Biochemical and Biophysical Research Communications xxx (2015) 1–8

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Salinomycin possesses anti-tumor activity and inhibits breast cancer stem-like cells via an apoptosis-independent pathway

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ARTICLE INFO

Article history:
Received 13 September 2015
Accepted 19 September 2015
Available online xxx

Keywords:
Salinomycin
Breast cancer stem cells
Mammosphere
ALDH1

ABSTRACT

Cancer stem cells (CSCs) play important roles in the formation, growth and recurrence of tumors, particularly following therapeutic intervention. Salinomycin has received recent attention for its ability to target breast cancer stem cells (BCSCs), but the mechanisms of action involved are not fully understood. In the present study, we sought to investigate the mechanisms responsible for salinomycin's selective targeting of BCSCs and its anti-tumor activity. Salinomycin suppressed cell viability, concomitant with the downregulation of cyclin D1 and increased $p27^{kip1}$ nuclear accumulation. Mammosphere formation assays revealed that salinomycin suppresses self-renewal of ALDH1-positive BCSCs and downregulates the transcription factors Nanog, Oct4 and Sox2. TUNEL analysis of MDA-MB-231-derived xenografts revealed that salinomycin administration elicited a significant reduction in tumor growth with a marked downregulation of ALDH1 and CD44 levels, but seemingly without the induction of apoptosis. Our findings shed further light on the mechanisms responsible for salinomycin's effects on BCSCs.

1. Introduction

Breast cancer is the most common cancer diagnosed in women [1]. It is a heterogeneous disease that is categorized by the expression of molecular markers, such as the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2). Although such subtyping is an essential tool for accurate prognosis and has led to significant improvements in overall survival, breast cancer remains the second most frequent cause of cancer-related deaths in women [1,2].

In recent years, it has been reported that larger malignant tumors invariably contain a smaller subset of cells with distinctive phenotypes, termed cancer stem cells (CSCs). CSCs harbor specific biological properties including self-renewal, the capacity to develop into multiple lineages, and an unlimited proliferative potential. As a result, these cells are a major determinant of tumor survival and resistance to anti-cancer therapies [3]. The targeting of CSCs may therefore provide new approaches for developing more effective therapeutic strategies.

Breast cancer stem cells (BCSCs) with the $CD44^{+}/CD24^{-}$ phenotype were first isolated from patient-derived pleural effusions [4]. Further studies have identified other BCSC markers including aldehyde dehydrogenase 1 (ALDH1) and SRY (sex determining region Y)-box 2 (Sox2) [5–7]. Recently, Ginestier and colleagues reported that a mere 500 ALDH1-positive cells was sufficient to generate a stable tumor via orthotopic injection into the mammary fat pads of NOD/SCID mice. However, ALDH1-negative cells cannot successfully generate a stable tumor even in numbers 100-fold higher than what is sufficient for ALDH-positive cells [5]. Clinical studies have shown that ALDH1 overexpression is linked to poorer clinical outcomes, suggesting its utility as a novel marker of aggressive phenotypes [8,9].

Salinomycin is a polyether ionophore with anti-microbial and anti-coccidial properties that has been used as an agricultural antibiotic [10]. A recent screen for novel anti-CSC activity involving 16,000 compounds of interest identified salinomycin as one of the most potent candidates [11]. Although previous studies have investigated the effect of salinomycin on cancer cell proliferation,

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http://dx.doi.org/10.1016/j.bbrc.2015.09.108
0006-291X/

apoptosis and stemness in various types of cancer cells [12–14], the precise mechanisms by which salinomycin inhibits breast cancer self-renewal remain to be elucidated.

In the present study, we sought to investigate the mechanisms responsible for salinomycin's anti-BCSC properties using mammosphere formation assays and a xenograft model.

2. Materials and methods

2.1. Reagents and antibodies

Salinomycin, paraformaldehyde (PFA), dimethyl sulfoxide (DMSO), Triton X-100, and phosphate buffered saline (PBS) tablets were obtained from Sigma–Aldrich (St.Louis, MO, USA). Phosphatase inhibitor and protease inhibitor cocktail tablets were purchased from Roche Applied Sciences (Penzberg, Germany). DMEM, RPMI1640, fetal bovine serum (FBS), streptomycin-penicillin and Fungizone were purchased from Gibco (Gaithersburg, MD, USA). Primary antibodies were obtained targeting the following proteins: cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); Nanog, Oct4 and Sox2 (Cell Signaling Technology, Beverly, CA, USA); Ki-67 and ALDH1 (Abcam, Cambridge, MA, USA); $p27^{kip1}$ and PE-conjugated anti-CD44 (BD Biosciences, Franklin Lakes, NJ, USA); and actin (Sigma–Aldrich). Secondary horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgG were obtained from Bio-Rad Laboratories, Hercules, CA, USA.

2.2. Breast cancer cell culture

The human breast cancer cell lines MCF7, T47D and MDA-MB-231 (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM or RPMI1640 containing 10% 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.

2.3. Cell viability assay

Cell viability was determined 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) according to the manufacturer's instructions. The quantity of formazan product was determined by measuring absorbance at 490 nm using a Spectramax Plus384 microplate analyzer (Molecular Devices, Sunnyvale, CA, USA).

2.4. Immunoblot analysis

Cells were solubilized in lysis buffer [30 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4)] containing phosphatase and protease inhibitor cocktail tablets. Supernatant was collected after centrifugation (14,000 g, 4 °C, 20 min) and protein concentrations were measured with a Bradford protein assay kit (Bio-Rad Laboratories). Equal quantities of protein (30 μg) were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane (GE Healthcare Life Science, Buckinghamshire, UK). The membranes were incubated overnight at 4 °C with primary antibodies diluted in 5% BSA [Nanog (1:1000), Oct4 (1:1000), Sox2 (1:1000), $p27^{kip1}$ (1:2000), cyclin D1 (1:2000) and actin (1:5000)], followed by incubation with horseradish peroxidase (HRP)-conjugated anti-rabbit and mouse IgGs (1:3000–1:10,000). Signal intensity was detected using an Enhanced Chemiluminescence Kit (Thermo Scientific Inc., Rockford, IL,USA) and X-ray film (Agfa Healthcare, Mortsel, Belgium) and quantitated using AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA).

2.5. ALDEFLUOR assay

The ALDEFLUOR assay kit (Stemcell Technology, Vancouver, Canada) was used to assess aldehyde dehydrogenase 1 (ALDH1) activity according to the manufacturer's protocol. Cells were incubated for 45 min at 37 °C in ALDEFLUOR assay buffer containing the ALDH1 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 to define the ALDEFLUOR population. ALDEFLUOR stained cells were analyzed with a Beckman Coulter Expo flow cytometer.

2.6. Mammosphere formation assay

Cells were plated in ultralow attachment dishes (CORNING, NY, USA) and cultured in HuMEC basal serum-free medium (Gibco), supplemented with B27 (1:50, Invitrogen, Carlsbad, CA), 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 agent, and 15 μg/mL gentamycin. The number and volume of mammospheres were determined under an Olympus IX 71 inverted microscope and photos were acquired with Olympus DP capture software. For the ALDEFLUOR assays, mammospheres were incubated with ACCUTASE™ (Stemcell technologies) for 15 min at 37 °C. The dissociated cells were filtered through a 40 μm cell strainer (BD Biosciences) and the ALDEFLUOR-positive population was analyzed using flow cytometry.

2.7. Immunofluorescence confocal microscopy

Cells on 8-well chamber slides (BD Biosciences) were fixed with 4% PFA, washed with PBS, and permeabilized with 0.2% Triton X-100 for 10 min. Primary antibodies ($p27^{kip1}$, 1:100) in antibody diluent (Dako, Glostrup, Denmark) were incubated overnight at 4 °C and then incubated with Alexa Fluor®-488 goat anti-mouse IgG (Invitrogen). Cells were mounted with ProLong Gold Antifade Reagent and DAPI (Life Technologies, Carlsbad, CA, USA). Images were acquired using a Carl Zeiss confocal microscope (Weimar, Germany).

2.8. RT-PCR analysis

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Amplification of transcripts was achieved by reverse transcriptase-polymerase chain reaction (RT-PCR) using 1 μg/μl total RNA, Molony Murine Leukemia Virus reverse transcriptase (MMLV; Gibco), and oligo-d(T)15 primer (Roche Applied Sciences). PCR amplification was performed using the following primers: Nanog, forward 5′-TTG TGC GCC TGA AGA AAA CTA TCC-3′, reverse 5′-CTG CGT CAC ACC ATT GCT ATT CTT-3′; Oct4, forward 5′-GAC AAC AAT GAG AAC CTT CAG GAG A-3′, reverse 5′-CTG GCG CCG GTT ACA GAA CCA-3′; Sox2, forward 5′-CCC CCG GCG GCA ATA GCA-3′, reverse 5′-TCG GCG CCG GGG AGA TAC AT-3′; actin, forward 5′-ACC CAG ATC ATG TTT GAG AC-3′, reverse 5′-GGA GTT GAA GGT AGT TTC GT-3′. The PCR products were separated on 1.2% agarose gels and visualized using a Gel Doc™ XR+ System (Bio-Rad Laboratories).

2.9. Animals, xenograft experiment and bioluminescence imaging (BLI)

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

Please cite this article in press as: H. An, et al., Salinomycin possesses anti-tumor activity and inhibits breast cancer stem-like cells via an apoptosis-independent pathway, Biochemical and Biophysical Research Communications (2015), http://dx.doi.org/10.1016/j.bbrc.2015.09.108