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RESEARCH ARTICLE

Effects of salinomycin on human ovarian cancer cell line OV2008 are associated with modulating p38 MAPK

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Abstract This study investigated the anticancer effect and mechanism of salinomycin, a selective inhibitor of cancer stem cell, on human ovarian cancer cell line OV2008 in vitro and in vivo. The growth inhibitory effect of salinomycin on ovarian cancer cell line OV2008 was determined by measuring cell viability using the resazurin reduction assay. Apoptotic nuclear morphology was visualized by 4,6-diamino-2-phenylindole staining technique. The percentages of apoptotic cells and cell cycle parameters were detected by flow cytometry. The activity of p38 mitogen-activated protein kinase (p38 MAPK) was analyzed by Bio-Plex phosphoprotein assay. In vivo activity of salinomycin was assayed through tumor growth. Salinomycin caused concentration- (0.01–200 μM) and time-dependent (24–72 h) growth inhibitory effects in OV2008. Cell nuclear morphology observations showed that salinomycin-treated OV2008 cells displayed typical apoptotic characteristics. Salinomycin significantly increased the percentages of apoptotic cells in OV2008, showing a concentration- and time-dependent manner. There was no cell cycle arrest in the G1/G0, S, and G2/M phases between salinomycin-treated cells and control cells. Salinomycin also enhanced the phosphorylation of p38 MAPK. Moreover, salinomycin significantly inhibited the growth of the ovarian xenograft tumors. Salinomycin exhibited significant growth inhibition and induction of apoptosis in the human ovarian cancer cell line OV2008. The data suggested that salinomycin-induced apoptosis in OV2008 might be associated with activating p38 MAPK and merits further investigations.

Keywords Salinomycin · Ovarian cancer · Growth inhibition · Apoptosis · p38 MAPK · Tumor xenografts

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Introduction

Salinomycin is a 751-Da monocarboxylic polyether antibiotic belonging to the group of ionophores isolated from *Streptomyces albus* (strain no. 80614) [1]. Salinomycin acts in different biological membranes, including mitochondrial and cytoplasmic membranes, as an ionophore with a stringent selectivity for monovalent cations and a considerable preference for potassium ions [2, 3]. Salinomycin exhibits a large spectrum antimicrobial activity including anticoccidial property [4, 5]. It is commonly used as a coccidiostat in poultry and other livestock and is fed to ruminants to improve nutrient absorption and feed efficiency [6]. Recently,

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salinomycin has been reported to selectively deplete human breast cancer stem cells from tumorspheres and to inhibit the mammary tumor growth and metastasis in vivo [7]. Another recent report showed that salinomycin induces apoptosis in human cancer cells, including those that display wild-type p53 or p53 mutation and multidrug resistance due to overexpression of Bcl-2, P-glycoprotein, or 26 S proteasomes with deregulated proteolytic activity [8]. These results strongly suggested that salinomycin should be regarded as an anticancer compound. The mechanism of anticancer action of salinomycin is not completely understood. One study showed that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest and independent of tumor suppressor protein p53, caspase activation, the CD95/CD95L system, and the proteasome [8]. More recently, salinomycin was reported to overcome ABC transporter-mediated multidrug and apoptosis resistance [9] and act as a potent inhibitor of multidrug resistance gp170 [10]. Furthermore, a recent study uncovered that salinomycin inhibits the activity of the Wnt signaling pathway, recently appointed as an essential regulator of cancer stem cell properties in chronic lymphocytic leukemia cells [11].

Ovarian cancer remains a leading cause of death from gynecological malignancy, with more than 204,000 new cases and 125,000 deaths each year, accounting for 4 % of all cancer cases and 4.2 % of all cancer deaths in women around the world [12]. The high mortality rate of women with ovarian cancer has been attributed both to the lack of early detection and to the development of chemoresistance during treatment [13, 14]. Current standard of care including surgery and chemotherapy has had very limited success in the treatment of patients diagnosed with late stage disease [15, 16]. The aims of this study were (1) to determine the anticancer biological activity of salinomycin toward human ovarian cancer cell line OV2008, (2) to derive mechanistic insights into the action of salinomycin, and (3) to determine whether salinomycin would significantly inhibit tumor growth in an in vivo model of ovarian cancer. The studies were conducted using human ovarian cancer cell line OV2008 and its murine xenograft model. The results showed that salinomycin inhibited cell growth and induced apoptosis in ovarian cancer cell line OV2008 in vitro and suppressed tumor growth in vivo as well. The salinomycin-induced apoptosis in ovarian cancer cell line OV2008 could be mediated through an increase in the activation of p38 mitogen-activated protein kinase (p38 MAPK).

Materials and methods

Cell line and culture

The OV2008 human epithelial ovarian cancer cell line was kindly supplied by Dr. Gaetano Marverti (University of Modena and Reggio Emilia, Italy) and routinely grown in a humidified condition at 5 % CO_2 and 37 °C and incubated with RPMI 1640 standard medium supplemented with 10 % fetal bovine serum, antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), and L-glutamine (2 mM). Exponentially growing cells were used throughout the study. All these reagents were supplied by Invitrogen (Carlsbad, CA, USA).

Growth inhibition assay

The growth inhibitory effect of salinomycin on OV2008 was determined by measuring cell viability using the resazurin reduction assay. Briefly, cells were seeded in 100 μl media in 96-well microtiter plates at a density of 5,000 cells/well. Following overnight incubation, cells were exposed to a range of different concentrations of salinomycin (Sigma-Aldrich, S4526 and 0.1 % DMSO as solvent control) and grown at 37 °C under a 5 % CO_2 atmosphere for 24–72 h. Five microliters of 0.02 % (*w*/*v*) resazurin (Sigma-Aldrich, R7017) in phosphate-buffered saline (PBS) was then added to each well and incubation was continued for an additional 2 h. Finally, fluorescence was read using a spectramax GEMINI XS microplate reader (λ_{exc}=544 nm, λ_{em}=590 nm).

Cell nuclear morphology observations

Exponentially growing cells were incubated with salinomycin for 12, 24, and 36 h, respectively, and equal volumes of solvent (0.1 % DMSO) as control. Apoptotic nuclear morphology was visualized by 4,6-diamino-2-phenylindole (DAPI) staining technique. Cells (1×10^5) were collected on the slide using cytospin, then fixed with 3.7 % of paraformaldehyde (#28906, Pierce) for 15 min at room temperature, washed three times with PBS, and immersed in 0.1 % of Triton X-100 (Sigma-Aldrich, T8787) for 15 min. Thus, paraformaldehyde-fixed cells were stained using 1:1,000 DAPI (1 mg/ml in ddH_2O; Invitrogen, D29410) in ddH_2O for 5 min in the dark at room temperature. After three times of washing with PBS, cells were coverslipped with a florescence mounting medium (Dako, S3023) and visualized using a fluorescence microscope (Olympus B×51, Japan).

Cell apoptosis detection

Cell apoptosis was studied by using the annexin V–fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis assay kit (BD Pharmingen) in combination with flow cytometry (CyAn ADP, Dako). After cells were incubated with salinomycin for 12, 24, and 36 h, respectively, and with solvent control (0.1 % DMSO) as well, they were harvested by quick trypsinization to minimize potentially high annexin V background levels in adherent cells. Cells were then washed twice with cold PBS and re-suspended in binding

buffer at a concentration of 1×10^6 cells/ml. One-hundred-microliter cells were taken to stain with 5 μl annexin V–FITC and 5 μl PI and incubated in the dark at room temperature for 15 min. Then, 400 μl binding buffer was added and cells were analyzed by flow cytometry. Cells negative for both annexin V and PI are viable, annexin V^+/PI^- cells are in early apoptosis, and annexin V^+/PI^+ cells are necrotic or in late apoptosis. The percentages of apoptotic cells were analyzed by the FlowJo software.

Cell cycle distribution analysis

To evaluate cell cycle profile, cells (about 1×10^6 cells), pretreated with salinomycin for 12 and 24 h (0.1 % DMSO as a solvent control), were harvested, washed twice with PBS, then fixed and stored in ice-cold 70 % (*v*/*v*) ethanol at −20 °C. Prior to analysis, samples were washed again with PBS and then incubated in propidium iodide/RNase staining buffer (BD Pharmingen) at room temperature in the dark for at least 15 min. After filtration to remove cellular debris, the single-cell suspensions were analyzed on a flow cytometer. Cell cycle parameters were analyzed using the FlowJo software.

Phosphoprotein assay

Phosphoprotein was measured in duplicate using a bead-based multiplex assay (Bio-Plex Phosphoprotein Detection, Bio-Rad, Hercules, USA), according to the manufacturer's instructions [17, 18]. After OV2008 cells were cultured with salinomycin or with solvent control (0.1 % DMSO) for the indicated time interval, cells were rinsed with ice-cold cell wash buffer and then lysed in lysing solution. The lysate was collected and centrifuged at 4,500×*g* for 20 min at 4 °C. The protein concentration was measured and calculated with a detergent-compatible protein assay (Bio-Rad). The Bio-Plex assay was applied to detect and quantify phosphoproteins of p38 MAPK. The prepared first antibody with coupled beads was captured under 96-well plates, and then the samples (15 μg proteins each) were incubated with the coupled beads overnight at room temperature. On the next day, after washing, the samples were incubated with biotin-labeled detection antibodies followed by further incubation with the PE-labeled streptavidin reporter. The level of phosphoproteins bound to the beads was indicated by the intensity of the reporter signal. The signal was acquired and analyzed using Bio-Plex Manager software (Bio-Rad) interfaced with a Bio-Plex 200 system (Bio-Rad). In this assay, the lysates of phosphotase-treated HeLa cells and UV-treated HEK293 cells, provided by the Bio-Plex phosphoprotein assay, were used as the background control and phospho-p38 MAPK (Thr180/Tyr182) positive control, respectively. This experiment was repeated in duplicate.

Ovarian cancer tumor xenografts in mice

Female mice of NOD/SCID were in-house-bred from the Animal Center (Tierversuchsstation) at the Department of Biomedicine, University Hospital of Basel and used at 6 weeks of age. All mouse procedures were approved by the Cantonal Veterinary Office (Kantonales Veterinäramt) and performed in accordance with the regulations concerning animal experiments. For in vivo salinomycin treatment study, cultured OV2008 cells (2×10^6 cells per mouse in 0.1 ml saline) were subcutaneously injected into the back of NOD/SCID mice. On the day after injection of tumor cells, mice were divided into two groups of five mice each. Treatment was initiated 24 h after injection. The two experimental groups were administrated with salinomycin (5 mg/kg) [7] and 5 % ethanol (vehicle), respectively, by intraperitoneal injection every other day for 3 weeks. The size of the tumor was measured every 2 days using a digital vernier caliper. Tumor volume was estimated by the following formula: volume=$(a\times b^2)\times\pi$ / 6, where a and b are the major and minor axes of the tumor.

Statistical analysis

All data were expressed as mean values±standard deviation. Growth inhibitory curves were analyzed using GraphPad Prism 5.01 software. Comparisons among groups were performed by Student's *t* test. The significance level was set at $P<0.05$.

Results

Growth inhibitory effect of salinomycin on ovarian cancer cell line OV2008

The growth inhibitory effect of salinomycin against OV2008 cell line is shown in Fig. 1. The effect of incubation time and

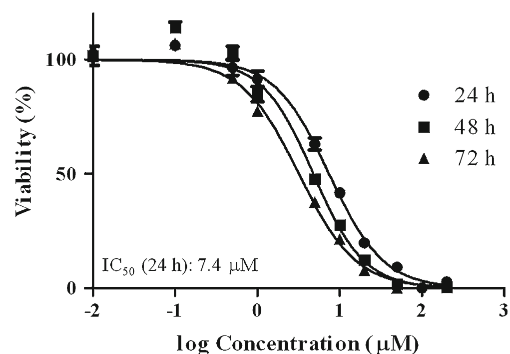

Fig. 1 Effect of salinomycin on cell viability in human ovarian cancer cell line OV2008. Cells were exposed to salinomycin at concentrations (0.01–200 μM, 0.1 % DMSO as solvent control) for 24, 48, and 72 h and cell viability was measured by resazurin reduction assay. Results are mean ± SD of quadruplicates from one of three independent experiments

concentration on viability of OV2008 cells by salinomycin was studied. Cells were exposed for 24, 48, or 72 h to salinomycin at the 0.01—200-μM concentration range, and cell viability was measured by the resazurin reduction assay. In this study, salinomycin inhibited the growth of OV2008 cells in a concentration- and time-dependent pattern. IC_{50} (95 % confidence interval) of salinomycin on OV2008 cell line for 24, 48, and 72 h was 7.44 (6.80 to 8.14), 4.78 (4.12 to 5.55), and 3.20 (2.90 to 3.53), respectively.

Effect of salinomycin on cell morphology and nuclear change

Treating OV2008 cells with 20 or 50 μM salinomycin for 12, 24, and 36 h, respectively, resulted in the detachment of cells from the tissue culture plates as well as cell death. In order to examine whether cells died due to apoptosis mechanisms, the nuclear morphological changes were observed under a fluorescence microscope. The nuclei in control cells exhibited equal distribution of the chromatin, while

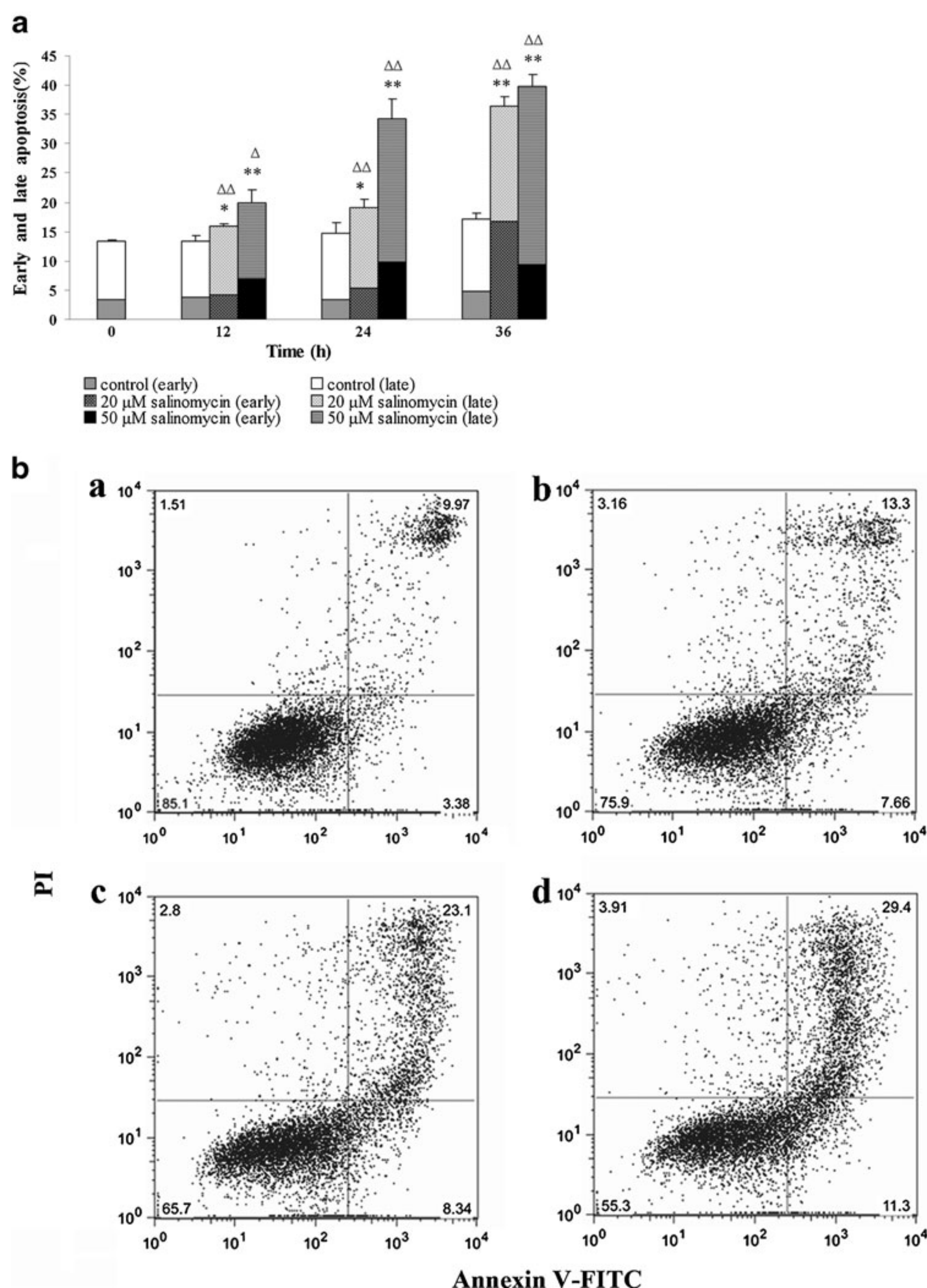

Fig. 2 Effect of salinomycin on cell apoptosis in OV2008 cells. After cells were treated with 20 or 50 μM salinomycin for 12, 24, and 36 h, respectively, and with solvent control (0.1 % DMSO) as well, the percentage of apoptotic subpopulations (**a**) was determined by flow cytometry analysis based on mean values obtained from three independent experiments. Results are expressed as mean± SD. $*P<0.05$ and $**P<0.01$: significant difference from control of time point, respectively. $\Delta P<0.05$ and $\Delta\Delta P<0.01$: significant difference from 0 h control, respectively. **b** Flow cytometry results showing a time-dependent increase in the number of apoptotic cells after cells were treated with 50 μM salinomycin for 12 (*b*), 24 (*c*), 36 (*d*), and 0 h as control (*a*)

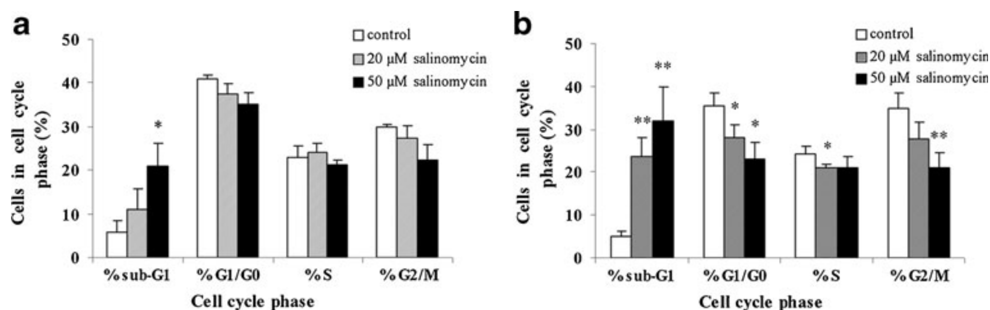

Fig. 3 Effect of salinomycin on cell cycle distribution in OV2008 cells. Cells were treated with 20 or 50 μM salinomycin for 12 (**a**) and 24 h (**b**) and with 0.1 % DMSO as a solvent control. The percentages of each cell cycle were evaluated by flow cytometry based on mean values obtained from three independent experiments. Results are expressed as mean±SD. *$P<0.05$ and **$P<0.01$: significant difference from control, respectively

salinomycin-treated cells showed the characteristic morphologic changes of apoptosis, such as condensed chromatin, nuclear fragmentation, and blebbing (Fig. S1). Therefore, these morphological changes suggested the occurrence of apoptosis in OV2008 cells after treated with salinomycin.

Effect of salinomycin on tumor cell apoptosis and cell cycle

Salinomycin-treated OV2008 cells were analyzed by flow cytometry which can distinguish between early apoptosis or late apoptosis of cells after cells were stained simultaneously with annexin V and PI. Compared to control, salinomycin treatment significantly increased the percentages of apoptotic cells in OV2008, showing a concentration- and time-dependent manner (Fig. 2a). In the control culture, 3.36±0.04 % cells were in the early apoptosis stage, whereas 10.09±0.16 % cells were in the late apoptosis stage. After cells were treated with 50 μM salinomycin for 12 h, the percentages of apoptotic cells at the early phase increased to 6.95±1.82 % and that of the late phase increased to 13.03±0.38 %. Whereas when cells were treated with salinomycin for 24 and 36 h, 9.70±1.77 and 9.35±1.79 % cells were in the early apoptosis, and 24.5±1.71 and 30.53±1.55 % cells were in the late apoptosis. A time-dependent increase in the number of apoptotic cells was observed (Fig. 2b). These results clearly indicate that salinomycin evoked apoptosis in OV2008 cells.

The effect of different concentrations of salinomycin (20 and 50 μM) on the cell cycle phases was investigated in OV2008 cells cultured over different times (12 and 24 h) by DNA content analysis and by flow cytometry. The results showed that the percentages of the cell population in the sub-G1 phase were significantly higher in salinomycin-treated OV2008 cells with a concentration-dependent fashion, whereas the percentages of cells in other phases (G1/G0, S, and G2/M phases) were almost reduced, in comparison with the control (Fig. 3). These effects were similar at 12 and 24 h (Fig. 3). The dramatic accumulation of cells in the sub-G1 phase was another marker for apoptosis, which further confirmed the results of annexin V/PI assay. Additionally,

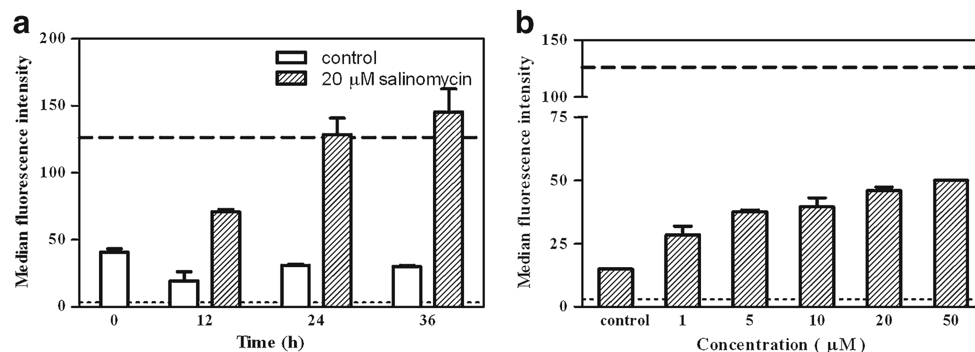

Fig. 4 Effect of salinomycin on phospho-p38 MAPK level in OV2008 cells. **a** The phospho-p38 MAPK level in OV2008 cells was assessed by the Bio-Plex assay at the indicated intervals after 20 μM salinomycin treatment (0.1 % DMSO as solvent control). After two washes with ice-cold cell wash buffer, monolayer cells were lysed. The fluorescence intensity of phospho-p38 MAPK in OV2008 was counted by the Bio-Plex suspension array system. **b** The OV2008 cells were incubated with salinomycin (1, 5, 10, 20, 50 μM) or with solvent control (0.1 % DMSO) for 24 h and phosphoprotein analysis was performed as described above. The *dotted line* shows the signal intensity of phosphotase-treated HeLa cells as a background control. The *dashed line* shows the signal intensity of positive control. Results are expressed as mean±SD

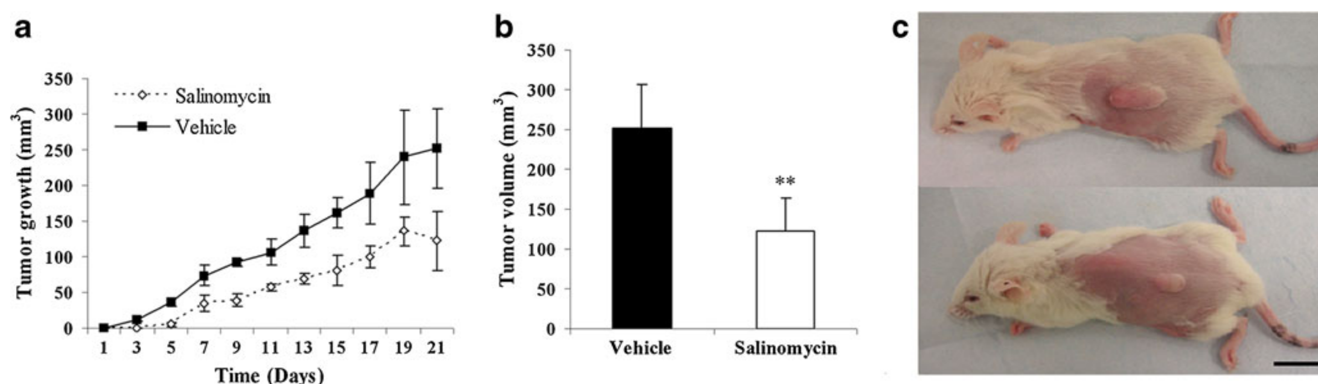

Fig. 5 Antitumor activity of salinomycin on NOD/SCID mice bearing human OV2008 cells. **a** Tumor growth curves of the mice treated with salinomycin (5 mg/kg) and vehicle control (5 % ethanol). **b** Final volume of tumors in salinomycin- and vehicle-treated animals on the 21st day after tumor injection. Data are presented as mean±SD of tumor volumes (n=5). **P<0.01: significant difference from vehicle control. **c** One of the vehicle control group mice (*up*) and one of the salinomycin-treated group mice (*down*). Tumor size in salinomycin-treated mouse (*down*) was significantly reduced relative to tumor in vehicle-treated mouse (*up*). *Black bar* indicates 1 cm

there was no cell cycle arrest in the G1/G0, S, and G2/M phases between salinomycin-treated cells and control cells, suggesting that salinomycin inhibits the cellular proliferation of OV2008 cells not accompanied by cell cycle arrest.

Effect of salinomycin on phosphorylation of p38 MAPK in OV2008 cells

To investigate the effect of salinomycin on p38 MAPK activity in ovarian cancer cell line OV2008, the regulation of p38 MAPK phosphorylation by salinomycin was examined using the Bio-Plex phosphoprotein assay. The results showed that phosphorylation of p38 MAPK in OV2008 cells was enhanced by salinomycin (20 μM) after 12, 24, and 36 h of incubation (Fig. 4a), while a marked concentration-dependent increase in the p38 MAPK phosphorylation was observed following salinomycin exposure for 24 h (Fig. 4b). These findings suggest that the salinomycin-induced growth inhibitory effect and apoptosis in OV2008 could be mediated through the alteration of phosphorylation of p38 MAPK.

Evaluation of antitumor activity of salinomycin in vivo

Based on the in vitro results, which showed significant cytotoxicity of salinomycin to human ovarian cancer cell line OV2008, the in vivo antitumor efficacy of salinomycin was further evaluated in a human ovarian tumor xenograft grown in the back of mice. The mice were treated with salinomycin, and the change in tumor volume after first injection was followed for 21 days (Fig. 5a). Compared with the vehicle-treated controls, a significant reduction in the tumor volume was observed in the mice treated with salinomycin (Fig. 5c). When the test came to an end, in OV2008 tumor model, the tumor volume of salinomycin therapy groups and controls was 122.3±41.4 and 252.2±55.29 mm^3, respectively (P<0.01; Fig. 5b).

Discussion

The present study demonstrated that salinomycin inhibited the growth of human ovarian cancer cell line OV2008 in vitro and in vivo. The growth inhibition effects of salinomycin and salinomycin-induced apoptosis in human ovarian cancer cell line OV2008 could correlate with modulating p38 MAPK.

Apoptosis, or programmed cell death, is an important homeostatic mechanism that balances cell division and cell death and maintains the appropriate cell number in the body [19]. Therefore, searching for agents which trigger apoptosis of tumor cells has become an attractive strategy in anticancer drug discovery [20]. Apoptosis is characterized morphologically by cell shrinkage and loss of contact with neighboring cells, formation of cytoplasmic vacuoles, plasma and nuclear membrane blebbing, chromatin condensation, and formation of apoptotic bodies [21]. In the present investigation, after OV2008 cells were treated with salinomycin, the fluorescence microscopic observations clearly indicated these apoptotic characteristics (Fig. S1). Furthermore, flow cytometry results, from both annexin V/PI assay (Fig. 2) and sub-G1 populations in cell cycle analysis (Fig. 3), further evidenced a concentration- and time-dependent increase in the percentage of apoptotic subpopulations after salinomycin treatment. These results provided evidence that salinomycin triggered apoptosis in OV2008 cells, which resembles a previous report on various human cancer cells such as leukemia cells [8, 9].

Cell cycle control plays a critical role in the regulation of tumor cell proliferation. Many anticancer agents and DNA-

damaging agents arrest the cell cycle at the G0/G1, S, or G2/M phase and then induce apoptotic cell death [22, 23]. From the results of the present study, it appeared that no cell cycle arrest in the G1/G0, S and G2/M phases was observed between salinomycin-treated OV2008 cells and control cells (Fig. 3), which confirms the previous finding that salinomycin activates a particular apoptotic pathway not accompanied by cell cycle arrest [8].

To better understand the signal pathways involved in salinomycin-induced growth inhibitory effect and apoptosis in OV2008, we investigated the possible involvement of p38 MAPK activity. To address this issue, phosphorylation of p38 MAPK was determined by Bio-Plex assays with Luminex technology, which contained dyed beads conjugated with monoclonal antibodies specific for a target protein or peptide such as a cytokine or a phosphoprotein. The antibodies used in these assays undergo rigorous optimization to ensure the highest degree of sensitivity, specificity, and reproducibility. Recently, using optimized standard operating procedures regarding sample size and total protein concentration range and monoclonal antibodies used for immunoanalysis, and on the basis of the US Food and Drug Administration guidelines, Bio-Plex phosphoprotein array intra-assay and inter-assay coefficients of variation revealed good reproducibility of the technique and the results achieved using Bio-Plex phosphoprotein array analyses significantly correlated ($P<0.001$) with those obtained with numerized western blot analyses [18]. Furthermore, Bland–Altman analyses clearly demonstrated that Bio-Plex phosphoprotein array could be used instead of western blot, providing a unique way of analyzing multiple phosphoprotein expression in small specimens.

The p38 MAPK pathway is implicated in cancer cell apoptosis and is induced by several chemotherapeutic drugs [24, 25]. We found that there are marked time-dependent and concentration-dependent increases in the phosphorylation of p38 MAPK following salinomycin treatment in OV2008 cells (Fig. 4). This result suggests that the activation of p38 MAPK appears to contribute to the proapoptotic effect of salinomycin in OV2008 cells and that the activation of the p38 MAPK pathway might play a causal role in the salinomycin-induced apoptosis in ovarian cancer cell line OV2008. However, detailed downstream and upstream signaling molecules of p38 MAPK modulated by salinomycin are not known and warranted further investigations.

In the present study, the xenografts of human OV2008 ovarian cancer model showed very good efficacy when treated with salinomycin (Fig. 5). Although we have not yet attempted to ascertain the mechanism of cell death in the xenograft tumor model, it remains possible that cell apoptosis induced by salinomycin may account for some of the observed reduction in tumor growth rate and needs further investigations. Additionally, considering one ovarian cell line was involved in the present study, we also believe that further in vitro and in vivo studies with salinomycin in different characterized ovarian cancer cell lines, such as p53 mutation cell lines, drug-resistant (MDR overexpression) cell lines are warranted to enhance our understanding of this promising antitumorigenic compound.

Overall, the results of this research demonstrated that salinomycin is a potent compound against human ovarian cancer cell line OV2008 in vitro and indicates significant in vivo efficacy in the tumor (OV2008) xenograft model. Salinomycin can inhibit the growth of ovarian cancer cell line OV2008 efficiently through induction of apoptosis, which is not accompanied by cell cycle arrest, but possibly is associated with activating p38 MAPK and merits further investigations.

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Conflicts of interest None.

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