Cancer Letters ■■ (2015) ■■–■■

Contents lists available at ScienceDirect

Cancer Letters

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

Original Articles

Salinomycin inhibits growth of pancreatic cancer and cancer cell migration by disruption of actin stress fiber integrity

Miriam Schenk [a], Berk Aykut [a], Christian Teske [b], Nathalia A. Giese [a], Juergen Weitz [b], Thilo Welsch [a,b,*,1]

[a] Department of General, Visceral and Transplantation Surgery, University of Heidelberg, 69120 Heidelberg, Germany
[b] Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, TU Dresden, Germany

ARTICLE INFO

Article history:
Received 29 September 2014
Received in revised form 2 December 2014
Accepted 13 December 2014

Keywords:
Pancreatic cancer
Salinomycin
Migration
Stress fibers
RhoA

ABSTRACT

Pancreatic ductal adenocarcinoma (PDAC) is characterized by aggressive growth, early metastasis and high resistance to chemotherapy. Salinomycin is a promising compound eliminating cancer stem cells and retarding cancer cell migration. The present study investigated the effectiveness of salinomycin against PDAC *in vivo* and elucidated the mechanism of PDAC growth inhibition.

Salinomycin treatment was well tolerated by the mice and significantly reduced tumor growth after 19 days compared to the control group (each n = 16). There was a trend that salinomycin also impeded metastatic spread to the liver and peritoneum. Whereas salinomycin moderately induced apoptosis and retarded proliferation at 5–10 μM, it strongly inhibited cancer cell migration that was accompanied by a marked loss of actin stress fibers after 6–9 h. Salinomycin silenced RhoA activity, and loss of stress fibers could be reversed by Rho activation. Moreover, salinomycin dislocated fascin from filopodia and stimulated Rac-associated circular dorsal ruffle formation.

In conclusion, salinomycin is an effective and promising compound against PDAC. Besides its known stem cell-specific cytotoxic effects, salinomycin blocks cancer cell migration by disrupting stress fiber integrity and affecting the mutual Rho-GTPase balance.

Introduction

The fate of patients with pancreatic ductal adenocarcinoma (PDAC) is largely determined by its early metastatic spread and high resistance to known chemotherapeutic agents. Even after microscopically complete tumor resection and with standard adjuvant chemotherapy, median survival time reaches only about 23 months, while palliative chemotherapy in advanced tumor stages allows overall survival of only 6 months [1,2]. Research efforts aim to improve the multimodal treatment of PDAC, covering new chemotherapeutic agents or small molecule inhibitors and novel cancer cell targets, *e.g.* cancer stem cells.

In the past few years, the polyether salinomycin has emerged from a high-throughput screen of substances that selectively deplete breast cancer stem cells (CSCs) [3]. Salinomycin is an antibiotic commonly used in poultry and known to act as an ionophore on cell membranes [4,5]. Recent experiments have now discovered several anti-cancer effects of salinomycin in various malignancies, *e.g.* inhibition of cancer cell proliferation and migration as well as induction of apoptosis and autophagy [6–9]. Inhibition of migration is most likely accountable for the decreased metastatic spread observed in a breast cancer mouse model [10]. Yet cancer cell migration is a complex subcellular process based on dynamic actin remodeling orchestrated by manifold intra- and extracellular signals, and key migratory processes are governed intracellularly by the three Rho-GTPases RhoA, cdc42 and Rac, which control actin stress fibers, filopodia and lamellipodia, respectively [11]. Where salinomycin targets cancer cell migration is largely unknown.

The aim of the present study was to investigate the use of salinomycin in a syngeneic PDAC mouse model and to track down the observed cytotoxic or anti-metastatic mechanisms.

Material and methods

Cell cultures and salinomycin treatment

Human pancreatic cancer cell lines AsPC-1, Colo357, MiaPaCa-2 and PANC-1 were purchased from ATCC (Rockville, MD, USA). The murine pancreatic cancer cell line Panc02 was obtained as a gift from A. Marten (Heidelberg, Germany). The cells were cultured under normoxic (5% CO_2; 21% O_2) conditions in RPMI-1640 (Invitrogen GmbH, Karlsruhe, Germany), supplemented with 10% fetal bovine serum (FBS, PAN Biotech, Aidenbach, Germany).

Cells were exposed to salinomycin (Sigma-Aldrich, Taufkirchen, Germany) at final concentrations of 0.1–100 μM diluted in dimethyl sulfoxide (DMSO) for *in vitro*

* Corresponding author. Tel.: +49 351 458 18283; fax: +49 351 458 4395.
E-mail address: thilo.welsch@uniklinikum-dresden.de (T. Welsch).
[1] Current address: Department of Visceral, Thoracic and Vascular Surgery, University Hospital Carl Gustav Carus, TU Dresden, Germany.

http://dx.doi.org/10.1016/j.canlet.2014.12.037
0304-3835/

experiments. The cells were harvested at different time points, depending on the experimental settings. For immunofluorescence analysis, cells were grown on collagen A (Biochrom AG, Berlin, Germany) coated glass coverslips.

Antibodies and reagents

The following antibodies were used: mouse monoclonal anti-β-catenin (BD Biosciences, Heidelberg, Germany), mouse monoclonal RhoA specific (Cytoskeleton Inc., TX, USA), mouse monoclonal anti-RhoA (Cell Biolabs, Inc., CA, USA), mouse monoclonal anti-phospho-myosin light chain 2 (MLC, Ser19) and rabbit monoclonal anti-GAPDH (Cell Signaling Technology, MA, USA), mouse monoclonal anti-human fascin (Dako GmbH, Hamburg, Germany), rabbit polyclonal anti-actinin4 (ImmunoGlobe Antikörpertechnik GmbH, Himmelstadt, Germany), goat anti-mouse IgG-HRP and goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, CA, USA), mouse monoclonal anti-α-actinin, mouse monoclonal anti-Vinculin, anti-mouse IgG-Cy3 and anti-rabbit IgG-Cy3 (Sigma-Aldrich, Taufkirchen, Germany).

Corn oil (Rewe Markt GmbH, Cologne, Germany) was used as vehicle control for animal experiments.

Functional studies required treatment of cells with the Rho-activators calpeptin or sphingosine-1-phosphate (both Sigma-Aldrich, Taufkirchen, Germany) at a final concentration of 5 μM (added 0.5 h and 1 h prior to experimental analysis, respectively). The ROCK inhibitor Y-27632 (Sigma-Aldrich, Taufkirchen, Germany) was applied at a final concentration of 10 μM for 1 h. The Rac1-inhibitor CAS 1177865-17-6 (Merck, Darmstadt, Germany) was used at 100 μM for 48 h.

Animal experiments

C57Bl/6 mice were purchased from Janvier, St. Berthevin Cedex, France. All mouse procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe, 35–9185.81/G-211/12) and were in accordance with the European Communities Council Directives. Mice were narcotized with 2–3 vol.-% isofluran and 600 mmHg O_2, and abdominal incision was performed. 1×10^5 Panc02 cells were resuspended in phosphate buffered saline (PBS) and orthotopically injected into the pancreas. Therapy was started 5 days after surgery with either salinomycin (5 mg/kg) or corn oil (vehicle) applied intraperitoneally (i.p.) daily for 14 days (Fig. 1A). Mice were sacrificed on day 19 and tumor volume was measured using a vernier caliper and calculated with the formula ½π × length × width × height (in mm^3).

Proliferation and apoptosis assays

To determine the effect of salinomycin on cell proliferation, we performed a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test. Cells were seeded in 96-well plates and treated with different concentrations of salinomycin (0.1–100 μM). The MTT reagent was added after 24 h for an additional 4 h and formed formazan crystals were dissolved in propanol. The optical density was measured at 570 nm.

Apoptosis was quantified using annexin/ propidium iodide (PI) flow cytometry. In brief, cells were exposed to salinomycin for 24 h. Then, 1×10^6 cells were washed with PBS and binding buffer (10 mM HEPES; 140 mM NaCl; 2.5 mM $CaCl_2$, pH 7.4) and resuspended in binding buffer containing annexin V-FITC (eBioscience, Frankfurt am Main, Germany) for 10 min, and PI (Sigma-Aldrich, Taufkirchen, Germany) was added immediately prior to flow cytometric analysis. Cells negative for both annexin V-FITC and PI were considered viable cells.

Migration assays

For the wound scratch experiments, cells were grown to a confluent monolayer in 12-well plates and serum starved overnight. The experiment was started by scraping a wound with a sterile pipette tip into the cell layer. Migration was stimulated by adding 30% FCS to the medium and cells were treated with indicated concentrations of salinomycin. Cells were fixed and stained with crystal violet after 21 h. Differences in wound width were documented after 0 h, 15 h and 21 h with a Leica DM IL microscope (Leica, Bensheim, Germany) and measured with AxioVision Software (Zeiss, Göttingen, Germany).

Boyden chamber assays were performed using commercial cell culture inserts (transparent PET membrane, 8.0 μm pore size, BD). Cells were seeded in the upper chamber in serum-free medium, and the bottom chamber contained medium with 30% FCS. Cells were treated with 5–10 μM salinomycin, 5 μM calpeptin or Y-27632, respectively. After 24 h, migrated cells were fixed and stained with crystal violet and cells were counted in 3 sequential visual fields under 100-fold magnification.

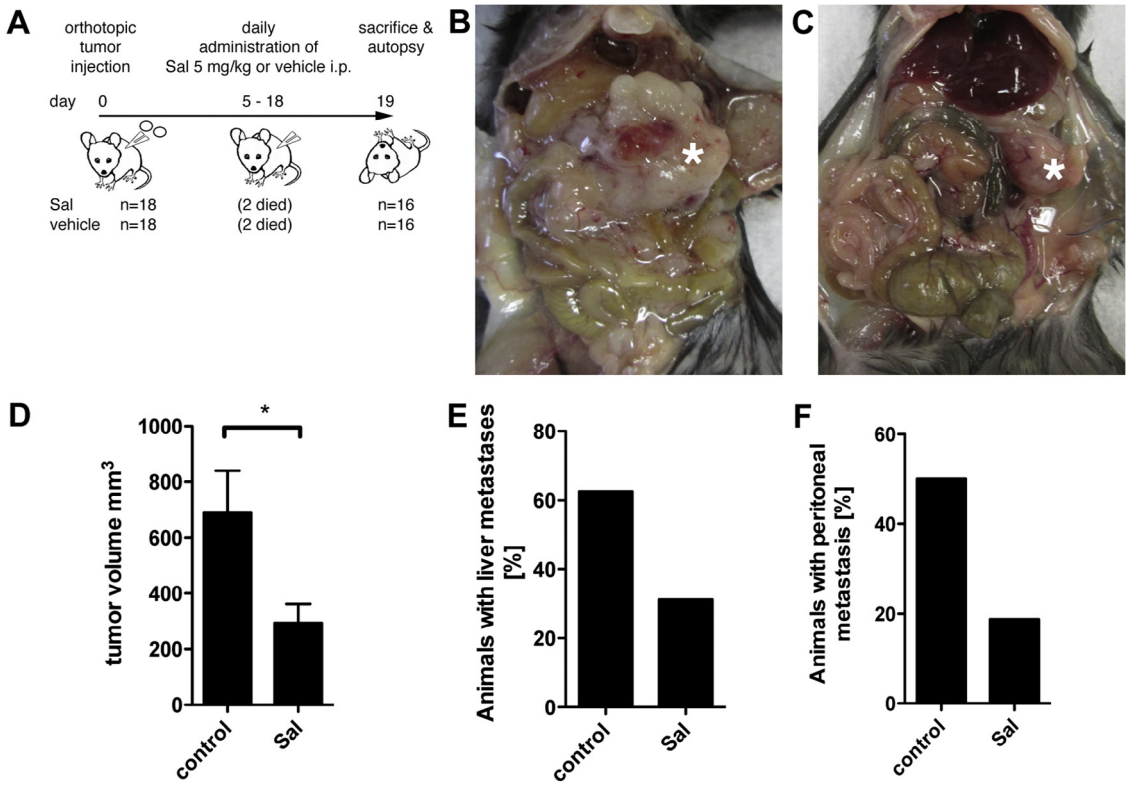

Fig. 1. Salinomycin treatment in a syngeneic orthotopic pancreatic cancer mouse model. (A) Schematic design of the mouse experiments. 10^5 tumor cells (Panc02) were injected orthotopically in the pancreatic head or body of syngeneic C57BL/6 mice. Treatment with salinomycin (Sal, 5 mg/kg) or control was started on day 5. Animals were sacrificed on day 19. (B) Postmortem situs of a control-treated mouse with a large pancreatic adenocarcinoma (asterix), and (C) with a smaller pancreatic tumor (asterix) after Sal treatment. (D) Tumor volume after treatment on day 19 in the control and Sal treatment group (each n = 16 animals). (E) Animals with macroscopic liver metastasis, and (F) with macroscopic metastatic spread on the parietal peritoneum (E, F: n = 16 animals in each treatment group, respectively). Bars represent means ± s.e.m. *$P < 0.05$ as determined by unpaired t-test.

Please cite this article in press as: Miriam Schenk, et al., Salinomycin inhibits growth of pancreatic cancer and cancer cell migration by disruption of actin stress fiber integrity, Cancer Letters (2015), doi: 10.1016/j.canlet.2014.12.037