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RESEARCH ARTICLE

# Effect of Salinomycin on Expression Pattern of Genes Associated with Apoptosis in Endometrial Cancer Cell Line

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**Abstract:** ***Background*:** Salinomycin is part of a group of ionophore antibiotics characterized by an activity towards tumor cells. To this day, the mechanism through which salinomycin induces their apoptosis is not fully known yet. The goal of this study was to assess the expression pattern of genes and the proteins coded by them connected with the process of programmed cell death in an endometrial cancer cell Ishikawa culture exposed to salinomycin and compared to the control.

***Materials and Methods*:** Analysis of the effect of salinomycin on Ishikawa endometrial cancer cells (ECACC 99040201) included a cytotoxicity MTT test (with a concentration range of 0.1-100 μM), assessment of the induction of apoptosis and necrosis by salinomycin at a concentration of 1 μM as well the assessment of the expression of the genes chosen in the microarray experiment (microarray HG-U 133A_2) and the proteins coded by them connected with apoptosis (RTqPCR, ELISA assay). The statistical significance level for all analyses carried out as part of this study was $p<0.05$.

***Results*:** It was observed that salinomycin causes the death of about 50% of cells treated by it (50.74±0.80% of all cells) at a concentration of 1μM. The decrease in the number of living cells was determined directly after treatment of the cells with the drug (time 0). The average percent of late apoptotic cells was 1.65±0.24% and 0.57±0.01% for necrotic cells throughout the entire observation period.

***Discussion*:** Microarray analysis indicated the following number of mRNA differentiating the culture depending on the time of incubation with the drug: H_12 vs C = 114 mRNA, H_8 vs C = 84 mRNA, H_48 *vs*. C = 27 mRNA, whereas 5 mRNAs were expressed differently at all times. During the whole incubation period of the cells with the drug, the following dependence of the expression profile of the analyzed transcripts was observed: Bax>*p53*>*FASL*>*BIRC5*>*BCL2L.*

***Conclusion*:** The analysis carried out indicated that salinomycin, at a concentration of 1 μM, stopped the proliferation of 50% of endometrial cancer cells, mainly by inducing the apoptotic process of the cells. The molecular exponent of the induction of programmed cell death was an observed increase in the transcriptional activity of pro-apoptotic genes: *Bax;p53;FASL* and a decrease in the expression of anti-apoptotic genes: *BCL2L2; BIRC5.*

**Keywords:** Salinomycin, apoptosis, microarray technique, RTqPCR, ELISA, assayed pro- and anti-apoptotic genes, MTT assay.

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## 1. INTRODUCTION

Salinomycin is part of a group of ionophore antibiotics, therefore it is an ionophore carrier. It was isolated in 1974 from a bacterial strain (*Streptomyces albus*). Its mechanism is related to distorting the ionic balance of $Na^+/K^+$ in cell membranes, which leads to the activation of programmed cell death (apoptosis) [1, 2]. In the first stage, condensation of the genetic material in the cell nucleus is observed. The following stage is the fragmentation of the cell nucleus and other organelles. The final stage of apoptosis is phagocytosis of the cell through macrophages [3, 4].

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This antibiotic is characterized by a high activity in relation to Gram-positive bacteria, mycobacteria, Staphylococcus aureus and HIV [5, 6]. However, in recent times, the anti-tumor traits of salinomycin have also been researched and observed; these traits were confirmed in both *in vitro* and *in vivo* models. This has indicated that the drug is an attractive therapeutic strategy in the case of tumors characterized by a multidrug resistance [7, 8].

The positive effect of salinomycin is described in for example breast cancer [9]; ovarian cancer [10]; or osteosarcoma [11]. Whereas, knowledge on the effect of salinomycin on the progression of endometrial cancer, being the sixth most diagnosed gynecological tumor, is fragmented [12]. Without a doubt, an advantage of salinomycin is the appearance of only insignificant, temporary side effects, such as hand tremors and an increased heart rate [1]. As of yet, the mechanism through which salinomycin induces apoptosis of tumor cells has not become fully known. Nonetheless, however, the high affinity between salinomycin and potassium cations has been confirmed, which results in their outflow from the mitochondria and the cytoplasm [13, 14]. A lowered level of potassium ions is most likely an important factor in the induction of apoptosis. It has also been confirmed that salinomycin causes changes on the molecular level. It was determined that there was an increase in the expression of caspases 3,8 and 9 of the pro-apoptotic protein Bax mainly as well as a lowered expression of the anti-apoptotic protein Bcl2, a Nuclear Factor of kappa B (NFkB) was observed [15].

The goal of this study was to assess the expression pattern of genes and the proteins those genes encode connected with the process of programmed cell death in endometrial cancer cell cultures of the Ishikawa cell line [16] when exposed to salinomycin, in comparison to the control. The potential of the cytotoxic drug was also assessed as well as its ability to induce apoptosis and necrosis in cells of cell line Ishikawa.

## 2. MATERIALS AND METHODS

### 2.1. Cell Culture

In this work, cells of the endometrial cancer cell line Ishikawa (European Collection of Authenticated Cell Cultures; ECACC 99040201) were used. These cells were grown in the Minimum Essential Medium (MEM) supplemented with 2 mM of glutamine, 1% Non-Essential Amino Acids (NEAA), and 5% Fetal Bovine Serum (FBS), according to the manufacturer's protocol. The cells were incubated at a constant temperature of 37ºC with a 5% $CO_2$ enriched atmosphere. All reagents were obtained from Sigma Aldrich, St Louis, MO, USA.

### 2.2. Cell Survival Assay

In order to determine cell viability, the MTT assay was utilized; the assay consists of measuring the absorbance of a violet solution of formazan (a product created by a reduction of the yellow tetrazole salt MTT that is water-soluble) through the use of mitochondrial succinate dehydrogenase of living cells. The quantity of reaction product (water-insoluble crystals of formazan) is proportional to the quantity of viable and metabolically active cells that are in the sample.

Firstly, it was decided to assess the effect of different concentrations of salinomycin (0.1 μM, 1 μM, 10 μM, 100 μM) on the vitality of endometrial cancer cells. To achieve this, the cytotoxicity MTT test was carried out (Sigma Aldrich, Merck, Cell Proliferation Kit I MTT). After 24 hours of the culture breeding, salinomycin was added in the concentration range of 0.1 μM-100 μM and subsequently incubated for 24 hours. After this time, the culture medium was removed, the monolayers of the cells were washed using PBS and a new medium was added. After 48 hours, the MTT test was done in accordance with the manufacturer's recommendations. After a gentle mix of the formazan solution, its absorbance was read at a wavelength of $\lambda = 580$ nm with a reference wavelength of $\lambda = 720$ nm. In order to calculate the percentage of viable cells in the sample, the absorbance values of the test samples were compared to the absorbance values collected from the control (the control was made up of cells not treated with compounds). The control absorbance was assumed to be 100%. Based on the obtained results, the concentration of salinomycin was determined, which decreased the number of living cells by 50% (IC50). The concentration of salinomycin chosen was used in the further parts of the experiment, during which the endometrial cancer cells were exposed to the drug for periods of 12, 24 and 48 hours and afterwards compared to the culture not treated with salinomycin (control).

### 2.3. Hoechst 33258 and Propidium Iodide Staining

Moreover, the fraction of apoptotic cells and necrotic cells was determined using the fluorescence microscopy technique (double staining of the cells with fluorescent dyes, Hoechst 33258 and propidium iodide) in the culture exposed to salinomycin at the concentration of IC50 for 12, 24 and 48 hour periods according to recommendations. The simultaneous use of two fluorescent dyes differing with the mechanism of entering the cell and in fluorescent spectrum allowed for identifying, in the same sample, 4 types of cell: alive (weak, matte light blue fluorescence); early apoptotic (bright light blue fluorescence); late apoptotic (pink-purple fluorescence); and necrotic (intense red fluorescence). Propidium iodide (PI) has a negative charge and can only enter a cell through a damaged cell membrane, which allows for the identification of necrotic cells or cells in the late stages of apoptosis. Whereas Hoechst 33258 can only enter through an undamaged cell membrane of a living cell or an early apoptotic cell. As a result of dye penetration through intact biological membranes, the nuclear DNA of the cell is dyed a light blue color. The intensity of the dye fluorescence is connected with the degree of DNA packing, which allows for, based on the intensity of the fluorescence of the fluorochrome in the cell nucleus, distinguishing highly fluorescent apoptotic cells, containing highly condensed chromatin, from weakly fluorescent living cells, containing looser chromatin. Both fluorochromes are excited by ultraviolet light- propidium iodide characterized by an orange-red fluorescence, whereas by Hoechst 33258 - blue. After a finished 10 minute incubation of the cells with two fluorochromes, the cell suspension, after a delicate and thorough stir, was applied to slides and analyzed under a fluorescent Olympus IX 70 microscope, using the UV 360-370 nm filter.

In each sample, there were 300 counted cells in the field of view. The sum of all cells was assumed to be 100%, and the content of living cells, early and late apoptotic as well necrotic was counted as a fraction of the whole.

### 2.4. Microarray, Real-Time PCR and ELISA Analysis

Molecular analysis included determining the changes in expression of genes connected with the apoptosis process based on the microarray technique mRNA HG-U 133A_2 (Affymetrix, Santa Clara, CA), according to the manufacturer's recommendation.

The first step was to prepare and add an exogenous poly-A RNA control to the template in order to control the RNA preparation and hybridization processes. The development of oligonucleotide microarrays was initiated through the synthesis of double-stranded cDNA on the matrix of the resultant ribonucleic acid extract. The GeneChip 30IVT Express Kit reagent kit and 250 ng total RNA were utilized for the synthesis of the double-stranded cDNA. 5µl of the total RNA mixture was combined with the poly-A control and 5µl of the First Strand Master Mix in tubes during the first stage. The incubation of this new mixture lasted for 2 hours at 42ºC. In the subsequent stage, 20µl of the Second Strand Master Mix was added to the reaction mixture and after that incubated for 1 hr at 16ºC and then again for 10 minutes at 65ºC. Subsequently, the biotinyl aRNA synthesis was performed utilizing the GeneChip 30IVT Express Kit. In order to achieve this, 30µl of IVTMaster Mix for cDNA was added. The incubation of this mixture then lasted for 16 hours at 40ºC. The aRNA was labeled as biotin; it was also purified using the aRNA binding mix, and after that fragmented using an array fragmentation buffer for 35 minutes at 94ºC. The HG-U133A_2 microarray was hybridized to the fragmented aRNA for a 16-hour period at 45ºC. The hybridization mixture was prepared with the GeneChip Hybridization kit, Wash and Stain Kit. The Affymetrix Gene ArrayScanner 3000 7G and GeneChip®Command Console®Software were utilized for analysis of the fluorescence intensity.

The validation of the results was done based on the Real-Time Quantitative Reverse Transcription Reaction (RTqPCR) with the use of SensiFast SYBR No-ROX One-Step Kit (Bioline, London, UK) for 5 mRNAs: *Bcl2l2; Bax; p53; BIRC5; FASL.* β- actin (*ACTB)* was used as an endogenous control. The oligonucleotide primers sequence for *Bcl2l2 (Forward 5`*- GTAGTTGGAGATGAGTTCGAGATTC-3`; Reverse 5`- TTCATCGAAAACCTAAATAAAACGT-3`), *Bax (5`*- Forward 5`- TTGTTTTAGGGTTTTATTTAGGATC-3`; Reverse 5`- ACTCCATATTACTATCCAATTCGTC-3`), *p53* (Forward 5`- ATTTTAGTGGTAATTTATTGGGACG-3`; Reverse 5`- TAATAAAACTCCCCTTTCTTACGAA-3`), *BIRC5* (Forward 5`- TTGTGGATTTTATTGGGTTTTTAA-3`,Reverse 5`- AATACATTTTCAATTATTTCTACCC-3`), *FASL* (Forward 5`- AGAAGGTTTGGTTAAAGGAGGTT-AT-3`, Reverse 5`- CATAAAAAACATCACAAAAAAAC-ACA-3`), *ACTB* (Forward :5'-TCACCCACACTGTGCC-CATCTACGA-3'; Reverse:5'-CAGCGGAACCGCTCATT-GCCAATGG-3').

In the thermal profile, RTqPCR can be used to determine the following steps: reverse transcription (45ºC; 10 min); polymerase activation (95ºC; 2 min); 40 cycles including denaturation (95ºC; 5 s); annealing (60ºC; 10 s); and elongation (72ºC; 5 s).

The last step of the molecular analysis was associated with evaluating changes in the concentration of the Bcl-2L2 protein (Catalog #EHBCL2L2), p53 (Catalog # BMS256TEN) using the ELISA technique (Thermo Fisher Scientific, CA, USA) according to the manufacturer's protocol. Changes in the expression of the assessed mRNA were presented as a Fold Change (FC) in the expression of the gene in the culture treated with salinomycin compared to the culture.

### 2.5. Statistical Analysis

Statistical analysis of the obtained results was done using the Transcriptome Analysis Console program (Thermo Fisher Scientific) and STATISTICA 13 PL software (Cracow, Poland). The ANOVA variance analysis test was done as well as the post-hoc Tukey test ($p<0.05$). The results of the changes in the expression of mRNA were presented as Fold Change (FC) of the gene expression in comparison to the control.

## 3. RESULTS

The results of the MTT assay are presented in Fig. (**1**). Salinomycin in the concentration of 1 µM -100 µM caused a cytotoxic effect in terms of endometrial cancer cells. It increased together with an increase in the concentration of salinomycin. Taking into account our results obtained by the MTT assay, it can be observed that salinomycin at a concentration of 0.1 µM causes the death of 13.02% of endometrial cancer cells (percentage of live cells 86.97±2.80). For a concentration of 1 µM, it was observed that salinomycin causes the death of about 50% of cells treated with it (50.74±0.80 % of all cells), therefore also this concentration of salinomycin was used in further stages of the experiment (IC50). For 10 µM concentration of the drug, 68.92% percentage of death cells was noticed with respect to live cells (31.08±1.07), whereas increasing concentration of salinomycin to 100 µM was associated with the death of 88.96% Ishikawa endometrial cancer cells percentage of live cells (11.04±0.025). Statistically significant differences were observed between the cells treated with salinomycin at a concentration of 1 µM in comparison to the control ($p=0.0001$); cells exposed to 10 µM /ml salinomycin in comparison to the control ($p<0.0001$) as well as between the culture exposed to the drug at a concentration of 100 µM in comparison to the control ($p<0.0001$). It was not determined that the difference between the culture incubated with salinomycin at a concentration of 0.1 µM and the control was statistically significant ($p>0.05$ Fig. **1**).

Then an analysis of the effect of salinomycin at a concentration of 1 µM on the induction of apoptosis and necrosis of endometrial cancer cells was carried out. A decrease in the number of cells was observed directly after treating the cells with the drug (time 0). Further extension of the incubation period resulted in a further decrease in the percentage of living cells and an increase in the fraction of early apoptotic cells. The average percent of late apoptotic cells was 1.65±0.24%, and of necrotic ones was 0.57±0.01% in the whole observation period. Immediately after the addition of salinomycin at a concentration of 1µM to the culture, it

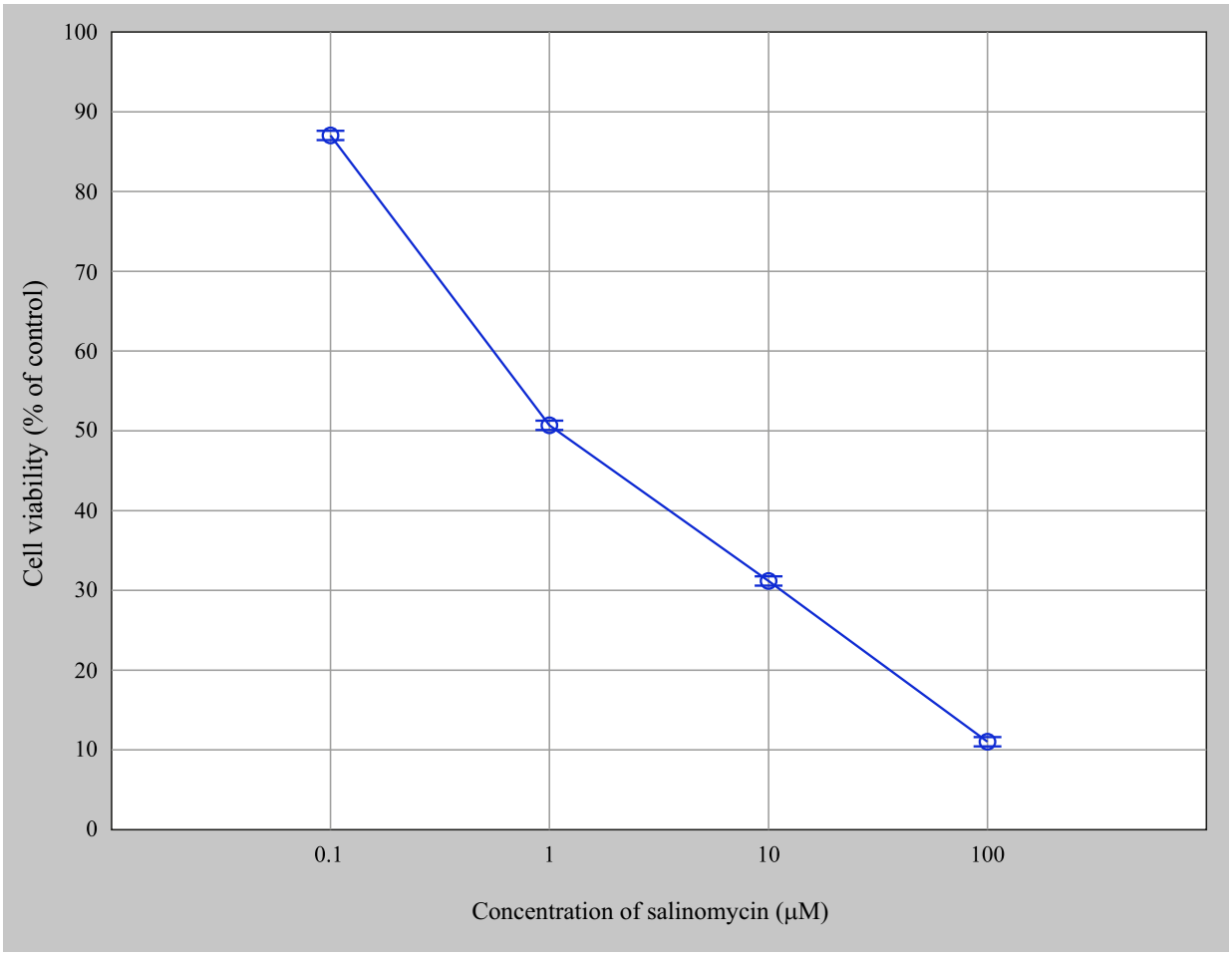


**Fig. (1).** Results of cytotoxicity of different concentrations of salinomycin (MTT assay) in endometrial cancer cells. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

could be determined that 94.56±0.53% of cells did not undergo any form of cell death; 2.89±0.59% were characterized as early apoptotic, 2.15±0.05% as late apoptotic, and 0.4±0.03% as cells that underwent necrosis. Whereas after 24-hour incubation of the cells with the drug, it was observed that 84.79±0.37% of cells did not undergo any form of cell death, 12,03±0.05% were qualified as early apoptotic, 2.43±0.38% as late apoptotic, and 0.79±0.01% as cells susceptible to necrosis. Increasing the exposition time to 24 hours caused the percentage of living cells to be 58,87±0.51%, early apoptotic- 39.55±0.49%, late apoptotic- 1.08±0.03%, and necrotic- 0.49±0.03%. Whereas when the endometrial cancer cells were incubated with salinomycin at a concentration of 1μM for two days (48h), the living cells were 58.99±0.11% of the whole population, early apoptotic- 39.99±0.13%, late apoptotic- 0.84±0.06%, and necrotic- 0.18±0.02% (Fig. **2**).

From 350 ID mRNA connected with the process of programmed cell death (the number of the probes were gained from Affymetrix NetAffx Analysis Center database after entering the query: "apoptosis" on 20$^{th}$ December 2019), 160 mRNA statistically significantly changed their expression under the influence of salinomycin added to the culture of endometrial cancer cells (analysis ANOVA $p<0.05$). The Tukey post-hoc test carried out afterward allowed for identifying the number of mRNAs differentiating depending on the time of the culture being exposed to the drug from the control: H_12 vs C = 114 mRNA, H_8 vs C = 84 mRNA, H_48 *vs*. C = 27 mRNA, whereas 5 mRNAs answering to genes *Bcl212, Bax, p53, BIRC5, ROCK1* differentiated the culture regardless of the exposition time of the cells to salinomycin in comparison to the control ($p<0.05$). As a large number of mRNAs statistically significantly changed transcriptional activity under salinomycin, we decided to narrow down the number of transcripts whose expression will be analyzed in detail in the paper. Analysis of mRNA differentiating the culture with a drug from a control irrespective of exposition time allowed us to indicate those mRNAs that have the best chance of aspiring to the name of a supplementary molecular marker for the assessment of apoptosis under the influence of salinomycin.

Analyzing the changes in expression of 5 mRNAs differentiating the culture irrespective of the time of the drug working can determine the silencing of 2 of the 5 analyzed genes: *BCL2L2; BIRC5*, however the transcriptional activity of *Bax, p53, FASL* increased under the influence of salinomycin. For all the analyzed transcripts, at least a 4-fold increase was noted for changes in expression caused by adding salinomycin to the culture of endometrial cancer cells. During the entire incubation period of the cells with the drug, the

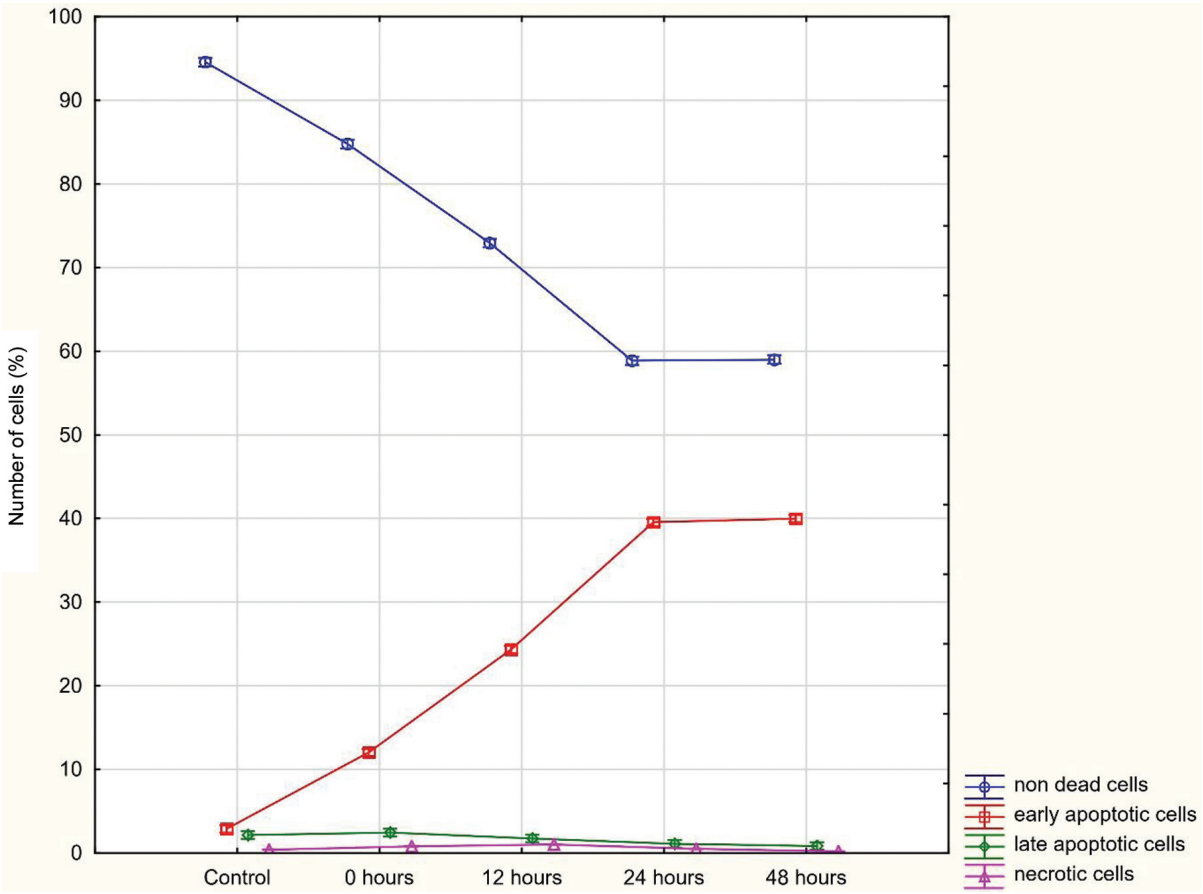


**Fig. (2).** The fraction of apoptotic and necrotic cells depending on the incubation time of cells with salinomycin at a concentration of 1 μM compared to untreated cells (control). (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

following dependency of the expression profile on the analyzed transcripts can be observed *Bax>p53>FASL>BIRC5>BCL2L.* In relation to the *Bax* gene, it can be observed that regardless of the incubation time under the influence of salinomycin, its expression was around 12-fold higher than in the control culture. The same tendency can be observed for the *p53* mRNA, wherein growth in transcriptional activity in the endometrial control culture exposed to salinomycin was on a level of 7-8-folds in expression in comparison to the control. Of the three assessed genes, changing its expression in a statistically significant way undergoing overexpression under the influence of salinomycin, the lowest fold change was noted for the *FASL* mRNA and when the incubation time was elongated, its expression decreased.

In turn, for the mRNAs *Bcl2l* and *BIRC5,* a decrease in the expression under the influence of 1 μM of salinomycin was noted when it was added to the culture of Ishikawa endometrial cancer cells irrespective of the time period. Moreover, it can be determined that the expression of these two genes was indirectly proportional to the exposition time of these cells with the drug. A trait shared by both changes in the expression pattern of mRNAs *Bcl2l* and *BIRC5* is the fact that the largest changes are visible already after 24 hours of adding salinomycin to the cell culture.

The expression pattern determined using the microarray technique of the 5 mRNAs differentiating the culture of cell line Ishikawa regardless of the incubation period of the cells with the drug from the control was confirmed using the RTqPCR method. It can be observed that the analysis of the gene expression using the RtqPCR method confirms the direction of these changes observed in the microarray experiment (Table **1**).

In the last stage of the molecular analysis, the effect of salinomycin on the concentration of BCL2L and p53 proteins was assessed (Table **2**).

The concentration profile of the BCL2L and p53 proteins is consistent with the pattern observed for it on the transcriptome level (microarray and RTqPCR techniques). Salinomycin statistically significantly decreased the expression level of BCL2L2 while the exposition time of the cells to the drug increased (from 1068.23±57.38 pg/ml after 12-hour exposition to 301.25±0.79 pg/ml after 48 hours of incubation of the cells with the drug; $p<0.05$). In addition, a lower level of BCL2L in the culture exposed to salinomycin when compared with a control ($p<0.05$) can be observed. Whereas in reference to the expression of p53, after adding salinomycin to the culture, an increase in the concentration of this protein in comparison to the control was determined ($p<0.05$). It is worth noticing that the expression of p53 increased with increasing duration of cell exposure to the drug (H_12 *vs.* H_48 increase by 0.98 U/ml). However, differences in the expression of p53 between the incubations lasting 24 and 48 hours respectively were not statistically significant ($p>0.05$; Table **2**).

**Table 1. Expression profiles of mRNAs *Bcl2l2, Bax, p53, FASL, BIRC5* in endometrial cancer cells exposed to 1 μM salinomycin compared to untreated cells (control).**

| Compared Group | H_12 *vs*. C | | | H_24 *vs*. C | | | H_48 *vs*. C | | |
|---|---|---|---|---|---|---|---|---|---|
| mRNA | Microarrays | RTqPCR | Post Hoc Test (p) | Microarrays | RTqPCR | Post Hoc Test (p) | Microarrays | RTqPCR | Post Hoc Test (p) |
| | Fold Change | | | Fold Change | | | Fold Change | | |
| *Bax* | +12.44 | +11.74 | 0.000000 | +13.09 | +12.89 | 0.000000 | +12.11 | +9.97 | 0.000000 |
| *P53* | +7.36 | +7.41 | 0.000000 | +8.09 | +8.54 | 0.000000 | +7.99 | +8.26 | 0.000000 |
| *FASL* | +5.97 | +6.09 | 0.000000 | +4.24 | +4.55 | 0.000000 | +4.31 | -4.42 | 0.000000 |
| *BCl2l* | -8.09 | -7.46 | 0.000000 | -11.07 | -11.98 | 0.000000 | -10.69 | -10.03 | 0.000000 |
| *BIRC5* | -6.98 | -6.10 | 0.000000 | -7.41 | -7.02 | 0.000000 | -6.89 | -6.98 | 0.000000 |

**Table 2. Changes in the level of BCL2L and p53 in endometrial cancer cells exposed to 1 μM of salinomycin at different times and in control (mean±standard deviation).**

| Protein | Control | H_12 | H_24 | H_ 48 |
|---|---|---|---|---|
| **BCL2L [pg/ml]** | 1480.35±1.57 | 1068.23±57.38[a,b] | 495.66±4.89[a,c] | 301.25±0.79[a,d] |
| **p53 [U/ml]** | 2.86±0.06 | 8.02±0.01[a,b] | 9.27±0.08[a] | 9.00±0.04[a,d] |

a – statistically significant differences compared to control ($p<0.05$).

b- statistically significant differences between H_12 *vs*. H_24 ($p<0.05$).

c- statistically significant differences between H_24 *vs*. H_48 ($p<0.05$).

d - statistically significant differences between H_12 *vs*. H_48 ($p<0.05$).

## 4. DISCUSSION

The search for effective therapeutic strategies in oncological illnesses is a constant challenge. More so when the drugs currently being used turn out to be ineffective in regards to tumor cells that are constantly becoming more resistant. Recent studies indicate that anticancer activity is demonstrated by antibiotics whose primary indication was bacterial infections. One example of such an antibiotic is salinomycin [5-8]. The development of molecular biology has allowed for a better understanding of the molecular mechanisms that make up the basis of cancer transformation and processes occurring during it, which became the search for finding new molecular markers characteristic of the given process, type of tumor, conditioning the resistance to cytostatics [17]. In addition, modern and sophisticated molecular methods, *i.e*. MALDI-TOF Mass Spectroscopy, supply new models for research as described by Zamani-Ahmadmahmudi *et al.* [18].

As part of this study, we decided to analyze the effect of salinomycin on the expression profile of genes and the proteins encoded by them on the apoptotic process using the endometrial cancer cell line Ishikawa research model. According to our knowledge, this is the first research of this sort on the effect of this antibiotic on the transcriptome and proteome of *in vitro* neoplastic endometrial cells [12, 16].

The first stage of our research was connected with deciding the concentration of salinomycin which would cause the death of half of the cells through the MTT test. It indicated that salinomycin at a concentration of 1 μM contributes to the decrease in the population of endometrial cancer cells by 50%. Zhang *et al.* analyzed the effect of salinomycin on the proliferation and apoptosis of ovarian cancer cells with a range of resistance to chemotherapy. In their work, salinomycin was used in the concentration range of 0.1 -200 μM, wherein the inhibitory effect of cell growth by 50% was noted at concentrations of salinomycin 1.7-7.4μM [19], which is higher than in the case of our analysis. This indicates that cells of endometrial cancer show a higher sensitivity to salinomycin, although, with the lowest concentration of 0.1 μM, the fraction of living cells fell by only 23% in comparison to the cells not treated with the antibiotic. However, Kaplan *et al.* observed that salinomycin at a concentration of 0.1 μM reduces the number of living ovarian cancer cells by 40%. These authors also noted just like us that there was an increase in the expression of Bax and a decrease in Bcl2 in response to salinomycin [20].

It is also worth noting that the pro-apoptotic effect of salinomycin at a concentration of 1 μM on endometrial cancer cells increases as the exposition time of the cells with the drug also increases, although this potential between 24 and 48 hours is similar. This indicates that salinomycin has the ability to exhibit and maintain a long-lasting therapeutic effect, which will without a doubt be a beneficial trait of treatment with this drug in routine practice. It will allow for increasing the time between subsequent drug administrations. It is also significant that on the basis of cell test and molecular analyses carried out, there was no sign that endometrial cancer cells were becoming resistant to salinomycin. Obviously, such assumptions should be checked, mainly on an endometrial cancer cell culture obtained directly from patients during a hysterectomy. Salinomycin induced mainly the early stage of the apoptotic process. The fraction of cells

that were necrotic was not significant. This is confirmation of the pro-apoptotic effect of the antibiotic. Cell death by apoptosis is a beneficial process because, unlike necrosis, it does not induce or develop an inflammatory process and therefore salinomycin appears to be a safe drug with anti-cancer properties. Zhao *et al.* in their research describe that salinomycin effectively stops the proliferation of promyelocytic leukemia cell line cells NB4 and HL-60 only after 48 hours when using the following concentrations of salinomycin: 0.8 μM; 1.6 μM; and 3 μM. They observed a sudden increase in the number of apoptotic cells. In the case of NB4 cell line cells unexposed, the culture of apoptotic cells was 6.01%, whereas in the presence of salinomycin, it grew to 32.30, 61.90 and 76.22%, and for HL-60 cell line cells, an increase from 3.16% to 37.45, 64.46 and 85.75% was observed. This suggests that the main signaling pathway responsible for the induction of apoptosis in these types of cells is the WNT/β-catenin pathway, and that salinomycin affects the transduction of signal along this signaling cascade [21]. Kusonoki *et al.* also suggest that salinomycin activates apoptosis through its effect on the WNT/β-catenin pathway in endometrial cancer cells [22].

The analysis carried out as part of this work also indicates that mRNA *BIRC5* codes survivin, a protein which in physiological conditions is practically absent in a gene that differentiates the culture exposed to the drug compared to the control. Whereas its concentration increases during the course of neoplastic changes [23-28]. It is included in a family of inhibitors of apoptosis, and its mechanism of working is connected with the stopping of the activity of initiator and effector caspases [29]. The silencing of its expression is another confirmation of the effectiveness of salinomycin at a concentration of 1 μM on endometrial cancer cells, although it has not yet been determined whether the reduction in the expression actually increases as the time of incubation with the drug increases. The expression of survivin was noted not only in the cytosol and cell nucleus but also in the mitochondria [30], which taking into consideration our previous observations regarding the expression of *BCL2L2 and Bax*, suggest that salinomycin is engaged in the mitochondrial death pathway in endometrial cancer.

Qin *et al.* suggest that changes in the expression of the p53 protein induced by salinomycin are connected with the fraction of cells directed at the necrosis path [31]. However, the results obtained by us do not indicate the key role of the p53 protein in the induction of necrosis in endometrial cancer cells exposed to salinomycin (the fraction of necrotic cells 0.57±0.01%). This does not change the fact that salinomycin affects the regulation of the cell cycle [32], in which the p53 protein plays an important role [33, 34].

It is also worth noticing that the expression of BCL2 and p53 on the mRNA and protein level indicated the same changes under the influence of salinomycin.

It might be suggested that the epigenetic mechanism of regulating mRNA *BCL2* and mRNA *p53* expression by micro RNA molecules (miRNA) has no influence. miRNAs are 18-25 nucleotide sequence molecules which play an extremely important role in regulating the expression pattern of mRNA. The influence of miRNA on gene expression is based on the principle of complementarity of nucleotide sequences. miRNAs are described not only as negative regulators of the expression of the gene whose expression is regulated but also as positive regulators. In such a situation, an increased expression level of a given gene can be observed at the transcriptome level, while its expression is reduced at the protein level or vice versa [35, 36]. Therefore, analyzing the expression pattern of Bcl2l and p53 at two stages of the flow of genetic information (mRNA and protein), the participation of miRNA in the regulation of their expression seems to be small, although it cannot be excluded with certainty.

In our work, it was noticed that after the addition of salinomycin to the cell culture, the expression of BCL2L was downregulated and the level of p53 was upregulated compared to the control, untreated Ishikawa endometrial cancer cell line cells.

A central role in the regulation of apoptosis is played by the suppressor *p53* gene (having pro-apoptotic properties), also called a guardian of the genome; additionally, mutations in this gene make up most of the widely described molecular changes in malignant tumors. In turn, the mitochondrial protein BCL2L (anti-apoptotic properties) is engaged in the late signal phase of apoptosis initiated by the p53 protein, acting as the final stage allowing for protection against the executive phase of apoptosis, in which caspases are engaged. The proteins of the pro-apoptotic interior pathway induced by p53 belong to two protein groups of the BCL-2 family (B-cell leukemia/lymphoma 2). The first group consists of the proteins BAX and BAK, which are responsible for the creation of channels in the inner membrane of mitochondria, through which pro-apoptotic factors escape to the cytoplasm. The second group contains the proteins: PUMA; NOXA; BID; or BIM. Under the influence of DNA and protein damage, the internal apoptosis pathway is induced. Proteins of the second group bind themselves to anti-apoptotic proteins BCL-2 and BCL-XL, which leads to their function being blocked and the release of the BAX and BAB proteins. The p53 protein can also, on the pathway independent of transcription, directly activate BAX ort BCL-2 acting as a result of its release through channels created by BAX and BAK in mitochondria, which leads to the release of cytochrome 3, alongside the APAF-1 protein (apoptotic protease activating factor 1) creating apoptosome in the cytoplasm, necessary for the activation of the caspase cascade. Overexpression of the oncoprotein BCL2L contributes to the prevention of the initiated apoptosis process, which explains the pro-oncogenic function of this protein [37-39]. For this reason, the expression profile of BCL2L and p53 in our study indicates that salinomycin has properties of inducing the cell death process of endometrial cancer.

In addition, in subsequent analyses, it would be reasonable to determine the effect of salinomycin on the induction of apoptosis in normal endometrial cells, since the main premise of anti-cancer therapies is the activation of apoptosis only in tumor cells [40] and in other endometrial cancer cell lines, *i.e*. HEC-1-A, HEC-1-B and KLE [41].

The microarray screening method for analyzing the expression of genes allowed for identifying the transcripts connected with apoptosis, which significantly changed their expression in response to salinomycin as well as selected those whose activity changes regardless of the treatment time of

endometrial cancer cells with salinomycin. This stage may pave the way for further research in regard to these mRNA and proteins, which changed their expression in the most significant and noticeable way.

## CONCLUSION

Salinomycin, the ionophore antibiotic was recently discovered as a promising strategy for treating tumors. The analysis carried out indicated that salinomycin at a concentration of 1μM stopped the proliferation of 50% of endometrial cancer cells, mainly through the induction of the apoptosis process of cells. The molecular exponent of the induction of programmed cell death was the observation of an increase in transcriptional activity of pro-apoptotic genes: *Bax; p53; FASL* and a decrease in the expression of anti-apoptotic genes: *Bcl212; BIRC5.* Taking into account, however, the pioneering character of our study in the context of endometrial cancer, further analysis regarding the molecular mechanisms of salinomycin is recommended.

## ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

## HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

## CONSENT FOR PUBLICATION

Not applicable.

## AVAILABILITY OF DATA AND MATERIALS

The authors confirm that the data supporting the findings of this research are available within the article.

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None.

## CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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