Our previous studies revealed that daintain/AIF-1 promoted the proliferation and migration of breast cancer cells. In the present study, we investigated the effect of daintain/AIF-1 on the cisplatin resistance of breast cancer cells. The results indicated that daintain/AIF-1 reinforced the resistance of breast cancer cells to cisplatin by inhibition of cell apoptosis and reduction of intracellular cisplatin accumulation.

Key words: daintain/AIF-1; cisplatin; breast cancer cells; apoptosis; efflux

Breast cancer is the most commonly occurring cancer among women and is second only to lung cancer as cause of cancer death in women. According to that report, in China, breast cancer rates have jumped more than 20 percent over the past 10 years. The situation is worse in big cities such as Shanghai and Beijing. The usual means of curing breast cancer are chemotherapeutics and radiotherapeutics. Cisplatin, a conventional effective chemotherapeutics drug, is known to induce DNA damage by forming platinum-DNA and DNA-protein adducts that trigger tumor cell apoptosis, but tumor cells sometimes do not accept the destruction of cisplatin passively. Several mechanisms of cisplatin resistance have been proposed, including increased DNA repair, increased levels of intracellular thiols, inactivation of intracellular cisplatin, evasion of apoptotic pathways, and decreased drug accumulation.

Allograft inflammatory factor-1 (AIF-1) was first identified in heterotopic cardiac allografts in rats and daintain was first isolated by us from porcine intestines. There was high homology between daintain and AIF-1. Hence, we called the peptide daintain/AIF-1. Daintain/AIF-1 is a 143-amino acid cytoplasmic, calcium-binding, inflammation-responsive scaffold that functions as a modulator of the immune response during macrophage activation. Previous studies have founded that daintain/AIF-1 plays important roles in many autoimmunity diseases. Our recent study revealed that it overexpressed in breast cancer tissues and promoted breast cancer proliferation via activation of the NF-κB/cyclin D1 pathway. We have also reported that it promotes breast cancer cell migration by upregulation of TNF-a by activating the p38 MAPK pathway. However, whether daintain/AIF-1 affects the resistance of breast cancer to antineoplastic drugs has not been reported. In the present study, we investigated the role of daintain/AIF-1 in cisplatin resistance. The results revealed that it decreased the apoptosis of breast cancer cell lines induced by cisplatin and enhanced the accumulation of cisplatin.

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from the American Type Culture Collection (ATCC), and were cultured in Dulbecco’s Modified Eagle’s Medium (DME) with 10% fetal bovine serum (FBS) containing 100 U/mL of penicillin and 100 mg/L of streptomycin at 37°C. The siRNA expression vector (pRNAT-DT) and reconstituted vector (pcDNA-DT) of daintain/AIF-1 were constructed in our laboratory. The reconstituted vector, siRNA expression vector, and the empty vectors (pcDNA and pRNAT) were transfected into MDA-MB-231 and MCF-7 cells respectively with lipofectamine 2000 reagent following the manufacturer’s instructions. Cisplatin was purchased from Sigma Chemical (St. Louis, MO). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Aptoptosis of cells was evaluated by flow cytometry using Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Kit (Beyotime Biotechnology, Nanging, China). For apoptosis analysis, wildtype (WT) and transfected cells were plated into 6-well plates at a density of 1 x 10⁶ cells/well. After adhesion, the cells were challenged with 10 μg/mL of cisplatin for 24 h and 48 h. At the end of treatment, the cells were collected and washed with PBS 3 times. Then they were resuspended with 195 μL of FITC binding buffer and incubated with 5 μL of Annexin V-FITC for 15 min at room temperature in the dark. After washing with PBS, the cell samples were co-stained with PI solution and analyzed by flow cytometry.

For Western blot analysis, the cell samples were lysed in 20 μL of cell lysis buffer containing 1 mM PMSF. The extracted proteins were separated by 15% SDS–PAGE and transferred to PVDF membranes by 2 h of electroblotting. The blots were blocked in 5% non-fat dry milk for 1 h at room temperature, and then incubated at 4°C overnight with the primary antibodies. The membranes were washed with TBS containing 0.05% Tween-20 (TBST) 3 times, and then incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at 37°C. Finally, they were developed with an enhanced chemiluminescence (ECL) kit (Pierce Biotechnology, Rockford, IL).

The amount of intracellular cisplatin was determined by HPLC. WT and transfected cells were seeded in 9-cm
dishes and cultured overnight. The next day, 10 μg/mL cisplatin was added to the dishes. Cell samples were then harvested and counted at various time points. The same number of cells were collected and lysed with cell lysis buffer, and nucleases were used to digest DNA. The supernatant was subjected to dose of cisplatin by RP-HPLC at 254 nm using a C18 column. The mobile phase was a mixture of water/methanol/acetonitrile (32:41:27/v:v:v), and the flow rate was 0.75 mL/min. Standard cisplatin was used as control.18)

To investigate drug efflux, cells were stimulated with 10 μg/mL of cisplatin for 1 h. Subsequently, they were washed with PBS and incubated for up to 2 h with drug-free, serum-free, phenol red-free DMEM medium. The cell supernatants were collected to determine the concentration of cisplatin.

All experiments were repeated at least 3 times. Data were expressed as mean ± SE, and were calculated by Student’s t test using GraphPad Prism version 5.0 (GraphPad software San Diego, CA). p-Values were considered significant at <0.05.

It has been suggested that anticancer drugs exert their effects by inducing cell apoptosis. Cisplatin has been confirmed to induce apoptosis in various types of cancer cells, including breast cancer cells.19) In our study, treatment of pcDNA-DT-transfected MDA-MB-231 cells with 10 μg/mL of cisplatin for 48 h caused a significant 18.6% reduction in cell apoptosis as compared to WT MDA-MB-231 cells (Fig. 1A). However, as compared to WT cells, incubation of pRNAT-DT-transfected MDA-MB-231 cells with 10 μg/mL of cisplatin for 24 h and 48 h resulted in increased cell apoptosis (Fig. 1A). Similar results were detained for the WT and transfected MCF-7 cells (Fig. 1B). The MDA-MB-231 cells were slightly more sensitive to cisplatin in inducing apoptosis than the MCF-7 cells. In addition, cells with pcDNA vector and pRNAT vector transfected had no effect on cisplatin-induced apoptosis (data not shown). These data suggest that daintain/AIF-1 contributes to cisplatin resistance in breast cancer cells by inhibiting cell apoptosis.

To elucidate the precise mechanisms of daintain/AIF-1 in the apoptosis of breast cancer cells, the expression of apoptotic-related protein in breast cancer cells was detected by Western blot. Incubation of the cells with 10 μg/mL of cisplatin for 24 h caused a 1.26-fold increase in Bcl-2 expression in the pcDNA-DT-transfected MDA-MB-231 cells and a 0.45-fold decrease in Bcl-2 expression in the pRNAT-DT-transfected MDA-MB-231 cells as compared to the WT MDA-MB-231 cells. In a contrast, after exposure to 10 μg/mL of cisplatin for 24 h, as compared to the WT MDA-MB-231 cells, a 1.89-fold p53 expression and a 1.31-fold Bax expression were observed in pRNAT-DT-transfected MDA-MB-231 cells, while the expression of p53 and Bax in the pcDNA-DT-transfected MDA-MB-231 cells decreased to 0.44 fold and 0.54 fold of the WT MDA-MB-231 cells (Fig. 2A). Similarly, after exposure of the cells to 10 μg/mL of cisplatin for 24 h, elevated Bcl-2 expression and reduced p53 and Bax expression were observed in pRNAT-DT-transfected MDA-MB-231 cells, while the expression of p53 and Bax in the pcDNA-DT-transfected MDA-MB-231 cells decreased to 0.44 fold and 0.54 fold of the WT MDA-MB-231 cells (Fig. 2A). Similarly, after exposure of the cells to 10 μg/mL of cisplatin for 24 h, elevated Bcl-2 expression and reduced p53 and Bax expression were observed in the pcDNA-DT-transfected MCF-7 cells; while in the pRNAT-DT-transfected MCF-7 cells, cisplatin induced significantly decreased Bcl-2 expression and sharply increased expression of p53 and Bax (Fig. 2B). The expression and activation of p53 and the Bcl-2 protein family has been reported to play an
important role in controlling apoptotic responses. Tumor suppressor p53 is a DNA-binding protein that has been suggested to play a critical role in some forms of apoptosis.8,20-22) It also has been found that p53 may be involved in cisplatin resistance in breast cancer cells, which cells became sensitive to cisplatin on p53 disruption.23) Bcl-2 is another apoptotic-related protein that functions as an anti-apoptotic signal. It has been confirmed to suppress cell apoptosis induced by a variety of stimuli, including chemotherapeutic drugs.23,24) Bax, a Bcl-2 family protein, serves as an essential effector of the mitochondrial apoptotic pathways and participates in executing p53-mediated apoptosis.4,25,26)

Decreased drug accumulation has been reported to be responsible for cisplatin resistance.5) Hence, we investigated the effect of daintain/AIF-1 on the accumulation of cisplatin in breast cancer cells. As shown in Fig. 3A, after exposure of 10μg/mL of cisplatin for 1 h, the amount of cisplatin in the MDA-MB-231 cells and MCF-7 cells reached a maximum, while a downward trend in the amount of intracellular cisplatin was observed when these cell lines were challenged with 10μg/mL of cisplatin for 3 h, especially in the cells transfected with pcDNA-DT. After stimulation for 6 h, 12 h, and 24 h with 10μg/mL of cisplatin, the amount of cisplatin in the pcDNA-DT transfected MDA-MB-231 and MCF-7 cells showed a more pronounced decrease, with the peak decrease at 12 h incubation, and the amount of cisplatin in the WT MDA-MB-231 and MCF-7 cells showed a modest reduction, while in the pRNAT-DT transfected cells, the accumulation of cisplatin showed no obvious change. Furthermore, after exposure for the same duration to 10μg/mL of cisplatin, the accumulation of cisplatin in the MDA-MB-231 cells was somewhat higher than in the MCF-7 cells, which is consistent with our observation that the MDA-MB-231 cells were more sensitive as regards cisplatin-induced apoptosis than the MCF-7 cells. A reduction in drug accumulation can result from decreased uptake or enhanced efflux. ATP-binding cassette transporters (ABC-transporters), MRP1, and cMOAT have been reported to be responsible for drug efflux.27-29) Copper transporters (CTR1, ATP7A, and ATP7B) are another type ATPase transporters. Evidence has confirmed that copper transporters play roles in the uptake and efflux of drug.30,31) In the present study, the pcDNA-DT transfected cells exhibited a more obvious elevation than the WT cells in the efflux of cisplatin, while the efflux of cisplatin from the pRNAT-DT transfected cells was much lower than that from the WT cells (Fig. 3B), but whether daintain/AIF-1 directly affects cisplatin efflux or indirectly promotes the efflux of cisplatin via enhancement of transporters activity is unknown.

Cisplatin is a common drug for tumors, but the acquisition of drug resistance by tumor cells largely limits its effectiveness. Based on existing research and our studies, we conclude that daintain/AIF-1 increases the resistance of breast cancer cells to cisplatin by promoting the efflux of intracellular cisplatin and inhibiting cisplatin-induced cell apoptosis through activation of Bcl-2 and suppression of p53 and Bax. However, more research should be done to investigate the underlying mechanism of Daintain/AIF-1 on cisplatin efflux.
Acknowledgment

This work was supported by the National Science Foundation of China (Grant 31040042).

References