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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

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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.

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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.

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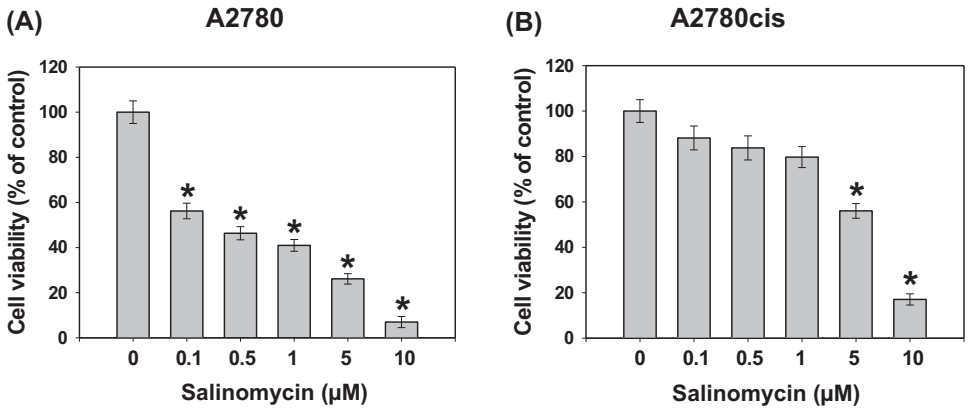

Fig. 1. Antiproliferative effect of salinomycin on cisplatin sensitive and resistant ovarian cancer cells (A2780 and A2780cis). Cells were treated with DMSO (control) or salinomycin (0.5, 1, 5 and 10 μM) for 48 h. Cell viability was measured by MTT assay and results were expressed in percentage of viable cells. Values are ±SD of three measurements $^{*}p < 0.05$.

2.9. Statistical analysis

The data are presented as mean ± SD. Statistical analysis was conducted using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test for a post hoc comparison by SPSS 17.0 (SPSS, Inc., an IBM Company, Chicago, Illinois, USA). Null hypotheses of no difference were rejected if *p*-values were less than .05.

3. Results

3.1. Growth inhibitory effect of salinomycin on A2780cis cells

This study evaluated the effect of salinomycin on the growth of cisplatin sensitive and resistant ovarian cancer cells. Salinomycin reduced the viability of A2780 and A2780cis cells in a dose dependent manner. The A2780cis cell growth was found decreased by about 55% in 5 μM in contrast with the untreated control (Fig. 1).

3.2. Effect of salinomycin on cell cycle

To assess whether salinomycin-induced inhibition is mediated via alternations in cell cycle, we examined the effect of salinomycin on cell cycle distribution. Propidium iodide staining was done so that the DNA content of untreated and treated cisplatin resistant ovarian cancer cells could be measured by flow cytometric analysis. DNA histograms showed that salinomycin augmented the percentage of cells in sub-G1 phase compared to untreated cells (Fig. 2).

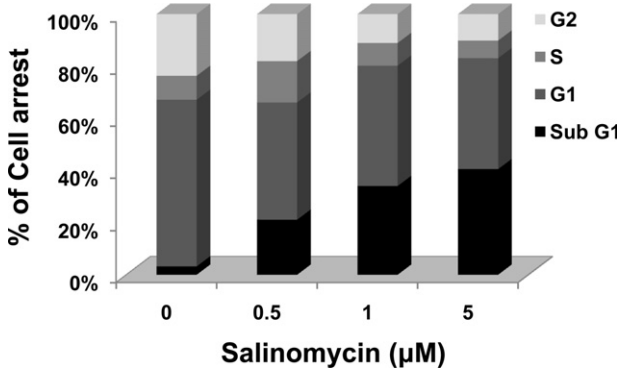

Fig. 2. Effect of salinomycin in cell cycle. A2780cis cells were treated with DMSO (control) or salinomycin (0.5, 1 and 5 μM) for 48 h. After treatment of salinomycin cells were harvested, fixed, stained with PI and analyzed by flow cytometric analysis. The values represent the number of cells in different phases of cell cycle progression as a percentage of total cells.

3.3. Role of salinomycin on apoptotic signaling pathway

The induction of apoptotic cell death could be in part due to inactivation of important survival genes and therefore the expressions of the proteins were evaluated. The expression of Akt, NF-κB proteins were decreased upon salinomycin treatment in A2780 and A2780cis. In addition, we observed the increased expression of I-κBα protein (Fig. 3A, B). Nuclear extraction of NF-κB sub unit p65 showed a time and dose dependent decrease (Fig. 4A, B). To obtain the evidence for the involvement Akt in mediating Akt/NF-κB pathway in salinomycin induced apoptosis, we used siRNA that specifically silence Akt. The NF-κB was attenuated by siRNA Akt which suggests the Akt may play a role in salinomycin induced apoptotic signaling (Fig. 4C).

There was no change in the expression of Bax but the Bcl-2 protein decreased. The caspase-3 and PARP protein cleavage increased after treatment. These results suggest that salinomycin induced cell growth inhibition and induction of apoptotic cell death results in part from the inhibition of survival factors, and inactivation of NF-κB via Akt/NF-κB pathway.

3.4. Induction of apoptotic cell death by salinomycin

The apoptosis assay was performed using A2780cis cells to evaluate the mechanism of the inhibition of cell growth using caspase-3 activity test and cell death detection kit. Activity of caspase-3, an executioner caspase of apoptosis, was increased following salinomycin treatment (Fig. 5A). ELISA showed significant increase of histone associated DNA fragments after 48 h exposure to salinomycin (Fig. 5B).

4. Discussion

Ovarian cancer patients have no specific symptoms in early stages. By the time most ovarian cancers are diagnosed, they are already at advanced stage. The women diagnosed at an early stage have longer survival, but the advanced stage disease has a poor prognosis due to chemoresistance developed during the platinum-based therapy. Despite the large number of individuals affected by this disease, the molecular mechanisms involved in the chemoresistance of this gynecologic cancer remain unknown. In this study, we investigated the potential effect of salinomycin to sensitize the

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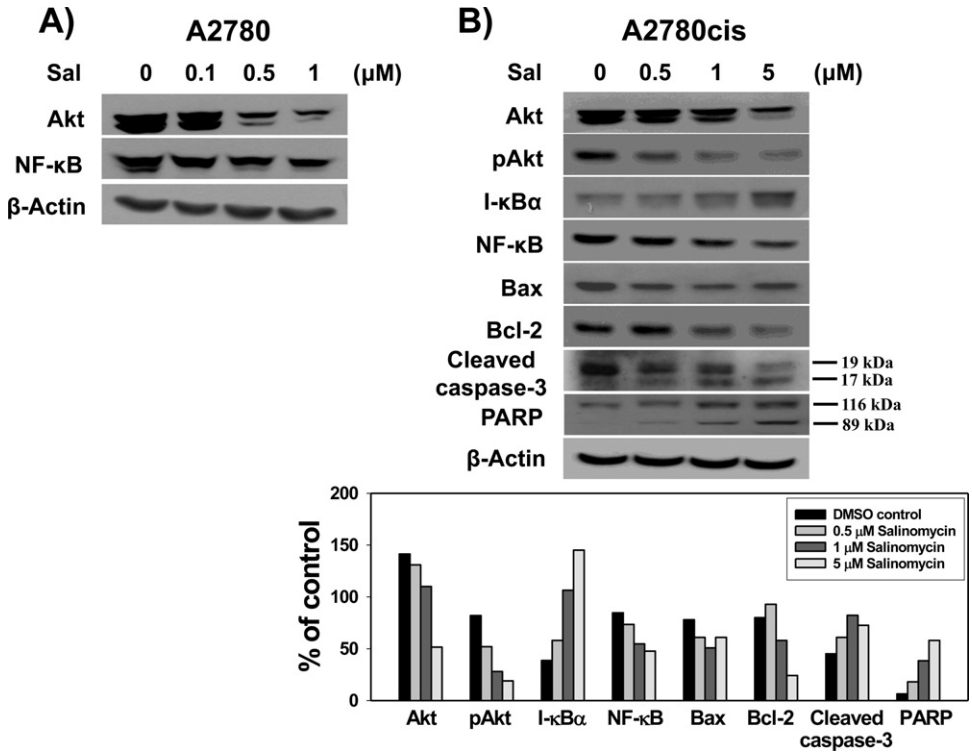

Fig. 3. Effect of salinomycin in apoptotic signal transduction pathway. Cells were treated with DMSO (control) or salinomycin (0.5, 1 and 5 μM) for 48 h. After treatment cell extracts were prepared and subjected for Western blot analysis. (a) Expression of Akt and NF-κB were measured in A2780 cells. (b) Expression of Akt, pAkt, I-κBα, NF-κB, Bax, Bcl-2, cleaved caspase-3 and PARP were measured. Beta-Actin was used as an internal loading control.

cisplatin-resistant ovarian cancer cells. We have showed that salinomycin can overcome cisplatin resistance through down-regulation of Akt/NF-κB signaling pathway.

Cell cycle control plays a critical role in the regulation of tumor cell proliferation. There is considerable evidence that the anticancer agent suppressed cell cycle progression in different phases and then induced apoptotic cell death. Salinomycin efficiently induced programmed cell death which we confirmed by the increased number of cells in the sub G1 phase of the cell cycle. Salinomycin significantly increased the amount of cells in

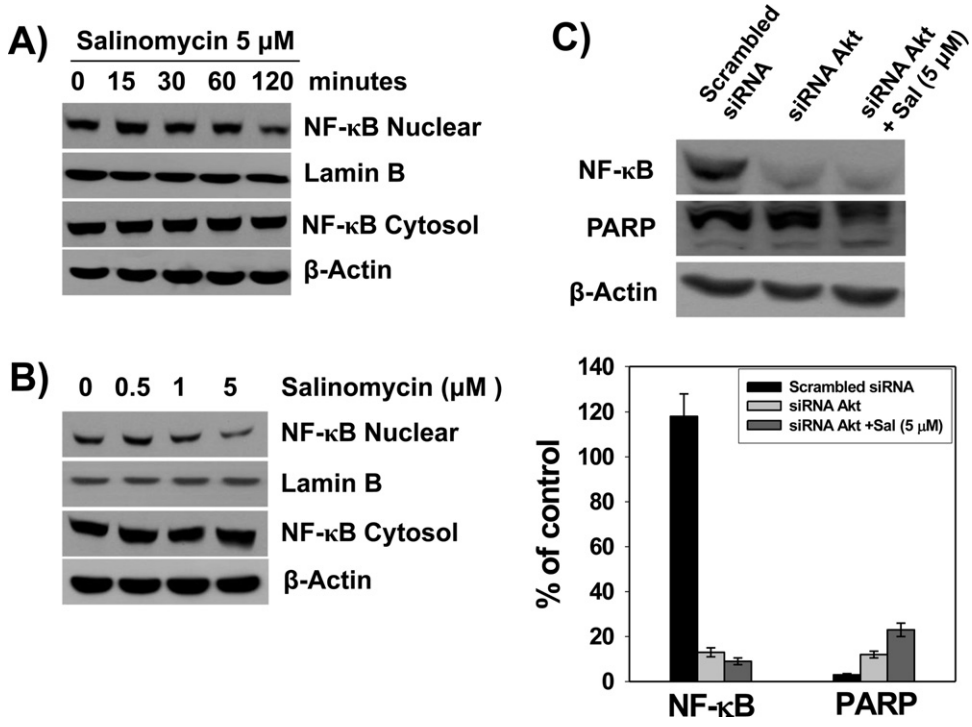

Fig. 4. Effect of salinomycin in NF-κB nuclear translocation. A2780cis cells were treated with salinomycin in the above mentioned time and doses. Inhibition of nuclear translocation (NF-κB) in (a) time and (b) dose dependent manner. (c) Effect of Akt siRNA on NF-κB and PARP expression in A2780cis cells. Cells were transfected with Akt siRNA and control siRNA. After 24 h transfection, cells were treated with salinomycin for 48 h and harvested for protein analysis.

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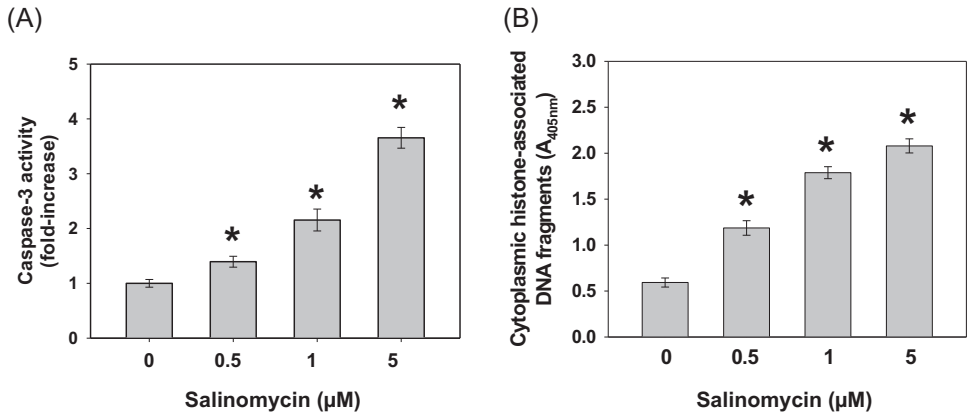

Fig. 5. Induction of apoptosis after salinomycin treatment in A2780cis cells. As describe in methodology section cells were exposed to DMSO (control) or salinomycin (0.5, 1 and 5 μM) for 48 h. Apoptosis was quantified by (a) caspase-3 activity and (b) ELISA. Values are ±SD of three measurements $^{*}p < 0.05$.

the sub G1 phase upon treatment while in S phase remained almost similar and that in G2/M phase decreased with increasing concentration of salinomycin treatment. This result showed that salinomycin causes apoptosis without causing cell cycle arrest which confirms the previous findings that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest [13].

In this study, we observed that salinomycin blocked the expression of Akt and consequently inhibited the nuclear translocation of NF-κB. The I-κB protein liberates NF-κB, allowing its translocation to the nucleus, blocking cell death pathway. In parallel, silencing of Akt had a similar effect on cisplatin resistant ovarian cancer cells. Cell survival role of Akt has been demonstrated in multiple cell lineages [14,15]. Akt is also involved in the regulation of NF-κB and apoptosis [16]. Constitutive activation of NF-κB induced by Akt may play a major role in the chemoresistant cells [17,18]. It has also been reported that the inhibition of pAkt enhanced the growth inhibitory and apoptotic effects of chemotherapeutic agents in gastric cancer cells [19]. Similarly, diminished malignant potential was observed when NF-κB was blocked in ovarian cancer and leukemia cells [20,21]. A recent study reported that salinomycin activated Akt signaling and induced changes in gene expression indicative of an EMT in HNSCC stem cells [22]. The difference in expression of Akt cannot be answered in the present work but a possible explanation can be outlined based on systemic differences and mechanism of kinase activities and merits further investigations in various cancers.

Apoptosis is critical in carcinogenesis and cancer progression. Increased apoptosis induced by salinomycin is an important feature of cellular response, which manifests the growth inhibitory effect on cisplatin resistant ovarian cancer cells. In our study, the expression of antiapoptotic protein Bcl-2 was downregulated. Caspase-3, one of the effector caspases, was activated thereby to induce PARP cleavage, the classical markers in apoptosis cascade. Early apoptosis is invariantly marked by a breakdown in mitochondrial membrane potential, which proceeds DNA fragmentation under apoptotic stimuli. The cytoplasmic histone-associated DNA fragments of the apoptotic cells were detected in the present study. Salinomycin can induce apoptosis in human lymphoma and leukemia cells [7,23]. Moreover, cancer cell specific apoptotic effects of salinomycin were documented in neuronal and colorectal cancer cells [24,25]. These findings show that the cell growth inhibition by salinomycin was through an apoptosis dependent mechanism.

In conclusion, this study has demonstrated that salinomycin suppressed cell proliferation by inducing apoptosis coupled with augmented sub-G1 arrest and DNA fragmentation. The apoptosis induction was associated with the inhibition of Akt and NF-κB expression. These results suggest that salinomycin is a promising antitumor agent in the cisplatin resistant ovarian cancer therapy.

Acknowledgments

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