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Salinomycin exerts anticancer effects on human breast carcinoma MCF-7 cancer stem cells via modulation of Hedgehog signaling

Ying Lu [a], Wei Ma [a], Jun Mao [a,b], Xiaotang Yu [a], Zhenhuan Hou [a], Shujun Fan [a], Bo Song [a], Huan Wang [a], Jiazhi Li [a], Le Kang [a], Pixu Liu [c], Quentin Liu [c], Lianhong Li [a,b,*]

[a] Department of Pathology, Dalian Medical University, Dalian 116044, PR China
[b] The Key Laboratory of Tumor Stem Cell Research of Liaoning Province, Dalian Medical University, Dalian 116044, PR China
[c] Institute of Cancer Stem Cell, Dalian Medical University, Dalian 116044, PR China

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ABSTRACT

Breast cancer tissue contains a small population of cells that have the ability to self-renew, these cells are known as breast cancer stem cells (BCSCs). The Hedgehog signal transduction pathway plays a central role in stem cell development, its aberrant activation has been shown to contribute to the development of breast cancer, making this pathway an attractive therapeutic target. Salinomycin (Sal) is a novel identified cancer stem cells (CSCs) killer, however, the molecular basis for its anticancer effects is not yet clear. In the current study, Sal's ability to modulate the activity of key elements in the Hedgehog pathway was examined in the human breast cancer cell line MCF-7, as well as in a subpopulation of cancer stem cells identified within this cancer cell line. We show here that Sal inhibits proliferation, invasion, and migration while also inducing apoptosis in MCF-7 cells. Interestingly, in a subpopulation of MCF-7 cells with the $CD44^+/CD24^-$ markers and high ALDH1 levels indicative of BCSCs, modulators of Hedgehog signaling Smo and Gli1 were significantly down-regulated upon treatment with Sal. These results demonstrate that Sal also inhibits proliferation and induces apoptosis of BCSCs, further establishing it as therapeutically relevant in the context of breast cancers and also indicating that modulation of Hedgehog signaling is one potential mechanism by which it exerts these anticancer effects.

1. Introduction

Breast cancer is the commonest type of cancer and the second leading cause of cancer-related deaths in American women [1]. Recently, a subpopulation known as BCSCs were isolated and identified with the markers $CD44^+/CD24^-$ as well as a high level of aldehyde dehydrogenase 1 (ALDH1) activity [2–3]. The high tumorigenicity and multidrug resistance found in this subpopulation make BCSCs an attractive target for potential therapeutics of breast cancer [4–6].

Mounting evidences have shown that several signaling pathways including Hedgehog (Hh), Notch and Wnt/β-catenin are involved in the regulation of BCSC self-renewal ability [7]. The Hh pathway operates as a sequence of inhibitory interactions. In the basal state, the twelve transmembrane domain receptor Patched (PTCH) antagonizes signal transduction by inhibiting the activity of the seven transmembrane domain receptor Smoothened (Smo). Upon binding of Hh ligands (SHh, IHh, or DHh, being the three known mammalian ligands) to PTCH, the inhibition of Smo by PTCH is relieved, and a series of intracellular signal transduction events are initiated and ultimately result in the nuclear translocation of the Hh transcription factor Gli1, by which the Hh-responsive genes are transcribed. The Hh signaling pathway functions as an organizer in embryonic development. Importantly, genetic analysis has demonstrated that this pathway plays a critical role in mammary gland development as well as in BCSC self-renewal.

Salinomycin (Sal), an antibiotics used in veterinary medicine, has been recently demonstrated to effectively inhibit the proliferation and induce the apoptosis of various types of cancer stem cells (CSCs), including breast cancer, a recent study showed that salinomycin kills breast cancer stem cells at least 100 times more effectively than paclitaxel in mice [9], gastric cancer [10], pancreatic cancer [11], hepatocellular carcinoma [12], ovarian cancer [13], prostate cancer, [14] and some additional malignancies [15]. Sal was found to overcome drug resistance in various human cancer cell lines being as an efflux pump p-glycoprotein inhibitor [16–19]. Sal may also sensitize cancer cells to radiation or to cytostatic drugs, such as

* Corresponding author at: Department of Pathology, Dalian Medical University, 9 West Lvshun Southern Road, Dalian 116044, PR China. Tel./fax: +86 411 8611 0050.
E-mail address: lilianhong9177@163.com (L. Li).

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etoposide or doxorubicin, by increasing apoptosis as a result of enhancing DNA damage and reducing the levels of CDKN1A/p21 protein [20–22]. Two recent studies reported that salinomycin selectively targets CSCs in the osteosarcoma and gastric cancer possibly by suppressing the Wnt/β-catenin signaling pathway [23–24]. Although the selective inhibition of Sal to the CSCs is explained partially by the above studies, limitations still exist. Based on the roles of Hh signaling pathway in the BCSC self-renewal, we reasoned that Sal may inhibit BCSCs through influencing Hh signaling expression.

In the present study, we demonstrate that Sal inhibits the proliferation and induces the apoptosis in both breast cancer MCF-7 cells and as well as in the subpopulation of BCSCs via modulating Hh signaling. Sal targeting Hh signaling may have important clinical applications in breast cancer therapy.

2. Materials and methods

2.1. Cell culture and reagents

The human breast cancer cell line MCF-7 was purchased from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Shanghai, China. The cells were maintained in Dulbecco's Modified Eagle's Medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (HyClone) at 37 °C in a humidified incubator with 5% CO_2. Sal was obtained from Sigma–Aldrich (St Louis, MO, USA), and dissolved in dimethyl sulfoxide (DMSO). An equal volume of media alone was used as the blank control group.

2.2. Cell proliferation assay

MCF-7 cells were harvested by trypsinization and plated in 96-well plates at 3×10^4 cells/well. The cells were treated with Sal in three replicates at concentrations of 0, 0.5, 1 and 1.5 μmol/L and quantified after 0, 24, 48, 72 and 96 h. To quantify the cells, 10 μl of the Cell Counting Assay Kit-8 solution (Sigma–Aldrich) was added to each well, and the cells were incubated for an additional 2 h. The absorbance was then measured with a microplate reader at 450 nm. To estimate the inhibition of cell growth, the drug concentration that inhibited 50% of the growth of control cells was calculated.

2.3. Cell apoptosis analysis

MCF-7 cells were plated at 1×10^6 cells/well in 6-well plates for 24 h and then treated with Sal (0, 0.5, 1 and 1.5 μmol/L). After incubation for 48 h, cells were harvested, washed twice with PBS, resuspended in 500 μl of binding buffer and stained with 5 μl of fluorescein isothiocyanate (FITC)–Annexin-V (BD Biosciences, San Jose, CA, USA) and 10 μl of propidium iodide (50 μg/mL, BD Biosciences) for 15 min at room temperature protected from light. Cells were analyzed by flow cytometry using the Aria cell sorter (BD, Franklin Lakes, NJ, USA). This experiment was repeated in triplicate.

2.4. Hoechst33342 staining

Morphological detection of apoptotic cells was observed using Hoechst33342 staining. MCF-7 cells were plated in 6-well plates. After 48 h, Sal treated cells (0, 0.5, 1 and 1.5 μmol/L) were harvested and washed twice with PBS. 10 μl of Hoechst33342 (10 μg/ml, Keygen Biotech, Nanjing, China) was added to each well and incubated for 30 min at 37 °C protected from light, then imaged using an inverted fluorescence microscope. Each treatment was performed in triplicate.

2.5. CD44/CD24 staining to identify BCSCs

After treatment with Sal for 48 h, MCF-7 cells were rinsed twice with PBS, and then stained with phycoerythrin-conjugated anti-human CD24 antibody (Invitrogen) and FITC-conjugated anti-human CD44 antibody (Invitrogen) for 30 min at room temperature according to the manufacturer's instructions. The labeled cells were then analyzed using a FACS Calibur flow cytometer and Cell Quest software (BD).

2.6. Mammosphere formation assay

Cells were plated in 6-well plates for 24 h and treated with Sal (0, 0.5, 1 and 1.5 μmol/L). After incubation for 48 h, cells were harvested and plated at 1×10^4 cells/well in ultra-low-attachment six-well plates (Corning, Acton, MA, USA) in serum-free DMEM/F12 supplemented with B27 (1:50, Invitrogen, Carlsbad, CA, USA), 20 ng/ml human recombinant epidermal growth factor (Sigma–Aldrich), 20 ng/ml basic fibroblast growth factor (Sigma–Aldrich), 4 μg/ml heparin (Sigma–Aldrich), and 5 μg/ml insulin (Sigma–Aldrich). Mammospheres were counted after 7 days of culture under a Nikon Eclipse TE2000-S microscope (Japan) and photographed with Meta Morph.

2.7. Migration and invasion assay

Tumor cell migration was analyzed using the Scratch assay. MCF-7 cells were cultured on 6-well plates in DMEM with 10% FBS. Upon reaching confluence, the medium was replaced with serum-free DMEM containing Sal (0, 0.5, 1 and 1.5 μmol/L) for an additional 48 h, and the cell layer was then wounded with 10 μL tips. After 48 h, the wound closures were photographed with a BZ-8100 microscope (Keyence, Japan).

Tumor cell invasion was analyzed using a transwell chamber (Corning Coster, Corning, NY, USA) with an 8 μm pore polycarbonate membrane and coated with Matrigel (Becton Dickinson Labware, Bedford, MA). The transwell was coated with 25 μl of diluted Matrigel (100 μl added to 300 μl DMEM) and allowed to set at 37 °C for 30 min. MCF-7 cells treated with Sal (0, 0.5, 1 and 1.5 μmol/L) for 48 h were placed at 5×10^5 cells/well in serum-free culture medium in the upper compartment of the transwell. The lower compartment was filled with 800 μL of DMEM containing 10% FBS. Chambers were incubated at 37 °C and 5% CO_2 for 24 h. Cells in the top compartment of the inserts were then removed with a cotton swab, and cells that had migrated to the underside of the inserts were stained with 2% crystal violet for 30 min. The membranes were analyzed under a light microscope and the migrated cells were counted in five randomly selected fields.

2.8. RNA extraction and RT-PCR of Smo and Gli1

Each sample was treated for 48 h with Sal. Total RNA was extracted using Trizol (Invitrogen, USA) according to the manufacturer's instructions. The quantities and qualities of isolated RNAs were evaluated using absorbance measurements at 260 and 280 nm. The first-strand cDNA was synthesized using the Reverse Transcriptase System (Invitrogen), and the target cDNAs were amplified using primer pairs for Smo, Gli1, and ALDH1. GAPDH was used as the internal standard. Primers were designed as: GAPDH (Forward, 5′-GGACCTGACCTGCCGTCTAG-3′; Reverse, 5′-GTAGCCCAGGATGCCCTTGA-3′), ALDH1 (forward, 5′-AATGGCATGATTCAGTGAGTGGC-3′; reverse, 5′-GAGGAGTTTGCTCTGCTGGTTTG-3′), SMO (Forward, 5′-CTTTGTCATCGTGTACTACGCC-3′; Reverse, 5′-CGAGAGAGGCTGGTAGGTG-3′), and Gli1 (Forward, 5′-GAACCCTTGGAAGGTGATATGTC-3′; Reverse, 5′-GGCAGTCAGTTTCATACACAGAT-3′). Real time PCR was done in the iCycler™ Real Time System (Bio-Rad