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Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization

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ABSTRACT

The anticancer activity of salinomycin has evoked excitement due to its recent identification as a selective inhibitor of breast cancer stem cells (CSCs) and its ability to reduce tumor growth and metastasis *in vivo*. In prostate cancer, similar to other cancer types, CSCs and/or progenitor cancer cells are believed to drive tumor recurrence and tumor growth. Thus salinomycin can potentially interfere with the end-stage progression of hormone-indifferent and chemotherapy-resistant prostate cancer. Androgen-responsive (LNCaP) and androgen-refractive (PC-3, DU-145) human prostate cancer cells showed dose- and time-dependent reduced viability upon salinomycin treatment; non-malignant RWPE-1 prostate cells were relatively less sensitive to drug-induced lethality. Salinomycin triggered apoptosis of PC-3 cells by elevating the intracellular ROS level, which was accompanied by decreased mitochondrial membrane potential, translocation of Bax protein to mitochondria, cytochrome *c* release to the cytoplasm, activation of the caspase-3 and cleavage of PARP-1, a caspase-3 substrate. Expression of the survival protein Bcl-2 declined. Pretreatment of PC-3 cells with the antioxidant *N*-acetylcysteine prevented escalation of oxidative stress, dissipation of the membrane polarity of mitochondria and changes in downstream molecular events. These results are the first to link elevated oxidative stress and mitochondrial membrane depolarization to salinomycin-mediated apoptosis of prostate cancer cells.

1. Introduction

Salinomycin is a potassium ionophore and a product of the bacterium *Streptomyces albus*. Its antibiotic activity has been exploited for many years to control coccidiosis in parasite-infected chickens and cows [1,2]. More recently, the anticancer property of salinomycin has been recognized based on its ability to induce apoptosis and cause growth inhibition in diverse types of apoptosis- and chemotherapeutic-resistant cancer cells [3]. Salinomycin-mediated apoptosis in these cells is independent of known mediators of the cell death signal pathway, such as the p53 tumor suppressor protein, the 26S proteasome and the CD95/DC95 ligand system. This drug also triggers apoptosis by overcoming ABC transporter-mediated multidrug resistance, as was observed in the case of KG-1a human leukemia cells [4,5]. Salinomycin caused massive tumor cell apoptosis and associated regression of breast tumor growth and metastasis *in vivo* in a mouse xenograft tumor model [6]. In fact, in high-throughput screening of ~16,000 small molecule chemicals, breast cancer stem cells (CSCs) were found to be inhibited selectively by salinomycin [6]. CSCs are a subpopulation of cells within the tumor mass that are thought to account for cancer recurrence by virtue of their refractivity to cytotoxic cancer treatment agents such as radiation and a wide variety of chemotherapeutic agents. Susceptibility of CSCs to salinomycin bolsters the possibility that this drug may target treatment-resistant advanced human cancers. Delineation of the mechanism(s) that underlies cancer cell apoptosis by salinomycin is needed in order to rigorously evaluate the potential of this drug as a novel cancer therapeutic.

Abbreviations: CSC, cancer stem cell; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; NAC, *N*-acetylcysteine; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; $DiOC_6$, 3,3-dihexyloxacarbocyanine; DCFH-DA, 2′-7′-dichlorodihydrofluorescein diacetate; PARP-1, poly (ADP-ribose) polymerase-1; FITC, fluorescein isothiocyanate; PI, propidium iodide.

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Apoptosis is a regulated cell death process that requires the cascaded activation and execution of a series of regulatory molecules and cysteine-aspartic proteases, known as caspases [7]. Stress agents, such as reactive oxygen species (ROS), ultraviolet radiation, viral infections, and anticancer agents are well-characterized apoptosis triggers. Mitochondria are the primary site of origin for the initiating signals of apoptosis, although a death receptor-dependent extramitochondrial apoptotic pathway also exists. Mitochondrially originated apoptotic signals include a change in the electron transport system, loss of mitochondrial membrane potential (MMP, $\Delta\Psi_m$), failure of Ca^{2+} flux homeostasis, generation of ROS, and release of caspase activators. Early apoptosis is invariantly marked by a breakdown in the MMP, which precedes DNA fragmentation in all cell types and under all types of apoptotic stimuli [8]. Production of endogenous ROS as mitochondrial byproducts of respiration is tightly controlled by MMP. Disruption in the ROS homeostasis plays a critical role in the regulation of mitochondrial dysfunction and apoptotic events [9].

Prostate cancer initially responds to androgen deprivation, which is a standard-of-care therapy when the androgen-dependent malignant cells meet with apoptotic death in an environment of low, castrate-level circulating androgens. Relapse, however, is a common occurrence at which point the recurrent cancer cells are castration resistant and have the ability to progress on chemotherapeutics to become completely therapy resistant [10]. How prostate cancer cells respond to salinomycin has been unknown.

In the present study, our results provide the first evidence that salinomycin induces apoptosis of prostate cancer cells by elevating oxidative stress through intracellular ROS production, which leads to the disruption of mitochondrial function and subsequent release of cytochrome *c* to the cytosol, activation of caspase-3, and cleavage of PARP-1 in androgen-independent, chemotherapeutic-refractive PC-3 human prostate cancer cells.

2. Materials and methods

2.1. Reagents and antibodies

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), *N*-acetylcysteine (NAC), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), 3,3-dihexyloxacarbocyanine ($DiOC_6$) and 2′-7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and salinomycin were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO). Annexin-V-Fluo Staining Kits and Caspase-3 Colorimetric Assay Kits were purchased from BD Biosciences (San Jose, CA) and R&D Systems Inc. (Minneapolis, MN), respectively. Mitotracker Red CMXRos was purchased from Invitrogen (Carlsbad, CA). The ECL Western Kit was purchased from Amersham (Piscataway, NJ). Antibodies for Bax, Bcl-2, and poly (ADP-ribose) polymerase-1 (PARP-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for caspase-3 and cytochrome *c* were purchased from Cell Signaling (Danvers, MA). The fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG secondary antibody was purchased from BioFX Laboratories (Owings Mills, MD).

2.2. Cell culture

Human prostate cancer cell lines, androgen-independent PC-3 and DU-145 cells and androgen-dependent LNCaP cells were obtained from the American Type Culture Collection (ATCC, Manassas, Va) and Korean Cell Line Bank (KCLB), respectively. These cells were maintained and cultured in Dulbecco's modified Eagle's medium (DMEM; WelGENE Inc.) and RPMI 1640 (WelGENE Inc.) supplemented with 10% fetal bovine serum (FBS) (WelGENE Inc.), 100 U/ml of penicillin and 100 μg/ml of streptomycin (WelGENE Inc.), respectively. Human prostate normal RWPE-1 cells from ATCC were maintained and cultured in keratinocyte serum-free media (K-SFM) containing 2.5 μg of epidermal growth factor (EGF), 25 mg of bovine pituitary extract (BPE)(GIBCO, 100 U/ml of penicillin and 100 μg/ml of streptomycin (WelGENE Inc., Daegu, South Korea). Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.3. Cell viability

Human prostate cell lines, PC-3 cells, DU-145 cells, LNCaP cells and RWPE-1 cells were treated with salinomycin of various concentrations at different time points. After incubation, these cells were added with 0.5 mg/ml of the MTT solution and the precipitates were dissolved in dimethylsulfoxide (DMSO). A colorimetric analysis was performed at 570 nm with a $VERSA_{max}$ microplate reader (Molecular Devices, Sunnyvale, CA). Cell viability was determined as relative percentage of treated cells to one of the untreated cells by comparing optical densities.

2.4. Annexin V/propidium iodide (PI) assay

Apoptotic cells were detected from annexin-V-Fluo staining [11], using a reagent kit (Roche Applied Science, Indianapolis, IN). PC-3 cells were cultured in 6-well plates at a density of 1×10^4/well and then exposed to salinomycin (0.15–4.00 μM). After exposing cells to salinomycin for 48 h cells were harvested, washed with PBS twice, and then the cells were mixed in 100 μl of 1× binding buffer and incubated with an annexin-V/PI double staining solution at room temperature for 15 min. The stained cells were analyzed by flow cytometry and the percentage of apoptotic cells was calculated using Cell Quest software (Becton Dickinson Co.).

2.5. Measurement of intracellular reactive oxygen species (ROS)

Intracellular ROS generation was measured using a DCFH-DA fluorescent dye (Molecular Probes/Invitrogen). PC-3 cells were cultured in 6-well plates at a density of 1×10^4/well. After treatment with an appropriate concentration of salinomycin, the cells were incubated with 10 μM DCFH-DA at 37 °C for 20 min and then washed twice with PBS. For each experiment, the cells were analyzed for fluorescence using a flow cytometer.

2.6. Measurement of the mitochondrial membrane potential (MMP)

MMPs ($\Delta\Psi_m$) were determined by the retention of the dye $DiOC_6$ [12]. Cells were collected 48 h after treatment with an appropriate concentration of salinomycin and incubated with 100 nM $DiOC_6$ at 37 °C for 30 min. Cells were washed twice with PBS and analyzed with flow cytometry.

2.7. Western blot analysis

Cell extracts were prepared by incubating the cells in lysis buffer [150 mM NaCl, 10 mM Tris (pH 7.4), 5 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 20 μg/ml aprotinin, 50 μg/ml leupeptin, 1 mM benzidine, and 1 mg/ml pepstatin]. Fifty micrograms of proteins was electrophoretically separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12–15% gel and transferred to a polyvinylidenefluoride (PVDF) membrane. After blocking with TBS-T buffer [20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20] containing 5% skim milk, membranes were incubated with primary and secondary antibodies. The membranes were then washed with TBS-T buffer and visualized with ECL Western blotting detection reagents. The density of each band

was determined with a fluorescence scanner (LAS 3000, Fuji Film) and analyzed with Multi Gauge V3.0 software.

2.8. Mitochondrial and cytosolic fractionation

Release of pro-apoptotic factors from the mitochondria to the cytosol was detected by Western blotting using a Qproteome™ Mitochondria isolation Kit (Qiagen) according to manufacturer's instructions. Western blotting was carried out as described above.

2.9. Caspase-3 activity

For detection of caspase-3 activity, a colorimetric assay kit (R&D Systems Inc.) was used according to the manufacturer's protocol. Equal amounts of protein (220 μg) were resuspended in reaction buffer containing substrate (Ac-DEVD-pNA) and then incubated at 37 °C for 4 h in the dark. Absorbance of the released pNA was measured at 405 nm using an ELISA reader.

2.10. Confocal microscopy

PC-3 cells were cultured in a glass-bottom dish. The cells were exposed to salinomycin and incubated for 30 min with 50 nM Mitotracker Red CMXRos (Invitrogen) at 37 °C prior to fixation. Fixed cells were incubated with primary Bax antibodies for 2 h at room temperature, washed with PBS, and incubated with FITC-conjugated anti-rabbit IgG secondary antibody (BioFX Laboratories). DAPI was used to counterstain the nuclei. Samples were visualized with a laser scanning confocal microscope (Olympus Fluoview FV1000).

2.11. Statistical validation

Experiments were repeated at least three times with consistent results. Unless otherwise stated, data are expressed as the mean ± SD. ANOVA was used to compare experimental groups to control values. Comparisons between multiple groups were performed using a Tukey's multiple comparison tests. Results were statistically significant at $p < 0.05$.

3. Results

3.1. Salinomycin reduced viability of prostate cancer cells at a lower dose than non-malignant prostate epithelial cells

Androgen-independent PC-3 and DU-145 and the androgen-dependent LNCaP prostate cancer cells and non-malignant RWPE-1 prostate epithelial cells were treated with increasing concentrations of salinomycin for different time periods, when salinomycin exposure reduced the viability of prostate cancer cells in a dose- and time-dependent manner (Fig. 1A, B and C). By comparison, RWPE-1 cells were relatively less sensitive to salinomycin, since at 0.15 μM concentration, the drug did not significantly inhibit viable cell number (Fig. 1D), unlike the all three cancer cells,

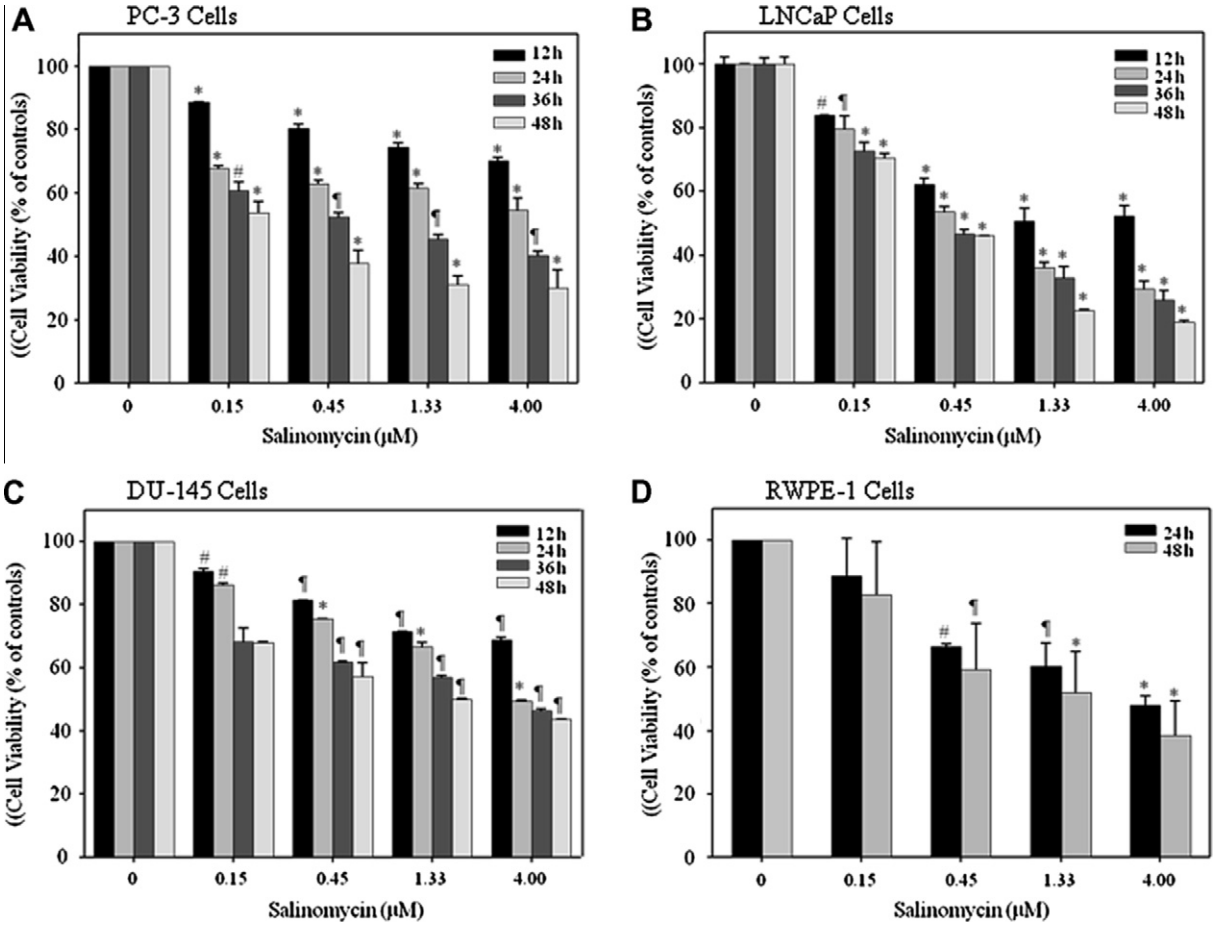

Fig. 1. Salinomycin inhibited viability of prostate cancer cells. (A) PC-3, (B) LNCaP, (C) DU-145, and (D) RWPE-1 cells. Cells/ml (5×10^4) were treated with salinomycin (0.15–4.00 μM) at different time points (12, 24, 36, and 48 h). Cell viability was determined by a 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Data are presented as mean ± SD ($n = 3$ in each group). $^{\#}p < 0.05$, $^{\P}p < 0.01$, $^{*}p < 0.001$ vs. the control group.

which showed significant drop in viability in MTT assay. To some extent, differential sensitivity to the drug was also seen for LNCaP vs. PC-3 and DU-145 cells, since at 1.33 μM of the drug, LNCaP cells manifested a stronger inhibition – viability reduced to ~55%, 38%, 35% and 22% (after 12, 24, 36 and 48 h, respectively), whereas >50% of PC-3 and DU-145 cells remained viable after 36 h treatment of the drug (at 1.33 μM), and even at 48 h, >30% of PC-3 cells and >50% of DU-145 cells remained viable (Fig. 1B vs. Fig. 1A and C). At 0.15 μM salinomycin, the three cell lines showed approximately similar sensitivity to the drug. To summarize, these results indicate that the chemo-resistance of the hormone-independent cancer cells to salinomycin is higher than that of the hormone-dependent cells, and compared to the cancer cells, non-malignant prostate cells (such as RWPE-1) are relatively more resistant to salinomycin. We next focused on the PC-3 cell model to investigate the molecular events associated with the salinomycin-induced loss of cell viability.

3.2. Salinomycin induced PC-3 cell apoptosis

To examine if the salinomycin effect is due to apoptosis, we examined PC-3 cells for the nuclear morphology, annexin V-FITC staining and induction of various apoptosis-related molecular events before and after salinomycin treatment. Laser scanning confocal microscopy of DAPI-stained PC-3 cells showed that in the absence of the drug, the nuclei were round and homogeneous, whereas salinomycin treatment caused a reduction of cell volume, nuclear condensation (a hallmark feature of apoptotic cells), and increased non-adherence of the cells to the culture surface (Fig. 2A). Induction of apoptosis was rigorously substantiated by examining the flow cytometry pattern of annexin V-FITC stained cells (Fig. 2B). Apoptotic cells accounted for 27.13% and 34.61% of the cells in early apoptosis (1st right quadrant) plus late apoptosis (4th right quadrant), and necrotic cells (3rd left quadrant) were 13.03% and 21.24% of total cells, in response to salinomycin treatment at 1.33 and 4.00 μM, respectively (Fig. 2B). Taken together, Fig. 2A and B show that salinomycin induced apoptotic cell death; at higher doses necrosis may also account for cell death.

3.3. Salinomycin differentially altered the levels of Bcl-2 family proteins and induced caspase-3 activation and PARP-1 cleavage in PC-3 cells

We examined the expression of Bax and Bcl-2, the apoptosis and cell survival related protein, respectively, and also cleavage of pro-caspase-3, and PARP-1 (a caspase-3 substrate) using Western blotting. Salinomycin increased Bax expression and decreased Bcl-2 expression in a dose-dependent manner within total cell lysates (Fig. 2C). Furthermore, declining pro-caspase-3 levels and increasing cleavage of PARP-1 were evident with increasingly higher salinomycin concentrations (Fig. 2D). Caspase-3 activity assay using an *in vitro* colorimetric method further confirmed caspase-3 activation in the presence of salinomycin. Treatment of PC-3 cells with the drug for 48 h resulted in a dose-dependent increase of caspase-3 activity (Fig. 2E). Thus, salinomycin mediated a cascaded series of molecular events that led to an attenuated level of Bcl-2, augmented level of the pro-apoptotic protein Bax, and activation of the executer apoptosis enzyme caspase-3.

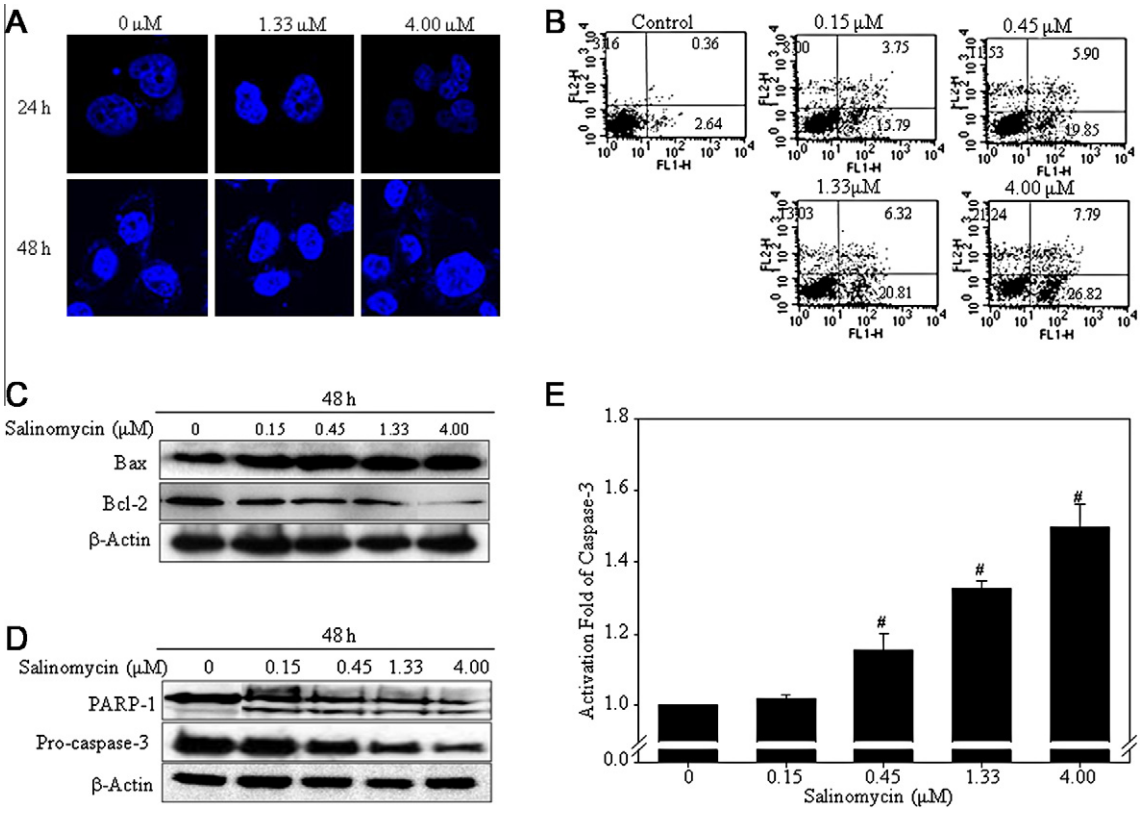

Fig. 2. Salinomycin induced apoptosis in PC-3 cells. (A) Morphological changes. After treatment with salinomycin (1.33 and 4.00 μM) for 24 and 48 h, nuclear fragmentation was observed by laser scanning confocal microscopy. Magnification at 1800×. (B) Flow cytometric analysis of annexin-V/propidium iodide (PI) staining. PC-3 cells were treated with various concentrations of salinomycin for 48 h. The dual parameter dot plots combining annexin V-fluorescein isothiocyanate (FITC) and PI fluorescence show the viable cell population in the lower left quadrant (annexin V^-PI^-), apoptotic cells in the lower right quadrant (annexin V^+PI^-) and the upper right quadrant (annexin V^+PI^+), and necrotic cells in the upper left quadrant (annexin V^-PI^+). (C) Bax and Bcl-2 expression in total cell lysates, detected by Western blotting. (D) Pro-caspase-3 and poly (ADP-ribose) polymerase (PARP-1, cleaved and uncleaved) levels. (E) Caspase-3 activity, determined by a colorimetric assay kit using the specific substrate Ac-DEVD-pNA. Data show mean ± SD ($n = 3$ in each group). $^{\#}p < 0.01$ vs. the control group.

3.4. Intracellular production of ROS in PC-3 cells increased markedly after salinomycin treatment

Cancer chemotherapy is known to induce tumor cell death in a variety of cell types in part by promoting the production of intracellular ROS. In order to know whether ROS production is associated with salinomycin-induced apoptosis of PC-3 cells, we assessed the state of ROS at various time points after salinomycin treatment by examining the fluorescence intensity of DCHF-DA-incubated cells. A representative fluorescence pattern from flow cytometry (Fig. 3A, upper panel) shows that the intracellular ROS level increased after 4 h of salinomycin treatment, and pretreatment of the cells with the antioxidant *N*-acetylcysteine (NAC), a known quencher of ROS, left shifted the fluorescence peak closer to the peak generated by cells with no treatment (control) or NAC treatment without subsequent exposure to salinomycin. The number of DCF-positive cells increased as early as 15 min following exposure to 1.33 μM salinomycin, and the peak production of ROS was after 4 h incubation of the drug (Fig. 3A, lower panel). As expected, pretreatment of the cells with NAC reduced the number of DCF-positive cells. NAC also increased the cell viability from 41.96% to 57.08% for 1.33 μM and from 25.4% to 41.21% for 4.00 μM of salinomycin (Fig. 3B). Salinomycin-induced caspase-3 activation in PC-3 cells was also inhibited by NAC (Fig. 3C). These findings suggest that intracellular ROS production is closely linked to caspase-3 activation and to the viability of PC-3 cells.

3.5. Salinomycin induced loss of mitochondrial membrane potential (MMP) in PC-3 cells

ROS is known to be involved in specific aspects of mitochondrial dysfunctions such as opening of the mitochondrial permeability transition pore that causes depolarization of the mitochondrial transmembrane potential ($\Delta\Psi_m$), release of apoptogenic factors and loss of oxidative phosphorylation. Flow cytometry of $DiOC_6$ fluorescence dye-labeled PC-3 cells showed progressive left shift of fluorescence intensity, indicating reduction in MMP, after treatment with 1.33 and 4.00 μM salinomycin (Fig. 4A, upper panel). Reduction in MMP was also prevented in NAC-pretreated cells, as shown in the results of intracellular ROS level (Fig. 4A, lower panel). These data suggest that dissipation of mitochondrial membrane potential (MMP) in salinomycin-treated PC-3 cells is dependent on intracellular ROS production.

3.6. Salinomycin promoted Bax translocation to mitochondria and cytosolic release of cytochrome c

Participation of mitochondrial components in salinomycin-induced apoptosis was determined by assessing the subcellular localization of Bax and cytochrome *c* before and after salinomycin treatment. The drug triggered Bax translocation onto the mitochondrial membrane (Fig. 4B, upper panel) and mitochondrial cytochrome *c* release into the cytosol (Fig. 4B, lower panel), revealed from Western blot assay. Bax translocation to mitochondria was visually confirmed by confocal microscopy (Fig. 4C), which showed a greatly enhanced staining for Bax in the mitochondrial compartment after treatment with salinomycin (1.33 μM) for 24 or 48 h. These data suggest that salinomycin plays a pivotal role in the mitochondrial uptake of Bax and concomitant release of cytochrome *c*.

4. Discussion

The pharmacologic action of salinomycin has garnered increased attention in recent years in view of its potential as a new cancer chemotherapeutic based on its activity as a selective

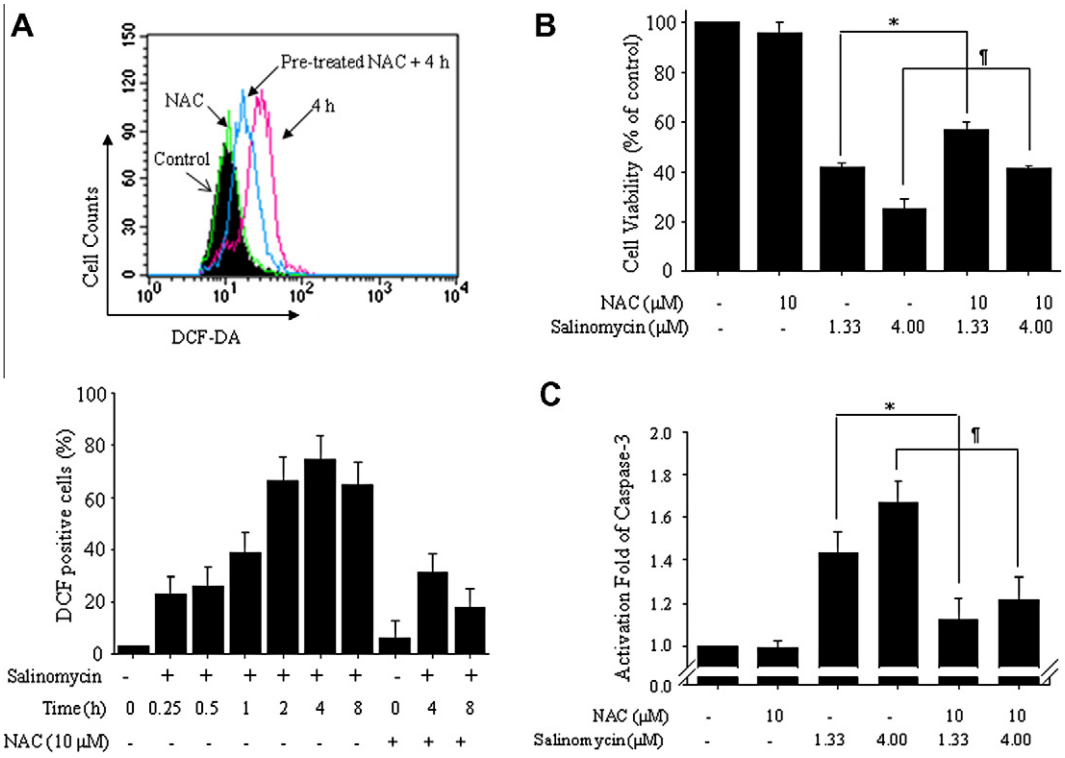

Fig. 3. Salinomycin mediated ROS-induced apoptosis. (A) The intracellular ROS level. Cells were treated with salinomycin (1.33 μM) for indicated time periods with or without prior 1 h incubation with *N*-acetylcysteine (NAC; 10 mM). The dichlorodihydrofluorescein (DCF) fluorescence intensity in the cells was detected by flow cytometry. (B) Cytotoxicity. Cells were treated with salinomycin for 48 h with or without pretreatment for 1 h with NAC (10 mM). (C) Caspase-3 activity. Cells were treated with salinomycin for 48 h with or without NAC (10 mM, 1 h). Data are presented as mean ± SD ($n = 3$ in each group). $^{\P}p < 0.01$, $^{*}p < 0.001$ vs. the control group.

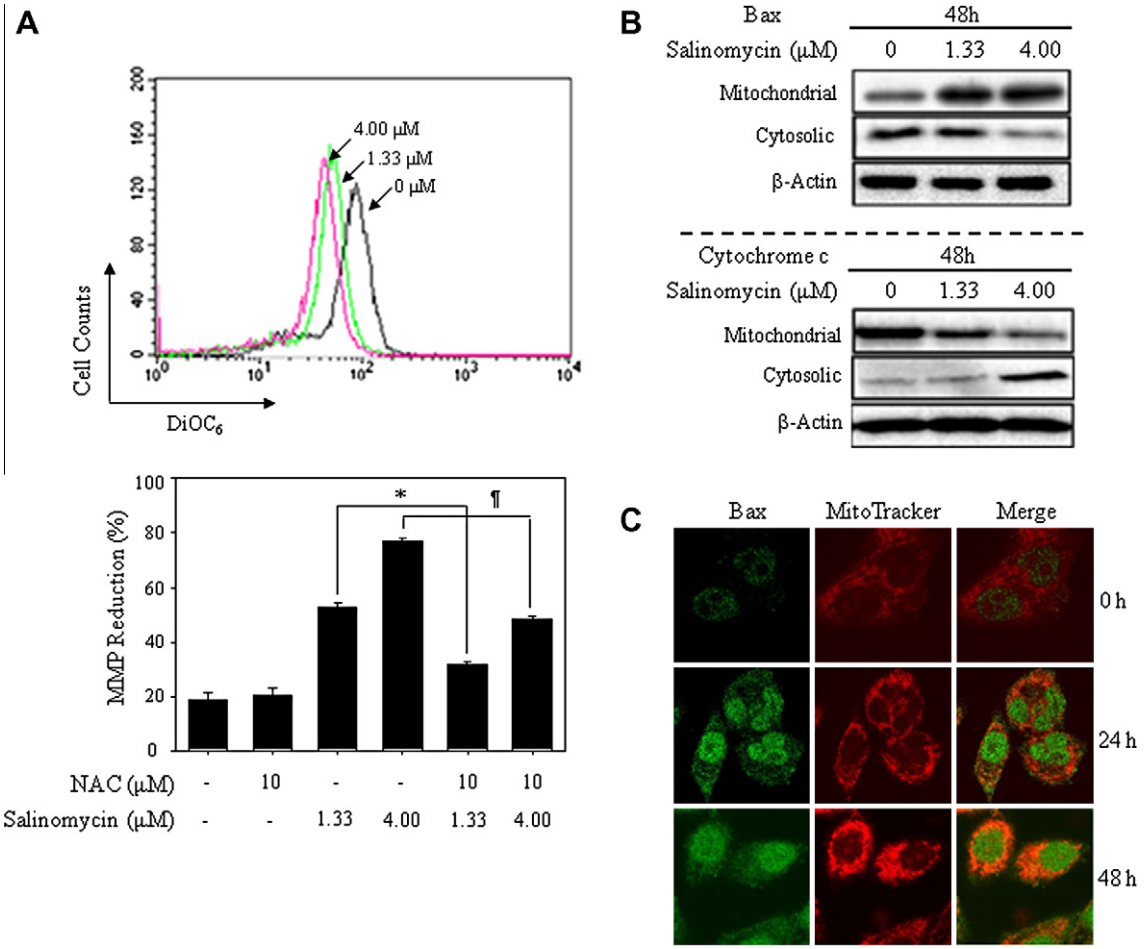

Fig. 4. Salinomycin induced dysfunctions of mitochondrial membrane in PC-3 cells. (A) Mitochondrial membrane potentials (MMP). Cells were treated with salinomycin (1.33 and 4.00 μM) for 48 h in the presence or absence of a prior 1 h incubation with NAC (10 mM). MMP changes were determined from $DiOC_6$ fluorescence, measured by flow cytometry analysis. Data are presented as mean ± SD ($n = 3$ in each group). $^{¶}p < 0.01$, $^{*}p < 0.001$ vs. the control group. (B) Bax translocation and release of cytochrome *c*. The levels of Bax and cytochrome *c* in the cytosol fraction and mitochondrial fraction were determined by Western blotting after prepared by commercial company's protocol. (C) Mitochondrial Bax translocation. Confocal microscopic images were observed by using the mitochondria staining dye Mitotracker Red CMXROS and anti-Bax antibody. Magnification at 1800×.

inhibitor of breast cancer stem cells. Salinomycin treatment also reduced formation of metastatic nodules by CSCs [6,13]. Since CSCs are inert to all current cancer therapy interventions, they are likely to drive tumor recurrence and progression. The absence of androgen receptor expression in the putative CSCs in prostate cancer suggests that targeting of the androgen receptor pathway will not yield lasting therapy for advanced prostate cancer. Our result that salinomycin is detrimental to the viability of androgen-dependent and androgen-independent prostate cancer cells due to the onset of apoptosis hints at the possibility that this drug or more likely, a significantly less cytotoxic derivative of this drug activity, may have clinical utility as part of a future treatment strategy for advanced prostate cancer.

Our present study shows (1) salinomycin decreased viability of the androgen-dependent LNCaP and androgen-independent PC-3 and DU-145 prostate cancer cells in MTT assay in a time- and dose-dependent manner. The non-malignant RWPE-1 prostate epithelial cells were resistant to the drug-induced lethality at a lower salinomycin dose, which was still effective in inhibiting LNCaP, PC-3 and DU-145 cells. (2) Early and late apoptosis and necrosis in salinomycin-treated PC-3 cells was revealed from the nuclear morphology of DAPI-stained cells and from flow cytometry of annexin V-FITC labeled cells. (3) Biochemical evidence of apoptosis came from the results that salinomycin activated caspase-3, induced cleavage of PARP-1 and caused a dose-dependent decreased expression of the survival protein Bcl-2 and increased expression of the pro-apoptotic protein Bax. (4) Bax was translocated to the mitochondria and cytochrome *c* was released into the cytosol of salinomycin-treated PC-3 cells, in agreement with the known coordinated events in the apoptosis pathway in which translocated Bax forms a transmembrane pore across the outer mitochondrial membrane, which in turn helps the cytosolic release of cytochrome *c*. (5) Finally, new evidence presented here shows that salinomycin promotes escalation of intracellular ROS levels which is accompanied by decreased mitochondrial membrane potential and increased caspase-3 activity of PC-3 cells and these effects of salinomycin were prevented by pretreatment of the cells with the antioxidant NAC.

Previously it was reported that cancer chemopreventive agents induce apoptosis in part through ROS generation and disruption of redox homeostasis [14]. It is also known that the pro-apoptotic signal(s) emanating from accumulated ROS triggers the mitochondrial release of caspase-activating proteins, such as cytochrome *c*, apoptosis inducting factor (AIF), and Smac/DIABLO to the cytosol [15]. ROS shows secondary messenger function because of its ability to influence MMP and mitochondrial function and to induce intracellular Ca^{2+} flux and eventual activation of the caspase cascade [16]. Although our results provide clear evidence of salinomycin-induced ROS generation, mitochondrial membrane depolarization and augmentation of caspase-3 activity in PC-3 cells, we did not detect any change in the intracellular Ca^{2+} level (data not shown).

In conclusion, the mechanistic implication of our data is that salinomycin-mediated ROS production, initiated upstream of mitochondrial dysfunction, is a determining event that commits the cancer cells to apoptotic death subsequent to the loss of MMP, cytosolic release of cytochrome *c* and activation of the caspase zymogen cascade. The link between ROS and apoptosis in salinomycin-exposed cells was also evident from the inhibition of apoptosis in NAC-pretreated PC-3 cells. The NAC inhibition hints at the possibility that the extent of salinomycin-induced cytotoxicity in a therapeutic setting may be controlled with the intermittent use of an antioxidant in the therapeutic regimen of prostate cancer treatment. Future extension of the studies described here will constitute evaluating the anticancer efficacy of salinomycin on prostate cancer xenografts and animal tumor models, and on patient-derived primary prostate tumor cells.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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References

[1] Y. Miyazaki, M. Shibuya, H. Sugawara, O. Kawaguchi, C. Hirsoe, Salinomycin, a new polyether antibiotic, J. Antibiot. 27 (1974) 814–821.
[2] H.D. Danforth, M.D. Ruff, W.M. Reid, R.L. Miller, Anticoccidial activity of salinomycin in battery raised broiler chickens, Poult. Sci. 56 (1977) 926–932.
[3] C. Naujokat, D. Fuchs, G. Opelz, Salinomycin in cancer: a new mission for an old agent, Mol. Med. Rep. 3 (2010) 555–559.
[4] D. Fuchs, A. Heinold, G. Opelz, V. Daniel, C. Naujokat, Salinomycin induces apoptosis and overcomes apoptosis resistance in human cancer cells, Biochim. Biophys. Res. Commun. 390 (2009) 743–749.
[5] D. Fuchs, V. Daniel, M. Sadeghi, G. Opelz, C. Naujokat, Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells, Biochim. Biophys. Res. Commun. 394 (2010) 1098–1104.
[6] P.B. Gupta, T.T. Onder, G. Jiang, K. Tao, C. Kuperwasser, R.A. Weinberg, E.S. Lander, Identification of selective inhibitors of cancer stem cells by high-throughput screening, Cell 138 (2009) 645–659.
[7] A. Degterev, J. Yuan, Expansion and evolution of cell death program, Nat. Rev. Mol. Cell Biol. 9 (2008) 378–390.
[8] M.J. Liu, Z. Wang, H.X. Li, R.C. Wu, Y.Z. Liu, Q.Y. Wu, Mitochondrial dysfunction as an early event in the process of apoptosis induced by woodfordin I in human leukemia K562 cells, Toxicol. Appl. Pharmacol. 194 (2004) 141–155.
[9] H.U. Simon, A. Haj-Yehia, F. Levi-Schaffer, Role of reactive oxygen species (ROS) in apoptosis induction, Apoptosis 5 (2000) 415–418.
[10] B. Seruga, A. Ocana, I.F. Tannock, Drug resistance in metastatic castration-resistant prostate cancer, Nat. Rev. Clin. Oncol. 8 (2011) 12–23.
[11] I. Vermes, C. Haanen, H. Steffens-Nakken, C.A. Reutelingsperger, Novel assay for apoptosis: flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V, J. Immunol. Methods 184 (1995) 39–45.
[12] R. Levenson, I.G. Macara, R.L. Smith, L. Cantley, D. Housman, Role of mitochondrial membrane potential in the regulation of murine erythroleukemia cell differentiation, Cell 28 (1982) 855–863.
[13] N.J. Maitland, A.T. Collins, Prostate cancer stem cells: a new target for therapy, J. Clin. Oncol. 26 (2008) 2862–2870.
[14] N. Hail, R. Lotan, Cancer chemoprevention and mitochondria: targeting apoptosis cells via the in transformed cells via disruption of mitochondrial bioenergetics/redox state, Mol. Nutr. Food Res. 53 (2009) 49–67.
[15] M.L. Circu, Y.A. Tak, Reactive oxygen species, cellular redox systems, and apoptosis, Free Radic. Biol. Med. 48 (2010) 749–762.
[16] J.D. Ly, D.R. Grubb, A. Lawen, The mitochondrial membrane potential (deltapsi(m)) in apoptosis; an update, Apoptosis 8 (2003) 115–128.