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RESEARCH ARTICLE

The Influence of Salinomycin on the Expression Profile of mRNAs Encoding Selected Caspases and MiRNAs Regulating their Expression in Endometrial Cancer Cell Line

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Abstract: ***Background*:** Apoptosis could take place in the pathway dependent on death receptors or pathways dependent on mitochondria. In both, a key role is played by enzymes with protease activity, known as caspases.

***Aim*:** The aim of this study was to assess the variances in the expression pattern of caspase-dependent signaling pathways in the endometrial cancer cell line when treated with salinomycin. Additionally, the changes in the level of miRNA that potentially regulate these mRNAs were evaluated.

***Materials and Methods*:** Endometrial cancer cells were treated with 1 μM of salinomycin for 12, 24 and 48 hours. Untreated cells made up the control culture. The molecular analysis consisted of screening mRNA and miRNA microarray expression profiles of caspases, and the evaluation of the expression of caspases 3,8 and 9 by RTqPCR, also on the protein level.

***Results and Discussion*:** It was observed that 5 of the 14 differentiating mRNAs were commonly found for all incubation times of the cells and they corresponded with *CASP3, CASP8,* and *CASP9* genes. The highest impact probability was determined between CASP3(up-regulated) and hsa- miR-30d (FC -2.01), CASP8 (down-regulated) and hsa-miR-21 (FC +1.39) and between CASP9 (up-regulated) and hsa-miR-1271 (FC +1.71).

***Conclusion*:** Salinomycin induces the apoptosis of endometrial cancer cells. The largest increase in activity was noted for caspases 3 and 9, while the expression of caspase 8 was decreased. Salinomycin causes a regulatory effect on the transcriptomes of mRNA and miRNA in *in vitro* endometrial cancer cells.

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1. INTRODUCTION

Caspase-cysteine-dependent aspartate specific proteases, also known as caspases, are cysteine proteases, which are synthesized, in the form of an inactive biological zymogen. There are 14 representatives of caspases, based on sequence analysis, the substrate specificity is divided into 3 main groups. The first group includes caspases-1, -4, -5, -11, -12,-13 and -14, which play a key role in cytokine activators taking part in the inflammatory process. Caspases in the second group are known as effector caspases, representatives of this type of protein include caspases-3, 6 and 7, whereas enzymes of this group undergo activation by other proteases, such as initiator caspases or other executive caspases. The third group comprises of initiator caspases, which are caspases-2, -8, -9, -10 and -12, recognized in tetrapeptide sequence substrates [1, 2]. The role of the caspases is described, above all, in the context of cell apoptosis [3, 4], the process that is disturbed in neoplastic illnesses [5, 6]. It has also been indicated that microRNA (miRNA) molecules play a role in this process, 17-25 nucleotide sequences are included, they affect

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the transcriptional activity of genes in the context of carcinogenesis [7, 8].

The activation of procaspases occurs as part of two different mechanisms: homoactivation and heteroactivation. The first of these involves the activation of initiator caspases such as -8/-10 and -9 through autoproteolysis and is connected with the formation of complexes, such as Death-Inducing Signaling Complex (DISC), which is significant in the course of the receptor and mitochondrial pathways of apoptosis, as well as Apoptosome. In the process of the forming of these complexes, the oligomerization of caspase molecules is observed. After that, molecular reorganization takes place of the remaining complexes, which results in autoproteolysis of initiator procaspases and the formation of their active heterotetramers. Full activation of caspases through oligomerization increases their activity, which results in the activation of further caspases (or caspase cascades). Whereas heteroactivation leads to the catalytic attack of initiator caspases (*e.g.* -8/-10,-9), or other proteases, such as granzyme B or calpain on exclusive caspases, as a consequence, the formation of active enzymatic caspases takes place.

Molecular reorganization of the DISC complex leads to the autoproteolysis of initiator procaspases (-2, -8, -9,-10) and the creation of their active forms (heterotetramers), through which the activation of regulator procaspases occurs. These last enzymes, either individually or together with other caspases, take part in protein proteolysis of cell substrates, which leads to the damaging of the structure and function of the cells as well as their death [9, 10]. Recent findings indicate that a promising anticancer strategy is a treatment using an ionophore antibiotic- salinomycin, its high effectiveness was observed in the treatment of gynecological cancers such as breast and ovarian cancer, among others, and also in the case of tumors characterized by a high risk of developing drug resistance to chemotherapy [11].

As of now, the exact mechanism through which salinomycin affects the apoptosis of changed cancer cells is not understood fully. Nonetheless, however, what has been described are the overexpression of caspases 3,8 and 9 of the proapoptotic protein Bax; the decrease in the expression of the anti-apoptotic protein Bcl2; Nuclear Factor kappa B (NFkB) under the influence of salinomycin [12].

The endometrial cancer cell lines, such as Ishikawa (correspond to histological grade 1 - G1 and type I endometrial cancer), EC-1-A, HEC-1-B (correspond to histological grade 2 – G2 and type II endometrial cancer) and KLE (correspond to histological grade 3 – G3 and type II endometrial cancer) are well-described, useful models for *in vitro* analysis. The Ishikawa cell line was originally obtained from a 39-year-old Japanese patient suffering from an endometrial adenocarcinoma. Cells of Ishikawa culture are estrogens and progesterone receptor positive. In turn, HEC-1-A and HEC-1-B were obtained from a 71-year-old patient with diagnosed endometrial adenocarcinoma, while the KLE culture was obtained from a 65-year-old woman in advanced disease [13].

Not much is known about the molecular working of salinomycin on endometrial cancer cells. This has become a premise for conducting research as part of this work.

The aim of this study was to analyze the variances in the expression pattern of signaling pathways that are caspase-dependent in endometrial cancer cells of the Ishikawa cell line when under the influence of salinomycin during treatment using the drug. Additionally, changes in the miRNA level that could potentially regulate these mRNAs were evaluated.

2. MATERIALS AND METHODS

As material in this project, endometrial cancer cells from cell line Ishikawa were used. The culture was carried out in Minimum Essential Medium (MEM) with 2 mM of glutamine, 1% Non-Essential Amino Acids (NEAA), and 5% Fetal Bovine Serum (FBS), as recommended by the manufacturer. The cells were in a 5% CO_2 enriched atmosphere at a constant temperature of 37^{o}C. All reagents that were used were purchased from the Sigma Aldrich company (St Louis, MO, USA).

In all parts of this study (cell culture, isolation of RNA, microarray, RTqPCR, evaluation of caspases activity) for each biological replicate, three technical replicates were performed.

The data represents the mean +/- SD of 3 separate experiments, each performed in triplicate.

In order to indicate and then assess the influence of salinomycin on the expression of mRNAs and miRNAs, 1 μM of the drug was added to the cells, and then the cells were cultured together with the drug for 12, 24 and 48 hours. The control culture was that cells were untreated by the drug. The total ribonucleic acid extraction was carried out using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. The evaluation of the expression pattern of mRNAs associated with the drug resistance was completed using oligonucleotide microarrays HG-U133A 2_0 (Affymetrix, Santa Clara, CA) just as the manufacturer recommended. The assessment of the expression profile of miRNAs that participated in the regulation of drug resistance genes was carried out using GeneChip® miRNA 2.0 Arrays (Affymetrix, Santa Clara, CA). The detailed protocol that was used by us for molecular analysis is described previously [14].

The third step of molecular analysis included the RTqPCR reaction, and it was performed to validate the changes observed in the transcriptional activity of mRNAs: *CASP3, CASP8, CASP9,* and the *miRNAs: hsa-miR-30d, hsa-miR-21, h-miR-1271.*

CASP3, CASP8, and *CASP9* were chosen for further molecular analysis because they differentiate endometrial cancer cells exposed to salinomycin compared to a control cell culture regardless of the incubation time of the cells with the drug.

In addition, in order to confirm the apoptosis *via* mitochondrial path or not, the changes in transcriptional activity of Bcl-2 genes family: *Bcl-2l* and *Bcl-w* (anti-apoptotic), *Bax* and *Bak* (pro-apoptotic) were evaluated *via* microarray and RTqPCR methods.

The reaction was conducted with the use of SensiFAST TM SYBR No-ROX One-Step Kit, (Bioline, London, UK),

where β-actin was used as the endogenous control, and the expression was presented as a fold change of the gene expression and compared to the control (the $2^{-\Delta\Delta Ct}$ method).

The evaluation of caspases -3, -8 and -9 was carried out using the caspase-8. -9 activity assays (Caspase-8/Caspase-9 Colorimetric Assay Kit (R&D Systems, Minneapolis, USA), the caspase-3 activity was determined in cell lysates (EnzChek® Caspase-3 Assay Kit #1 (Molecular Probes. Minneapolis. Willow Creek Rd. USA) in accordance with the manufacturer's protocol. The measurement of the absorbance at λ = 405 nm (caspase-8 and 9) and λ = 520 nm (caspase-3) permitted the level of caspase activity in cell lysates to be determined.

Statistical analysis of the microarray data was carried out with the use of the Transcriptome Analysis Console (Thermo Fisher, USA) and STATISTICA 13 PL (Cracow, Poland) software to analyze the results obtained using RTqPCR. The one-way ANOVA and Tukey post-hoc tests were used in order to determine the differentially expressed genes (|FC≥1; $p< 0.05$). In order to show which of the miRNAs indicated in microarray analysis were engaged in the regulation of the expression of the selected mRNAs, the microRNA database (http://microrna.org) was used as suggested in the protocol. The miRanda-mirSVR algorithm was used to find a relation to target mRNA for the relevant miRNA molecules (miRanda) and designate a potential interaction between mRNA: miRNA (mirSVR). miRanda target sites and mirSVR scores are available at http://www.microRNA.org. Based on the miRNA SVR score parameter, the relationship between the respective miRNA and the mRNA was determined. The cut-off of −0.1 or lower is used as the cut-off point for this parameter based on the data from the literature [15].

3. RESULTS

Based on the Affymetrix database, it was confirmed that 59 from the 22227 mRNAs present on the microarray plate were connected to caspase-dependent signaling pathways. The one-way analysis using the ANOVA variance test with Benjamini-Hochberg's procedure was used for the comparison of all examined transcriptome groups with the control and determining which of these noted variances in the expression of mRNA were statistically significant ($p<0.05$). It was observed that out of 59 mRNAs, 14 mRNAs differentiated the cell culture when exposed to salinomycin in comparison to the control endometrial cancer culture ($p<0.05$). After that, the post-hoc Tukey test was carried out. This step of the statistical analysis allowed the indication of the number of mRNAs that specifically differentiated the culture with the drug in comparison to the control (Table **1**).

Based on the displayed results, it is worth noting that the number of mRNAs differentiating the endometrial cancer cells exposed to salinomycin, and compared with the control cell culture for every incubation time of the cells with the drugs are as follows: H-12 *vs.* C - 8 mRNAs, H_24 *vs.* C –10 mRNAs, H_48 *vs.* C - 3 mRNA.

It was observed that 5 of the 14 differentiating mRNAs were common for all of the incubation time of the cells, and they correspond with the following genes: *CASP3, CASP8,* and *CASP9* (Differentially Expressed Genes - DEG). For this reason, further molecular analysis was focused on the three caspases mentioned.

After 12 hours of exposition of the endometrial cancer cells to the drug, only 2 mRNAs (*CASP1, CASP2*) were specifically Differentially Expressed Genes (DEG). After 24 hours, only 1 of the mRNAs was characteristic for this incubation time of the cells with salinomycin: *CASP6* (DEG). No mRNA characteristic for 48-hours of incubation of the cells with the drug was noted (Table **2**).

Differences in the transcriptional activity of *CASP3, CASP8,* and *CASP9,* depending on the exposition of cells to salinomycin obtained through RTqPCR are shown in Figs. (**1-3**). The expression pattern of these three genes designated using the microarray technique was verified using the RTqPCR technique.

The next stage was connected with the indication of micro RNA molecules (miRNAs) that could potentially regulate the expression of *CASP3, CASP8,* and *CASP9*. It should be noted that the miRNA SVR score parameter relation between the respective miRNA and mRNA was conclusive. The highest probability of influence was determined between *CASP3* and hsa- miR-30d (average FC -2.01, miRSVR score -0.3918), *CASP8* and hsa-miR-21 (average FC +1.39, miRSVR score -0.7787), and between *CASP9* and hsa-miR-1271 (average FC +1.71, miRSVR score -0.5220). The validation of the microarray expression profile of three miRNAs (hsa-miR-30d, hsa-miR-21, hsa-miR-1271) that most likely regulate expression (miRanda: miRSVR algorithm) of *CASP3, CASP8* and *CASP 9* was determined through the RTqPCR method (Table **3**).

The last step of our analysis was associated with evaluating caspase-3, -8 and -9 activity in endometrial cancer cells

Table 1. List of the mRNAs differentiating the individual transcriptome groups indicated with the use of the post-hoc multi comparison Tukey test ($p<0.05$).

Transcriptome Group	H-C	H-12	H-24	H-48
H-C	14	8	10	3
H-12	9	14	10	9
H-24	7	7	14	10
H-48	13	8	7	14

Table 2. List of the mRNAs associated with the caspase-dependent signaling path, differentiating endometrial cancer cells treated with salinomycin, compared to the control culture.

ID	Gene Symbol	12 Hours (Up/Down)	FC (12 Hours *vs.* C)	24 Hours (Up/Down)	FC (24 Hours *vs.* C)	48 Hours (Up/Down)	FC (48 Hours *vs.* C)
202763_at	*CASP3*	up	+2.11	up	+2.08	up	+1.74
207686_s_at	*CASP8*	down	-1.41	down	-1.55.	up	+1.08
213373_s_at	*CASP8*	down	-1.45	down	-1.43	up	+1.02
203984_s_at	*CASP9*	up	+2.16	up	+2.84	up	+2.55
210775_x_at	*CASP9*	up	+1.89	up	+2.41	up	+2.44
206011_at	*CASP1*	up	+1.36	up	+1.22	down	-1.01
211366_x_at	*CASP1*	up	+1.32	up	+1.25	down	-1.06
211367_s_at	*CASP1*	up	+1.38	up	+1.21	up	+1.02
211368_s_at	*CASP1*	up	+1.32	up	+1.33	up	+1.08
208050_s_at	*CASP2*	up	+1.22	up	+1.02	down	-1.09
209811_at	*CASP2*	up	+1.18	up	+1.03	down	-1.07
34449_at	*CASP2*	up	+1.19	up	+1.05	down	-1.01
211464_x_at	*CASP6*	down	-1.26	down	-1.09	up	+1.12
205467_at	*CASP10*	up	+1.11	up	+1.19	up	+1.04

(+) overexpression of gene (increased level of mRNAs); (-) suppressed gene expression (decreased level of mRNAs); ID - ID of the probe on a microarray; FC - fold change; C - control culture; 12h. 24h. 48h of exposure to salinomycin.

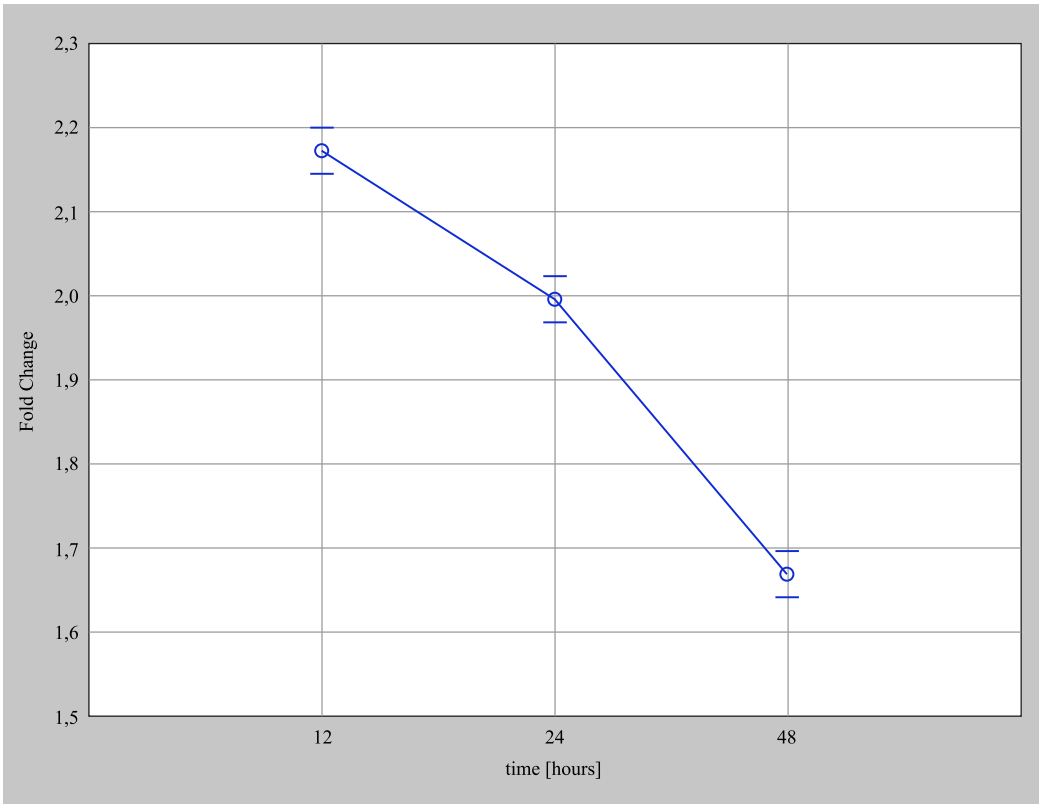

Fig. (1). Expression of CASP3, depending on the exposure time of the Ishikawa line cells to salinomycin (RT-qPCR). The data represents the mean +/- SD of 3 separate experiments, each performed in triplicate. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

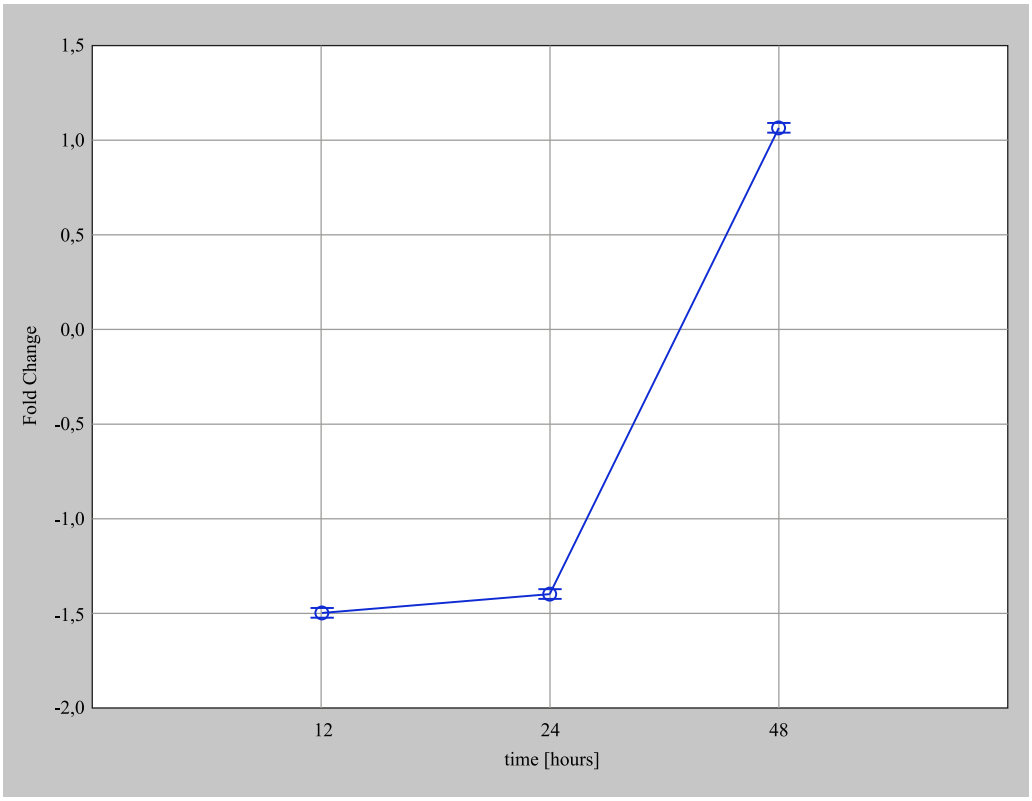

Fig. (2). Expression of CASP8, depending on the exposure time of the Ishikawa line cells to salinomycin (RT-qPCR). The data represents the mean +/- SD of 3 separate experiments, each performed in triplicate. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

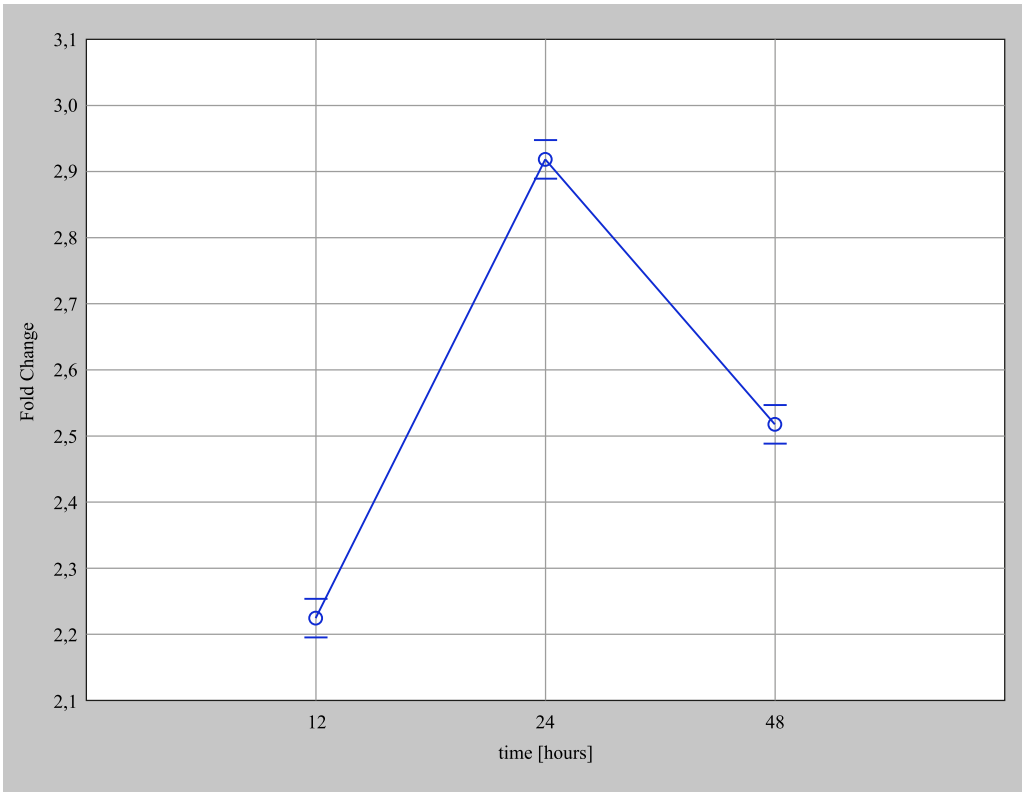

Fig. (3). Expression of CASP9, depending on the exposure time of Ishikawa line cells to salinomycin (RT-qPCR). The data represents the mean +/- SD of 3 separate experiments, each performed in triplicate. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 3. The expression profile of hsa-miR-30d, hsa-miR-21, hsa-miR-1271 obtained by microarray and RTqPCR assays.

Compared Group	miRNA	Expression (FC)	
		Microarray	RTqPCR
H_12 *vs*. C	hsa-miR-30d	-1.04	-1.11
H_24 *vs*. C		-2.01	-2.06
H_48 *vs*. C		-2.98	-2.96
H_12 *vs*. C	hsa-miR-21	+1.38	+1.32
H_24 *vs*. C		+1.27	+1.28
H_48 *vs*. C		+1.52	+1.44
H_12 *vs*. C	hsa-miR-1271	+4.96	+4.83
H_24 *vs*. C		-4.41	-4.69
H_48 *vs*. C		+4.58	+4.32

Table 4. The activity of caspases 3, 8 and 9 in endometrial cancer cells treated with 1 μM salinomycin for 12, 24 and 48 hours in comparison to a control culture (p<0.05; Control - cells treated with PBS; 100%).

Time [Hours]	Caspase-3 [%]	Caspase-8 [%]	Caspase-9 [%]
12	136.52±0.46*	102.70±0.71	125.33±0.48*
24	146.89±1.52*	101.89±0.13	141.41±2.11*
48	156.53±0.42*	101.19±0.23	131.04±0.09*

* - p<0.05 when compared with a control.

exposed to salinomycin for 12, 24 and 48 hours. To determine the level of these caspases, commercially available assays were used. This stage allowed us to examine the pro-apoptotic properties of salinomycin. Statistically significant differences were observed in the level of caspase-3 and -9 ($p<0.05$). However, changes in caspase-8 were not statistically significant ($p>0.05$). In Table **4** and Fig. (**4**), the results of caspase activity obtained by using commercially available assays were presented.

The last stage of our work was associated with confirming or exclusion induce apoptosis *via* mitochondrial path by salinomycin. Therefore, expression changes of Bcl-2 genes family: *Bcl-2l* and *Bcl-w* (anti-apoptotic), *Bax* and *Bak* (pro-apoptotic) were assessed (Table **5**).

The obtained results indicated that after adding salinomycin to endometrial cancer cell culture, pro-apoptotic members of Bcl-2 genes family were over expressed, while anti-apoptotic members were silenced. The statistical analysis showed that changes in expression of selected BCL-2 genes family were significant in comparison with a control (untreated with salinomycin cell culture) (Table **5**; $p<0.05$). This part of the molecular analysis suggests that salinomycin induces the programmed cell death *via* mainly mitochondrial-related pathway.

4. DISCUSSION

Research carried out as part of this work aimed to define the effect of salinomycin on the expression profile of caspases and miRNA molecules that are potentially engaged in the regulation of their expression in endometrial cancer. Currently, the largest number of studies on the effect of salinomycin in the course of neoplastic illnesses concentrates around ovarian and breast cancer carried out on an *in vitro* and *in vivo* model [11, 12]. However, there is a lack of studies about salinomycin in endometrial cancer, which is the sixth most commonly diagnosed gynecological cancer in women after the endometrium. It was confirmed that salinomycin effectively decreases the number of cancerous cells, including cancer stem cells, however, the induction mechanism of programmed cell death by this drug remains unclear [16].

Caspases are proteolytic enzymes with a significant role in the course of apoptosis. This process could take place through an external pathway induced by the interaction with a ligand, such as the tumor necrosis factor α with the death receptor, which in the next stage undergoes trimerization, and allows the activation of the death pathway. The activation of caspases -8/-10 begins the proteolysis of enzymes and proteins of cell structure, which is cascading. Whereas in the interior pathway (mitochondrial), a key role is played by caspases - as well as the executive caspases -3, -6, -7. The activation of procaspase 9 in the result of proteolysis leads to the activation of procaspase -3 or -7 which in turn activates the caspase cascades and proteolysis of cell proteins, in which calpains and also caspase-activated DNase are engaged [17].

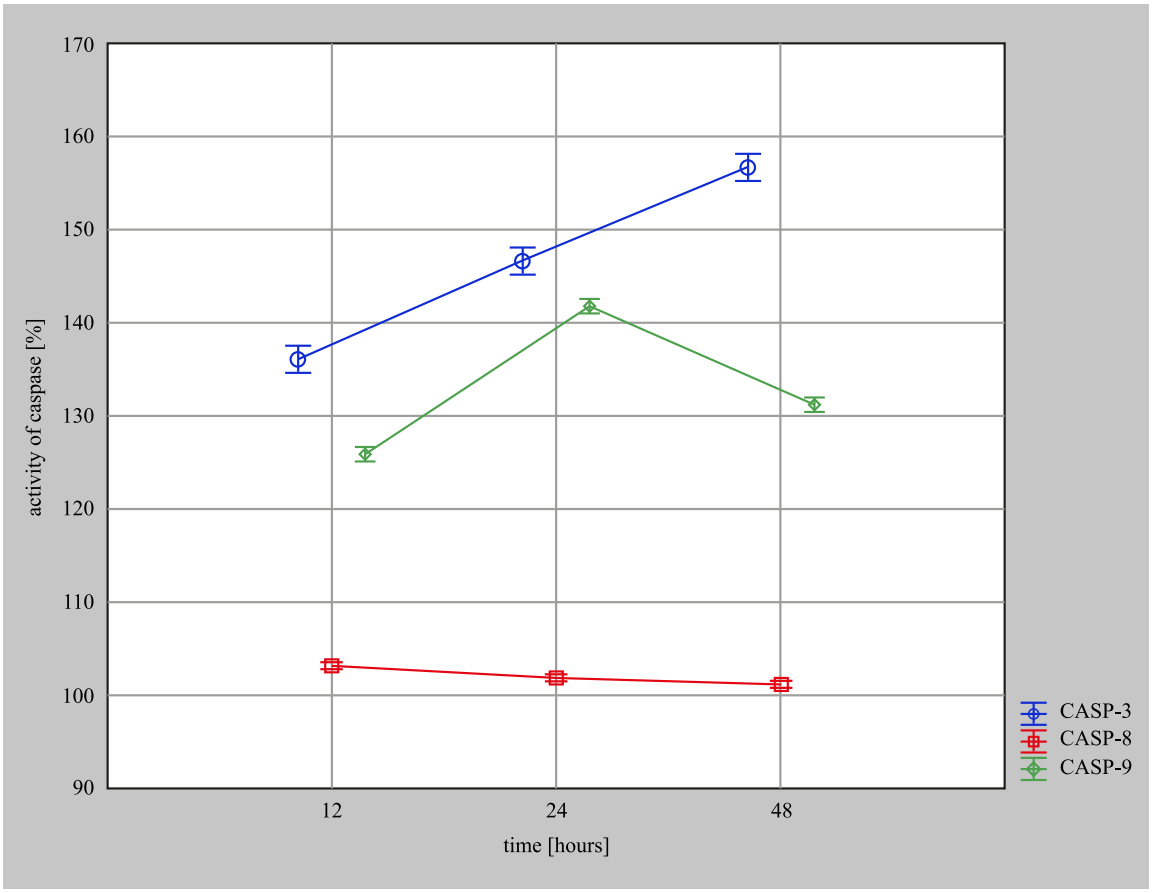

Fig. (4). Evaluating caspase-3, 8 and 9 activity in endometrial cancer cells exposed to salinomycin for 12, 24 and 48 hours. [The data represents the mean +/- SD of 3 separate experiment, each performed in triplicate]. (*A higher resolution/colour version of this figure is available in the electronic copy of the article*).

Table 5. Expression profile of *Bcl-2l* and *Bcl-w* (anti-apoptotic), *Bax* and *Bak* (pro-apoptotic) genes in endometrial cancer cells treated with µM salinomycin for 12, 24 and 48 hour periods in comparison to a control culture (p<0.05).

Compared Group	H_12 *vs.* C		H_24 *vs.* C		H_48 *vs.* C	
mRNA	**Microarrays**	**RTqPCR**	**Microarrays**	**RTqPCR**	**Microarrays**	**RTqPCR**
	Fold Change		**Fold Change**		**Fold Change**	
Bcl2l	-8.09	-7.46	-11.07	-11.98	-10.69	-10.03
Bcl-w	-6.11	-5.14	-8.14	-7.35	-8.99	-9.65
Bax	+12.44	+11.74	+13.09	+12.89	+12.11	+9.97
Bak	+14.36	+12.74	+15.02	+14.01	+13/84	+12.26

Microarray analysis of the profile indicated that *CASP3*, *CASP8* and *CASP9,* which encode caspases -3,-8 and -9 respectively, change their activity under the influence of 1 µM of salinomycin added to the *in vitro* endometrial cancer cell culture to the largest degree. The results were confirmed using the RTqPCR technique. Ho *et al.*, in research regarding the influence of berberine in the context of tongue cancer, also observed statistically significant changes in the expressions of the same caspases as we did in our research. They determined that together with an increase in the exposition time of the tongue cancer cells to berberine, an increase in the activity of caspases -3,-8 and -9 in comparison to the control and shorter incubation time, which is partly convergent with our observations [18]. As a result, it might seem that salinomycin induces apoptosis through the interior pathway (mitochondrial) [16]. Observations on the transcriptome level were also confirmed on the protein level, where only caspases -3 and -9 were noted to have a significant increase in their activity, whereas the changes in the expression of caspase-8 were not statistically significant. What is significant, from the point of assessing the effectiveness of salinomycin therapy as a result of endometrial cancer, is that

in vitro analysis suggests that this ionophore antibiotic permanently induces changes, including cell death, as even with the longest exposition time of the cells to salinomycin, the overexpression of the caspases -3 and -9 continues in comparison to the control culture [19]. Zhang *et al.* were assessing the influence of salinomycin on the expression profile of caspases and other proteins of the apoptosis pathway in a prostate cancer model. They determined that an increased level in caspase-12 under the influence of salinomycin as well as a decreased expression of caspase-3 when the cells were treated simultaneously with salinomycin and 4-phenylbutyrate. Based on the obtained results, Zhang *et al.* confirmed that salinomycin increases cell stress, which leads to changes in the endoplasmic reticulum and apoptosis. Fu *et al.* also indicate an increase in the concentration of caspase-3 in the case of breast cancer as a result of treating the cancer cells with salinomycin. They determined that the expression of caspases was over 4 times higher in comparison to cells unexposed to the drug [20], whereas our analysis indicated a 2-fold change higher than the expression in the Ishikawa cell culture with salinomycin. The potential cause of these differences in the fold change of the expression of caspase-3 presented in the research by Fu *et al.* [20] and our research could be, not only a different cell model, but also with a longer incubation time with salinomycin, which lasted 6 days. The concentration of salinomycin - 1 μM - in the cited work [20] and in our research was identical. Moreover, it can be determined that salinomycin is characterized by the speed of action, as changes in the expression of the assessed mRNA could be observed already after 12 hours of incubation. It can also be seen that the transcriptome is a compound characterized by dynamism and plasticity, and the chosen experimental model- the Ishikawa cell line - is a useful experimental model.

The observations of Gao *et al.* are also interesting, as they confirmed the induction of cell death of cardiomyocytes by salinomycin on an animal model - that of chickens. They also determined that the drug most strongly caused changes in the expression of caspases -3 and -9 (increase in activity) on the gene and protein levels in comparison to the expression of caspase-8 [21]. Therefore, it may be assumed that salinomycin causes cell apoptosis through the interior pathway (mitochondrial) not only regardless of the type of cancer, but also the type of cell and the chosen experimental model. This is an important report, as it indicates that changes in the expression of caspases -3 and -9 could be considered as new supplementary markers of the answer to salinomycin treatment. However, further analysis using caspase-3 or -9 inhibitor is necessary to confirm this point of view.

The second part of our research included checking whether salinomycin affects miRNA transcriptome of endometrial cancer cells exposed to salinomycin as well as what is the regulatory effect of the chosen miRNA of expression proliferation *CASP3, CASP8,* and *CASP9.* MiRNA affects mRNA on the basis of nucleotide sequence complementarity, wherein the effect of their action could be both a decrease as well as an increase in the transcriptional activity of the target transcript [22]. Based on microarray analysis of the miRNA profile, it can be determined that salinomycin affects the epigenomes as well as that the determined changes in the expression pattern of the chosen caspases could be a result of not only the workings of the drug but also a result of the regulatory role of the miRNAs.

The fact that the selected miRNAs can have a real effect on the expression of target mRNA in the experiment is also evidenced by the fact that the mRNA:miRNA relationship was determined based on the moRSVR Score parameter, providing information about the complementarity between the hybrid molecules [15].

The highest impact probability was determined between CASP3 (up-regulated) and hsa- miR-30d (FC -2.01), CASP8 (down-regulated) and hsa-miR-21 (FC +1.39), and between CASP9 (up-regulated) and hsa-miR-1271 (FC +1.71).

In relation to hsa-miR-30d, a decrease in its expression was determined which was accompanied by an increase in the activity of *CASP3*. The observed expression relationship between the mRNA *CASP3* and the hsa-miR-30d mRNA and the level of CASP3 protein suggests that salinomycin may affect the level of methylation of CpG islands within the *CASP3* nucleotide sequence, which translates into reduced mRNA expression. In contrast, accompanying a decrease in *CASP3* expression at the transcriptome level, an increase in protein concentration may be the result of a decrease in the activity of hsa-miR-30d, which is potentially involved in the regulation of mRNA *CASP3* expression [22].

It was confirmed that hsa-miR-30d is an important, useful marker for assessing the advancement of neoplastic changes in prostate cancer. An increase in the level of this miRNA molecule was described in patients with worse prognosis, which demanded a more aggressive treatment [23]. Moreover, it was noted that the expression pattern of hsa-mIR-30d indicates the effectiveness of salinomycin, the sensitivity of cells to salinomycin as well as an increase in *CASP3*, contributing to changed cancer cell apoptosis. Furthermore, the role of hsa-miR-30d is broadly discussed in the context of its effect on the proliferation of cells. The observation that the miRNA molecule alone affects the proliferation, differentiation and cell death seems reasonable [24, 25]. Whereas, the observation made by Muhammad *et al.* in the context of large intestine cancer indicates that a decreased expression of hsa-miR30d was a factor that promoted the proliferation of cancer cells [26]. Therefore it suggests the dualism of action that hsa-miR30d possesses in the process of the neoplastic transformation. Ipso facto, when assessing whether an increase or rather silencing in the activity of hsa-miR30d is a useful factor, should include the biological context, as well as the type of tumor, and also the location, such as in relation to the representatives of the Transforming Growth Factor-beta (TGFβ) superfamily [27]. Similar observations are presented by Kuo *et al.*, who assessed the effect of salinomycin on the expression profile of miRNA in head and neck squamous cell carcinoma stem cells [28].

In turn, for two of the following differentiating cultures exposed to salinomycin compared to the control, an increase in the expression of miRNA molecules - hsa-miR-21 and hsa-miR-1271 was noted. The role of hsa-miR-21 is described in the context of the death pathway receptor, activated by the FAS ligand, whereas hsa-miR-1271 is connected with the gaining of resistance to treatment with cisplatin

[29]. However, only concerning hsa-miR-21 was the first characterized function of mRNA confirmed, as a negative regulator of gene expression, including in the case of *CASP8*.

An increased expression of hsa-miRNA-21 (FC=2.3) was observed by Torres *et al.* in endometrial cancer, comparing biopsies to each other obtained from 20 female patients with clippings obtained from 10 volunteers (control) [30]. Our analysis indicates overexpression of this mRNA in comparison to the control on the level of FC=1.3, which would suggest that the difference in the fold change on the expression between observations of Torres *et al.* [30] and those obtained in this work could be a result of the effect of salinomycin. Also, Bin *et al.* describe the direct relationship between hsa-miR-21 and *CASP8* and the effect of them on its expression caused by this miRNA molecule. An overexpression of hsa-miR-21 was connected with the increase in the survival and proliferation of cancer cells with the simultaneous inhibition of apoptosis [31]. As a result of this, although it seems that salinomycin affects, above all, on the mitochondrial death pathway, it can be assumed that, to a lesser extent, it affects the regulation of the receptor pathway. Furthermore, Liu *et al.*, based on their research, postulate that the role of hsa-miR-21 in two types of pathways leading to cell apoptosis as a consequence [32]. This shows the complex picture of the cancer process as well as the multidirectional effect of salinomycin.

The last of the differentiating miRNA for which the largest probability of effecting CASP9 was observed is hsa-miR-1271, indicated as a molecule, whose changes in expression are a reflection of the cancer cell's sensitivity to chemotherapy, and also in proliferation, apoptosis and metastasis [33, 34]. Moreover, Yao *et al.* noted an increase in the proapoptotic activity of the Bax protein and caspase 9, with a simultaneous decrease in the expression of anti-apoptotic proteins, such as Bcl-2 in miR-1271-mimic-treated colorectal cancer cells [34]. Observations on the role of hsa-miR-1271 in comparison to the expression of caspase-9 are also confirmed in the research done by Xie *et al.* [35]. It is worth noting that the obtained results of hsa-miR-1271 expression in the endometrial cell culture exposed to salinomycin showed that after 12 and 48 hours, the level of hsa-miR-1271 is higher than in a control culture while after 24 hours incubation the silencing of hsa-miR-1271 expression can be observed. Silencing the expression of hsa-miR-1271 consequently leads to a higher level of *CASP9* after 24 hours incubation cells with salinomycin, this may result from the activation of adaptive mechanisms by cells or/and the effect of other signaling pathways on the expression of the analyzed mRNA and miRNA [36].

In conclusion, the molecular analysis carried out as part of this work indicates that salinomycin induces the apoptosis of endometrial cancer cells.

It should be noted that in the process of programmed cell death, in addition to the caspase pathway, other signaling pathways are involved, including with p21 or p53 protein. Therefore, it is reasonable to conduct further molecular analyses regarding the effect of salinomycin on the process of apoptosis in endometrial cancer [37].

CONCLUSION

Salinomycin induces the apoptosis of endometrial cancer cells. The largest increase in activity was noted for caspases 3 and 9, while the expression of caspase 8 was decreased. Salinomycin causes a regulatory effect on the transcriptomes of mRNA and miRNA in *in vitro* endometrial cancer cells. It was also confirmed that the influence of 3 mRNA molecules on the expression profile of the mentioned caspases namely: CASP3 (up-regulated) and hsa- miR-30d (FC -2.01); CASP8 (down-regulated) and hsa-miR-21 (FC+1.39); between CASP9 (up-regulated) and hsa-miR-1271 (FC +1.71).

The carried out analysis indicated that salinomycin has a regulatory effect on mRNA and miRNA transcriptomes in *in vitro* endometrial cancer cells. Further studies are suggested, including those based on an *in vivo* model.

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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CONFLICT OF INTEREST

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

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SUPPLEMENTARY MATERIAL

Supplementary material is available on the publisher's website along with the published article.

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