

Apoptotic effects of salinomycin on human ovarian cancer cell line (OVCAR-3)

Fuat Kaplan¹ · Fulya Teksen²

Received: 16 June 2015 / Accepted: 9 October 2015 / Published online: 18 October 2015
© International Society of Oncology and BioMarkers (ISOBM) 2015

Abstract In this study, we studied the apoptotic and cytotoxic effects of salinomycin on human ovarian cancer cell line (OVCAR-3) as salinomycin is known as a selectively cancer stem cell killer agent. We used immortal human ovarian epithelial cell line (IHOEC) as control group. Ovarian cancer cells and ovarian epithelial cells were treated by different concentrations of salinomycin such as 0.1, 1, and 40 μM and incubated for 24, 48, and 72 h. Dimethylthiazol (MTT) cell viability assay was performed to determine cell viability and toxicity. On the other hand, the expression levels of some of the apoptosis-related genes, namely anti-apoptotic Bcl-2, apoptotic Bax, and Caspase-3 were determined by quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, Caspase-3 protein level was also determined. As a result, we concluded that incubation of human OVCAR-3 by 0.1 μM concentration of salinomycin for 24 h killed 40 % of the cancer cells by activating apoptosis but had no effect on normal cells. The apoptotic Bax gene expression was upregulated but anti-apoptotic Bcl-2 gene expression was downregulated. Active Caspase-3 protein level was increased significantly ($p < 0.05$).

Keywords Ovarian cancer · Apoptosis · Salinomycin · Cell viability

✉ Fuat Kaplan
fuat_kaplan@hotmail.com

✉ Fulya Teksen
fteksen58@windowslive.com

¹ Health Science Institute, Biochemistry, Hacettepe University, Ankara, Turkey

² Medical Biology Department, Faculty of Medicine, Ankara University, Ankara, Turkey

Introduction

Ovarian cancer is one of the most common gynecologic cancers in Western countries [1]. The treatment protocol is generally surgery and chemotherapy, but again, the survival rate is not more than 30 % [2]. Apoptosis is the programmed cell death that response to DNA damage, hypoxia, or oncogene overexpression. There are two major pathways, the extrinsic death receptor pathway and intrinsic mitochondrial pathway [3]. The extrinsic pathway is triggered by binding of cell membrane death receptors (TNF, Fas, etc.) with their ligands. After this interaction, caspase-8 is activated and it activates caspase-3 [4]. The intrinsic pathway is triggered by mitochondrial proteins such as Bcl-2 family (proapoptotic: Bax, Bak, Bim, etc. and anti-apoptotic: Bcl-2, Bcl-xL, etc.) and cytochrome-c. When the exterior mitochondrial membrane becomes permeable, cytochrome-c passes the cytosol and recruits apoptosome complex. This activation triggers caspase 9/3 signaling cascade. Caspase-3 is activated at the end and apoptosis begins [5, 6]. The releasing of cytochrome-c from mitochondria is controlled by anti-apoptotic Bcl-2 and proapoptotic Bax proteins [7].

Salinomycin is an antibiotic which is isolated from *Streptomyces albus* [8]. It affects different biological membranes such as mitochondrial membrane and cell membrane, by promoting the mitochondrial and cellular potassium efflux and inhibiting mitochondrial oxidative phosphorylation [9]. Generally, it is used as an anticoccidial drug in poultry and shown to be effective in nutrition [10–12]. Besides, salinomycin has recently been shown to be selectively effective in inhibiting the breast cancer stem cells in mice. The mechanism could not be explained but it was suggested that salinomycin could be used as an anticancer drug [13]. In another study, the importance of a decrease in intracellular potassium concentration for the induction of apoptosis in human lymphoma cells was

Table 1 qRT-PCR primers

Hprt	F: TGACACTGGCAAAAACAATGCA R: GGTCCTTTTCACCAGCAAGCT
Bcl-2	F: CGGTGGGGTCATGTGTGTGGA R: GGGGCAGGCATGTTGACTTCACT
Bax	F: TGGCAGCTGACATGTTTTCTGAC R: TCACCCAACCACCTGGTCTT
Caspase-3	F: ACAAATGGACCTGTTGACCTGA R: ACACCACTGTCTGTCTCAATGC

HPRT gene was used as housekeeping control gene

emphasized [14]. Fuchs et al. demonstrated that salinomycin had the capacity of overcoming ATP-binding cassette (ABC) transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like cells [15]. Also in another study, it was revealed that salinomycin induced apoptosis and overcame apoptosis resistance in various human cancer cells from different origins [16]. It was also shown that salinomycin was effective in inducing apoptosis independent of p53 gene [16].

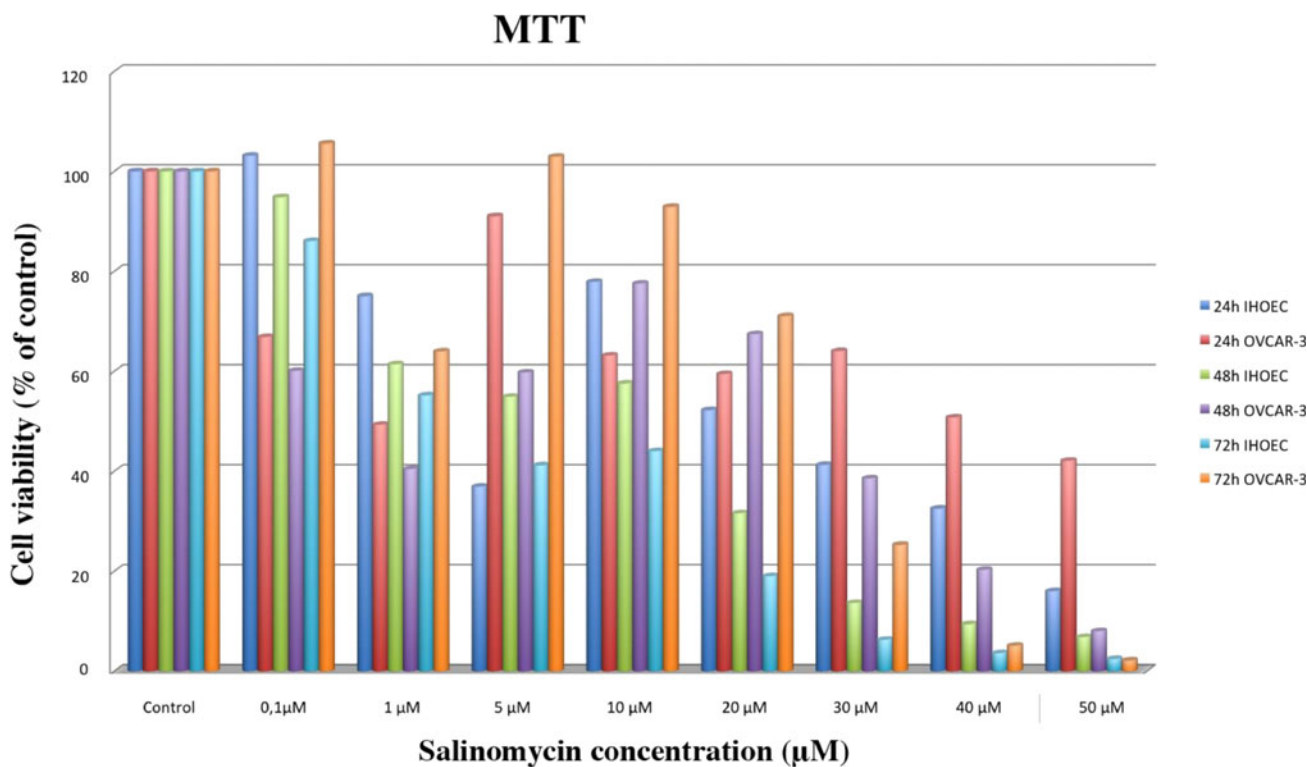
Fig. 2 Bcl-2, Bax, and Caspase-3 gene expression profiles at 24 h of incubation with salinomycin for different doses

As all these findings suggested that salinomycin could be used as an anticancer agent in many kind of cancers including ovarian cancer, we aimed to investigate the apoptotic effects of salinomycin on human ovarian cancer cell line (OVCAR-3) in this study.

Material and method

Cell lines and cell culture

The OVCAR-3 was obtained from the American Type Culture Collection (ATCC) and incubated at 5 % CO₂ and 37 °C. The cell culture was performed with RPMI 1640 medium containing L-glutamine (Sigma Aldrich), 20 % fetal bovine serum, and antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin) (Sigma Aldrich).



	Control	0,1µM	1 µM	5 µM	10 µM	20 µM	30 µM	40 µM	50 µM
24h IHOEC	100,00	103,15	75,04	36,93	77,91	52,23	41,28	32,54	16,03
24h OVCAR-3	100,00	66,86	49,32	91,03	63,15	59,45	64,05	50,77	42,11
48h IHOEC	100,00	94,84	61,40	54,93	57,56	31,61	13,65	9,42	6,84
48h OVCAR-3	100,00	60,12	40,46	59,77	77,56	67,46	38,59	20,29	8,02
72h IHOEC	100,00	86,06	55,19	41,18	44,03	19,03	6,29	3,61	2,45
72h OVCAR-3	100,00	105,57	63,98	102,95	92,91	71,06	25,29	5,12	2,15

Fig. 1 MTT cell viability results for immortal human ovarian cancer cells (IHOEC) and OVCAR-3 cancer cells

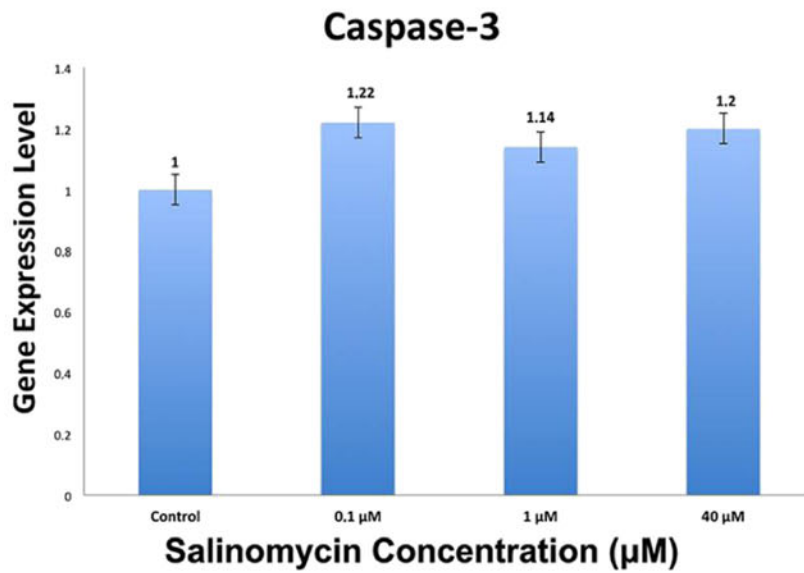
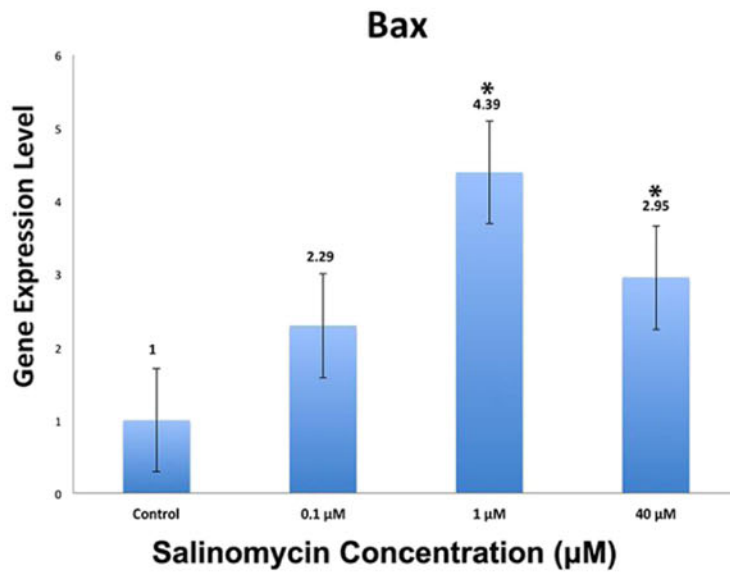
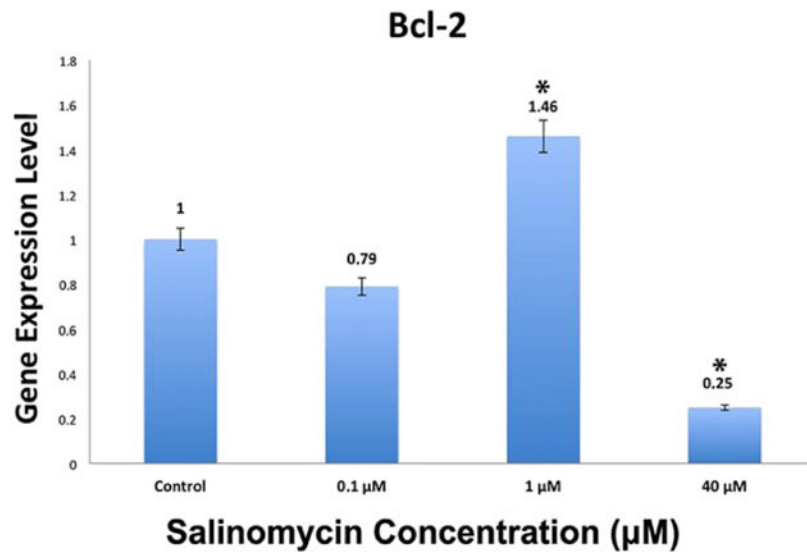


Table 2 Bax, Bcl-2, and Caspase-3 genes expression profiles

Gene	0.1 μ M of salinomycin Control=1	1 μ M of salinomycin Control=1	40 μ M of salinomycin Control=1
Bcl-2	0.79 ($p=0.14$)	1.46 ($p=0.02$)	0.25 ($p=0.04$)
Bax	2.29 ($p=0.16$)	4.39 ($p=0.036$)	2.95 ($p=0.001$)
Caspase-3	No change	No change	No change

For the control group, immortal human ovarian epithelial cell line (IHOEC) was obtained from the Applied Biological Materials Inc. (ABM) and grown in a special Prigrow I Medium (ABM) with 10 % FBS.

Cell viability and toxicity

Both cancer cells and epithelial cells were incubated in 96-well plates and treated by different concentrations (0.1–1–5–10–20–30–40–50 μ M) of salinomycin (Sigma Aldrich, S4526), and 0.1 % methanol was used as control solution. Cells were incubated for 24, 48, and 72 h. Dimethylthiazol (MTT) assay (Roche) was used to determine cell viability and toxicity [17]. A colorimetric analysis was performed at 550–690 nm.

Gene expression analysis

OVCAR-3 cells were treated with 0.1, 1, and 40 μ M of salinomycin and after 24 h of incubation; cells were collected for RNA isolation. Transcriptor High Fidelity cDNA synthesis Kit (Roche) was used for cDNA synthesis, and LightCycler 480 Syber Green I Master Kit (Roche) was used for quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The apoptosis-related genes Bcl-2, Bax, and Caspase-3 were determined by qRT-PCR.

The designed primers were given in Table 1.

Caspase-3 activity and apoptosis

OVCAR-3 cells were treated with 0.1, 1, and 40 μ M of salinomycin. After 24 h of incubation, cells were collected and Trizol Reagent (Sigma Aldrich) was used for protein isolation. Caspase-3 protein level was measured by using Bradford assay with bovine serum albumin (BSA). Caspase-3 Colorimetric kit was used for detecting active protein level. Colorimetric data analysis was performed at 405 nm.

Statistical analysis

MTT results were analyzed by ANOVA test; qRT-PCR and Caspase-3 colorimetric assay results were analyzed by *t* test. Results were statistically significant ($p<0.05$).

Results

Cell viability

Salinomycin reduced the cell viability of ovarian cancer cells at lower doses and had no effect on the ovarian epithelial cells. OVCAR-3 and immortal human ovarian epithelial cells (IHOEC) were treated by different doses of salinomycin for 24, 48, and 72 h. It was observed that 0.1 and 1 μ M salinomycin concentrations were the most effective doses for cancer cells and 24 h was the best incubation time. After 24 h incubation of 0.1 μ M salinomycin treatment, cancer cell viability was 66.85 % and epithelial cell viability was 100 %. For 1 μ M salinomycin treatment, cancer cell viability was 49.32 and epithelial cell viability was 75.04 %. Higher doses of salinomycin killed the cancer cells effectively, but at the same time, the normal cells also died (Fig. 1).

Gene expression levels of apoptosis-related genes

OVCAR-3 cells were treated by 0.1, 1, and 40 μ M salinomycin for 24 h. During the apoptosis, apoptotic Bax gene and anti-apoptotic Bcl-2 gene expressions have an important roles and Bax/Bcl2 rate is also used as an important apoptotic marker. Salinomycin at 0.1 and 1 μ M was our effective doses and 24 h was found to be the best incubation time.

Our results showed that after salinomycin treatment, Bcl-2 gene was downregulated and Bax gene was upregulated. As shown in Fig. 2 and Table 2, there was no change in gene expression level for caspase-3 gene in all doses of salinomycin treatment.

We treated cancer cells with 0.1, 1, and 40 μ M concentrations of salinomycin for 24 h, and after incubation time, gene expression results showed that Bax/Bcl-2 rate was increased significantly for all doses as given in Table 3.

Table 3 Bax/Bcl-2 ratio in cancer cells

Control (μ M)	Bax/Bcl-2=1
0.1	2.88, $p<0.05$
1	3.00, $p<0.05$
40	11.84, $p<0.05$

Active Caspase-3 protein level

During the apoptosis, activated caspase proteins are responsible for DNA damage and cell death. For that reason, Caspase-3 protein plays a key role during apoptosis and can be used as a marker [18]. After 0.1, 1, and 40 μM of salinomycin treatment, we have measured active Caspase-3 protein level in cancer cells against the control group (Fig. 3). We indicated increased Caspase-3 level for 0.1 and 1 μM doses ($p < 0.05$), but there was no change with the 40 μM treatment of salinomycin. We also observed that 40 μM was highly toxic for normal cells.

Discussion

It is a known fact that potassium is highly effective during apoptosis [19], and salinomycin helps to initiate apoptosis by decreasing intercellular potassium level.

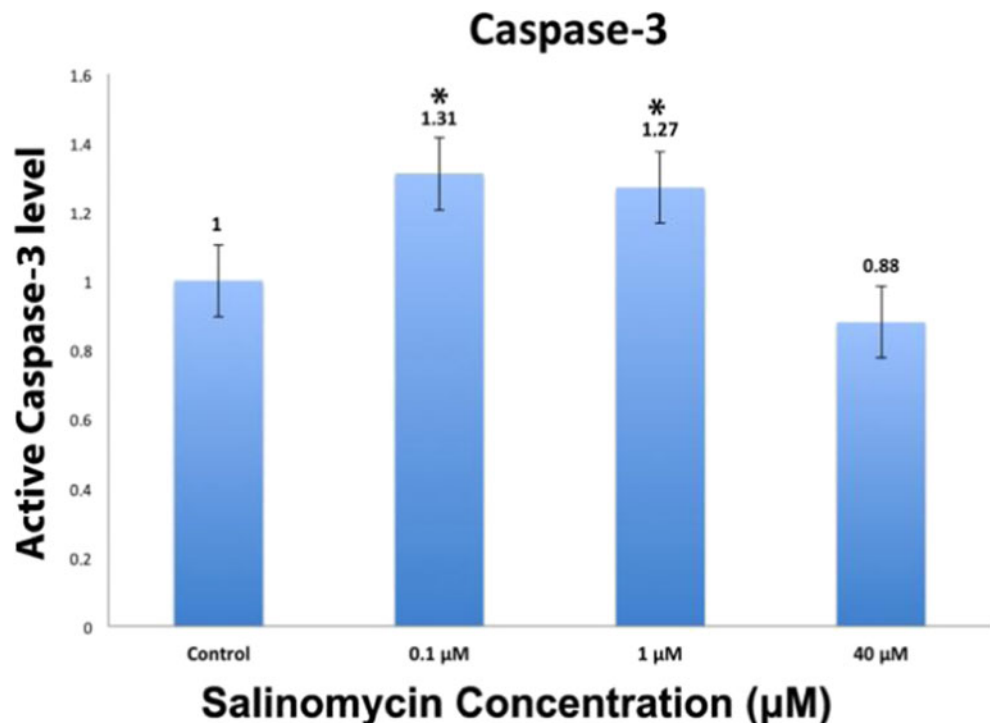
In their study, Parajuli and his group used A2780 and cisplatin-resistant type A2780 cis ovarian cancer cell lines and reported that salinomycin killed 40 % of the cells at 0.1 μM and 60 % of cells at 1 μM concentration [20]. In another study, it was shown that lower doses of salinomycin such as 0.15, 0.45, and 1.33 μM did not effect on normal prostate cells (RWPE-1) but were highly effective on human prostate cancer cells (PC-3 and DU-145) [21]. Ketola and his group treated prostate cancer cells and reported that prostate cancer epithelial cells incubated with 1 μM of salinomycin stopped the cell proliferation but had shown no effect on normal epithelial cells [22]. Similarly, we

also found that the doses of 0.1 and 1 μM were effective on cancer cells. On the other hand, higher doses of salinomycin also killed normal cells.

Parajuli and his group incubated the ovarian cancer cells (A2780 and cisplatin-resistant A2780cis) by salinomycin, and they determined Bax and Bcl-2 protein levels by Western blot technique. They found that Bcl-2 level was decreased, and there was no change in gene expression of Bax gene [20]. Another study showed that after 0.15–4 μM of salinomycin treatment on PC-3 prostate cancer cells, Bax level was increased and Bcl-2 gene level was decreased [21], which was quite similar to our results. Similarly, in another study, cisplatin-resistant colorectal cancer cell line SW620 was treated by 0.1–80 μM of salinomycin and qRT-PCR analysis showed that Caspase-3, Caspase-9, and Bax gene expressions were upregulated, but on the other hand, Bcl-2 expression was downregulated. Also, these results were supported by Western blot technique [23]. Our results were almost close to these results, but distinctively, we found no change on Caspase-3 gene expression. Shuang-ging and his group used gleevec-resistant chronic myeloid cancer cell line (K562/Glv) exposed to 2 μM salinomycin, and Western blot analysis showed that Bcl-2 protein level was decreased and Bax level was increased [24]. They also used Bax/Bcl-2 rate. These results were found to be in concordance with our study.

In a similar study in which Western blot analysis was performed, it was shown that after the treatment of 2 μM salinomycin on lung adenocarcinoma cells (A549/DDP), Bax protein level was increased but Bcl-2 level was decreased and the Bax/Bcl-2 rate was also increased [25].

Fig. 3 Active Caspase-3 protein levels for OVCAR-3 cells treated by 0.1, 1, and 40 μM of salinomycin



Salinomycin induced apoptosis on nasopharyngeal carcinoma cells (CNE-1) that were treated with 0, 1, 2, 8, and 16 μM salinomycin for 24 h. As a result, Bcl-2 protein level was decreased and Bax level was increased [26].

Wang, et al. have shown that salinomycin induced apoptosis on hepatocellular carcinoma cell (HepG2, SMMC-7721, BEL-7402) and the Bax/Bcl-2 rate was increased [27]. Our results were in concordance with this result, that we found increased Bax gene expression and decreased Bcl-2 gene expression.

It was reported that after 0.1 and 1 μM of salinomycin treatment on human ovarian cancer cells (OV2008) [20], and after 2 μM of salinomycin treatment on gleevec-resistant CML cells (K562/Glv) [24], active Caspase-3 protein level was increased significantly ($p < 0.05$). In another study, 2 μM of salinomycin treatment induced apoptosis and increased Caspase-3 level on cisplatin-resistant lung cancer cells (A549/DDP) [25]. A similar study indicated that salinomycin induced apoptosis on prostate cancer cells (PC-3, DU-145) and increased caspase-3 level [21]. Our results were almost close to these results that we found increased Caspase-3 protein level for 0.1 and 1 μM doses of salinomycin treatment.

In addition, a study in 2014 showed that salinomycin inhibited proliferation and invasion of NPC (nasopharyngeal carcinoma) cell lines and triggered apoptosis by activating caspase-3 and caspase-9 [26]. Another study in 2014 demonstrated that salinomycin and paclitaxel combined treatment reduced CD44⁺ ovarian cancer stem cells and induced apoptosis in ovarian cancer and cancer stem cells [28].

Finally, it was concluded that lower doses of salinomycin (0.1 and 1 μM) effect on cancer cells but not on normal epithelial cells. Our qRT-PCR analysis results showed that anti-apoptotic Bcl-2 gene was downregulated and apoptotic Bax gene was upregulated. Besides, the apoptotic Caspase-3 protein level was also increased.

In the light of these findings, it could be concluded that salinomycin being an ionophore molecule decreases intercellular potassium level and is capable of inducing apoptosis by the effect of apoptosis-related genes.

As far as we know, this is the first study in our country performed by salinomycin, over cancer. For further evaluation of the in vitro and in vivo effects of salinomycin on cancer cells, and the usage of salinomycin as a drug in humans, more planned prospective studies are needed.

Acknowledgments This Study was funded by the Ankara University BAP Project No: 13B3330001 and accepted as Master Thesis, by The Institute of Health Sciences.

References

- Jemal A et al. Cancer statistics, 2006. *CA Cancer J Clin.* 2006;56(2):106–30.
- Aletti GD et al. Current management strategies for ovarian cancer. *Mayo Clin Proc.* 2007;82(6):751–70.
- Eum KH, Lee M. Crosstalk between autophagy and apoptosis in the regulation of paclitaxel-induced cell death in v-Ha-ras-transformed fibroblasts. *Mol Cell Biochem.* 2011;348(1–2):61–8.
- Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer.* 1972;26(4):239–57.
- Engel T, Henshall DC. Apoptosis, Bcl-2 family proteins and caspases: the ABCs of seizure-damage and epileptogenesis? *Int J Physiol Pathophysiol Pharmacol.* 2009;1(2):97–115.
- Ghobrial IM, Witzig TE, Adjei AA. Targeting apoptosis pathways in cancer therapy. *CA Cancer J Clin.* 2005;55(3):178–94.
- Tsujimoto Y. Role of Bcl-2 family proteins in apoptosis: apoptosomes or mitochondria? *Genes Cells.* 1998;3(11):697–707.
- Miyazaki Y et al. Salinomycin, a new polyether antibiotic. *J Antibiot.* 1974;27(11):814–21.
- Mitani M, Yamanishi T, Miyazaki Y. Salinomycin: a new monovalent cation ionophore. *Biochem Biophys Res Commun.* 1975;66(4):1231–6.
- Butaye P, Devriese LA, Haesebrouck F. Antimicrobial growth promoters used in animal feed: effects of less well known antibiotics on gram-positive bacteria. *Clin Microbiol Rev.* 2003;16(2):175–88.
- Callaway TR et al. Ionophores: their use as ruminant growth promotants and impact on food safety. *Curr Issues Intest Microbiol.* 2003;4(2):43–51.
- Danforth HD et al. Anticoccidial activity of salinomycin in battery raised broiler chickens. *Poult Sci.* 1977;56(3):926–32.
- Gupta PB et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. *Cell.* 2009;138(4):645–59.
- Bortner CD, Hughes Jr FM, Cidowski JA. A primary role for K⁺ and Na⁺ efflux in the activation of apoptosis. *J Biol Chem.* 1997;272(51):32436–42.
- Fuchs D et al. Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. *Biochem Biophys Res Commun.* 2010;394(4):1098–104.
- Fuchs D et al. Salinomycin induces apoptosis and overcomes apoptosis resistance in human cancer cells. *Biochem Biophys Res Commun.* 2009;390(3):743–9.
- Morgan DM. Tetrazolium (MTT) assay for cellular viability and activity. *Methods Mol Biol.* 1998;79:179–83.
- Abu-Qare AW, Abou-Donia MB. Biomarkers of apoptosis: release of cytochrome c, activation of caspase-3, induction of 8-hydroxy-2'-deoxyguanosine, increased 3-nitrotyrosine, and alteration of p53 gene. *J Toxicol Environ Health B Crit Rev.* 2001;4(3):313–32.
- Park IS, Kim JE. Potassium efflux during apoptosis. *J Biochem Mol Biol.* 2002;35(1):41–6.
- Parajuli B et al. Salinomycin inhibits Akt/NF-kappaB and induces apoptosis in cisplatin resistant ovarian cancer cells. *Cancer Epidemiol.* 2013;37(4):512–7.
- Kim KY et al. Salinomycin-induced apoptosis of human prostate cancer cells due to accumulated reactive oxygen species and mitochondrial membrane depolarization. *Biochem Biophys Res Commun.* 2011;413(1):80–6.
- Ketola K et al. Salinomycin inhibits prostate cancer growth and migration via induction of oxidative stress. *Br J Cancer.* 2012;106(1):99–106.
- Zhou J et al. Salinomycin induces apoptosis in cisplatin-resistant colorectal cancer cells by accumulation of reactive oxygen species. *Toxicol Lett.* 2013;222(2):139–45.
- Xu S-q, Z A-z, Liu C-c, Xu T-t, Chen X-y, Liu G-x. Salinomycin inhibits proliferation and induces apoptosis of Gleevec-resistant chronic myeloid leukemic cell line K562/Glv. *Chin J Pathophysiol.* 2012;28:1208–1212.

25. Zeng J, Liu C-c, Zhu A-z, Chen X-y, Tan G-x, Liu G-x. Salinomycin inhibited proliferation and induced apoptosis of cisplatin-resistant human lung adenocarcinoma cell line A549/DDP.pdf. *Chin J Pathophysiol*. 2012;28:834–8.
26. Wu D et al. Salinomycin inhibits proliferation and induces apoptosis of human nasopharyngeal carcinoma cell in vitro and suppresses tumor growth in vivo. *Biochem Biophys Res Commun*. 2014;443(2):712–7.
27. Wang F et al. Salinomycin inhibits proliferation and induces apoptosis of human hepatocellular carcinoma cells in vitro and in vivo. *PLoS One*. 2012;7(12):e50638.
28. Shin S-J et al. Salinomycin have antiproliferative and apoptotic effects on ovarian cancer stem-like cell. In: *Proceedings of the 105th annual meeting of the american association for cancer research*. 2014. *Cancer Res* 2014: 5–9.