RESEARCH ARTICLE

Evaluation of Variances in VEGF-A-D and VEGFR-1-3 Expression in the Ishikawa Endometrial Cancer Cell Line Treated with Salinomycin and Anti-Angiogenic/Lymphangiogenic Effect

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Abstract: Background: In an cancer, an excessive and uncontrolled process of creating new blood and lymphatic vessels that play a key role in the metastasis process can be observed. The Vascular Endothelial Growth Factor (VEGF-A,-B,-C,-D) family together with their specific receptors (VEGFR-1,-2,-3) plays a key role in these processes, therefore, it would be reasonable to determine the correct pattern of their expression.

Objectives: The study aimed to assess the use of salinomycin as an anti-angiogenic and anti-lymphangiogenic drug during endometrial cancer by examining changes in the expression pattern of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2 and VEGFR-3 depending on the treatment period of the Ishikawa endometrial cancer cells with salinomycin in comparison to the control culture.

Materials and Methods: To determine how influential salinomycin was on the expression of both mRNAs, 1 µM of the drug was added to the cell culture and then it was cultured all together for 12, 24 and 48 hour periods. The cells that made up the control culture were not treated with salinomycin. To determine the changes in the expression profile of the selected genes, we used the microarray techniques: RTqPCR and ELISA (p<0.05).

Results: For all isomers of VEGF-A-D as well as receptors of VEGFR-1-3, a decrease in expression under the influence of salinomycin was noted. For VEGF-A and VEGF-1, the difference in the expression between the culture treated with salinomycin in comparison to the control was statistically significant (p=0.0004). In turn, for VEGF-B, the difference between the culture exposed for 24 hours in comparison to the control (p=0.00000) as well as the comparison between H48 vs. C (p=0.00000) was statistically significant. In reference to VEGF-C, VEGF-2 and VEGF-3, the statistical analysis showed the significant difference in expression between the culture incubated with the drug for 12, 24 and 48 hours in comparison to the control as well as between the selected times. For all of these comparisons, p=0.00000 was utilized.

Conclusion: Salinomycin changes the expression pattern of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 in endometrial cancer cells. The obtained results suggest that salinomycin might exert the effect via VEGF signaling pathways.

Keywords: Salinomycin, angiogenesis, lymphangiogenesis, anti-angiogenic and anti-lymphangiogenic endometrial cancer’s drug, molecular diagnostic.

1. INTRODUCTION

Angiogenesis, which is the process of forming new blood vessels, is an inseparable process accompanying the neoplastic transformation process [1, 2]. Their development creates a compensatory and adaptive mechanism of cancer cells in answer to changing conditions in their microenvironment within the forming tumor. Together with the unrestrained potential for proliferation of changed cancer cells [3], at the moment when the growing tumor reaches a critical size, the inflow of nutrients and oxygen on the way to diffusion is…
made more difficult [4]. It is then observed that there are outbreaks of hypoxia in the microenvironment [5]. Therefore, to counteract them, the cancer cells stimulate the formation of their web of cancerous blood vessels [6]. Other than functions connected with supplying cells with nutrients and oxygen, new blood vessels create an invasion route for primary cells of the tumor mass to other organs, which as a consequence, leads to metastasis [7, 8].

The second significant process that accompanies both carcinogeneses, and also angiogenesis is lymphangiogenesis. In this process, what is mainly involved are macrophages, which show an ability to secrete growth factors, cytokines responsible for the induction, and the development of the discussed process [9, 10]. The role of lymphatic vessels is highlighted in the progression of the neoplastic transformation [11, 12].

A significant role in the aforementioned processes is played by the Vascular Endothelial Growth Factor Family (VEGF-A,-B,-C,-D) together with the receptors specific to them, with tyrosine kinase activity (VEGFR-1,-2,-3). For the process of angiogenesis, the expression of isoforms VEGF-A, VEGF-B, as well as receptors of VEGFR-1 and VEGFR-2, is significant [13, 14].

Treatment of endometrial cancer includes surgery, radiotherapy, radiochemotherapy, as well as chemotherapy [15]. One of these promising drugs is the ionophore antibiotic with an antibacterial effect as well as inhibition of proliferation of cancer stem cells- salinomycin [16, 17]. However, knowledge on the subject of using this drug in the case of endometrial cancer is in the initial stage [18]. The study aimed to evaluate changes in the expression profile of mRNA and protein of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 in Ishikawa endometrial cancer cells treated with salinomycin.

2. MATERIALS AND METHODS

2.1. Cell Culture

As material in this study, we used Ishikawa cell line endometrial cancer cells (European Collection of Authenticated Cell Cultures, ECACC 99040201). The culture was carried out with the use of the Minimum Essential Medium (MEM) with 2 mM of glutamine, 1% Non-Essential Amino Acids (NEAA), and 5% Fetal Bovine Serum (FBS), as the manufacturer’s protocol recommended, Catalog number: 51411C. The cells were incubated with 2 mM of glutamine, 1% Non-Essential Amino Acids (NEAA), and 5% Fetal Bovine Serum (FBS), as the manufacturer’s protocol recommended, Catalog number were obtained from the Affymetrix NetAffx™ Analysis Center database after entering the phrase “VEGF” (http://www.affymetrix.com/analysis/index.affx; accessed on 18th December 2020).

Microarray analysis can be included in the most important stages:

1) Synthesis of double-stranded cDNA on the matrix of the RNA extracts.
2) A mixture of the total RNA mixture with the poly-A control and First Strand Master Mix.
3) Incubation for 2 hours at 42ºC.
4) Adding the Second Strand Master Mix to the reaction mixture.
Table 1. The nucleotide sequence of primers used to amplify VEGF-A-D and VEGFR-1-3 genes via the RTqPCR reaction.

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Primers Sequence (Forward, Reverse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>GGCCAGCAGACATTAGAGAGAT ACAGTTCAAGGACTATACCG</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>GCTCCTCCATTTGAGCAAGTG AGAAGCTCCCTCAAGCATTCAG</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>CTCCTCCTCAAGGCCCGAAA AGTCATCTCCAGACGTCGAG</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>AGTATTGGACTCTCAGCTG TGGTGTACTCTCAGTCAGCAG</td>
</tr>
<tr>
<td>VEGF-R1</td>
<td>GTCTGTAAAGAGTGGACACG GCAGATTTCAGTCAGTGAG</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>TTACTGAGGGGAGACGGAGG TCCCGGATGAAGCAGTTCG</td>
</tr>
<tr>
<td>VEGF-R3</td>
<td>GGAGAGCTGTGGCTTGAAACT AGTTTTGAGTGGTTCAGGAT</td>
</tr>
</tbody>
</table>

5) Incubation for 1 hour at 16°C and next for 10 minutes at 65°C.
6) Synthesis of biotinyl aRNA.
7) Incubation for 16 hours at 40°C.
8) Labeling aRNA with biotin and purifying.
9) Hybridization of aRNA specimens with microarray probes.
10) Reading of the hybridization signal.
11) Data analysis.

2.5. Reverse-Transcription Quantitative Polymerase Chain Reaction

In the second stage of molecular analysis, the RTqPCR reaction was carried out to validate the microarray data.

This was conducted with the use of the SensiFAST™ SYBR No-ROX One-Step Kit, (Bioline, London, UK). β-actin was used as the endogenous control. The thermal profile of the reaction was as follows:

- Reverse transcription (temperature: 45°C, time: 10 minutes).
- Activation of the polymerase (temperature: 95°C, time: 2 minutes).
- 40 cycles including denaturation (temperature: 95°C, time: 5 seconds).
- Annealing (temperature: 60°C, time: 10 seconds).
- Elongation (temperature: 72°C, time: 5 seconds).

If the value of FC is lower than 1.0, it means the downregulation of genes expression of genes in comparison to a control, and if the FC value is lower than 1.0, it means the downregulation of genes compared to control culture.

The sequence of primers was presented in Table 1.

2.6. ELISA Assay

The next step of the molecular analysis was to evaluate changes in the expression pattern of VEGF-A and D and also VEGFR-1-3 at the proteome level via ELISA method according to the manufacturer’s protocol (ThermoFisher, CA, USA) using the kits: VEGF-A Human ELISA Kit Catalog number BMS277-2; VEGF-D (FIGF) Human ELISA Kit Catalog number EHFIGF; VEGF Receptor 1 (Soluble) Human ELISA Kit Catalog number BMS268-3; VEGF Receptor 2/KDR Human ELISA Kit Catalog number BMS2019TEN; VEGF Receptor 3/FLT4 Human ELISA Kit Catalog number BMS2064. For detecting VEGF-B, we used the VEGF-B ELISA kit: Human Vascular Endothelial Growth Factor B (VEGF-B) ELISA Kit, Catalog number MBS268048, while for VEGF-C, VEGF-C elisa kit Human VEGF-C ELISA Kit, Catalog number MBS4503300 was used (My Biosource Inc, San Diego, CA 92195-3308 USA).

2.7. siRNA Targeted to VEGF

The last stage of our experiment was associated with confirming if salinomycin exerts via the VEGF signal pathway. To do this, the VEGF siRNA molecule (sequence of sense strand 5’-GGAGUACCCUGAUAGAACUU-3’ and sequence of anti-sense strand 5’-GAUCUCAACGAGGGAUAAUCU-3’) complementary with mRNA VEGF (GenBank Reference number AB021221) was constructed. Since the homology of human genes was not known, siRNAscr (sequence of sense strand 5’-GGAGUACCCUGAGGGAUAAU-3’ and sequence of anti-sense strand 5’-GUAAA-UUCCCGGUAUCGUU-3’) was used as a negative control of the experiment (Invitrogen, USA). Next, VEGF siRNA, siRNAscr were transfected to endometrial cancer cells according to the protocol described by Ge et al. [19]. In this part of the research, untreated endometrial cancer cells were also used as a second negative control.

2.8. Statistical Analysis

The statistical analysis of the obtained microarray data was done using the Transcriptome Analysis Console (Thermo Fisher, USA). The STATISTICA 13.5 PL (Cracow, Poland) software was used to analyze data obtained from the RTqPCR and ELISA reactions.

Statistical analysis was performed for the significance level \( p < 0.05 \). In the first stage of statistical analysis, it was assessed whether the obtained data met the assumptions of normal distribution. For this purpose, the Shapiro-Wilk test was carried out. The analysis indicated that the data distribution is consistent with the assumptions of normal distribution. Therefore, further analyses were performed using parametric methods. Subsequently, the one-way ANOVA variance analysis test and Tukey’s post-hoc test were performed, which assessed whether the differences in the expression profile of VEGF-A and VEGF1-4 change statistically significantly depending on the exposure time of the endometrial cancer cells to 1 μM salinomycin in comparison to a control culture (FC>1; \( p < 0.05 \)). The results were presented as a mean ± standard deviation of 3 separate experiments, each performed in triplicate.
3. RESULTS

The cytotoxicity test showed that salinomycin has an influence on Ishikawa endometrial cancer cell viability. For the 0.1 µM concentration, the average percentage of viable cells was 84.74%±0.14%, for 1 µM - 50.02%±0.04%, for 10 µM – 32.4%±0.09% and for 100 µM - 14.74% ±0.41%. The differences in the percentage of viable cells treated with different concentrations of salinomycin in comparison to the untreated cells (control) were statistically significant (p<0.05). Considering these results, salinomycin in the 1 µM concentration was used to treat endometrial cancer cells in the next steps of our analysis.

The microarray analysis indicated changes in the expression pattern of the following mRNA: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 in the culture of neoplastic endometrial cells exposed to salinomycin for 12, 24 and 48 hour periods and compared to the control culture. Concerning VEGF-A, a decrease in its expression was observed after 12 hours of adding the drug to the culture. Further incubation resulted in a decrease in the activity of this transcriptome also, to a value of FC=−1,36 after 48 hours. In turn, treating the endometrial cancer cells for 12 hours with salinomycin resulted in small change in the expression of mRNA VEGF-B. Only after 24 and 48 hours of incubation of the cells with salinomycin a 2-fold silencing in comparison to the control was noticed. The expression of mRNA VEGF-C and VEGF-D no matter the incubation period of the cells with salinomycin is decreased in comparison to the control culture, whereas for VEGF-C, differences in the transcriptional activity between the times of 12 and 24 hours of incubation are larger than that noted for VEGF-D. Based on the carried out analysis, it can be determined that salinomycin causes a decrease in the expression of three assessed receptors, namely - mRNA VEGFR-1, VEGFR-2, and VEGFR-3. Nonetheless, for VEGF-1, it can be observed that the exposure to the drug for 24 and 48 hours causes an expression close to that noted in the control culture. The microarray profile of gene expression encoding proteins of the VEGF family and its receptors were validated using the RTqPCR technique. Statistical analysis indicated that the appearance of statistically significant differences in the transcriptional activity of the analyzed transcripts (p<0.05). For VEGF-A and VEGFR-1, the difference in expression between the culture treated with salinomycin in comparison to the control was statistically characteristic (p=0.0004). In turn, for VEGF-B, the difference between the culture exposed for 24 hours in comparison to the control (p=0.00000) was significant as well as for the comparison between H48 vs. C (p=0.00000). In relation to VEGF-C, VEGFR-2, and VEGFR-3, statistical analysis showed the significant difference between the expressions of the culture incubated with the drug for 12,24 and 48 hours in comparison to the control between the selected times. For all of these comparisons, p=0.00000 was used. The results of the microarray analysis were presented in Table 2, while the outcomes of RTqPCR were shown in Fig. (1).

In the next stage of analysis, we examined the changes in the expression profile of VEGF-A, VEGF-D, VEGFR-1, VEGFR-2, and VEGFR-3 at the protein level. The analysis showed that the expression pattern of the analyzed proteins was similar to that indicated at the transcriptome level. The results indicated that regardless of the incubation time of the endometrial cancer cells with the drug, the level of VEGF-A-D and VEGFR-1-3 isoforms were silenced in comparison with control culture. It is also worth noting that as the time of cell exposure to salinomycin was prolonged, the expression of the evaluated isoforms was lower than at shorter incubation times. The differences in the protein level were statistically significant (p<0.05; Table 3).

Table 2. The microarray expression profile of mRNA VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGFR-1, VEGFR-2, VEGFR-3 in cells exposed to 1 µM of salinomycin.

<table>
<thead>
<tr>
<th>ID Probe</th>
<th>mRNA</th>
<th>H12 vs. C</th>
<th>H24 vs. C</th>
<th>H48 vs. C</th>
</tr>
</thead>
<tbody>
<tr>
<td>210512_s_at</td>
<td>VEGF-A</td>
<td>-1.53*</td>
<td>-1.12</td>
<td>-1.36</td>
</tr>
<tr>
<td>210513_s_at</td>
<td>VEGF-A</td>
<td>-1.52*</td>
<td>-1.09</td>
<td>-1.38</td>
</tr>
<tr>
<td>211527_x_at</td>
<td>VEGF-C</td>
<td>-1.56*</td>
<td>-1.16</td>
<td>-1.22</td>
</tr>
<tr>
<td>212171_x_at</td>
<td>VEGF-C</td>
<td>-1.57*</td>
<td>-1.03</td>
<td>-1.31</td>
</tr>
<tr>
<td>203683_s_at</td>
<td>VEGF-B</td>
<td>+1.12</td>
<td>-1.98*</td>
<td>-2.02*</td>
</tr>
<tr>
<td>209946_at</td>
<td>VEGF-C</td>
<td>-2.88*</td>
<td>-4.07*</td>
<td>-4.11*</td>
</tr>
<tr>
<td>206742_at</td>
<td>VEGF-D</td>
<td>-3.01*</td>
<td>-3.69*</td>
<td>-3.41*</td>
</tr>
<tr>
<td>210287_s_at</td>
<td>VEGFR-1</td>
<td>-1.55*</td>
<td>-1.22</td>
<td>-1.08</td>
</tr>
<tr>
<td>222033_s_at</td>
<td>VEGFR-1</td>
<td>-1.54*</td>
<td>-1.18</td>
<td>-1.17</td>
</tr>
<tr>
<td>204406_at</td>
<td>VEGFR-2</td>
<td>-1.48*</td>
<td>-1.23</td>
<td>-1.12</td>
</tr>
<tr>
<td>203934_at</td>
<td>VEGFR-2</td>
<td>-1.77*</td>
<td>-2.03*</td>
<td>-1.88*</td>
</tr>
<tr>
<td>210316_at</td>
<td>VEGFR-3</td>
<td>-2.11*</td>
<td>-2.8*</td>
<td>-3.01*</td>
</tr>
</tbody>
</table>

(*) 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 time of exposure to salinomycin. The data showed the mean +/- SD of 3 separate experiments, each performed in triplicate, RTqPCR.

* Statistically significant differences in the expression of the gene in the endometrial cancer culture with salinomycin in comparison to the cell culture (p<0.05).
Fig. (1). The expression relationships between the assessed VEGF-A-D and VEGFR-1-3 isoforms in endometrial cancer cells exposed to salinomycin. The data showed the mean +/- SD of 3 separate experiments, each performed in triplicate, RTqPCR. (A higher resolution/colour version of this figure is available in the electronic copy of the article).

Table 3. Expression profile of proteins VEGF-A, VEGF-D, and VEGFR-1-3 in endometrial cancer cells exposed to salinomycin.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Control</th>
<th>H12 vs. C</th>
<th>H24 vs. C</th>
<th>H48 vs. C</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A [pg/ml]</td>
<td>398.17±2.01</td>
<td>214.03±2.14*</td>
<td>125.14±1.98*</td>
<td>100.22±1.75*</td>
</tr>
<tr>
<td>VEGF-B [pg/ml]</td>
<td>682.03±4.09</td>
<td>401.99±1.98*</td>
<td>236.44±2.74*</td>
<td>101.06±3.65*</td>
</tr>
<tr>
<td>VEGF-C [pg/ml]</td>
<td>3204.14±3.58</td>
<td>2584.01±6.29*</td>
<td>1855.94±1.84*</td>
<td>1590.76±1.04*</td>
</tr>
<tr>
<td>VEGF-D [ng/ml]</td>
<td>7.89±0.11</td>
<td>5.02±0.08*</td>
<td>4.33±0.07*</td>
<td>3.01±0.09*</td>
</tr>
<tr>
<td>VEGFR-1 [ng/ml]</td>
<td>2.58±0.19</td>
<td>2.00±0.14*</td>
<td>0.95±0.04*</td>
<td>0.85±0.06*</td>
</tr>
<tr>
<td>VEGFR-2 [pg/ml]</td>
<td>658.96±4.15</td>
<td>520.11±6.84*</td>
<td>425.08±5.11*</td>
<td>300.14±8.74*</td>
</tr>
<tr>
<td>VEGFR-3 [ng/ml]</td>
<td>11.85±0.18</td>
<td>7.46±0.36*</td>
<td>5.85±0.14*</td>
<td>3.22±0.18*</td>
</tr>
</tbody>
</table>

C - Control culture; 12h, 24h., 48h time of exposure to salinomycin.
* - Statistically significant differences in the expression of the analyzed protein in the endometrial cancer culture with salinomycin in comparison to the cell culture (p<0.05). The data showed the mean +/- SD of 3 separate experiments, each performed in triplicate.

Table 4. The effect of transfecting the endometrial cancer cell line exposed to the drug after transfecting for VEGF165 siRNA or siRNAscr on the expression of VEGF-A-D and VEGFR1-3.

<table>
<thead>
<tr>
<th></th>
<th>Cells with Salinomycin and VEGFsiRNA</th>
<th>Cells with Salinomycin and siRNAscr</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A [pg/ml]</td>
<td>101.22±1.14*</td>
<td>310.13±3.08</td>
</tr>
<tr>
<td>VEGF-B [pg/ml]</td>
<td>84.16±0.98*</td>
<td>514.89±4.68</td>
</tr>
<tr>
<td>VEGF-C [pg/ml]</td>
<td>752.14±1.36*</td>
<td>3011.21±2.74</td>
</tr>
<tr>
<td>VEGF-D [ng/ml]</td>
<td>2.11±0.14*</td>
<td>7.11±0.76</td>
</tr>
<tr>
<td>VEGFR-1 [ng/ml]</td>
<td>0.99±0.09*</td>
<td>2.96±0.48</td>
</tr>
<tr>
<td>VEGFR-2 [pg/ml]</td>
<td>250.11±3.54*</td>
<td>699.84±3.45</td>
</tr>
<tr>
<td>VEGFR-3 [ng/ml]</td>
<td>5.02±0.066*</td>
<td>14.09±0.44</td>
</tr>
</tbody>
</table>

* - Statistically significant differences in the expression of the VEGF's isoforms in the endometrial cancer culture with salinomycin and VEGF siRNA in comparison to the untreated cells and cells with siRNAscr (p<0.05). The data showed the mean +/- SD of 3 separate experiments, each performed in triplicate.
In turn, to determine if salinomycin might have influence via VEGF paths, the level of VEGFs and their receptors were evaluated in the endometrial cancer cell line exposed to the drug after transfecting for VEGF165 siRNA or siRNAscr (as a negative control). Table 4 presents the results of this stage of the experiment. In turn, in Table 5, the effect of transfecting endometrial cancer cells, which were not exposed to the drug by VEGF165 siRNA or siRNAscr was presented. The obtained results showed that salinomycin might affect VEGF paths. Transfection cells with VEGF siRNA caused silencing of the VEGF expression in comparison to the control (p<0.05, Tables 4 and 5).

4. DISCUSSION

In reference to salinomycin, the phrase “old drug, new viewpoint” can be used. The mechanism of action that salinomycin has, is one that has a high affinity with potassium ions, causing their translocation from the mitochondrion to the cytoplasm of the cell. It is one of the mechanisms that probably induces the apoptosis of cells through salinomycin [19, 20]. The positive effects of salinomycin’s actions in probably induces the apoptosis of cells through salinomycin the cytoplasm of the cell. It is one of the mechanisms that ions, causing their translocation from the mitochondrion to

<table>
<thead>
<tr>
<th>-</th>
<th>Cells with VEGFsirNA</th>
<th>Cells with siRNAscr</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A [pg/ml]</td>
<td>196.25±1.19*</td>
<td>373.13±3.77</td>
</tr>
<tr>
<td>VEGF-B [pg/ml]</td>
<td>204.44±0.85*</td>
<td>401.22±1.07</td>
</tr>
<tr>
<td>VEGF-C [pg/ml]</td>
<td>1095.47±2.01*</td>
<td>1458.01±1.62</td>
</tr>
<tr>
<td>VEGF-D [ng/ml]</td>
<td>4.01±0.49*</td>
<td>7.80±0.86</td>
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<tr>
<td>VEGFR-1 [ng/ml]</td>
<td>1.19±0.40*</td>
<td>2.46±0.88</td>
</tr>
<tr>
<td>VEGFR-2 [pg/ml]</td>
<td>350.11±3.22*</td>
<td>622.94±3.41</td>
</tr>
<tr>
<td>VEGFR-3 [ng/ml]</td>
<td>8.09±0.74*</td>
<td>11.09±0.55</td>
</tr>
</tbody>
</table>

*p* Statistically significant differences in the expression of the VEGFs isoforms in the endometrial cancer culture with salinomycin and VEGF siRNA in comparison to the untreated cells and cells with siRNAscr (p<0.05). (The data showed the mean +/- SD of 3 separate experiments, each performed in triplicate.

conduct at the protein level [25], whereas ours was conducted in regards to the mRNA level. Based on the results obtained as part of this work, it can be noted that salinomycin at a concentration of 1 µM already after 12 hours affects the transcriptome of endometrial cancer cells. A decrease in the transcriptional activity of VEGF-A and VEGFR-2 was determined, wherein, as the incubation time with the drug increased, the expression of VEGF-A gradually returned to the level observed in the control culture, whereas the expression for VEGFR-2 continued to decrease. If the expression of both these genes increases in time, it could be assumed that the cause of the observed changes is a decrease in the drug working on the endometrial cancer cells, or possibly cells developing changes to adapt to a changing environment. It is worth remembering, however, that the cancer cells do not have to express all isoforms of VEGF and VEGFR simultaneously. The analysis carried out by Wang et al. indicated that only 63% of 76 endometrial cancers studied by them expressed VEGF-A, 55% - VEGFR-2, and only 26% - VEGFR-3 [26]. Furthermore, it could be that salinomycin changes the proportion of expression of VEGF-A/VEGFR-2 and the observed changes in the expression profile of these transcriptomes are a result of the fact that a smaller number of cells of the Ishikawa cell line exposed to salinomycin were characterized by an expression of VEGF-A in comparison to VEGFR-2.

Acting as an inhibitor of new blood vessels forming, salinomycin blocks the flow of nutrients and oxygen to the cancer cells [7, 8], which leads to the disturbance of their metabolism. This can serve as a new possible way of utilizing salinomycin. The VEGF-A/VEGFR-2 compound is described as the key to angiogenesis [27]. The traits that inhibit angiogenesis were described by Bi et al. on a glioma model. These authors indicated that salinomycin is an inhibitor of the complex signaling pathway VEGF-VEGFR2-AKT/FAK. In conclusion, these authors determined that salinomycin is active in both in vitro and in vivo conditions concerning glioma cells, inhibiting its proliferation by influencing the VEGF-VEGFR2-AKT/FAK pathway [28]. This shows the multidirectional character of the actions of the analyzed drug on the molecular level as well as that it confirms the complex picture that accompanies carcinogenesis.
Moreover, we observed that salinomycin also causes the inhibition of VEGF-B and VEGFR-1 expression, wherein, the most noticeable changes for VEGF-B can be observed after a longer incubation time of the cells with the drug, and VEGFR-1 after exposition that lasted 12 hours. VEGF-B is characterized by a much smaller pro-angiogenic potential than VEGF-A. In recent times, the importance of VEGF-B as a protective factor of cells has been highlighted as well as tissue under oxidative stress, as it affects the increase in the activity of key enzymes with an antioxidant effect [29]. The expression of VEGF-B is characteristic of many types of cancer [30]. Hanrahan et al. noted significantly higher levels in the expression of VEGF-A, VEGF-B, VEGF-C, and VEGF-D in glioma samples of the large intestine in comparison to the control, indicating simultaneously the difficulty in assigning the given receptor - VEGF-R1, VEGFR-2, VEGFR-3 - the dominating role in the angiogenic or lymphangiogenesis process [31]. Therefore a decreased expression of VEGF-B could be a result of the programmed cell death being activated, which is connected with the oxidative stress phenomenon caused by salinomycin. Therefore, the smaller number of VEGF-B transcripts only after 24 and 48 hours in comparison to the control allows for the assumption that after this time the protective mechanisms of the cancer cells have been exhausted, resulting in an increase in the Reactive Oxygen Species (ROS), intensification of oxidative stress and the death of cancer cells [32]. Also, the decreased expression of VEGFR-1 suggests the beneficial effect caused by salinomycin, as this receptor has a significant role in cells gaining the potential for metastasis [33]. Also, Atozri et al. indicate that the increased expression of VEGFR-1 is characteristic for cancer cells that have a phenotype that presents sensitivity to treatment, with a higher capability for metastasis [34]. As part of this work, we also analyzed the expression profiles of VEGF-C, VEGF-D, and VEGF-3, factors characteristic for lymphangiogenesis, under the influence of 1 µM of salinomycin. Sopo et al. indicate that the overexpression of VEGF-C, VEGF-D, and a decrease in the level of VEGF-3 is an unfavorable prognosis marker for patients with ovarian cancer [35]. Also, Zajkowska et al., indicate higher expression of VEGF-A, VEGF-C, and VEGF-D in breast cancer [36]. On the other hand, however, in research regarding changes in the expression of VEGF-3 in endometrial cancer, overexpression was noted, which negatively correlated with the length of survival [37]. Furthermore, it was observed that the level of VEGF-3 is not dependent on the presence of ligands, indicating the role of this receptor in the remodeling process of blood vessels [38]. This might also suggest that the expression profile of VEGF-3 is dependent on the type of cancer, wherein in one type of cancer, a negative prognosis could be the inhibition of expression, but in another type, it might be an increase in its expression [35-38]. It seems significant then to take into account the context of the observed changes when interpreting them. Taking into account the observations of Wang et al. [26], a lowered expression of the discussed reporter indicates the therapeutic effect caused by salinomycin.

Based on the changes in the transcriptional activity of mRNAs induced by salinomycin observed in this study, it can be assumed that also in vivo, this drug would have anti-angiogenic and anti-lymphangiogenic effects. Thus, the number of modern therapeutic options available to women with endometrial cancer would increase. In addition, it seems that changes in the expression of the genes we analyzed can be considered as new, complementary molecular markers in the diagnosis and response to salinomycin treatment for endometrial cancer.

It is worth highlighting that this is the first study that assesses the changes of angiogenic factors and lymphangiogenesis of VEGF and their receptors in in vitro endometrial cancer when treated with salinomycin. In addition, transcriptome analysis was performed using two methods: mRNA microarray and RTqPCR (validation of a microarray experiment). This analysis allowed us to investigate whether salinomycin, so far best known for its bactericidal properties, has the potential to become a new drug in anti-angiogenic/anti-lymphangiogenic oncology. Nevertheless, in subsequent stages of research, it would be advisable to evaluate changes in the expression pattern of the analyzed genes also in vivo, as well as to extend the analysis to other genes associated with the process of angiogenesis and lymphangiogenesis. This would also indicate the effectiveness of salinomycin working depending on the degree of the differentiated endometrial cancer (G1-G3) [39, 40]. It would also be interesting to determine the changes in the expression profile of VEGFs and their VEGFRs receptors at the protein level, as well as to indicate whether the epigenetic mechanisms of regulating gene expression affect the expression profile of the assessed mRNAs observed in our study.

CONCLUSION

The term “old drug, a new idea” perfectly characterizes salinomycin, whose anticancer qualities are only now becoming known and intensely researched. Microarray analysis of the expression profile of VEGF-A, VEGF-B, VEGF-C, VEGFR-1, VEGFR-2, VEGFR-3 confirmed using RTqPCR in an endometrial cancer culture, which confirms the influence that salinomycin has on the expression of the assessed mRNA. The obtained results suggest that salinomycin might exert the effect via VEGF signaling pathways. However, further research is recommended.

ETHICS APPROVAL AND CONSENT TO PARTICI- PATE

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 data will not be shared due to the fact the third-party rights and commercial confidentiality.
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The authors declare no conflict of interest, financial or otherwise.

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