducted to support this.

DS-induced vacuoles were derived from the ER and triggered by oxidative stress

DS induced the production of ROS, as can be seen in Fig. 5. Immunofluorescent staining with the ER chaperone calreticulin, which mainly engages in the ER lumen [21], demonstrated that DS-induced vacuoles were derived from the ER (Fig. 6A). N-acetylcysteine (NAC, Fig. 6B), a type of ROS inhibitor, blocked the formation of vacuoles, suggesting that oxidative stress likely played an important role in the novel pathway. To prove our assumption, we examined cell morphology and cell viability with Giemsa staining and the MTT assay, respectively, after pretreatment NAC. NAC abolished the ability of DS to induce cytoplasmic vacuolation (Fig. 7A) and increased the number of living cells (Fig. 7B). Similarly, ER stress (i.e., GRP78 and GADD153) and the increase in LC3B-II and ubiquitinated proteins that were induced by DS were abolished by the treatment with NAC (Fig. 7C). This suggests that the cytoplasmic vacuolation-mediated cell death pathway was triggered by oxidative stress.

DS inhibited tumor growth *in vivo*

The PC-3 xenograft tumor model was conducted to determine the effects of DS treatment on nude mice with PC-3 tumors. Treatment with the chemotherapeutic cyclophosphamide was used as a positive control for successful treatment of established tumors. Treatment with 200 mg/kg of DS inhibited tumor growth by 39.13% compared with the vehicle (Fig. 8A). Mice treated with DS did not undergo significant weight loss (Fig. 8B). Tumor samples were examined for their expression of proteins related to ER stress and autophagy by Western analysis. Consistent with the effects of the DS treatment on protein expression *in vitro*, in the tumor, DS

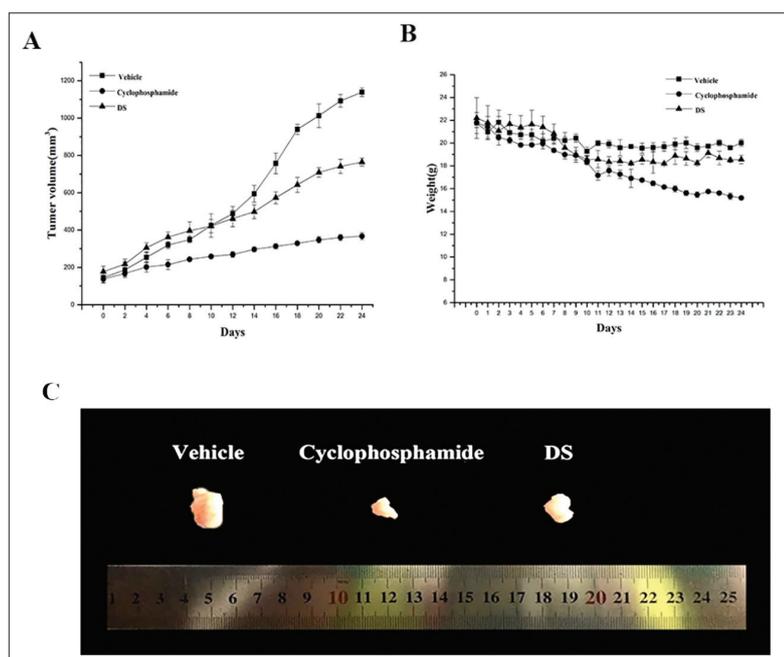


Fig. 8. DS inhibited PC-3 tumor growth in nude mice. **A** – Tumor volume. **B** – Body weight. **C** – Image of tumors of various groups.

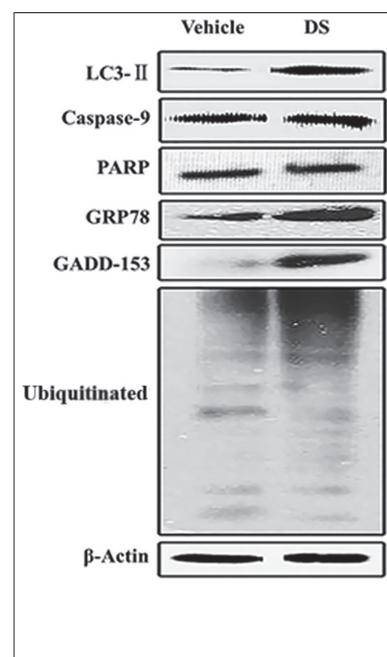


Fig. 9. Effect of DS on the expression of proteins *in vivo*. Tumor proteins were extracted and analyzed by Western blotting.

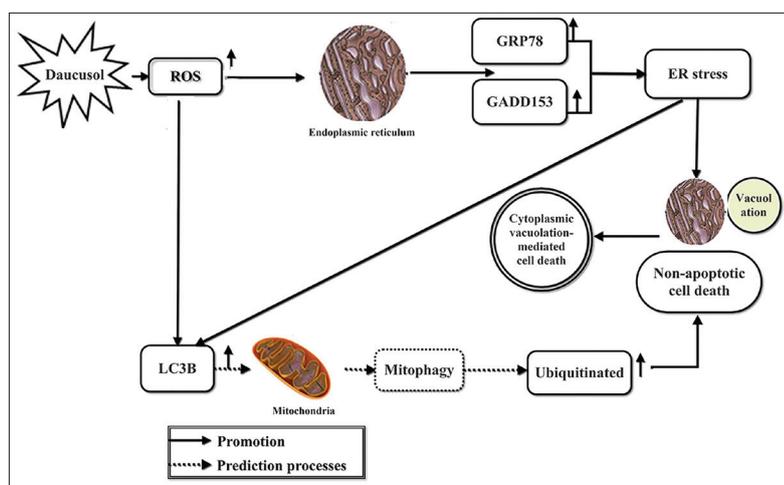


Fig. 10. Diagram of the cytoplasmic vacuolation-mediated mechanism by which DS induces PC-3 cell death.

upregulated the expression of GRP78 and GADD153 proteins, which points to the activation of ER stress. DS also increased the level of protein ubiquitination in the tumor, but had no effect on the levels of PARP-1 and caspase-9 (Fig. 9).

The novel mechanism of cell death mediation by DS in cancer cells is summarized in Fig. 10. DS, a novel guaiane-type sesquiterpenoids natural com-

pound has been shown to be capable of inducing cytoplasmic vacuolation-mediated PC-3 cells death by triggering oxidative stress, which was related to ER stress and upregulation of LC3B-II and ubiquitinated proteins without caspase-dependent activation *in vitro* and *in vivo*. This pathway could represent a new target in the development of therapeutic approaches for prostate cancer.

This study provides new information about the mechanisms of non-apoptotic cell death which are not well understood. Structural modification of DS which would enhance its anti-tumor efficacy could promote its future development as a new type of anti-prostate cancer drug.

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Conflict of interest: The authors report no conflicts of interest.

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