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Apoptosis and inactivation of the PI3-kinase pathway by tetrocarcin A in breast cancers

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Abstract

A survival kinase, Akt, is a downstream factor in the phosphatidylinositide-3′-kinase-dependent pathway, which mediates many biological responses including glucose uptake, protein synthesis and the regulation of proliferation and apoptosis, which is assumed to contribute to acquisition of malignant properties of human cancers. Here we find that an anti-tumor antibiotic, tetrocarcin A, directly induces apoptosis of human breast cancer cells. The apoptosis is accompanied by the activation of a proteolytic cascade of caspases including caspase-3 and -9, and concomitantly decreases phosphorylation of Akt, PDK1, and PTEN, a tumor suppressor that regulates the activity of Akt through the dephosphorylation of polyphosphoinositides. Tetrocarcin A affected neither expression of Akt, PDK1, or PTEN, nor did it affect the expression of Bcl family members including Bcl-2, Bcl-X_L, and Bax. These results suggest that tetrocarcin A could be a potent chemotherapeutic agent for human breast cancer targeting the phosphatidylinositide-3′-kinase/Akt signaling pathway.

Keywords: Tetrocarcin A; PTEN; PI3-kinase; PDK1; Akt; Breast cancer; Apoptosis; Phosphorylation; Dephosphorylation; Survival factor; Bcl-2; BcL-x_L; Caspase

Because loss of growth control and resistance to apoptosis aberrantly prolong cell viability and contribute to cancer by facilitating the mutations that lead to malignancy, therapeutic agents for human cancers are developed by targeting molecules that regulate signal transduction pathways controlling the cancer cell's fate. Akt, also known as protein kinase B, acts as one of the major factors in this transduction pathway through protein phosphorylation [1]. Overexpression of Akt is believed to contribute to malignant expansion of various human cancers, including breast cancers [2–6].

Three isoforms of Akt have been identified, and phosphorylation at Thr^{308} (Akt1), Thr^{309} (Akt2) or Thr^{305} (Akt3), and Ser^{473} (Akt1), Ser^{474} (Akt2) or Ser^{472} (Akt3) is required for full activation, which subsequently regulates biological responses including glucose uptake, protein synthesis, and inhibition of apoptosis [1]. 3′-Polyphosphoinositide-dependent kinase-1 (PDK1) phosphorylates these threonines [7,8], and target of rapamycin (TOR)–rictor complex [9,10] and DNA-dependent protein kinase [11] are reported as kinases for phosphorylation at these serines. PDK1 exists in an active, phosphorylated configuration under basal conditions [12], and association with PtdIns(3,4)P_2 or PtdIns(3,4,5)P_3 through its C-terminal PH domain is necessary for activation [7]. Akt also has a PH domain that preferentially binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2, and association with these polyphosphoinositides is required for membrane anchoring and activation through phosphorylation by PDK1. Thus, activation of phosphatidylinositide-3′-kinase (PI3K), which generates these polyphosphoinositides, activates Akt [13].

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Phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which dephosphorylates polyphosphoinositides, antagonizes Akt through the regulation of inositol lipid phosphorylation [14]. PTEN protein consists of an N-terminal phosphatase domain, a lipid-binding C2 domain and a 50-amino acid C-terminal domain. The C-terminal domain is rich in putative phosphorylation sites, and phosphorylation of this domain increases the stability and function of the phosphatase [15–17]. Most cellular PTEN appears to be phosphorylated, and dephosphorylation makes PTEN highly susceptible to proteolysis, increases its affinity for anionic lipids and enhances its localization to the plasma membrane. As a result, dephosphorylation greatly enhances biological activity in cells, presumably due to co-localization with the membrane-incorporated substrate. Inactivation of PTEN results in excessive activation of the PI3K/Akt signaling pathway [18,19]. Consistent with its critical role in regulating Akt activity, PTEN is one of the tumor suppressor genes most frequently mutated or inactivated in human cancers, including breast cancers [20].

Tetrocarcin A, an antibiotic active against Gram-negative bacteria, is isolated from the culture broth of *Micromonospora chalcea* [21,22]. It shows anti-tumor activity in murine experimental models such as sarcoma 180, P388 leukemia and B16 melanoma [21,23]. Recent studies have suggested tetrocarcin A induces apoptosis in tumor cells in a cell type-dependent manner. In HeLa cells, tetrocarcin A antagonizes anti-apoptotic factors Bcl-2 and Bcl-X_L in the apoptosis induced by Fas-stimulation, TNFα, staurosporine, and irradiation [24,25]. In contrast, tetrocarcin A directly induces caspase-dependent apoptosis in lymphomas [26,27]. Although treatment with tetrocarcin A induces activation of both death-inducing signaling-complex and mitochondria-dependent apoptosis pathways, deficiency of caspase-8 and ectopic expression of Bcl-2 family proteins hardly affect the apoptosis. In cDNA array microchip analysis, tetrocarcin A specifically enhances transcription of HSP70, suggesting that tetrocarcin A induces apoptosis through endoplasmic reticulum stressors, a premise that is further supported by the observation that tetrocarcin A activates caspase-12.

In this study, we examine the effect of tetrocarcin A on the PI3K/Akt signaling pathways of human breast cancer cell lines. We find that tetrocarcin A inhibits phosphorylation of factors involved in the PI3K/Akt signaling pathway, including PTEN, PDK1, and Akt. Our results suggest that tetrocarcin A could be a potent chemotherapeutic agent for human breast cancers with excess PI3K/Akt activity.

Materials and methods

Cell culture. The human breast cancer cell line KPL-1 was kindly provided by Dr. Kurebayashi (Kawasaki Medical College, Okayama, Japan) [28]. MDA-MB-231 and ZR75-1 were purchased from American Type Culture Collection (Manassas, VA, USA). All cells were cultured in D-MEM (Nissui, Tokyo, Japan) containing 1% penicillin–streptomycin (Nacalai Tesque, Kyoto, Japan) and 10% fetal bovine serum (BioWhittaker, MD, USA).

Reagents. Tetrocarcin A was kindly provided by Fujisawa Asteras Pharmaceutical (Osaka, Japan). Hoechst 33342 (Sigma, St. Louis, MO, USA) was dissolved in DMSO at 1 mM as a stock solution, and propidium iodide (Sigma) was dissolved in PBS (pH 7.4) at 1 mg/ml. Antibodies for Bcl-2, Bcl-X_L, poly(ADP-ribose)polymerase (PARP), and anti-β-actin were purchased from Santa Cruz (Santa Cruz, CA, USA). Antibodies for caspase-3 and caspase-9 were purchased from MBL (Nagoya, Japan). Antibodies against phosphorylated form and total protein for PDK1, Akt and PTEN were purchased from Cell Signaling Technol. Inc. (Danvers, MA, USA).

Western blotting. Immunodetection was performed as described previously [29]. Briefly, cells (1×10^6) were lysed in 50 μl 2× SDS sample buffer (120 mM Tris, 4% SDS, 20% glycerol, 0.1 mg/ml bromophenol blue, pH 6.8) containing 100 mM dithiothreitol and boiled for 5 min. Each sample (10 μg protein) was resolved on SDS–PAGE. Proteins in each gel were transferred to Immobilon-P membrane (Milipore). Blots were blocked for 1 h with 5% nonfat dry milk, probed with the indicated primary antibodies for 1 h, and then with the appropriate horseradish peroxidase-conjugated secondary antibody (MBL, Nagoya, Japan). Signals were visualized by fluorescence emission using commercial detection kits (Roche Diagnosis, Mannheim, Germany) according to the manufacturer's instructions.

MTT-dye reduction assay. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [30], as described previously [31]. In brief, cells were suspended in a complete medium, plated at 100 μl/well on 96-well, flat-bottomed plates (Nunc, Roskilde, Denmark) at an initial density of 1×10^5 cells/ml, allowed to adhere for 24 h, and exposed to various concentrations of tetrocarcin A for 48 h. After cells were further incubated with 1 mg/ml MTT for 4 h, the medium was removed and 100 μl of 0.04 M HCl in isopropanol was added to extract the reduced formazan product. The resulting optical density at 590 nm was determined.

DNA fragmentation assay. DNA fragmentation was detected using TACSTM Apoptotic DNA Laddering Kits (Trevigen, Inc., MD, USA), according to the manufacturer's instructions. Briefly, cells (1×10^6 cells) were lysed using lysis buffer and their DNA was extracted to be separated by 2% agarose-gel electrophoresis, followed by ethidium bromide staining (500 ng/ml) to visualize laddered DNA.

Assay for apoptotic morphology. Cells were mixed with an equal volume of 3 μg/ml of Hoechst 33342 for 15 min, followed by adding 1.5 μg/ml propidium iodide. Cells were mounted on a slide glass and the morphology of cells was observed using a fluorescence microscope (Olympus, Tokyo, Japan), as described previously [32].

Results

Cytotoxic effect of tetrocarcin A on human breast cancer cell lines

We evaluated the therapeutic efficacy of tetrocarcin A on three different human breast cancer cell lines. MDA-MB-231 expresses no estrogen receptor, whereas ZR75-1 and KPL-1 are typical estrogen receptor-positive cell lines. Tetrocarcin A above 5 μM dramatically reduced viability of MDA-MB-231 cells after 48-h incubation, as judged by MTT assay. IC_{50} of tetrocarcin A for the cytotoxic effect on this breast cancer cell line is about 2.5 μM. Paclitaxel, a clinical anti-breast cancer agent that interferes with the depolymerization of polymerized tubulin, was less effective compared to tetrocarcin A. Tetrocarcin A also reduced the viability of estrogen receptor-positive KPL-1 and ZR75-1 cells, suggesting that tetrocarcin A has a cytotoxic effect

on breast cancer cells independent of estrogen receptor expression. Again, paclitaxel was less effective on these two cell lines, compared to tetrocarcin A.

Direct induction of apoptotis by tetrocarcin A in human breast cancer cell lines

To determine whether the cytotoxic effect of tetrocarcin A on breast cancer cells is due to the direct induction of apoptosis, we analyzed nuclear DNA from breast cancer cells treated with 2.5 μM tetrocarcin A for 48 h. Analysis by agarose-gel electrophoresis showed a DNA ladder with a nucleosome unit (189 bp) characteristic of apoptotic cells in the three different breast cancer cell lines treated with tetrocarcin A (Fig. 2). We further confirmed the apoptotic character through observation of nuclear morphology by staining cells with Hoechst 33342 and propidium iodide. All three breast cancer cell lines treated with 2.5 μM tetrocarcin A for 48 h had fragmented and/or condensed chromatin, one of the characteristic features of apoptotic cells [33], as compared to untreated cells which had rounded, intact nuclei (Fig. 2B). It should be noted that almost all of the apoptotic cells could eliminate propidium iodide, suggesting that apoptosis occurs without impairing membrane permeability. These results indicate that tetrocarcin A directly and effectively induces apoptosis in human breast cancer cell lines.

Dephosphorylation of PI3K pathway components by tetrocarcin A

To characterize the properties of tetrocarcin A-mediated apoptosis in human breast cancer cell lines, we analyzed the protein expression of cells treated with 2.5 μM tetrocarcin A for 48 h. Among Bcl-family proteins, expression of Bcl-2 was highest in KPL-1, intermediate in ZR75-1 and lowest in MDA-MB-231, correlated to sensitivity to apoptosis induced by tetrocarcin A (Fig. 1) (although tetrocarcin A did not affect their expression levels at all). Expression levels of Bcl-X_L and Bax were almost the same among these cell lines, and were not affected by treatment with tetrocarcin A. The active forms of caspase-9 (35/37 kDa) and caspase-3 (15/17 kDa) were generated from the corresponding proenzymes by the treatment with tetrocarcin A, and poly(ADP-ribose)polymerase (116 kDa), one of the endogenous substrates for caspase-3 [34], was

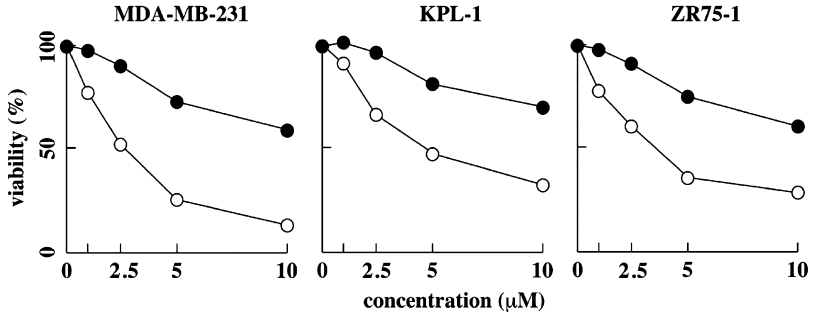

Fig. 1. Cytotoxic effect of tetrocarcin A on human breast cancer cells. Human breast cancer cells were treated with indicated concentrations of tetrocarcin A (open circles) or paclitaxel (closed circles) for 48 h. MTT reagent was added 4 h prior to termination of the culture. Formazan deposits were quantified by absorbance at 590 nm. Each point represents the average of independent triplicate cultures.

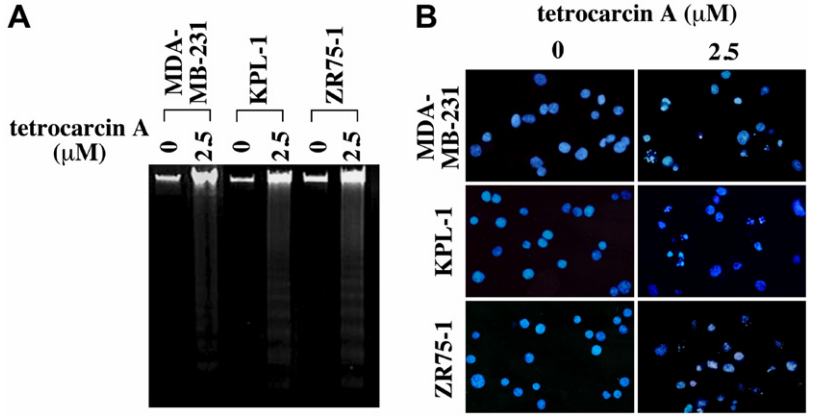

Fig. 2. Apoptosis induced by tetrocarcin A in human breast cancer cells. Human breast cancer cells were treated with 2.5 μM tetrocarcin A. After 48 h of culture, cells were harvested and their extracted DNA were run in 2% agarose gel (A), and the nuclear morphology of cells stained with Hoechst 33342 and propidium iodide was observed under a fluorescent microscope (B).

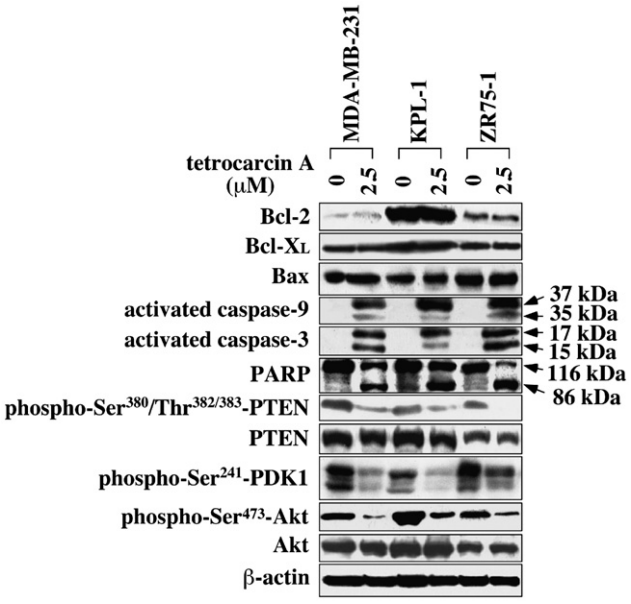

Fig. 3. Protein expression of human breast cancer cells treated with tetrocarcin A. Human breast cancer cells were treated with 2.5 μM tetrocarcin A. After 48 h of culture, cells were harvested and their protein expression was analyzed by Western blot.

cleaved to generate a 86-kDa product, suggesting that apoptosis induced by tetrocarcin A is associated with the activation of a proteolytic cascade of caspases (see Fig. 3).

Because the PI3K signal transduction pathway regulates cell death and survival [1,13,14], we further determined the activity of Akt, PDK1, and PTEN, major factors in PI3K pathways, with phospho-amino acid-specific antibodies. Significant amounts of these factors were expressed in the three breast cancer cell lines, and they were phosphorylated in the basal state. Treatment with 2.5 μM tetrocarcin A significantly reduced phosphorylation of PTEN at Ser^{380}/$Thr^{382/383}$ on the C-terminal tail, PDK1 at Ser^{241} and Akt at Ser^{473}. Tetrocarcin A did not affect the expression levels of these proteins. These results suggest that tetrocarcin A induces apoptosis of human breast cancer cells through the inhibition of survival signals produced by the PI3K pathway.

Discussion

Because the malignant transformation of cells requires signals suppressing apoptosis in addition to signals promoting cell proliferation, suppression of survival factors such as Bcl-2 family proteins and components of the PI3K pathways is a promising strategy for cancer chemotherapy. An anti-tumor antibiotic, tetrocarcin A, affects apoptosis of cells in a manner dependent on cell type. In HeLa cells, tetrocarcin A induces apoptosis that is ordinarily suppressed by ectopic expression of anti-apoptotic factors such as Bcl-2 and Bcl-X_L upon a variety of stimuli, including anti-Fas antibody, staurosporine, DNA break caused by irradiation and TNFα. However, tetrocarcin A does not induce apoptosis in the absence of these stimuli [24,25]. On the contrary, tetrocarcin A directly induces caspase-dependent apoptosis in lymphomas through the induction of endoplasmic reticulum stress [26,27].

In this report, we evaluate apoptosis induced by tetrocarcin A in human breast cancer cell lines, and find that tetrocarcin A effectively induces apoptosis as judged by DNA fragmentation, nuclear morphology, and activation of caspases without affecting expression levels of Bcl-2 family members including Bcl-2, Bcl-X_L, and Bax. Induction of apoptosis by tetrocarcin A is more efficient than by paclitaxel, an anti-cancer drug generally used in breast cancer therapy. Apoptotic nuclear morphology is observed in cells retaining membrane permeability, suggesting that the cytotoxic effect of tetrocarcin A on breast cancer cells results mainly from apoptosis. This finding suggests that the apoptotic properties induced by tetrocarcin A in breast cancer cells are similar to those induced in lymphomas, but not those in HeLa cells, even though breast cancer cell lines, like HeLa cells, are firmly adherent to plastic dishes.

Akt and its regulators in the PI3K pathway produce important survival signals in cancer cells and play an essential role in the acquisition of malignant properties [1,13,14]. Therefore, to elucidate the mechanism of apoptosis induction in breast cancer cells by tetrocarcin A, we examined the activities of PI3K signal pathway factors including Akt, PDK1, and PTEN. Frequent overexpression and activation of Akt are reported in various human cancers [2–6], and overexpression of HER-2/neu renders cancer cells resistant to chemotherapeutic agents through activation of the PI3K/Akt pathway [6]. Our results demonstrate that tetrocarcin A suppresses phosphorylation of these molecules without affecting their expression levels. Although the dephosphorylation of PTEN reduces the stability of the protein by increasing its susceptibility to proteolysis, this greatly enhances its biological activity through the enhancement of localization on the plasma membrane [14–17]. Activation of PTEN suppresses the generation of polyphosphoinositides, resulting in the inactivation of kinases in the PI3K cascade, including PDK1 and Akt [14]. Thus, it is possible that tetrocarcin A inactivates the PI3K pathway by targeting PTEN phosphorylation. However, it is necessary to further elucidate the molecular mechanism for suppression of the PI3K pathway by tetrocarcin A.

Activation of the PI3K pathway suppresses apoptosis induced by diverse pathways: Akt suppresses p53-mediated apoptosis by phosphorylation and inactivation of Mdm2 [35], a negative feedback regulator of p53 [36]. Akt suppresses mitochondrial-dependent apoptosis by phosphorylation and inactivation of caspase-9 [37] and Bad [38], or by phosphorylation and stabilization of the anti-apoptotic protein XIAP [39]. Akt suppresses death-inducing signaling-complex-mediated apoptosis by inhibiting Forkhead family transcription factors [40]. Nerve growth factor attenuates endoplasmic reticulum stress-mediated apoptosis via suppression of caspase-12 through activation of the PI3K pathway [41]. The antagonistic effect of tetrocar-

cin A on anti-apoptotic factors Bcl-2 and Bcl-X_L is demonstrated in the mitochondria-dependent apoptosis of HeLa cells induced by various signals, whereas tetrocarcin A directly induces apoptosis of lymphoma cell lines by the activation of caspase-12 that results from endoplasmic reticulum stress. It is possible that inhibition of the PI3K pathway is involved in the upstream signaling for apoptosis induced by tetrocarcin A.

In conclusion, our results demonstrate that tetrocarcin A directly induces apoptosis of breast cancer cells via dephosphorylation and inactivation of PI3K pathway components, which suggests that tetrocarcin A can be a potent clinical agent for chemotherapy against human breast cancers that have aberrantly elevated activity in the PI3K pathway.

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