Biochemical and Biophysical Research Communications 484 (2017) 833–838

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Diallyl trisulfide, a chemopreventive agent from *Allium* vegetables, inhibits alpha-secretases in breast cancer cells

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ARTICLE INFO

Article history:
Received 31 January 2017
Accepted 31 January 2017
Available online 2 February 2017

Keywords:
Alpha secretases
Notch ligands
Breast cancer
Diallyl trisulfide

ABSTRACT

Breast cancer affects one in eight women throughout the course of their lifetime creating a demand for novel prevention strategies against this disease. The Notch signaling pathway is often aberrantly activated in human malignancies including breast cancer. Alpha secretases, including ADAM (A Disintegrin and Metalloprotease) −10 and −17, are proteases that play a key role in the cleavage of cell surface molecules and subsequent ligand-mediated activation of Notch signaling pathway. High expression levels of ADAM10 and 17 have been clinically associated with a lower disease-free survival in breast cancer patients. This study was undertaken to determine the effect of diallyl trisulfide (DATS), a bioactive organosulfide found in garlic and other *Allium* vegetables, on alpha secretases in breast cancer cells. Here we report for the first time that DATS inhibits the expression of ADAM10 and ADAM17 in estrogen-independent MDA-MB-231 and estrogen-dependent MCF-7 breast cancer cells, and in Harvey-*ras* (H-Ras) transformed MCF10A-H-Ras breast epithelial cells. We also show that DATS induces a dose-dependent reduction in colony formation ability of MDA-MB-231 and MCF-7 cells, suggesting a long-term effect of DATS on growth inhibition of breast cancer cells. Furthermore, we show that DATS inhibits the Notch ligands Jagged-1 and Jagged-2 involved in activation of Notch signaling pathway. Collectively, these findings show that DATS targets Notch pathway components overexpressed in breast cancer tumors and may serve as a functionally relevant bioactive for breast cancer prevention.

1. Introduction

Breast cancer is the most frequently diagnosed cancer among US women [1]. Epidemiological data show an inverse relationship between consumption of *Allium* vegetables, including garlic and onions, and development of several types of cancer [2–4]. Specifically, a case-control study in France examining development of breast cancer reported an odds-ratio of 0.52 (95% CI: 0.34–0.78) among women consuming 7–10 weekly servings of garlic and onions compared to women consuming fewer than 6 weekly servings [2].

Anticancer effects of *Allium* vegetables have been attributed to several organosulfur compounds [e.g., diallyl sulfide, diallyl disulfide, diallyl trisulfide, S-allyl cysteine, ajoene, etc.] [5]. Multiple reports indicate that diallyl trisulfide (DATS) is a more potent inhibitor of cancer cell survival compared to its close analogs diallyl disulfide or diallyl sulfide [6,7]. Several mechanisms of action of DATS in cancer cells have been identified [5–11]. In breast cancer cells, DATS treatment resulted in accumulation of reactive oxygen species and induced apoptosis in estrogen receptor (ER)-positive and triple-negative cells [6,8,9]. DATS treatment inhibited invasion, migration and mammosphere formation of breast cancer cells [7,11]. In a MCF-7 xenograft model, mice treated with 5 μmol DATS/kg body weight by oral gavage twice weekly had significantly smaller tumors than control-treated animals [6]. In a SUM159 xenograft model, mice treated by oral gavage with 2 mg DATS three times per week had reduced tumor incidence and tumor weight compared to control-treated mice [7]. Tumor tissue from DATS-treated SUM159 xenograft mice displayed reduced ALDH1 activity compared to tissue from untreated mice [7], suggesting that DATS targets the cancer stem cells population.

ADAMs (A Disintegrin and Metalloproteases) are a broad family of proteases responsible for cleavage of several substrates relevant to cancer progression, including Notch [12]. ADAM proteins are first

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http://dx.doi.org/10.1016/j.bbrc.2017.01.184
0006-291X/

synthesized as zymogens and are processed by the proprotein convertases (PC) furin and PC7 to yield active proteases [13]. ADAM10 constitutively cleaves Notch upon ligand binding, and ADAM17 is capable of cleaving Notch under ligand-independent conditions [14,15].

Notch is a juxtacrine signaling pathway in which membrane-bound Notch ligands activate Notch receptors on neighboring cells. Notch ligands, including Jagged-1, Jagged-2, Delta-like (DLL)-1, −3, and −4, bind to Notch receptors (Notch-1, -2, -3, -4), permitting cleavage of the Notch extracellular domain (NECD). Cleavage of NECD is achieved by ADAM10 in a ligand-dependent fashion [14]. Cleavage of NECD permits the gamma secretase complex to cleave Notch intracellular domain (NICD). NICD translocates to the nucleus where it binds recombinant signal binding protein for immunoglobulin kappa J region (RBP-Jκ) and recruits the co-activator mastermind-like (MAML), thereby activating transcription. Downstream targets of the Notch pathway include canonical targets Hes and Hey, as well as context-dependent targets such as cyclin D1, c-myc, and Nanog [16]. Notch signaling is aberrantly activated in breast cancer, and plays a key role in cell proliferation and differentiation [17,18]. Cells with high levels of active Notch signaling display increased tumorigenic capacity *in vivo*, and Notch contributes to maintenance of cancer stem cells [19]. In osteosarcoma cells, DATS treatment was shown to suppress protein expression of the Notch pathway components Notch-1 and Hes-1, and the Notch downstream target cyclin D1 [20].

In this study we report a novel mechanism of action of DATS by targeting alpha secretases and Notch ligands in breast cancer cells. We show that DATS treatment has a long-lasting effect on breast cancer cells by impairing the colony formation ability of both estrogen-dependent and estrogen-independent breast cancer cells. We also report that DATS targets key components of the Notch signaling pathway frequently overexpressed in breast cancer including alpha secretases ADAM10 and ADAM17, and Notch ligands Jagged-1 and Jagged-2.

2. Materials and methods

2.1. Cell culture and reagents

Human breast cancer cell lines MDA-MB-231 and MCF-7 were purchased from American Type Culture Collection (ATCC, Manassas VA). MDA-MB-231 cells were cultured in RPMI 1640 (Corning, Manassas VA) supplemented with 10% fetal bovine serum (FBS, Corning) and 1% penicillin/streptomycin antibiotic mixture (PS, Corning). MCF-7 cells were cultured in Eagle's Minimum Essential Medium (Corning) with 0.01 mg/ml human recombinant insulin (Sigma-Aldrich, St. Louis MO), 10% FBS, and 1% PS. MDA-MB-231 cells do not express ER, PR, or HER2 receptors and were derived from a 51 y old female patient with breast adenocarcinoma. MCF-7 cells are ER-positive and were derived from a 69 y old female patient with breast adenocarcinoma.

Characteristics of the MCF10A-H-Ras cell line are well described in the literature and have recently been reviewed [21]. Briefly, the MCF10A-H-Ras cell line is a modified version of normal-like MCF-10A breast epithelial cells, with a T24-mutated Harvey-*ras* stable gene insertion. These cells are capable of forming ducts *in vivo* that could progress to invasive ductal carcinoma [21]. MCF10A-H-Ras cells were a kind gift from Dr. Dorothy Teegarden, Purdue University. MCF10A-H-Ras cells were cultured in Dulbecco's Modified Eagle's Medium/Ham's F-12 50/50 Mix (Corning), supplemented with 20 ng/ml human recombinant EGF (StemCell Technologies, Vancouver BC), 100 ng/ml cholera toxin (Calbiochem, Billerica MA), 0.01 mg/ml human recombinant insulin, 500 ng/ml hydrocortisone (Sigma Aldrich), 5% donor horse serum (Corning), and 1% PS. All cells were maintained at 37 °C with 5% carbon dioxide.

Diallyl trisulfide (DATS, ≥ 98% purity) was purchased from LKT Laboratories (St. Paul, MN). DMSO was obtained from Fisher (Pittsburgh, PA) and PBS was from Corning. Trypan blue was purchased from Mediatech (Manassas, VA) and crystal violet was purchased from Amresco (Boise, ID). Antibodies against ADAM10, ADAM17, Jagged-1, and Jagged-2 were purchased from Cell Signaling (Danvers, MA), and antibody against Actin was purchased from Sigma-Aldrich.

2.2. Trypan blue exclusion assay

Cells were seeded into 12-well plates at a density of 10,000 cells/well for MDA-MB-231 cells, or 50,000 cells/well for MCF-7 and MCF10A-H-Ras cells, and allowed to attach for 24 h. Cell culture medium was then replaced with medium containing DATS (20, 40, 60, 80, 100 μmol/L) or vehicle control (DMSO, <0.55% v/v) and incubated for 24 h. Cells were counted in 30 μL trypan blue (0.4% w/v) under a hemocytometer.

2.3. Clonogenic assay

MDA-MB-231 and MCF-7 breast cancer cells (1×10^6) were plated in 100 mm plates and allowed to attach for 24 h. Cells were then treated for 24 h with 20, 40, or 60 μmol/L DATS or vehicle control. After 24 h of DATS treatment, cells were reseeded in the absence of DATS into 6 well plates with 300 cells/well. MDA-MB-231 cells were incubated for 10 d and MCF-7 cells were incubated for 14 d in the absence of DATS. Growth medium was changed every 3 d. Colonies were fixed with methanol and stained with 0.5% (w/v) crystal violet prior to counting.

2.4. Western blotting

Cells were treated with 20, 40, or 60 μmol/L DATS or control (DMSO) for 16 or 24 h. Protein levels were determined using a BioRad (Hercules, CA) protein assay kit as previously described [22]. Equal amounts of protein were then separated on polyacrylamide gels and transferred to PVDF membranes. Membranes were incubated in primary antibody overnight at 4 °C with gentle rocking and developed with ECL Prime Detection Reagent (GE Amersham, Batavia, IL). Band quantification was completed with UN-SCAN-IT software (Silk Scientific, Orem, UT).

2.5. Statistical analysis

All statistical analysis was completed using GraphPad Prism 6.0 software (La Jolla, CA). Trypan blue assay data were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons to determine differences between groups. Clonogenic assay data were analyzed by Student's *t*-test. *P*-values < 0.05 were considered significant.

3. Results

3.1. Diallyl trisulfide reduces viability of human breast cancer cells

We first examined the ability of DATS to inhibit viability of cultured breast cancer cells. Cells were treated with DATS (20, 40, 60, 80, 100 μmol/L) or control (DMSO) and viability was determined after 24 h using trypan blue exclusion assay. Treatment with 40 μmol/L DATS reduced cell viability by approximately 53% in MDA-MB-231 and MCF-7 breast cancer cells (Fig. 1A), indicating an IC50 value of DATS between 30 and 40 μmol/L. Treatment of MCF10A-H-Ras cells with 40 μmol/L DATS reduced viability by