Cancer Letters 379 (2016) 39–48

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Disulfiram targets cancer stem-like properties and the HER2/Akt signaling pathway in HER2-positive breast cancer

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ARTICLE INFO

Article history:
Received 24 March 2016
Received in revised form 22 May 2016
Accepted 23 May 2016

Keywords:
HER2
p95HER2
Disulfiram
ALDH1A1
Apoptosis
Trastuzumab resistance

ABSTRACT

HER2-positive breast tumors are known to harbor cancer stem-like cell populations and are associated with an aggressive tumor phenotype and poor clinical outcomes. Disulfiram (DSF), an anti-alcoholism drug, is known to elicit cytotoxicity in many cancer cell types in the presence of copper (Cu). The objective of the present study was to investigate the mechanism of action responsible for the induction of apoptosis by DSF/Cu and its effect on cancer stem cell properties in HER2-positive breast cancers *in vitro* and *in vivo*.

DSF/Cu treatment induced apoptosis, associated with a marked decrease in HER2, truncated p95HER2, phospho-HER2, HER3, phospho-HER3 and phospho-Akt levels, and p27 nuclear accumulation. This was accompanied by the eradication of cancer stem-like populations, concomitant with the suppression of aldehyde dehydrogenase 1 (ALDH1) activity and mammosphere formation. DSF administration resulted in a significant reduction in tumor growth and an enhancement of apoptosis, as well as HER2 intracellular domain (ICD) and ALDH1A1 downregulation. Our results demonstrate that DSF/Cu induces apoptosis and eliminates cancer stem-like cells via the suppression of HER2/Akt signaling, suggesting that DSF may be potentially effective for the treatment of HER2-positive cancers.

Introduction

Human epidermal growth factor receptor-2 (HER2)-positive breast cancer accounts for approximately 20–30% of all breast cancer cases, and amplification of the gene correlates with aggressive tumor growth and recurrence [1,2]. Members of the HER family play a critical role in regulating cell survival, growth, differentiation and apoptosis of cancer cells. HER2 is activated by homo- or hetero-dimerization with corresponding family members including EGFR and HER3, which drive downstream signaling via phosphatidylinositol-3 kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK) pathway activation, contributing to a poor prognosis [3–5]. HER2-amplified breast cancers can express both full-length p185HER2 and amino-terminal truncated domain p95HER2 that lacks the extracellular trastuzumab-binding domain. The latter form of oncogenic p95HER2 can exhibit constitutive kinase activity, triggering continuous activation of the PI3K/Akt pathway, and enabling trastuzumab resistance [6–8].

Amplified HER2 is an important determinant of survival for breast cancer stem cells (BCSCs) and is frequently associated with elevated levels of ALDH1 and the CD44+high/CD24-low phenotype which has been associated with a higher risk of tumor recurrence, chemo- and radiation-resistance, and metastasis [9–11]. In order to overcome these issues, various chemotherapeutic approaches and combination therapies have focused on the suppression of HER2, the prevention of hetero-dimerization, and the specific targeting of cancer stem cells or inhibiting key receptor tyrosine kinases [12,13]. However, HER2-positive tumors are typically heterogeneous and their associated trastuzumab resistance is a multi-factorial phenomenon limiting the effectiveness of pharmacological therapy [14]. Therefore, new drugs that effectively inhibit activation of the HER receptor family and target cancer stem cells are needed.

Over the past several decades, disulfiram (DSF) has been widely used for the treatment of alcohol dependence, and clinical trials have demonstrated its exceptional safety profile [15]. Recent studies have investigated the copper (Cu)-dependent antitumor properties of DSF in breast, brain, ovary, liver and lung cancers [16–21]. The apoptogenic mechanism of action of DSF/Cu is thought to arise from its effects on proteasome inhibition, caspase activation, suppression of NF-kB activity, and ROS production [16–18,22]. Although cumulative studies have investigated the apoptotic effect and

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http://dx.doi.org/10.1016/j.canlet.2016.05.026
0304-3835/

cytotoxicity of DSF/Cu against cancer cells *in vitro* and *in vivo*, its effect on HER2-positive breast cancers has not been elucidated.

In the present study, our objective was to characterize the mechanism of action of DSF/Cu on the induction of apoptosis and its effect on cancer stem-like properties in HER2-positive breast cancer *in vivo* and *in vitro*. We hypothesize that DSF/Cu targets HER2-positive BCSCs by inhibiting the HER2/Akt signaling pathway.

Materials and methods

Reagents and antibodies

Disulfiram, copper chloride, paraformaldehyde, Triton X-100 and propidium iodide (PI) were obtained from Sigma-Aldrich (St Louis, MO). Phosphatase inhibitor, protease inhibitor cocktail tablets and trastuzumab were purchased from Roche Applied Sciences (Penzberg, GER). zVAD-fmk was obtained from Tocris Bioscience (Ellisville, MO). The antibodies that were used include: ALDH1A1, XIAP and p27 (Abcam, Cambridge, MA); total-Akt, phospho-Akt (Ser473), pro-PARP, cleaved-PARP, procaspase 3, cleaved-caspase 3, E-cadherin, HER2, phospho-HER2 (Tyr1221/1222), phospho-HER3 (Tyr1289) and HER3 (Cell Signaling, Beverly, CA); HER2 clone 4B5 (Ventana Medical Systems, Tucson, AZ); CB11 (Thermo Fisher Scientific Fremont, CA); vimentin clone V9 (Dako, Glostrup, Denmark); horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgG (Bio-Rad Laboratories, Hercules, CA); and Alexa Fluor-488 and -594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA).

Breast cancer cell culture

The human breast cancer cell lines MCF7, T47D, SKBR3, BT474, MDA-MB-453 and MDA-MB-231 (American Type Culture Collection) were cultured in RPMI 1640 or DMEM containing 10% fetal bovine serum (FBS), streptomycin-penicillin (100 U/ml) and Fungizone (0.625 μg/ml). Cells were incubated at 37 °C in an atmosphere of 5% CO_2.

Cell viability assay

Cell viability was measured using the CellTiter 96* Aqueous One Solution Cell Proliferation Assay [MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (Promega, Madison, WI, USA), as previously described [23].

Annexin V/PI assay

Cells were stained using a FITC-conjugated Annexin V apoptosis detection kit (BD Biosciences, NJ) according to the manufacturer's protocol. Stained cells were analyzed by flow cytometry using a Beckman Coulter Expo (Brea, CA).

Aldefluor assay

The Aldefluor assay kit (Stemcell Technology, Vancouver, BC) was used to assess ALDH1 activity according to the manufacturer's protocol. Cells were incubated for 45 min at 37 °C in Aldefluor assay buffer containing the ALDH protein substrate BODIPY-aminoacetaldehyde (BAAA, 1 μM per 0.5×10^6 cells). As a specific inhibitor of ALDH1, 50 mM diethylamino-benzaldehyde (DEAB) was used for defining the Aldefluor-positive population. Aldefluor stained cells were analyzed with a Beckman Coulter Expo flow cytometer.

Immunofluorescence confocal microscopy

Cells on 8-well chamber slides were fixed with 4% paraformaldehyde for 1 h, washed with PBS, and incubated with 0.2% Triton X-100 for 10 min and washed with PBS. Cells were blocked with 3% BSA for 1 h. Primary antibodies in antibody-diluent (Dako, Glostrup, Denmark) were incubated overnight at 4 °C before incubation with fluorescence-conjugated secondary antibodies (Alexa-Fluor-594 or -488) at room temperature for 2 h. Cells were counterstained with ProLong Gold Antifade Reagent with DAPI (Life Technologies, Carlsbad, CA). Images were acquired using a Carl Zeiss confocal microscope (Weimar, Germany). Intensity of fluorescence was analyzed using fluorescence profiling, as previously described [24].

Western blot analysis

The procedures were performed as previously described [23,24]. Primary antibody dilutions were as follows: [HER2 (1:5000), phospho-HER2 (1:1000), HER3 (1:2000), phospho-HER3 (1:2000), Akt (1:2000), phospho-Akt (1:1000), pro-PARP (1:5000), cleaved-PARP (1:2000), XIAP (1:2000), pro-caspase-3 (1:2000), cleaved-caspase 3 (1:1000), ALDH1A1 (1:2000), p27 (1:2000) or β-actin (1:5000)] and subsequently with HRP-conjugated rabbit or mouse secondary antibody (1:3000–1:10,000). Signal intensity was detected using a Chemiluminescence Kit (Thermo Fisher Scientific Fremont, CA) on X-ray film (Agfa Healthcare, Mortsel, Belgium) and quantitated using AlphaEaseFC software (Alpha Innotech, San Leandro, CA).

Mammosphere formation assay

Mammosphere-forming ability was analyzed, as previously described [25]. BT474 cells were plated in ultralow attachment dishes and cultured in HuMEC basal serum free medium (Gibco, Gaithersburg, MD), supplemented with B27 (1:50, Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF, Sigma-Aldrich), 20 ng/ml human epidermal growth factor (EGF, Sigma-Aldrich), 4 μg/ml heparin, 1% antibiotic-antimycotic, and 15 μg/ml gentamycin at 37 °C in an atmosphere of 5% CO_2.

Animals, xenograft experiments

All animal procedures were carried out in accordance with animal care guidelines approved by the Korea University Institutional Animal Care and Use Committee (IACUC). Five-week-old female BALB/c nude mice were obtained from the Shizuoka Laboratory Animal Center (Shizuoka, Japan) and housed in a specific pathogen-free environment. The animals were acclimated for 1 week prior to the study and had free access to food and water. Mice were inoculated with 17β-Estradiol pellets (1.72 mg, Innovative Research of America, USA) 48 h before injection of cells. BT474 cells (8×10^6) in 100 μl of culture medium were mixed with 100 μl of Matrigel and implanted subcutaneously in the right flank of 6-week-old BALB/c nude female mice (n = 10/each group). After 1 week, solvent control (cremophor/DMSO/ethanol/PBS, 1.5:0.5:0.5:7.5) [16] or DSF (50 mg/kg/day) was administered intraperitoneally 5 days per week, and tumor volumes were measured using a caliper and calculated using the formula $V = (\text{Length} \times \text{Width}^2)/2$.

For xenografting of DSF/Cu-treated tumor cells, BT474 cells were pretreated with DSF/Cu (0.3 μM) for 72 h. The cells were harvested and cell viability was determined by trypan blue exclusion (viable cells >95%). Sub-G1 accumulation was further confirmed by flow cytometry (sub-G1, 4.66%). After normalizing the number of viable cells, the control or DSF/Cu-treated cells (8×10^6) suspended in 100 μl of culture medium were mixed with 100 μl of Matrigel and injected into the left and right flanks of BALB/c nude female mice (6-week-old, n = 5), respectively.

Immunohistochemistry and in-situ localization of apoptosis (TUNEL)

At sacrifice, the tumors were removed, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Tissue sections of 4-μm thickness were mounted on positively charged glass slides and then deparaffinized with xylene and dehydrated through a graded alcohol series to water. For antigen retrieval, sections were boiled in citric acid buffer (pH 6.0) and subjected to immunofluorescence analysis. Tissue sections with primary antibodies in antibody-diluent were incubated overnight at 4 °C. For secondary antibody reactions, the sections were incubated with fluorescent-conjugated secondary antibody at RT for 2 hours, followed by ProLong gold antifade reagent with DAPI. *In situ* TUNEL was carried out on tissue sections using a TUNEL kit (Roche Applied Sciences, Penzberg, GER) in accordance with the manufacturer's instructions.

Statistical analysis

All data were analyzed using GraphPad Prism 5.0 statistical software (San Diego, CA). The results are presented as mean ± SEM of at least three independent experiments. Data were analyzed by student's *t* test, and one- or two-way ANOVA as appropriate. Significance between multiple experimental groups was determined using the Bonferroni *post hoc* test and defined at $p < 0.05$.

Results

DSF induces apoptosis in a copper (Cu)-dependent manner in HER2-positive breast cancer cells

To investigate the relationship between HER2 amplification and cancer stem-like properties, Aldefluor-positivity and HER2, phospho-Akt (Ser473), Akt and ALDH1A1 levels were determined in several HER2-positive (SKBR3, BT474 and MDA-MB-453), and HER2-negative (MCF7, T47D and MDA-MB-231) breast cancer cell lines. HER2 amplification was associated with high levels of Akt phosphorylation (Fig. 1A and B). ALDH1Al is an established marker for BCSCs [9,10,26], and we thus sought to investigate whether HER2 amplification is associated with increased levels of ALDH1Al. Of the HER2-positive cell lines, SKBR3 and BT474 harbored a higher Aldefluor-positive population when compared to the HER2-negative breast cancer cells, while MDA-MB-453 exhibited limited ALDH1A1 expression (Fig. 1C and D).