Cancer Epidemiology 37 (2013) 512–517

Contents lists available at SciVerse ScienceDirect

Cancer Epidemiology

The International Journal of Cancer Epidemiology, Detection, and Prevention

journal homepage: www.cancerepidemiology.net

Salinomycin inhibits Akt/NF-κB and induces apoptosis in cisplatin resistant ovarian cancer cells

Bidur Parajuli [a,1], Hyun-Gyo Lee [b,1], Sang-Hoon Kwon [a], Soon-Do Cha [a,c], So-Jin Shin [a], Gun-Ho Lee [d], Insoo Bae [e], Chi-Heum Cho [a,b,c,*]

[a] Department of Obstetrics and Gynecology, Keimyung University, School of Medicine, Daegu, Republic of Korea
[b] Institute for Cancer Research, Keimyung University, School of Medicine, Daegu, Republic of Korea
[c] Institute for Medical Science, Keimyung University, School of Medicine, Daegu, Republic of Korea
[d] Department of Obstetrics and Gynecology, Cha Gumi Medical Center, Cha University, Gyeongsangbukdo, Republic of Korea
[e] Department of Oncology, Lombardi Cancer Center, Georgetown University, USA

ARTICLE INFO

Article history:
Received 14 August 2012
Received in revised form 31 January 2013
Accepted 20 February 2013
Available online 29 March 2013

Keywords:
Salinomycin
Akt
Cisplatin resistance
Ovarian cancer
Apoptosis

ABSTRACT

Background: Despite advances in treatment, ovarian cancer is the most lethal gynecologic malignancy. Therefore significant efforts are being made to develop novel strategies for the treatment of ovarian cancer. Salinomycin has been shown to be highly effective in the elimination of cancer stem cells both in vitro and in vivo. The present study focused on investigating important cell signaling molecules such as Akt and NF-κB during salinomycin-induced apoptosis in cisplatin resistant ovarian cancer cells (A2780cis).

Methods: MTT assay was performed to determine cell viability. Flow cytometry and DNA fragmentation assay were performed to analyze the effect on cell cycle and apoptosis. The expression of apoptosis related proteins was evaluated by Western blot analysis.

Results: The cell viability was significantly reduced by salinomycin treatment in a dose dependent manner. The flow cytometry result showed an increase in sub-G1 phase. Salinomycin inhibited the nuclear transportation of NF-κB, and downregulated Akt expression. Declined Bcl-2, activation of caspase-3 and increased PARP cleavage triggered apoptosis. Moreover, DNA fragmentation assay also revealed apoptotic induction.

Conclusion: The result suggested that salinomycin-induced apoptosis in A2780cis was associated with inhibition of Akt/NF-κB. It may become a potential chemotherapeutic agent for the cisplatin resistant ovarian cancer therapy.

1. Introduction

Ovarian cancer is the most common cause of cancer death from gynecologic malignancies. Maximal surgical cytoreduction followed by platinum-based chemotherapy is the standard treatment for patients with ovarian cancer. However, there is a distinct problem with this therapy in that ovarian cancer cells are resistant to the cisplatin, leading to a survival rate of less than 30% in patients with advanced ovarian cancers [1]. Reduced accumulation of the drug, enhanced DNA repair and alterations in apoptosis are believed to be the major cause of the chemoresistance. No specific treatment has been reported to reduce the resistance in ovarian cancer. Therefore, there is a dire need to develop novel therapies preferably based on the understanding of the molecular mechanism of the chemo-resistant ovarian cancer.

Salinomycin was found most effective agent against breast cancer stem cells in high throughput screening of 16,000 natural and commercial chemical compounds. It was 100 times more effective than the paclitaxel [2]. Salinomycin was originally used as antimicrobial agent to kill bacteria, fungi and parasites and to increase feed efficacy of ruminant animals [3–5]. It is a monocarboxylic polyether antibiotic derived from *Streptomyces albus* and acts as an ionophore [6]. As an ionophore with strict selective alkali ions and a strong preference for potassium, it interferes with transmembrane potassium potential. Currently, it is reported that salinomycin can overcome drug resistance in human cancer cells [7]. A combination treatment of paclitaxel and salinomycin produced strong antitumor efficacy for the eradication of breast cancer and cancer stem cells [8]. Salinomycin can inhibit Wnt signaling in leukemia [9] and function as a Pgp inhibitor [10].

* Corresponding author at: Department of Obstetrics and Gynecology, Keimyung University, School of Medicine, 194 Dongsan-dong, Jung-gu, Daegu 700-712, Republic of Korea. Tel.: +82 53 250 7518; fax: +82 53 250 7599.
E-mail address: c0035@dsmc.or.kr (C.-H. Cho).

[1] These authors contributed equally to this study.

1877-7821/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.canep.2013.02.008

Akt, also known as protein kinase B is a serine/threonine-specific protein kinase that is important in multiple processes such as glucose metabolism, apoptosis, cell proliferation, transcription and cell migration [11,12]. Akt regulates cellular survival and metabolism by binding and regulating many downstream effectors, such as NF-κB and Bcl-2 family proteins. Akt can activate NF-κB via regulating I-κB kinase (IKK), resulting in transcription of pro-survival genes. The activation of Akt is one of the most frequent alterations observed in human cancer and tumor cells. Therefore, understanding the Akt/NF-κB pathway is important for the creation of better therapies to treat cancer cells.

The present study examined the growth inhibition effect of salinomycin on cisplatin resistant ovarian cancer cells. We also investigated its influence on cell cycle distribution and apoptosis regulatory genes. Our result provided the first evidence that salinomycin inhibited Akt/NF-kB and induced apoptosis. This offers a new promising therapeutic approach in overcoming cisplatin resistant ovarian cancer.

2. Materials and methods

2.1. Reagents and cell line

Dulbecco's modified eagle medium was obtained from GIBCO BRL (Grand Island, USA). Salinomycin was purchased from Sigma–Aldrich (St. Louis, USA) and dissolved in dimethylsulfoxide (DMSO). The ovarian cancer cells (A2780) and cisplatin resistant ovarian cancer cells (A2780cis) were obtained from European collection of cell cultures (ECACC, UK). The cells were cultured and incubated at 37 °C in 5% CO_2.

2.2. Cell viability assay

The number of viable cells exposed to salinomycin were evaluated by a colorimetric 3-(4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (MTT) assay. Initially, cells were seeded in 96-well plate, and then cultured for 24 h to allow their adhesion to the plate. After pre-incubation, the culture medium was changed to experimental medium supplemented with salinomycin (0.1, 0.5, 1, 5 and 10 μM) or DMSO (0.1%) for 48 h. The intensity of the purple color formed by this assay is proportional to the number of viable cells. MTT reagent was added and incubated for an additional 4 h at 37 °C. The optical density was measured at 495 nm. The mean value and their standard deviation were calculated from triplicate experiments.

2.3. Determination of cell cycle distribution

To determine the distribution of cells in the different phases of cell cycle profile FACS analysis was performed. After 48 h treatment of salinomycin, cells were harvested by trypsinization and centrifugation, washed with cold phosphate buffer saline (PBS), and were fixed in ice-cold 70% ethanol at 4 °C for 24 h. Ethanol fixed cells were washed and treated with RNase A for 30 min at 37 °C and were stained with propidium iodide and incubated for 30 min at room temperature. DNA fluorescence was measured by flow cytometer using a FACS Calibur cell sorter (Becton-Dickinson, USA). The percentage of cells in each cell cycle phase was determined using the ModFit LT™ software (Becton-Dickinson) based on the DNA histogram.

2.4. Protein isolation and immunoblotting

Cell (2×10^5 ml^{-1}) extracts were prepared in lysis buffer [10 mM Tris (7.4) 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, serine protease inhibitor phenylmethylsulphonyl fluoride (10 μg/mL), leupeptin (10 μg/mL), aprotinin (10 μg/mL), 5 mM phenanthroline, and 28 mM benzamidine–HCl]. Protein concentrations were measured using Bio-Rad Protein Assay Reagent (Bio-Rad, USA) following the manufacture's protocol. Aliquots of protein were separated by 8–15% SDS-PAGE and transferred to polyvinylidine difluoride membrane (Millipore, USA). The membrane was blocked with Tris buffered saline containing 5% skim milk. After washing, the membranes were incubated with primary antibodies of Bcl-2 and β-actin (Santa Cruz Biotech, USA), Bax (BD Pharmingen, USA), I-κBα, NF-κB, Akt, pAkt, cleaved caspase-3 and PARP (Cell Signaling, USA). After reaction with horseradish peroxidase conjugated secondary antibodies (Santa Cruz Biotech) bands on the membranes were visualized by an enhanced chemiluminescence system (Thermo Scientific, USA). The density of respective bands was analyzed by the Chemi-Doc XRS imaging system (Bio-Rad, USA). The data were presented as percentage of controls.

2.5. siRNA transfection

Transfection of siRNA was performed using ON-TARGETplus (Dharmacon, USA) kit, according to manufacturer's instruction. SMARTpool sequenced siRNA targeting Akt or non specific control pool (siRNA negative control) was diluted to a working stock solution of 5 μM in RNase free water. Transient transfection was done using the DharmaFECT transfection reagents. Control experiments consisted for transfection with the non-targeting siRNA scrambled control or non-transfected cells. After 24 h of post transfection, cells were treated with salinomycin for 48 h and harvested for protein analysis.

2.6. Extraction of cytoplasmic and nuclear extract

Cells treated with salinomycin were fractionated into cytoplasmic and nuclear extracts using NE-PER nuclear extraction reagents (Thermo Scientific) according to the manufacturer's protocol.

2.7. Caspase-3 activity assay

After drug treatment, cells were harvested with lysis buffer. 2× Reaction buffer and DTT Mix was added in supernatants and incubated on ice for 30 min. Caspase-3 substrate (DEVD-pNA; 50 μM final concentrations) was added and incubated at 37 °C for one hour in water bath. Caspase-3 activity was measured as the absorbance at 405 nm of the cleaved substrate pNA followed by ApoAlert Caspase Colorimetric Assay kits User Manual (Clontech Laboratories, USA).

2.8. DNA fragmentation assay

Fragmented nucleosomal DNA was quantified by Cell Death Detection ELISA Plus kit (Roche, Germany) as described in the manufacture's manual. Briefly, 2×10^5 cells/ml were plated in 6 well plates in the presence of 10% FBS with antibiotics (streptomycin and penicillin). The cells were exposed to salinomycin or DMSO for 48 h. Lysates were transferred to 1.5 ml Eppendorf tubes and centrifuged at $200 \times g$ for 10 min to obtain low molecular weight DNA from apoptotic cells. Supernatant (20 μl) was used to detect apoptosis with microplate reader (Tecan, USA) at 405 nm. Background values were subtracted and OD values representing nucleosomal DNA fragments in salinomycin treated samples were compared with those values obtained from control cells and expressed as percentage (%) of control.