

A novel adriamycin analogue derived from marine microbes induces apoptosis by blocking Akt activation in human breast cancer cells

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Abstract. 1403P-3 is a novel anthracenedione derivative isolated from the secondary metabolites of endophytic fungus from the South China Sea. In previous studies, 1403P-3 was found to exhibit potent cytotoxicity against human cancer cells, but its molecular target and the mechanisms mediating its cytotoxicity remain unknown. In this study, we showed that 1403P-3 markedly inhibited the survival of the human breast cancer cells MCF-7 and MDA-MB-435 in a dose-dependent manner, with an IC_{50} of 9.5 and 7.6 μ M, respectively. Apoptosis induced by 1403P-3 was detected, as indicated by Annexin V-FITC/PI staining, elevated activated caspase-8 and -9, and cleaved PARP determined by Western blot analysis. It is of note that the phosphorylation level of Akt was significantly reduced in 1403P-3-treated cells in a dose- and time-dependent manner. Taken together, our data demonstrated that 1403P-3 induced breast cancer cell apoptosis by blocking Akt activation, suggesting that 1403P-3 may be a promising candidate compound for anti-tumor drug development.

Introduction

Breast cancer is the most common malignant disease in women and the second most frequent cancer in the world, with a rising incidence. On average, the number of diagnosed breast cancer patients increases by 1.05 million annually, accounting for 22% of all new cancer cases worldwide, and more than 410,000 women die from the disease each year (1,2).

It is widely acknowledged that disordered regulation of apoptosis is a major mechanism of tumorigenesis and drug resistance (3,4). During the apoptotic process, pro-apoptotic inducers trigger a cascade of reactions that eventually lead to the cleavage of poly (ADP-ribose) polymerase (PARP) into 89- and 24-kDa fragments, which respectively contain the active site and the DNA-binding domain of the enzyme, leading to the apoptotic death of a variety of cell types (5,6).

The phosphorylation of Akt modulates multiple molecular targets involved in the inhibition of apoptosis, cell growth and metabolism. In general, the decrease of Akt phosphorylation causes cells to undergo apoptosis, cell cycle arrest or homeostasis under low nutrient conditions. Moreover, the activation of the PI3K/Akt signaling pathway confers resistance to many types of cancer therapy and is a poor prognostic factor for numerous neoplastic lesions, making Akt a promising target for innovative cancer treatment (7).

Anthracyclines represent an important class of anti-cancer chemotherapeutic drugs, and have shown a wide spectrum of anti-cancer activities (8,9). As a novel adriamycin analogue derived from marine microbes, 1403P-3 has been shown to cause the apoptosis of KB cells and MDR KBv200 oral carcinoma cells (10). The mechanism underlying such anti-cancer effects of 1403P-3, as well as its effects on other cancer types, are not clear. The present study was performed to investigate the potential role of 1403P-3 in the growth inhibition of breast cancer cells, and to characterize the signaling pathway affected by 1403P-3 using a breast cancer model.

Materials and methods

Chemicals and reagents. Pure 1403P-3 compound was prepared and purified as described previously (11). Before use,

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulphoxide; FITC, fluorescein isothiocyanate; PI, propidium iodide; PARP, poly (ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PBS, phosphate-buffered saline; IETD-pNA, L-isoleucyl-L-glutamyl-L-Threonyl-L-aspartic-p-nitroanilide acid amide; LEHD-pNA, L-leucine-L-glutamyl-L-histidyl-L-aspartic-p-nitroaniline acid amide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling

Key words: marine, adriamycin analogue, breast cancer, apoptosis, Akt

the compound was initially dissolved in 0.5% (v/v) dimethylsulphoxide (DMSO) at 1 mM, and was then diluted in cell culture medium according to experimental requirements.

Cell lines and cell culture. The human breast cancer cell line MDA-MB-435 was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 mg/ml streptomycin and 100 U/ml penicillin (Invitrogen). Cells were grown at 37°C in a humidified 5% CO₂ atmosphere.

MTT assay for cell viability assessment. The dose-dependent effect of 1403P-3 on the viability of the human breast cancer cell line MDA-MB-435 was evaluated with varying concentrations (0, 0.1, 1, 10 and 100 μM) of 1403P-3 treatment using the MTT assay. The MTT assay is based on the conversion of a yellow substrate to dark blue formazan by mitochondrial dehydrogenases in living cells. In this assay, cells were incubated with fresh medium containing MTT (final concentration 1 mg/ml) for 4 h at 37°C. The dark blue formazan crystals formed in intact cells were solubilized with DMSO (Sangon Biotech, Shanghai, China) and the absorbance was measured at 570 nm with a reference wavelength of 630 nm using a microplate reader (BioTek Synergy2; BioTek, Winooski, VT, USA). The percentage cell growth inhibition rate was calculated using the formula: Inhibition rate (%) = [(1 - OD of treated cells)/OD of control cells] × 100%, where OD stands for the optical density (12). The half maximal inhibitory concentration (IC₅₀) was calculated using Bliss's software (13).

Annexin V-FITC/PI dual staining. Cells were grown for 2-3 days prior to drug treatment [final DMSO concentration, 0.5% (v/v)], then maintained at 37°C for the indicated additional time periods. According to the manufacturer's protocol (Keygen Biotech, Nanjing, China), the cells were washed twice with PBS and incubated in 500 μl binding buffer containing Annexin V-FITC and PI. Then, the cells were left standing for 10 min at 22°C in the dark. The chamber slide was washed twice with PBS, then the specimen was air-dried and a coverslip was applied with antifade mounting solution. The cells were examined with an inverted microscope (Zeiss Axiovert 100M; Carl Zeiss, Germany) with epifluorescence and appropriate filters. Ten randomly selected microscopic fields were captured. Experiments were performed in triplicate.

Caspase activity assay. The activity of caspase-8 and -9 was measured using a caspase colorimetric assay (Keygen Biotech). After treatment with 1403P-3 at different concentrations for 48 h, the cells were harvested, washed with PBS and then resuspended in chilled lysis buffer. Following incubation on ice for 40 min, the cells were centrifuged to collect the supernatant and protein concentrations were determined using the Bradford protein assay (Keygen Biotech). Subsequently, 150 μg of each protein sample was diluted with 50 μl lysis buffer and added to 50 μl of 2X reaction buffer containing 10 mM dithiothreitol in a 96-well plate. A colorigenic substrate (5 μl), IETD-pNA (L-isoleucyl-L-glutamyl-L-threonyl-L-aspartic-p-nitroanilide acid amide) or LEHD-pNA (L-leucine-L-glutamyl-L-histidyl-L-aspartic-p-nitroaniline acid amide), was added to each well,

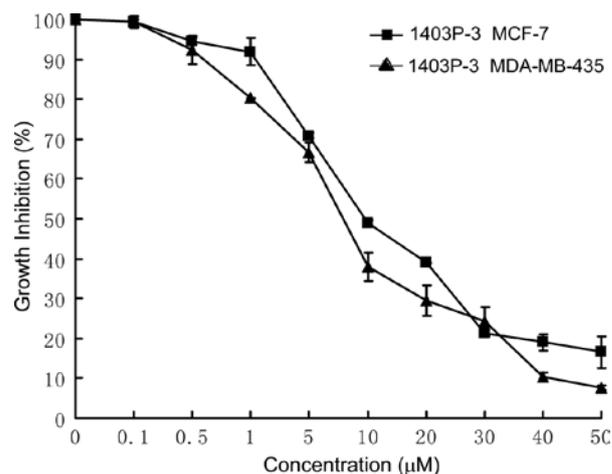


Figure 1. Growth inhibitory effect of 1403P-3 treatment at the indicated concentrations for 24 h on the human breast cancer cell line MDA-MB-435. Cell viability was measured by the MTT assay. Data were obtained from three separate experiments conducted with triplicate samples. Statistical analysis used the Student's t-test.

and the plate was incubated at 37°C in the dark for 4 h. ODs were determined at 405 nm using a microplate reader (BioTek Synergy2; BioTek).

Western blot analysis. Cells were harvested in sampling buffer [62.5 mmol/l Tris-HCl (pH 6.8), 10% glycerol and 2% SDS] and heated for 5 min at 100°C. The protein concentration was determined by the Bradford assay using a commercial kit purchased from Bio-Rad Laboratories (Hercules, CA, USA). Equal quantities of protein were separated electrophoretically on 10% SDS/polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Roche, Mannheim, Germany). The membrane was probed with rabbit anti-human PARP, rabbit anti-human phospho-Akt (ser473) or rabbit anti-human Akt antibodies (Cell Signaling Technology, Beverly, MA, USA). The expression of phospho-Akt was determined with horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (1:3,000) and enhanced chemiluminescence (Pierce, Rockford, IL, USA) according to the manufacturer's suggested protocols. The membranes were stripped and re-probed with an anti-Actin mouse monoclonal antibody (Sigma, St. Louis, MO, USA) as a loading control.

Statistical analysis. Statistical analyses were carried out using the SPSS 10.0 statistical software package. The data are expressed as the means ± SD. For the MTT assay, statistical analyses were performed using Student's t-test. The statistical significance of the differences between groups was determined by one-way analysis of variance (ANOVA) followed by a Newman-Keuls *post hoc* test. P ≤ 0.05 was considered statistically significant.

Results

Inhibitory effect of 1403P-3 on human breast cancer MDA-MB-435 cell growth in vitro. It has been reported that 1403P-3 exert potent cytotoxicity against oral squamous carcinoma KB and KBv200 cells (10). To further investigate its

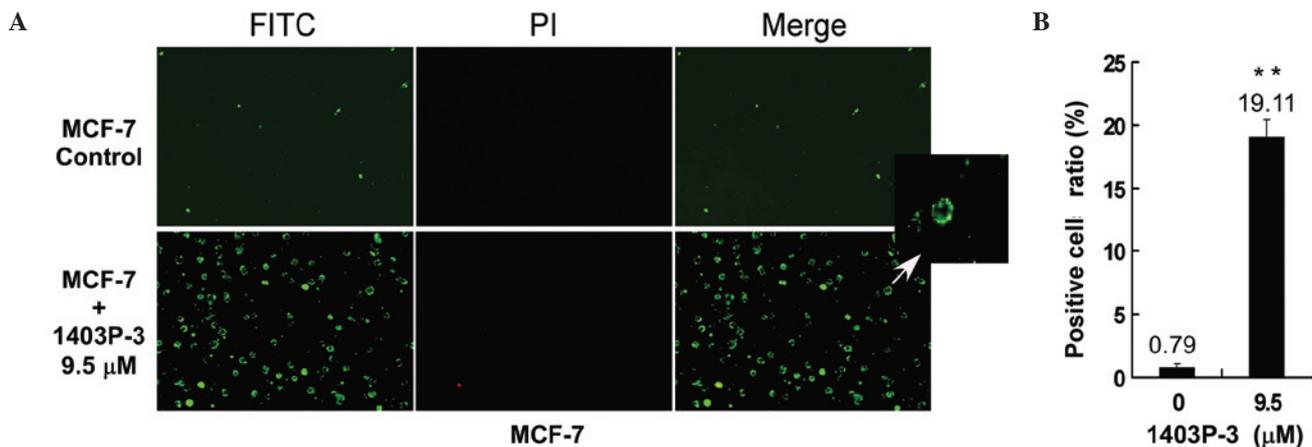


Figure 2. Induction of apoptosis of MCF-7 breast cancer cells treated with 1403P-3, detected by Annexin V-FITC/PI staining. (A) Cells were exposed to 9.5 μM 1403P-3 for 8 h. The apoptotic cells were Annexin V-FITC⁺/PI⁻ (green) and the dead cells were labeled with PI (red). Original magnification, $\times 200$. Ten representative stained fields for each section were analyzed to determine the apoptotic index. (B) Apoptotic index as determined by counting and calculating the percentage of FITC-positive cells in ten fields. Data for the quantitative assessment of apoptosis are expressed as the means \pm SD. ** $P < 0.01$ compared to the control. FITC, fluorescein isothiocyanate; PI, propidium iodide.

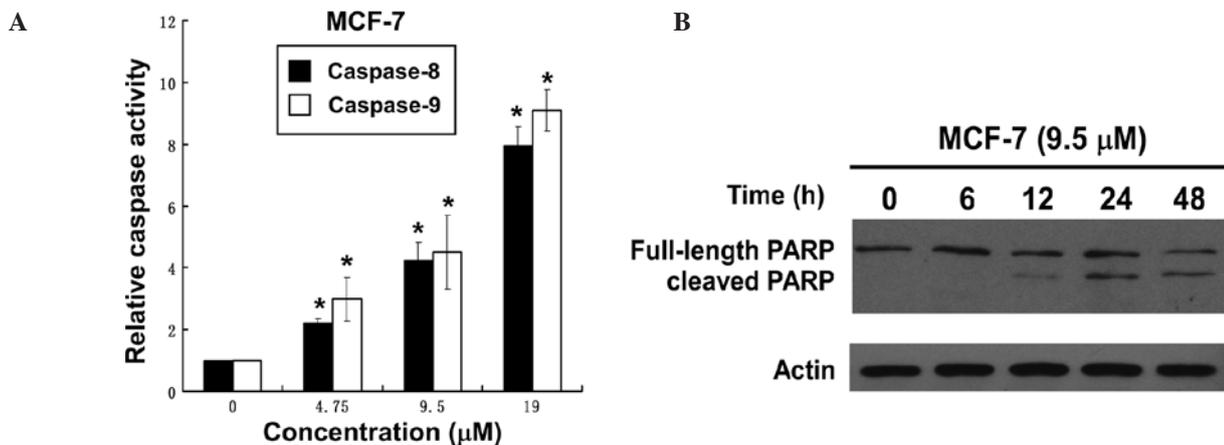


Figure 3. Activation of caspase-8 and -9 and poly (ADP-ribose) polymerase (PARP) in MCF-7 cells treated with 1403P-3. (A) Enzymatic activity of caspase-8 and -9 in MCF-7 cells treated for 24 h with 1403P-3 at the indicated concentrations, as assessed by colorimetric assay. Fold increases in the activity of caspase-8 and -9 were determined by comparison to those of the vehicle-treated control cells. Results are presented as the means \pm SD. * $P < 0.05$ significant differences compared to the control. (B) Effect of 1403P-3 on the cleavage of PARP in MCF-7 cells, as analyzed by Western blotting. Cells were exposed to 9.5 μM of 1403P-3 for the indicated times and the total cell lysates were analyzed for proteolytic cleavage of PARP. Actin was used as a loading control.

anti-tumor effects in human breast cancer, the cytotoxicity of 1403P-3 was measured by the MTT assay. As shown in Fig. 1, 1403P-3 exhibited a marked inhibition of the survival of MCF-7 and MDA-MB-435 cells in a dose-dependent manner. The IC_{50} value was 9.7 μM for MCF-7 and 7.6 μM for MDA-MB-435.

Induction of an irreversible apoptotic process by 1403P-3 in human breast cancer cells. To determine whether the observed decrease in the survival of breast cancer cells was due to the induction of apoptosis, MCF-7 breast cancer cells were treated with 1403P-3 at a concentration of $\text{IC}_{50} \sim 9.5 \mu\text{M}$ for 8 h. The apoptosis of the cells was detected by Annexin V-FITC/PI dual staining. The results showed that the treatment with 1403P-3 induced apoptosis, exhibiting a ratio of Annexin V-FITC⁺/PI⁻ cells of 19.11%, which was significantly higher than that of the control cells ($P < 0.01$) (Fig. 2).

To further confirm the above-mentioned observation, we characterized the effect of 1403P-3 on apoptosis protec-

tion by assessing its impact on the enzymatic activation of caspase-8 and -9 and the cleavage of PARP proteins, which are critical for cell apoptosis. The activity of caspase-8 and -9 was measured by a caspase colorimetric assay. The enzymatic activity of the caspases showed a dose-dependent increase in conjunction with 1403P-3 treatment (Fig. 3A). Concurrently, the cleavage of PARP in the 1403P-3-treated MCF-7 cells was detectable by Western blotting. Cleavage of PARP from 116 to 85 kDa was clearly demonstrated in the MCF-7 cells exposed to 1403P-3 (Fig. 3B). Collectively, these data suggest that 1403P-3 induces apoptosis in human breast cancer cells.

1403P-3 reduced Akt phosphorylation levels in human breast cancer cells. To investigate the signaling cascade leading to apoptosis induced by 1403P-3 in breast cancer cells, changes in the phosphorylation levels of Akt in cells treated with 1403P-3 were examined. As shown in Fig. 4, 1403P-3 treatment resulted in a dose- and time-dependent decrease in

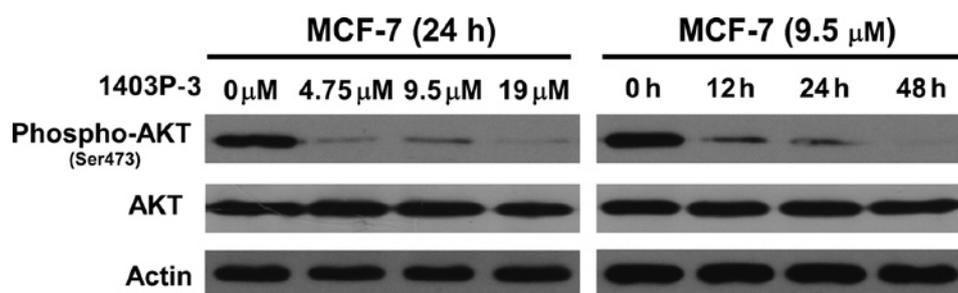


Figure 4. 1403P-3-induced apoptosis was accompanied by decreased phosphorylation of Akt proteins in a dose- and time-dependent manner in breast cancer cells. After cells were treated with indicated concentrations of 1403P-3 for 48 h, the whole-cell lysates were assayed by Western blotting and corresponding antibodies. The significant reduction of the phosphorylation level of Akt at Ser473 site was observed in MCF-7 cells with the total Akt unchanged.

phospho-Ser473-Akt in the MCF-7 cells, while the expression of total Akt remained unchanged. Together, these data indicate that 1403P-3 inactivated Akt, which in turn may modulate the downstream pathways and target genes, finally triggering apoptosis.

Discussion

Although a thorough understanding of apoptotic defects and clinical multidrug resistance remains to be developed (14), the hypothesis for the causal relationship between apoptosis and drug-induced cytotoxicity has been widely accepted (3). Developing an apoptosis-inducing new drug represents a promising direction in anti-cancer therapy. Unfortunately, many tumor types evade drug-induced death signals (15). Among the several apoptosis-related signaling molecules, Akt has been suggested to be an important modulator in sustaining survival against the apoptosis of cancer cells, and its function is frequently found altered in a variety of human cancers (16,17). It has been shown that the up-regulation of Akt is a common event in many human cancer types, and that the activation of the PI3K/Akt pathway contributes to chemotherapeutic resistance in human cancers (18-20). Therefore, studying the mechanism of the blockage of Akt activation may reveal promising targets for anti-cancer therapy.

To date, chemotherapy has played an important role in breast cancer treatment. Adriamycin and other anthracyclines are currently preferred among treatments for breast cancer. However, multidrug resistance and severely adverse side effects (e.g., cardiotoxicity and myelosuppression) limit the use of anthracycline (21,22). The development of new chemotherapeutic agents with improved pharmacological properties is essential (23).

1403P-3 is a novel compound derived from the secondary metabolites of the mangrove endophytic fungus no. 1403 from the South China Sea, and is an anthracenedione derivative (11). As the present data show, 1403P-3 possesses a significant inhibitory effect on the survival of human breast cancer cells. Notably, the phosphorylation level of Akt was significantly reduced in 1403P-3-treated cells, and apoptotic morphological changes occurred during the exposure of breast cells to 1403P-3. Although the exact target triggering the apoptosis of breast cancer cells requires further study, our data suggest that 1403P-3 is a candidate compound that merits a thorough pre-clinical evaluation and mechanistic studies in the laboratory.

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