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Salinomycin induces apoptosis and senescence in breast cancer: Upregulation of p21, downregulation of survivin and histone H3 and H4 hyperacetylation

Yusra Al Dhaheri [a], Samir Attoub [b], Kholoud Arafat [b], Synan AbuQamar [a], Ali Eid [a,1], Nesreen Al Faresi [a], Rabah Iratni [a,*]

[a] Department of Biology, College of Science, UAE University, P.O. Box: 17551, United Arab Emirates University, Al-Ain, P.O. Box 15551, United Arab Emirates
[b] Department of Pharmacology & Therapeutics, Faculty of Medicine & Health Sciences, United Arab Emirates University, Al-Ain, P.O. Box: 17666, United Arab Emirates

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ABSTRACT

Background: In the present study, we investigated the effect of Salinomycin on the survival of three human breast cancer cell lines MCF-7, T47D and MDA-MB-231 grown in adherent culture conditions.
Methods: Cell viability was measured by CellTiter-Glo and Trypan blue exclusion assay. Apoptosis was determined by caspase 3/7 activation, PARP cleavage and Annexin V staining. Cell cycle distribution was assessed by propidium iodide flow cytometry. Senescence was confirmed by measuring the senescence-associated β-galactosidase activity. Changes in protein expression and histone hyperacetylation was determined by western blot and confirmed by immunofluorescence assay.
Results: Salinomycinwas able to inhibit the growth of the three cell lines in time- and concentration-dependent manners. We showed that depending on the concentrations used, Salinomycin elicits different effects on theMDA-MB-231 cells. High concentrations of Salinomycin induced a G2 arrest, downregulation of survivin and triggered apoptosis. Interestingly, treatment with low concentrations of Salinomycin induced a transient G1 arrest at earlier time point and G2 arrest at later point and senescence associatedwith enlarged cellmorphology, upregulation of p21 protein, increase in histone H3 and H4 hyperacetylation and expression of SA-β-Gal activity. Furthermore, we found that Salinomycin was able to potentiate the killing of the MCF-7 and MDA-MB-231 cells, by the chemotherapeutic agents, 4-Hydroxytamoxifen and frondoside A, respectively.
Conclusion: Our data are the first to link senescence and histone modifications to Salinomycin.
Significance: This study provides a new insight to better understand the mechanism of action of Salinomycin, at least in breast cancer cells.

1. Introduction

Breast cancer is the most common cancer seen in women worldwide accounting for more than 1.5 million new cases diagnosed in 2010, with one million deaths each year worldwide. Remarkable progress has been made in the treatment of breast cancer over the years such as hormonal therapy for estrogen receptor and/or progesterone receptor positive tumors, as well as other targeted therapies for selected subgroups of patients. However, despite these advances, breast cancer remains a frequent cause of death in women.

Salinomycin is a monocarboxylic polyether antibiotic, isolated from *Streptomyces albus*, widely used as an antibiotic to prevent anticoccidiosis in poultry and as growth promoter for ruminants and swine. Screening for compounds that target specifically cancer stem cells has led to the identification of Salinomycin as a potential anticancer agent. Salinomycin was shown to (i) selectively induce cell death of breast cancer stem cells in cell culture assay and (ii) inhibit breast tumor growth in mice [1]. More recent investigations have reported that Salinomycin possess potent anticancer activities against other cancer stem cells such as the ALDH positive A549 lung cancer cells [2], ALDH gastric cancer cells [3] and CD133 + subpopulations in human CRC HT29 and SW480 colorectal cancer cells [4]. However, the mechanisms of action of Salinomycin on cancer stem cells are yet to be elucidated.

More recent studies reported that Salinomycin shows an efficacy against other human cancer cells including those displaying drug

Abbreviations: **4**-HT, 4-hydroxy-tamoxifen; DMEM, Dulbecco Minimal Essential Medium; DMSO, Dimethyl sulfoxide; FACS, Fluorescence-activated cell sorting; Fr, Frondoside A; PARP, Poly (ADP-ribose) polymerase; RIPA, Radioimmunoprecipitation assay buffer; SA-β-Gal, Senescence-associated β-galactosidase; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TBST, Tris buffered saline containing/0.05% Tween 20; X-gal, 5-bromo-4-chloro-indolyl-β-D-galactopyranoside

* Corresponding author. Tel.: +971 3 713 6526/6731; fax: +971 3 713 4927.
E-mail address: R_iratni@uaeu.ac.ae (R. Iratni).
[1] Present address: Department of Biological and Environmental Sciences, College of Arts and Sciences, Qatar University, P.O. Box: 2713, Doha, Qatar.

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resistances [5]. Salinomycin was shown to act as a p-glycoprotein inhibitor to overcome apoptosis resistance in cancer cells [6]. Lu et al. have recently shown that Salinomycin inhibits Wnt signaling and selectively induces apoptosis in chronic lymphocytic leukemic cells [7]. Salinomycin was also shown to induce apoptosis in human prostate cancer cells by inducing an elevation in the intracellular reactive oxygen species (ROS) levels and mitochondrial membrane depolarization [8]. Very recent studies have also shown that Salinomycin inhibits prostate cancer growth and migration through induction of oxidative stress [9].

Combination treatment of Salinomycin with gemcitabine has proven to be more effective than the individual drugs against pancreatic cancer cells [10]. It appears that Salinomycin sensitizes cancer cells to the effects of etoposide and doxorubicin treatment by enhancing apoptosis, increasing DNA damage and reducing the level of p21 protein [11]. Indeed, Salinomycin sensitizes radiation-treated cells by inducing G2 arrest and causing DNA damage [12]. More recently, Salinomycin was reported to sensitize cancer cells to antimitotic drugs (paclitaxel, docetaxel, vinblastin and colchicines) by enhancing apoptosis as well as preventing G2 arrest [13].

Despite the recent findings, the mechanisms underlying the anticancer activity of Salinomycin are still poorly understood. In the present study, we have investigated the effect of Salinomycin on the viability of MCF-7 and T47D and the triple negative (TNBC) MDA-MB-231 human breast cancer cell lines. We found that Salinomycin was able to induce growth inhibition, permanent cell cycle arrest, apoptosis and senescence of breast cancer cells. Salinomycin activities correlated with, induction of histone H3 and H4 hyperacetylation and upregulation of the level of p21 protein. Furthermore, we showed that Salinomycin was able to enhance the antiproliferative activity of 4-Hydroxytamoxifen and frondoside A in MCF-7 and MDA-MB-231 breast cancer cells, respectively.

2. Materials and methods

2.1. Cell culture and reagents

Human breast cancer cells MDA-MB-231, MCF-7 were maintained in DMEM (Hyclone). The T47-D were maintained in RPMI 1640 (Hyclone). All media were supplemented with 10% fetal bovine serum (invitrogen), 100 U/ml penicillin/streptomycin (invitrogen). Salinomycin and 4-Hydroxytamoxifen were purchased from (Sigma-Aldrich, Saint-Quentin Fallavier; France). Antibodies to p21 (556431), PARP (556494) were obtained from BD Pharmingen. Antibodies to phosphor-H2A.X (ser139) (07-164), acetyl-Histone H3 (06-599), acetyl-Histone H4 (06-866), phosphor-Histone H3 (ser10) (05-1336), P53 (E26, 04-241), Cyclin B1 (05-73) and cyclin D1 (04-1151) were obtained from Millipore, and survivin (sc-1779), β-actin (C4, sc-47778), goat anti-mouse IgG-HRP (sc-2005) and goat anti-rabbit IgG-HRP (sc-2004) were obtained from Santa Cruz Biotechnology, Inc. AlexaFluor 488 goat anti-rabbit IgG (H + L) (A11008) and AlexaFluor 594 goat anti-mouse IgG (H + L) (A11005) were obtained from Invitrogen.

2.2. Cellular viability

Cells were seeded in triplicate in 96-well plates at a density of 5000 cells/well. After 24 h of culture, cells were treated with increasing concentrations of Salinomycin and incubated for the indicated time periods. Control cells were exposed to DMSO at a concentration equivalent to that of the Salinomycin-treated cells. In combination experiments, MCF-7 and MDA-MB-231 cells were subjected to simultaneous treatment with 20 μM 4-hydroxy-tamoxifen and 1 μM frondoside A, respectively, plus DMSO or increasing concentrations of Salinomycin for 48 h. The effects of the Salinomycin, alone or in combination, on cell viability were determined using a CellTiter-Glo Luminescent Cell Viability assay (Promega Corporation, Madison, USA), based on quantification of ATP, which signals the presence of metabolically active cells. Luminescent signal was measured using Berthold FB12 Luminometer. Data were presented as proportional viability (%) by comparing the treated group with the untreated cells, the viability of which is assumed to be 100%.

Cell viability was also assessed by Trypan blue exclusion assay. Briefly, cells were plated onto 24-well plates (20×10^4 cells/well). The day of treatment cells were counted to estimate the approximate number of cells per well. Following Salinomycin treatment at indicated times, cells were trypsinized, pelleted by centrifugation and resuspended in serum free DMEM and stained with Trypan blue. Dead (stained blue) and live (unstained) cells were counted on Neubauer hemacytometer. Three independent assays were performed in triplicate, and results are reported as the means ± S.E.M.

2.3. Caspase 3/7 activity

MDA-MB-231 and MCF-7 cells were seeded at a density of 5000 cells/well into 96-well plate in triplicate and treated with Salinomycin for 24 h and 48 h. Caspase-3/7 activity was measured using a luminescent caspase-Glo 3/7 assay kit (Promega Corporation, Madison, USA) following the manufacturer's instructions. Briefly, caspase reagents were added to triplicate 96 wells. The plate was mixed on an orbital shaker and incubated for 2.5 h at room temperature in the dark. Luminescent signal was measured as described above.

2.4. Senescence associated-β-galactosidase (SA-β-gal) staining

2.5×10^5 MDA-MB-231 cells were cultured in 60 mm culture dishes for 24 h and then exposed to Salinomycin 10 μM or DMSO for 96 h. Treated and control cells were then washed in PBS, and fixed with 2% formaldehyde/0.2% glutaraldehyde for 5 min at room temperature. The SA-β-gal staining was performed as previously described [14].

2.5. Flow cytometric analysis of cell cycle

MDA-MB-231 cells (1.8×10^6) were seeded in 100 mm culture dishes and cultured for 24 h before addition of Salinomycin. After incubation, cells were harvested, washed twice with ice-cold PBS, resuspended in 500 μl PBS, fixed with an equal volume of 100% ethanol and incubated for at least 12 h at −20 °C. Cells were then pelleted, washed twice with PBS, permeabilized in 0.1% Triton X-100/PBS for 15 min on ice, pelleted and then resuspended in PBS containing 40 μg/ml propidium iodide and 25 μg/ml RNase A, at 37 °C for 30 min. Cells were analyzed using BD FACSCanto II (Becton Dickinson). Data acquisition was performed using FACSDiva 6.1 software. Percentage of cells in G1, S and G2/M phases was determined using the FlowJo software.

2.6. Quantification of apoptosis by annexin V/propidium iodide (PI) staining

DMSO and Salinomycin-treated MDA-MB231 (1.8×10^6) cells grown in 100 mm culture dishes were harvested by trypsin release and washed twice with ice-cold PBS. Apoptotic cell death was determined using the BD FITC annexin V apoptosis detection kit II (BD Biosciences) according to the manufacturer's instructions. Cell samples were analyzed on the BD FACSCanto II (Becton Dickinson).

2.7. Immunofluorescence staining

MDA-MB 231 cells (4×10^4) were grown on 4 well labtek chamber slide (Becton Dickinson) for 24 h, then treated with Salinomycin or DMSO for 24 h at the concentrations indicated. Cells were then fixed in 10% formalin solution (4% paraformaldehyde) (Sigma-Aldrich; Saint-Quentin Fallavier, France) for 5 min at room temperature followed by permeabilization in PBS containing 0.1% Triton X-100 for 5 min at