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Salinomycin induces apoptosis in cisplatin-resistant colorectal cancer cells by accumulation of reactive oxygen species☆

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HIGHLIGHTS

- Cisp-resistant SW620 cells maintained as a relative quiescent state (G0/G1 arrest).
- Cisp-resistant SW620 cells displayed more stem-like signatures.
- Cisp-resistant SW620 cells were sensitive to salinomycin.
- Salinomycin induced more apoptosis in Cisp-resistant SW620 cells.

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ABSTRACT

Postoperative chemotherapy for Colorectal cancer (CRC) patients is not all effective and the main reason might lie in cancer stem cells (CSCs). Emerging studies showed that CSCs overexpress some drug-resistance related proteins, which efficiently transport the chemotherapeutics out of cancer cells. Salinomycin, which considered as a novel and an effective anticancer drug, is found to have the ability to kill both CSCs and therapy-resistant cancer cells. To explore the potential mechanisms that salinomycin could specifically target on therapy-resistant cancer cells in colorectal cancers, we firstly obtained cisplatin-resistant (Cisp-resistant) SW620 cells by repeated exposure to 5 μmol/l of cisplatin from an original colorectal cancer cell line. These Cisp-resistant SW620 cells, which maintained a relative quiescent state (G0/G1 arrest) and displayed stem-like signatures (up-regulations of Sox2, Oct4, Nanog, Klf4, Hes1, CD24, CD26, CD44, CD133, CD166, Lgr5, ALDH1A1 and ALDH1A3 mRNA expressions) ($p < 0.05$), were sensitive to salinomycin ($p < 0.05$). Salinomycin did not show the influence on the cell cycle of Cisp-resistant SW620 cells ($p > 0.05$), but could induce cell death process ($p < 0.05$), with increased levels of LDH release and MDA contents as well as down-regulations of SOD and GSH-PX activities ($p < 0.05$). Our data also showed that the pro-apoptotic genes (Caspase-3, Caspase-8, Caspase-9 and Bax) were up-regulated and the anti-apoptotic gene Bcl-2 were down-regulated in Cisp-resistant SW620 cells ($p < 0.05$). Accumulated reactive oxygen species and dysregulation of some apoptosis-related genes might ultimately lead to apoptosis in Cisp-resistant SW620 cells. These findings will provide new clues for novel and selective chemotherapy on cisplatin-resistant colorectal cancer cells.

1. Introduction

Colorectal cancer (CRC) is the third most common malignancy worldwide with increasing incidence over the past years (Sung et al., 2005). Surgical resection remains the most effective treatment for CRC. However, after the radical surgery, the overall five-year survival rate still remains poor. Most of the CRC patients die from recurrence and metastasis (Stillwell et al., 2011). Furthermore, postoperative chemotherapy for CRC patients is not all effective. A small proportion of patients are naturally tolerant to

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some traditional chemotherapeutics, and the main reason might lie in cancer stem cells (CSCs) (Morrison et al., 2011).

CSCs are a small proportion of cancer cells that exist in the cancer cell population, that have the ability to self-renew and undergo differentiation. And CSCs are found to be resistant to conventional cancer treatments, including chemotherapeutic drugs and radiation therapy (Dalerba et al., 2007; Santin, 2009; Tan et al., 2006). CSCs highly express ATP-binding drug transporters. Standard chemotherapy or radiation therapy is effective in killing the bulk of the tumor but not the CSCs. The mortality of cancer remains high, because conventional therapies often fail to eradicate the CSC population, allowing relapse to occur (Lu et al., 2011). Alternatively, tumor drug-resistant cells induced by traditional chemotherapeutics also present cancer stem-like characteristics (Gopalan et al., 2013). So novel therapeutic strategies targeting specifically on CSCs are urgently needed.

In 2009, Gupta et al. identified salinomycin by a high throughput screening that could potentially be used to target breast CSCs, and it killed breast CSCs at least 100 times more effectively than paclitaxel in mice (Gupta et al., 2009). Emerging evidences showed that salinomycin could effectively kill various types of cancer stem-like cells, including gastric cancer (Zhi et al., 2011), pancreatic cancer (He et al., 2013; Zhang et al., 2011), hepatocellular carcinoma (Wang et al., 2012), ovarian cancer (Zhang et al., 2012), prostate cancer(Ketola et al., 2012; Kim et al., 2011) and some other types of malignancy (Kuo et al., 2012; Scherzed et al., 2013). However, the mechanism underlying salinomycin and drug-resistant cells in CRC has not been studied yet.

In our study, we continously treated the coloretal cancer cells by 5 μmol/l of cisplatin and successfully obtained the Cisp-resistant SW620 cells. Then we detected some specific stem cell markers to confirm whether Cisplatin-resistant SW620 cells displayed a stem-like signature. In order to discuss the killing effect of salinomycin on Cisplatin-resistant SW620 cells, we treated Cisplatin-resistant SW620 cells and orginal SW620 cells with a certain concentration of salinomycin, and observed the change of cell cycle and apoptosis. Meanwhile, we also evaluated the levels of oxidative stress markers (LDH release, MDA contents, SOD and GSH-PX activities) and apoptosis-related genes (Caspase-3, Caspase-8, Caspase-9, Bcl-2 and Bax) to explore the possible mechanism of salinomycin on Cisp-resistant SW620 cells.

2. Materials and methods

2.1. Chemicals and cell culture

Cisplatin and salinomycin were obtained from Sigma-Aldrich (Sigma, USA). Human colorectal cancer cell line SW620 was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (Gibco, Invitrogen) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells were in the logarithmic phase of growth for all experiments.

2.2. 50% inhibiting concentration (IC50) and cell proliferation analysis by cell counting kit-8

For cisplatin or salinomycin IC50 analysis in SW620 cells or Cisp-resistant SW620 cells, cells (1×10^4/well) were cultured in 96-well plates and treated with different chemotherapeutics (cisplatin, salinomycin) in different concentrations for 48 h. Then 20 μl of cell counting kit-8 (CCK-8, Dojindo, Kumamoto, Japan) was added into each of the 96-wells. After 4 h incubation at 37 °C, the optical density (OD) values were detected at 450 nm using the scan reader (Labsystems, Santa Fe, NM, USA). Cell growth inhibiting rates were described as cell inhibiting curves and the IC50 parameters (inhibiting concentration of 50% cells) were evaluated by Xlfit 5.2 software (IDBS, UK). For cell proliferation analysis, SW620 cells or Cisp-resistant SW620 cells (5×10^3/well) were also seeded in 96-well plates in serum-containing medium and treated with cisplatin (5 μmol/l, according to the calculated IC50 values of cisplatin in SW620 cells) for 0, 12, 24, 48, 72 and 96 h. Then 20 μl cell counting kit-8 was added into each of the 96-wells. After 4-h incubation at 37 °C, the coloring reactions were also quantified at 450 nm.

2.3. Continuous treatment with cisplatin

SW620 cells were grown in 10-cm culture dishes and treated with proper concentration of cisplatin (5 μmol/l) according to the results of IC50. We replaced the culture medium every 7 day. After continuous exposure to non-lethal levels of cisplatin for 3 months, the Cisp-resistant SW620 cells were obtained (Fig. 1).

2.4. Real-time PCR detection

Total RNA was extracted using Trizol solution (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. Then reverse transcription (RT) was performed in a 20-μl reaction system using the ReverAid First Stand cDNA Synthesis (Thermo Scientific, Mountain View, CA, USA). Primer sequences for GAPDH were 5′-GGACCTGACCTGCCGTCTAG-3′ (Forward) and 5′-GTAGCCCAGGATGCCCTTGA-3′ (Reverse). Other primer sequences were all listed in Table 1. RT-PCR with SybGreen I (Generay Bio Co., Shanghai, China) was performed using the 7500 real-time PCR system (Applied Biosystems, Hayward, CA, USA) with the follow program: initial denature at 95 °C for 5 min, followed by 40 cycles for 95 °C for 15 s, annealing at 60 °C for 60 s, and 72 °C for 30 s. All samples were processed in triplicate. The mRNA expression of each gene was calculated using the relative quantitative $2^{-\Delta\Delta CT}$ method.

2.5. Cell cycle analysis

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l) or not, SW620 cells and Cisp-resistant SW620 cells were collected, washed twice in 1x PBS, mixed in 200 μl of 1x binding buffer, and incubated at room temperature for 15 min with 250 μg/ml propidium iodide (PI) (Sigma, St. Louis, USA) and 5 μg/ml Rnase (BD Biosciences, USA). The cell cycle was analyzed by FACSscan flow cytometer. Each treatment was performed in triplicate.

2.6. Hoechst33342 staining

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l) or not, SW620 cells and Cisp-resistant SW620 cells (2×10^5 ml^{-1}) were harvested and added 10 μl of Hoechst33342 solution (Keygen Biotech, Nanjing, China) for 15 min in the dark at 37 °C. After centrifuging for 5 min and washing with 1x PBS, 1 ml of Buffer A solution (Keygen Biotech, Nanjing, China) was added at room temperature and mixed. Finally, 20 μl of cell pellet was dropped on a glass slide and the image was observed under an inverted phase-contrast fluorescence microscope (Olympus, Japan).

2.7. Annexin-V-PE staining

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l) or not, SW620 cells and Cisp-resistant SW620 cells were collected. Cells were double stained with Annexin-V-PE (BD Pharmingen, San Jose, CA, USA) and propidium iodide (PI) (Sigma, St. Louis, USA) following the manufacturer's instructions. Cell apoptosis was determined by FACSscan flow cytometer. Each treatment was performed in triplicate.

2.8. LDH release assay

LDH activity was evaluated using a colorimetric LDH assay. Cell were seeded at 1×10^4cells/200 μl in each 96-well plate and pre-treated with salinomycin (0.25 μmol/l) for 48 h. Briefly, 100 μl supernatant was transferred from each well to a 96-well plate and 100 μl freshly reaction mixture was added to each well. After 30 min of incubation at room temperature in the dark, the optical density (OD) values were detected at 490 nm using the scan reader (Labsystems, Santa Fe, NM, USA). The amount of LDH was calculated as a percent compared to the total amount of LDH present in cell treated with 1% Triton-X 100 (Solarbio, Shanghai, China).

2.9. Detections of oxidative stress markers (MDA, SOD and GSH-PX)

Cells were seeded at 5×10^4 per well in 6-well plates in triplicate. 48 h after salinomycin treatment (0.25 μmol/l), cells were harvested and the extracted proteins were quantified. We measured the MDA contents, SOD and GSH-PX activities with commercial reagent kits purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

2.10. Western blot analysis

Cells were harvested and lysed with mammalian protein extraction reagent (Pierce, Rockford, IL, USA). Total protein (30 μg) was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to Immobilon-P Transfer Membrane (Millipore, Ballerica, USA). Then the membranes were blocked for 2 h with 5% non-fat dry milk in PBS and incubated with primary antibodies (Caspase-3, Caspase-8 and Caspase-9, Bioworld Technology, USA; Bax and Bcl-2, Cell Signaling Technology, USA). Second antibodies were conjugated to