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Autophagy inhibition enhances antiproliferative effect of salinomycin in pancreatic cancer cells

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ABSTRACT

Background: Salinomycin has cytotoxic effects on various types of malignancy and induces autophagy. However, it has not been clarified whether autophagy induced by salinomycin treatment has a protective or cytotoxic role. We investigated whether salinomycin affects autophagy in pancreatic cancer cells and whether autophagy induced by salinomycin treatment has a protective or cytotoxic role in these cells.
Methods: We investigated the effect of salinomycin using three pancreatic cancer cell lines. We investigated effect on proliferation and the CD133 positive fraction using flow cytometry. In addition, we monitored the change in autophagic activity after salinomycin treatment using fluorescent immunostaining, western blotting, and flow cytometry. Finally, knockdown of ATG5 or ATG7 by siRNA was used to investigate the impact of autophagy inhibition on sensitivity to salinomycin.
Results: Salinomycin suppressed the proliferation of pancreatic cancer cells in a concentration dependent manner, and reduced the CD133 positive fraction. Salinomycin enhanced autophagy activity in these cells in a concentration dependent manner. Autophagy inhibition made pancreatic cancer cells more sensitive to salinomycin.
Conclusions: Our data provide the first evidence indicating that autophagy induced by salinomycin have a protective role in pancreatic cancer cells. A new therapeutic strategy of combining salinomycin, autophagy inhibitors, and anticancer drugs could hold promise for pancreatic cancer treatment.

Introduction

Pancreatic cancer is one of the most aggressive malignancies, and the fourth leading cause of cancer-related deaths in western countries [1]. Conventional therapy for pancreatic cancer is ineffective and the 5-year survival rate of patients with pancreatic cancer is approximately 6% [1]. Although new therapeutic strategies using drug combinations have shown some benefit [2,3], the improvement in overall response rate remains unsatisfactory. Cancer stem cells (CSCs), which are defined as tumor-initiating cells with self-renewal capacity [4], are considered primarily responsible for therapy resistance and recurrence [5,6]. Therefore, CSCs are a promising therapeutic target for cancer treatment.

Salinomycin is an oral ionophore which is used as an antibiotic in poultry [7]. Recently, it was shown that salinomycin has cytotoxic effects on CSCs from various cancers [8–17]. The combination of salinomycin and conventional therapy was reported to be effective in vitro and the clinical application of this inexpensive oral drug in combination is expected [12]. However, there are only a few reports detailing salinomycin treatment of pancreatic cancer cells [12,18,19].

Autophagy is a process necessary to maintain homeostasis, especially during metabolic stress [20–22]. It is involved in various human diseases [20,22], and the role of autophagy in cancer has been reported to be both protumorigenic and tumor suppressive,

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depending on the cancer type and stage [22,23]. In addition, although autophagy is mainly considered to promote cell survival in established cancerous cells, excess or prolonged autophagy can lead to autophagic cell death. In pancreatic cancer, autophagy is considered to contribute to the aggressiveness of pancreatic cancer and autophagy activation is associated with poor outcomes [24]. Therefore, inhibition of autophagy suppresses the growth of pancreatic cancer and enhances the effect of anticancer agents [23–25].

There are numerous reports showing that salinomycin treatment induces autophagy and in all cases this plays a protective role in cancer cells [15–17,26–30]. In these reports, autophagy inhibition using drugs [15,27–30], siRNA [15,29,30], shRNA [26,31] or cells derived from knockdown mice [28] enhanced the toxic effect of salinomycin. However, whether salinomycin affects autophagy in pancreatic cancer cells is not fully elucidated. So we investigated the hypothesis whether autophagy induced by salinomycin treatment has a protective or cytotoxic role in pancreatic cancer cells. Here, we showed that salinomycin induces autophagy in pancreatic cancer cells. We also show autophagy inhibition enhances the antiproliferative effect of salinomycin, suggesting that autophagy plays a protective role against salinomycin.

Materials and methods

Cell culture conditions and treatment

Pancreatic cancer cell lines, Panc1 (RIKEN BioResource Center, Ibaraki, Japan), SUIT-2 (Japan Health Science Research Resources Bank (JCRB), Osaka, Japan), and Capan-2 (American Type Culture Collection (ATCC), Manassas, VA, USA) were purchased and maintained as previously described [32]. Salinomycin was purchased from Sigma-Aldrich (#S4526, St. Louis, MO, USA). The powder was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 10 mM, and stored at −20 °C until use.

Cell proliferation assay

Cell proliferation was evaluated by CellTiter-Glo luminescent cell viability assay or by cytometry. For the CellTiter-Glo assay, pancreatic cancer cells were seeded in 96-well polystyrene cell culture microplates (#655083, Greiner Bio-One International, Kremsmunster, Austria) at a density of 1×10^3 cells/well. After incubation for 24 h, the 100 μl of medium was replaced with fresh DMEM containing 10% fetal bovine serum (FBS) with the indicated concentration of salinomycin or DMSO. The emission value was measured as a control according to the manufacturer's instructions by a microplate reader (Infinite F200, Tecan, Mannedorf, Switzerland). The emission value was measured after culture for 24, 48, 72, and 96 h. Cell proliferation was evaluated as a ratio to the control.

For cytometry, pancreatic cancer cells were seeded in 96-well cell culture plates (#0030730119, Eppendorf, Hamburg, Germany) at a density of 1×10^3 cells/well. After incubation for 24 h, the 100 μl of medium was replaced with fresh DMEM containing 10% FBS with indicated concentrations of salinomycin or DMSO. Cell proliferation was evaluated after culture for 96 h, by counting the number of cells using CDA-100B (Sysmex Corporation, Kobe, Japan). Each experiment was carried out in triplicate wells and repeated three times.

siRNA silencing of Atg5 or Atg7

Pancreatic cancer cells at 90% confluence were transfected with siAtg5-1 (#SI00069251, all from QIAGEN, Hilden, Germany), siAtg5-2 (#SI02655310), siAtg7-1 (#02655373) or siAtg7-2 (#SI04344830) siRNA by electroporation using a Nucleofector System (Lonza, Basel, Switzerland) according to the manufacturer's recommendations. To verify knockdown specificity, we used a control siRNA (#1027310). Transfected cells were used in subsequent experiments 24–72 h after transfection.

Flow cytometry

To assay CD133, 48 h after incubating at the indicated concentration of salinomycin or DMSO, cultured cells were harvested by exposure to trypsin for 5 min at 37 °C. Harvested cells were washed in 10% FBS/DMEM, and suspended in ice-cold 2% FBS/phosphate buffered saline (PBS) solution with or without anti-CD133-PE antibody (# 130-090-853, Miltenyi Biotec, Bergisch Gladbach, Germany). After incubating for 10 min in the dark, cells were washed by 2% FBS/PBS, suspended in 2% FBS/PBS, and analyzed using a flow cytometer (EC800, Sony, Tokyo, Japan). Eclipse analysis software (EC800, version1.3.1, Sony) was used to quantify the fluorescent signals and set the logical electronic gating parameters. To analyze autophagy, the CYTO-ID autophagy detection kit (#51031-K200, Enzo Life Sciences, Farmingdale, NY, USA) was used and measurements taken using a flow cytometer (EC800, Sony) according to the manufacturer's instructions.

Western blotting

After incubation for 48 h with the indicated concentrations of salinomycin or DMSO, proteins were extracted using PRO-PREP Protein Extraction Solution (#17081, iNtRON biotechnology, Kyungki, Korea) according to the manufacturer's instructions. The concentration of the proteins was measured using NanoDrop 1000 Spectrophotometer (version3.8.1, Thermo Fisher Scientific, Wilmington, DE, USA). For SDS-PAGE, 20 μg of total protein per well was separated by gel electrophoresis on 4–15% Mini-PROTEAN TGX Precast Gels (#456–1084, Bio-Rad laboratories, Hercules, CA, USA) and transferred to *Trans*-Blot Turbo Mini PVDF Transfer Packs (#170–4156, Bio-Rad Laboratories) using a *Trans*-Blot Turbo Transfer Starter System (Bio-Rad Laboratories). The membrane was incubated at 4 °C overnight with anti-Atg5 (#2630S, Cell Signaling Technology, Danvers, MA, USA; 1:1000), anti-Atg7 (#2631S, Cell Signaling Technology; 1:1000), anti-LC3B (#2775S, Cell Signaling Technology; 1:1000), anti-p62 (#BML-PW9860, ENZO life sciences; 1:1000) or anti-β-actin (#8227, Abcam, Cambridge, UK; 1:5000) antibodies. Membranes were then probed with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoblot detection was performed using chemiluminescence with a ChemiDoc XRS System (Bio-Rad Laboratories). Each experiment was repeated three times.

Immunofluorescence staining

Cells were seeded in glass-bottomed dishes (#81156, Ibidi, Martinsried, Germany) at a density of 2×10^4 cells/well and incubated for 48 h at indicated concentrations of salinomycin or DMSO. Then cells were fixed with −20 °C ethanol for 10 min, blocked with 3% BSA in PBS for 30 min at room temperature and incubated with anti- LC3B (#2775S, Cell Signaling Technology; 1:400) antibody for 2 h at room temperature. Subsequently, cells were incubated with 488-conjugated anti-rabbit IgG (#A-11034, Thermo Fisher Scientific) for 1 h. Nuclear DNA was counterstained with 4′,6-diamidino-2-phenylindole (1 μg/mL, #D523, Dojindo, Kumamoto, Japan). After washing with PBS, images were acquired using a laser-scanning

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