Research Article

Pamukkale Medical Journal
doi:https://dx.doi.org/10.31362/patd.1490977

The effects of lithium, metformin and everolimus substances on cell growth in 2D and 3D Ishikawa endometrial carcinoma cell culture

Lityum, metformin ve everolimus maddelerinin 2D ve 3D Ishikawa endometrial karsinom hücre kültüründe hücre büyümesi üzerine etkileri

Emine Tural, Nazlı Çil, Mücahit Seçme, Gülçin Abban Mete, Hakan Darıcı, Ayhan Bilir, Erdal Karaöz

Posted date:28.05.2024 Acceptance date:03.07.2024

Abstract

Purpose: Our aim is to study the effects of the single and combined treatments of Everolimus, Metformin, and Lithium Chloride in two-dimensional (2D, monolayer) and three-dimensional (3D, spheroid) cell cultures of Ishikawa cells, which comprise the endometrial cancer cell line.

Materials and methods: As part of the study, the effects of single and combined forms of Everolimus, Metformin, and Lithium Chloride were determined on cell viability, invasion, colony formation and apoptosis, and PI3K/AKT/mTOR pathway. Cell viability was assessed using XTT assay. *CASP3, CASP8, CASP9, FASL, FADD, TNF, TRADD, BAX, TP53, PI3KCA, PI3KCB, PTEN, MTOR, AKT1* genes were evaluated with RT-PCR, apoptosis was evaluated by flow cytometry and 3D spheroid results were evaluated with invert microscope analysis.

Results: Everolimus, metformin, and lithium's IC50 levels were found at 48 hours to be 37.46 nM, 48.59 mM, and 100 μM, respectively. It was determined that the invasive capacities of Ishikawa cells in treatment groups, as well as cell colony formation were significantly reduced. In addition, Ishikawa spheroid cells were significantly suppressed compared with the control groups. RT-PCR results revealed that substances and their combinations affect genes associated with PI3K/AKT/mTOR pathway and apoptosis. Flow cytometry results showed notably increased apoptosis by single and combined treatments.

Conclusion: As a result, the single and combination forms of everolimus, metformin, and lithium have reduced cell proliferation, induced apoptosis, and decreased mTOR activation through various mechanisms in Ishikawa cells. However, our study has shown that Eve alone and triple combination therapy (Eve+Met+Lit) are more effective than other therapies in the treatment of endometrial cancer.

Keywords: Endometrial cancer, PI3K/AKT/mTOR pathway, everolimus, metformin, lithium.

Tural E, Cil N, Secme M, Abban Mete G, Darici H, Bilir A, Karaoz E. The effects of lithium, metformin and everolimus substances on cell growth in 2D and 3D Ishikawa endometrial carcinoma cell culture. Pam Med J 2024;17:560-576.

Öz

Amaç: Amacımız endometrium kanser hücre hattı olan Ishikawa hücrelerinin iki boyutlu (2D, monolayer) ve üç boyutlu (3D, spheroid) hücre kültürlerinde Everolimus, Metformin ve Lityum Klorür'ün tekli ve kombine tedavilerinin etkilerini incelemektir.

Gereç ve yöntem: Çalışma kapsamında, Everolimus, Metformin ve Lityum Klorürün tekli ve kombine formlarının hücre canlılığı, invazyon, koloni oluşumu ve apoptoz ve PI3K/AKT/mTOR yolağı üzerindeki etkileri belirlendi. Hücre canlılığı XTT testi kullanılarak değerlendirilmiştir. *CASP3, CASP8, CASP9, FASL, FADD, TNF, TRADD, BAX, TP53, PI3KCA, PI3KCB, PTEN, MTOR, AKT1* genleri RT-PCR ile, apoptoz flow sitometri ile ve 3D sferoid sonuçları invert mikroskop analizi ile değerlendirildi.

Bulgular: Everolimus, metformin ve lityumun IC50 seviyeleri 48 saatte sırasıyla 37,46 nM, 48,59 mM ve 100 μM olarak bulundu. Tedavi gruplarındaki Ishikawa hücrelerinin invazyon kapasitelerinin yanı sıra hücre koloni oluşumunun da önemli ölçüde azaldığı tespit edilmiştir. Ayrıca, Ishikawa sferoid hücreleri kontrol gruplarına kıyasla önemli ölçüde baskılanmıştır. RT-PCR sonuçları, maddelerin ve kombinasyonlarının PI3K/AKT/mTOR yolağı ve apoptoz ile ilişkili genleri etkilediğini ortaya koymuştur. Flow sitometri sonuçları tekli ve kombine tedavilerin apoptozu belirgin şekilde arttırdığını göstermiştir.

Emine Tural, M.D. Department of Histology and Embryology, Faculty of Medicine, Medeniyet University, Istanbul, Türkiye, e-mail: eminetural@gmail.com (https://orcid.org/0000-0003-3624-1378)

Nazlı Çil, Assoc. Prof. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli Türkiye, e-mail: ncil@pau.edu.tr (https://orcid.org/0000-0002-2164-8688) (Corresponding Author)

Mücahit Seçme, Assoc. Prof. Department of Medical Biology, Ordu University, Ordu, Türkiye, e-mail: mehtersecme@gmail.com (https://orcid.org/0000-0002-2084-760X)

Gülçin Abban Mete, Prof. Department of Histology and Embryology, Faculty of Medicine, Pamukkale University, Denizli, Türkiye, e-mail: gabban@pau.edu.tr (https://orcid.org/0000-0001-6794-3685)

Hakan Darıcı, Asst. Prof. Department of Histology and Embryology, and 3D Design&Prototyping Center, Faculty of Medicine, Istinye University, Istanbul, Türkiye, e-mail: hdarici@istinye.edu.tr (https://orcid.org/0000-0001-9393-554X)

Ayhan Bilir, Prof. Department of Histology and Embryology, Faculty of Medicine, Atlas University, Denizli, Türkiye, e-mail: ayhan.bilir@atlas.edu.tr (https://orcid.org/0009-0009-9399-5927)

Erdal Karaöz, Prof. Regenerative Medicine and Stem Cell Center, Liv Hospital, Ulus, Istinye University, Istanbul, Türkiye, e-mail: ekaraoz@hotmail.com (https://orcid.org/0000-0002-9992-833X)

Sonuç: Sonuç olarak, everolimus, metformin ve lityumun tekli ve kombinasyon formları, Ishikawa hücrelerinde çeşitli mekanizmalar yoluyla hücre çoğalmasını azaltmış, apoptozu indüklemiş ve mTOR aktivasyonunu azaltmıştır. Bununla birlikte, çalışmamız tek başına Eve ve üçlü kombinasyon tedavisinin (Eve+Met+Lit) endometriyal kanser tedavisinde diğer tedavilerden daha etkili olduğunu göstermiştir.

Anahtar kelimeler: Endometriyal kanser, PI3K/AKT/mTOR yolağı, everolimus, metformin, lityum.

Tural E, Çil N, Seçme M, Abban Mete G, Darıcı H, Bilir A, Karaöz E. Lityum, metformin ve everolimus maddelerinin 2D ve 3D Ishikawa endometrial karsinom hücre kültüründe hücre büyümesi üzerine etkileri. Pam Tıp Derg 2024;17:560-576.

Introduction

Cancers are ranked second after cardiovascular disease-related deaths due to disease. Endometrial cancer is the most common gynecological cancer in developing countries [1-3].

According to biological, molecular, and clinical characteristics endometrial cancers divide into two types. Type I tumors are low-grade International Federation of Gynecology and Obstetrics grades 1 and grade 2 (FIGO grade1, 2). These patients typically develop endometrial hyperplasia. Type II (FIGO grade 3) contains histological subtypes that are not endometrioid. They generally appear on the atrophic endometrium base, are not estrogen-dependent, and have a poor prognosis with a high grade [4-7].

To improve the treatment of endometrial cancer, signaling pathways which may play a role in the development of cancer are targeted. The PI3K/AKT/mTOR is important in endometrial cancers as well as in many other cancers. The PI3K/AKT/mTOR pathway regulates various cellular functions. PI3K/AKT/mTOR overactivation is implicated in developing endometrial cancer [8]. It is important to elucidate the antitumour effects of different PI3K/AKT/mTOR pathway inhibitors and to identify the patient populations in which these inhibitors may be most effective, based on preclinical and clinical studies [4-10].

A number of studies have investigated the role of mTOR inhibition as a single agent in the recurrence of endometrial cancer [10, 11]. Everolimus (Eve) is an effective, selective, and orally active mTOR inhibitor [12]. In previous clinical studies of EC patients with progressive or recurrent phases I and II, Eve was shown to be promising [11-13].

It has been reported that single-agent treatment such as mTOR inhibitors and its analogous activates the negative feedback and as a result, the mechanisms that lead to the development of resistance [14, 15]. Combined treatments have been applied to overcome this problem and to maximally inhibit this pathway.

It has been hypothesised that drugs targeting glucose metabolism may be effective in preventing or treating endometrial cancer because of the association between obesity, diabetes, hyperinsulinemia and endometrial cancer. A prominent drug in this area was Metformin (Met) (1.1-dimethylbiguanid), a first-line oral antihyperglycemic agent used in treating type 2 diabetes [13, 14]. Met has a direct and indirect effect on cell development and metabolism. Its direct effect activates AMP-Activated protein kinase (AMPK) and causes phosphorylation of tuberous sclerosis 2 protein and mTOR inhibition. Its indirect effect increases the glucose intake of cells, thereby reducing insulin circulation. The decrease in IGF-1 and insulin inhibit cell proliferation [15, 16].

With a well-known confidence interval, Lithium (Lit) chloride is used to treat psychotic diseases, particularly bipolar disorder. Studies with Lit have shown that it has antineoplastic effects in various cancers, including colorectal cancer, stomach cancer, and neuroblastoma [17-22]. In addition, limited clinical studies have suggested that Lit may increase the therapeutic efficacy and reduce the side effects of some anti-cancer drugs. Investigation of the antitumor effects of Lit may be important for combination treatment of endometrial cancers. This is the first study to investigate the use of Lit alone or in combination with Eve and Met for the treatment of endometrial cancer.

In this study, we investigated whether Eve, Met and Lit single and combined treatments affect the human endometrial carcinoma cell line (Ishikawa), and their potential mechanisms of action.

Materials and methods

Cell culture

This study was performed using the human endometrial adenocarcinoma cell line (Ishikawa). Cells were cultured in an RPMI 1640 nutrient environment containing 10% fetal bovine serum (FBS), 1% L-glutamine, penicillin (100 U/mL), streptomycin (100 µg/mL) in a stove under 95% humidity at 37°C and 5% CO_2. Studies were performed when the cell density reached 80-90%, studies were performed. In this study, single, dual, and triple combinations of Eve, Met and Lit were used.

The experimental groups were as follows:

Group1: Control

Group2: Everolimus 37.46 nM IC50 (Eve)

Group3: Metformin 48.59 mM IC50) (Met)

Group4: Lithium 100 µM IC50 (Lit)

Group5: Everolimus IC50 + MetforminIC50 (Eve+Met)

Group6: Everolimus IC50 + Lithium 100 µM IC50) (Eve+Lit)

Group7: Everolimus IC50 + Metformin IC50 + Lithium 100 µM IC50 (Eve+Lit+Met)

Cell viability assay

Cytotoxicity, dose, and time-dependent effects of the substances that we used were studied with the Biological Industries Cell Proliferation Kit XTT based Colorimetric Assay (CellTiter-Glo® luminescent cell viability assay REF:20-300-1000, LOT:2002010). The powdered substances were dissolved in 1/1000 DMSO in an RPMI 1640 nutrient environment containing 10% FBS at various doses, and their effects were investigated by adjusting their concentrations. The selected concentration range was determined according to the test kit protocol, considering information available in the literature. The cells were seeded in 96-well plate within RPMI 1640 with 2,000 Ishikawa cells in each well and were kept 24 hours in the incubator containing 5% CO_2 at 37°C. At the end of 24h, the medium was aspirated. Then, study of following concentrations: 0.1 mM, 1 mM, 5 mM, 10 mM, 20 mM, 50 mM, 100 mM for Met; 50 µM, 100 µM, 200 µM, 400 µM for Lit; 2 nM, 10 nM, 25 nM, 50 nM, 100 nM, 200 nM and 300 nM for Eve were prepared in the medium containing 10% FBS and the effects at 24, 48 and 72h were determined.

After 24 h, 100 µL medium was added to each well, 50 µL reagent solution A, 1 µL XTT activator mixture was prepared, and 150 µL was added per well. The cells were then incubated for 4h in an incubator containing 5% CO_2 at 37°C, and the absorbance levels of the groups were determined in the ELISA. IC50 ratio was calculated as follows:

Cell viability (%)= (Absorbance value of the substance-applied group/Absorbance value of the control group) x 100

Spheroid formation with three-dimensional (3D) cell culture method

The liquid overlay technique was used to create an *in vitro* Ishikawa spheroid model. Ishikawa cells were first seeded in a T75 flask with RPMI 1640 medium and were incubated in a stove with 95% humidity and 5% CO_2 at 37°C. When the cells were 90% confluent, they were washed with PBS, thoroughly purged from dead cells and cell waste, and aspirated. Trypsin was added to allow the cells to leave the flask. In 1500 g, the cell-media mix in the flask was centrifuged for 4 min, and the supernatant was removed. Then cells with 100% viability were cultured using RPMI 1640 to a 3% Noble agar-covered six-well culture plate with 1×10^6 cells in each well, and images of the cells were taken in the inverted microscope in certain ranges and measured in size.

Colony formation analysis

Ishikawa cells were seeded at 6-well plates at $1x10^3$ cell/well. After 24h of incubation, the cells were exposed to the single and combined concentrations of Eve, Met and Lit agents for 48h. The cells were incubated at 5% CO_2, 37°C. At the end of 14 days, the cells were fixed for 10 min with methanol and incubation. The colonies were painted with crystal violet and counted.

Matrigel invasion analysis

Ishikawa cells were seeded at a concentration of $5x10^5$ cells/wells on a Matrigel membrane with 8 µm pores and incubated overnight in a non-serum environment. They were put in RPMI 1640 24 well plates with 10% FBS containing serum. After the cells were incubated overnight, Met, Eve and Lit and combined doses were applied to the medium without serum. The cells passing through the membrane were detected by methanol, painted with crystal violet and counted.

Apoptosis detection with annexin V

The apoptotic index was evaluated using flow cytometric annexin-V-fluorescence isothiocyanate/propidium iodide (Annexin-V-FITC/PI) (BD Pharmingen™ FITC, catolog No:5565447). After the instructions in the kit's manual, the cells were washed twice with PBS and re-suspended with the 0.01 M HEPES, 0.14 mM NaCl, and 2.5 mM $CaCl_2$ containing binding pad. The cells in the cell suspension were incubated with 5 µL Annexin V (BD Pharmingen) FITC-labelled stain and PI for 15 min at room temperature in the dark. PI fluorescence and Annexin V were measured at the same time in a (BD FACS Calibur™, Cat. No:349227) and analyzed with the operating software of the device.

Real-time PCR analysis

RNA isolation was performed in the groups to assess expression at the gene level. Cells were seeded at a density of $3×10^6$ cells/well. After 24h of incubation, Lit, Eve, Met, and their combination were applied to each well, except the control well, and incubated for 48h. Complementary DNA (cDNA) was synthesized using the cDNA Synthesis Kit (WizScript™). *CASP3, CASP8, CASP9, PI3KCA, PI3KCB, FASL, FADD, TNF, TRADD, BAX, TP53, PTEN, mTOR, and AKT* expression analyses were performed using the StepOnePlus quantitative real-time PCR (Thermo Scientific PikoReal 96) according to the expression analysis SYBR Green (Thermo Scientific, USA). Values were normalised to *ACTB* levels for each gene (Table 1).

Statistical evaluation of data

PCR data were analysed by the ΔΔCT method and quantified by a computer programme. The Volcano Plot analysis provided by the RT² Profiler; PCR Array Data Analysis programme was used to compare the groups. Student's t-test was used to statistically evaluate the comparison between groups. SPSS 24.0 package program was used for data analysis. Continuous variables were presented in terms of mean ± standard deviation and medians (minimum and maximum), and categorical variables in terms of numbers and proportions. The Shapiro-Wilk test was used to examine the conformity of the data for normal distribution. The Kruskal-Wallis Analysis of Variance (Post-hoc, Mann-Whitney U-test with Bonferroni adjustment) was used to compare independent group differences (p<0.05, considered statistically significant).

Results

Eve, Met and Lit and their combined forms decreased cell proliferation of Ishikawa cells

In the results of the 24h cell viability test of the applied substances, dose rates at 48h were considered IC50 as cell proliferation did not fall below 50%. The varying dose ranges and overtime effects of Eve, Met, Lit alone, and combined treatments are shown in Figure 1.

Eve, Met and Lit and their combination reduced Ishikawa 3D spheroids

In the 48h images, which represent the IC50 time of the doses, a reduced number of spheroids, shrinking sizes, and border irregularities were detected. The control group presented a large number of spheroids with an average size of 170 µm, ranging from 120 µm to 225 µm. In treatment groups, average of spheroid size in the Eve treatment group was 71.33 µm, average of spheroid size in the Met treatment group was 97.5 µm, in the Lit treatment group was 90 µm, in the Eve+Met treatment group was 96.4 µm, in the Eve+Lit, treatment group was 97 µm, in the Eve+Met+Lit treatment group was found to be 80 µm (Figure 2).

Table 1. Primary list of genes analyzed on RT-PCR

Genes	Primer Sequence
ACTB	F: CACCATTGGCAATGAGCGGTTC R: AGGTCTTTGCGGATGTCCACGT
CASP3	F: GGAAGCGAATCAATGGACTCTGG R: GCATCGACATCTGTACCAGACC
CASP8	F: AGAAGAGGGTCATCCTGGGAGA R: TCAGGACTTCCTTCAAGGCTGC
CASP9	F: GTTTGAGGACCTTCGACCAGCT R: CAACGTACCAGGAGCCACTCTT
MTOR	F: GCTTGATTTGGTTCCCAGGACAGT R: GTGCTGAGTTTGCTGTACCCATGT
AKT1	F: TTCTGCAGCTATGCGCAATGTG R: TGGCCAGCATACCATAGTGAGGTT
PIK3CA	F: GGTTGTCTGTCAATCGGTGACTGT R: GAACTGCAGTGCACCTTTCAAGC
PIK3CB	F: TTGTCTGTCACACTTCTGTAGTT R: AACAGTTCCCATTGGATTCAACA
BAX	F: TCAGGATGCGTCCACCAAGAAG R: TGTGTCCACGGCGGCAATCATC
PTEN	F: TGAGTTCCCTCAGCCGTTACCT R: GAGGTTTCCTCTGGTCCTGGTA
FADD	F: GCTGGCTCGTCAGCTCAAA R: ACTGTTGCGTTCTCCTTCTCT
TRADD	F: GCTGTTTGAGTTGCATCCTAGC R: CCGCACTTCAGATTTCGCA
FASL	F: GGTTCTGGTTGCCTTGGTAGGA R: CTGTGTGCATCTGGCTGGTAGA
TNFα	F: CTCTTCTGCCTGCTGCACTTTG R: ATGGGCTACAGGCTTGTCACTC
TP53	F: ATCTACAAGCAGTCACAGCACAT R: GTGGTACAGTCAGAGCCAACC

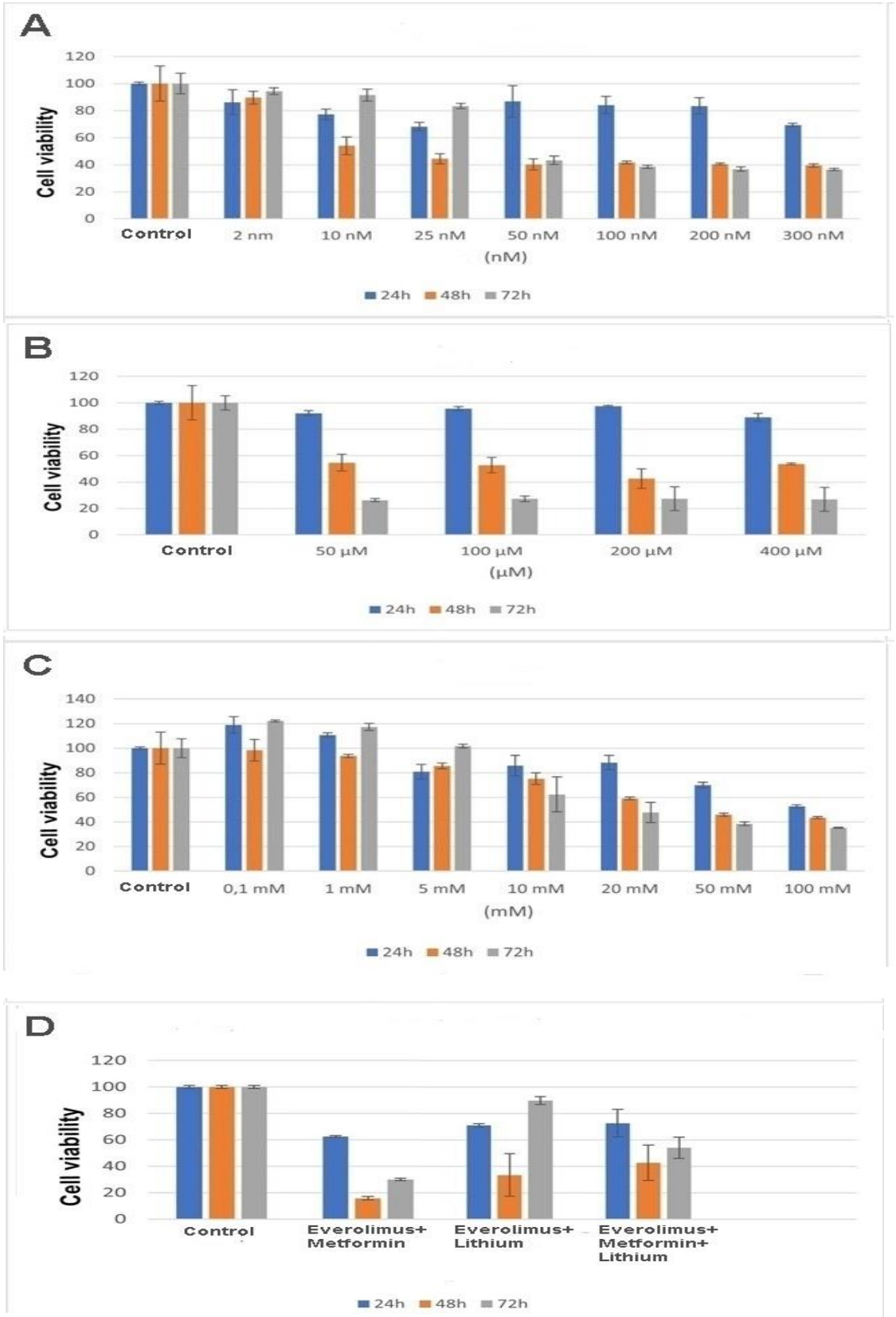

Figure 1. The effect of Everolimus, Lithium, Metformin, and combined doses on Ishikawa cell viability.
A) IC50 doses of Everolimus; Ishikawa cells were detected 37.46 nM at the 48th hour
B) IC50 doses of Metformin; Ishikawa cells were detected 48.59 mM at the 48th hour
C) IC50 doses of Lithium; Ishikawa cells were detected 100 mM at the 48th hour
D) IC50 doses of combined treatment; Ishikawa cells were detected at the 48th hour

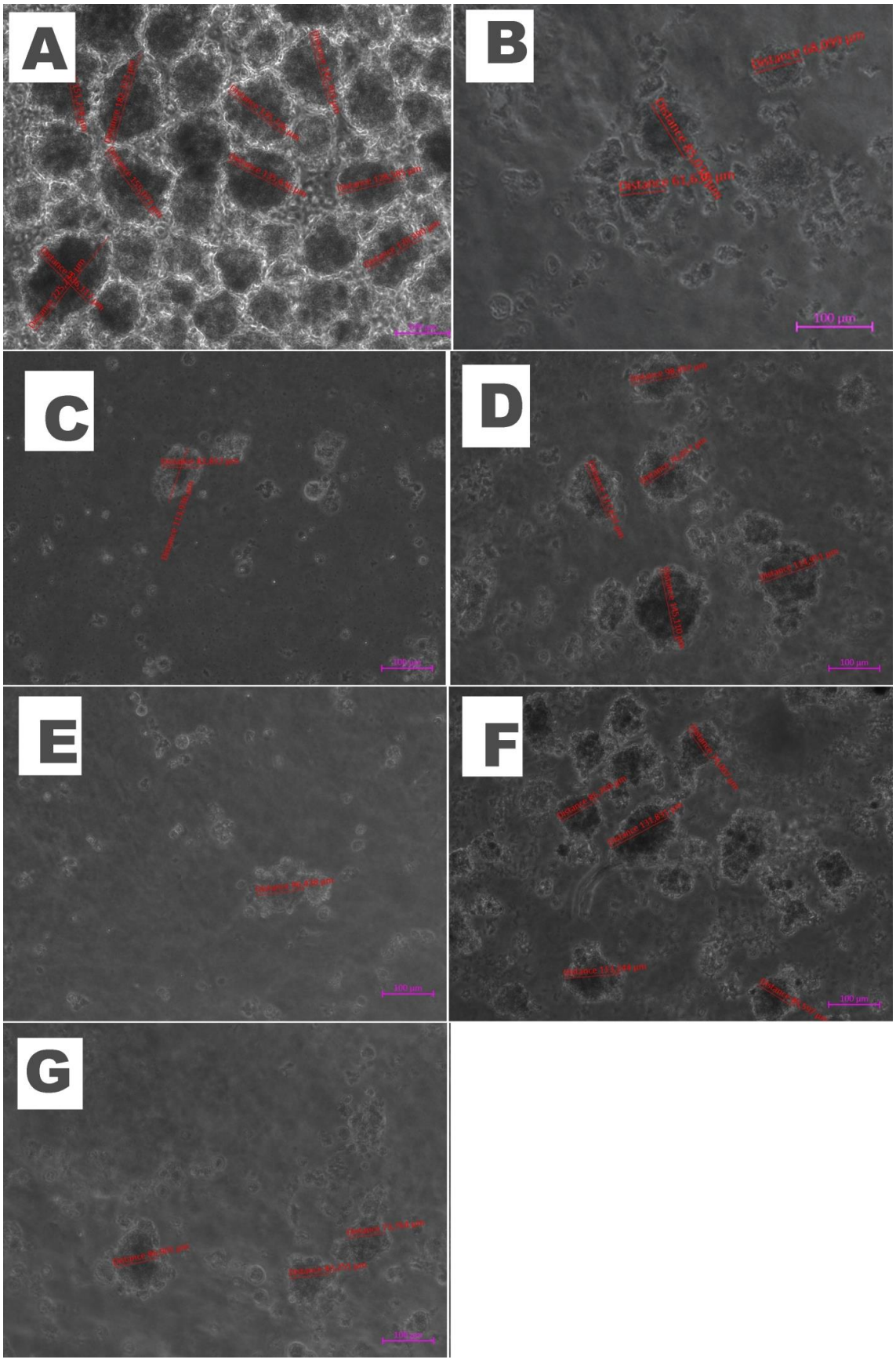

Figure 2. Images of spheroids consisting of endometrial cancer cells. A: Control, B: Everolimus, C: Metformin, D: Lithium, E: Everolimus + Metformin, F: Everolimus + Lithium, G: Everolimus + Metformin + Lithium. Bar:100µm

Eve, Met and Lit and their combined forms reduced colony formation capacity of Ishikawa cells

In all groups colony numbers in Ishikawa cells was significantly reduced with respect to the control group. The colony formation data of the groups in the Ishikawa cell array was shown as follows, respectively: Control (382.33±20.53), Eve (195±13.23), Met (126±7), Lit (251.67±12.58) Eve+Met (48.33±13.01), Eve+Lit (210±43.59) Eve+Met+Lit (62.67±15.37) Eve+Met was the lowest in the colony formation analysis compared to the control group (Figure 3).

Eve, Met and Lit and their combined forms inhibits migration of Ishikawa cells

As a result of the Matrigel invasion experiment, single and combined of Eve, Met and Lit administered Ishikawa cells noticeably decreased according to the control group of the invasion. The invasive data of the groups in the Ishikawa cell array were as follows, respectively: Control (1420±5), Eve (311±3.61), Met (10±0), Lit (47.33±2.52), Eve+Met (10±0), Eve+Lit (210±43.59), Eve+Met+Lit (351±3) (Figure 4).

Eve, Met and Lit and their combined forms induces apoptosis on Ishikawa cells

Flow cytometry results

According to the flow cytometry results, compared to the control group, late apoptosis increased significantly in the treatment groups. The highest rate of late apoptosis was observed in the Eve+Met+Lit and Eve+Lit groups (Figure 5).

Eve, Met and Lit and their combined forms changes mRNA expressions genes

In the group administered Eve, *FASL* and *CASP3* showed a significant increase. In the group administered with Met, *FASL, PTEN, CASP9*, and *TRADD* increased significantly ($p<0.05$), whereas *CASP8* decreased significantly ($p<0.05$) in the Lit administered group. In the group administered Eve+Met, there was a significant increase in *FADD, CASP9, and BAX* values, and the Eve+Lit group showed a significant increase in *FADD* and *TNF* values ($p<0.05$). *PTEN*, *FADD, TNF*, and *TP53* levels increased significantly ($p<0.05$) in the Eve+Met+Lit group (Table 2, 3).

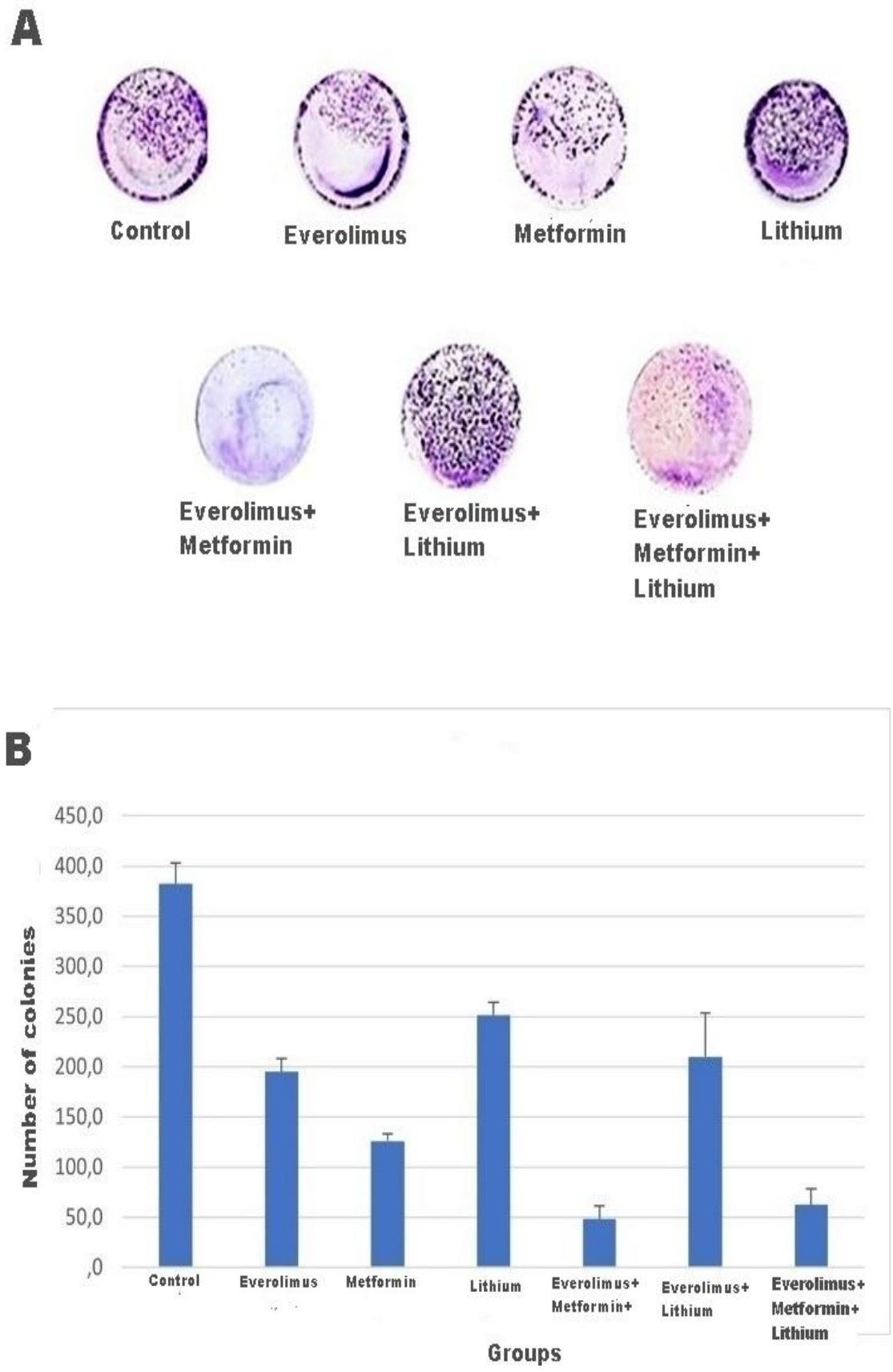

Figure 3. A: Everolimus, Lithium, Metformin and combined doses decrease colony formation in Ishikawa cells. Colonies were stained with crystal violet. B: Data was presented as mean ± SD

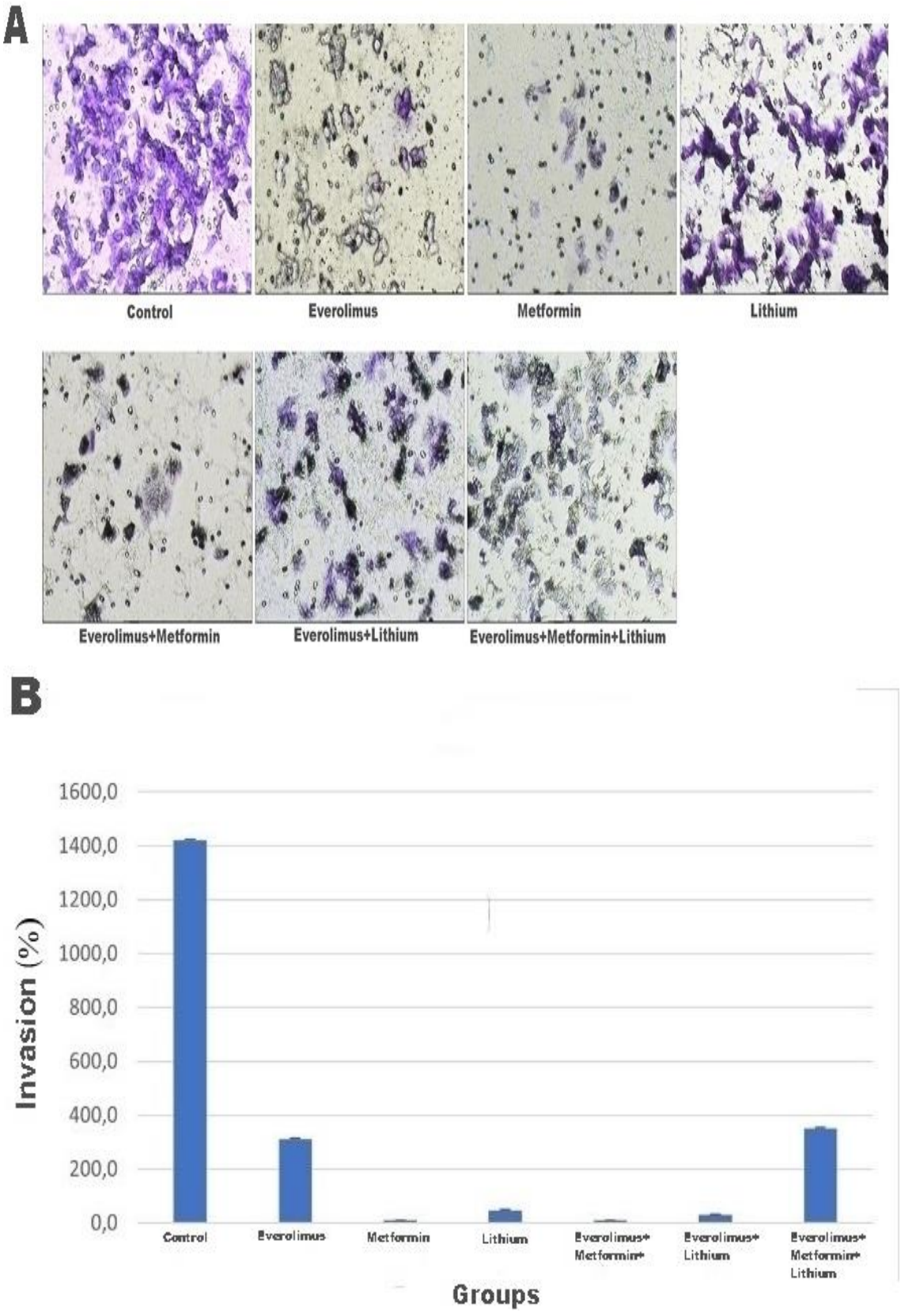

Figure 4. A: Migration and invasion assay results of Ishikawa cells were showed. Cells that passed through the membrane were counted in 10 representative areas. Graph showing invasion for control and dose groups. B: Graph showing invasion for both control and dose groups. Data were presented as mean ± SD

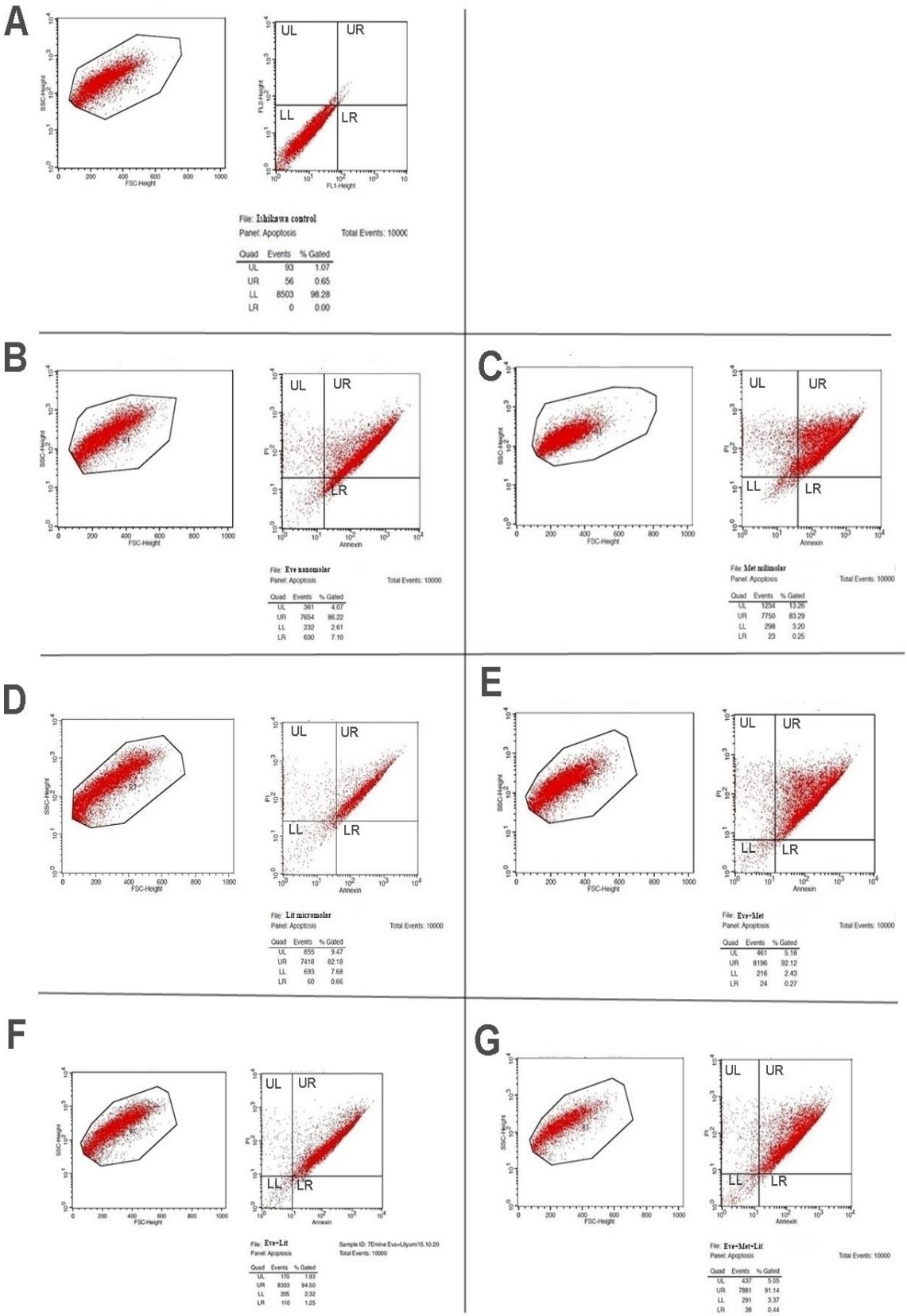

Figure 5. The effect of Everolimus, Metformin, Lithium, and combined doses on apoptosis in Ishikawa cells. A: Control, B: Everolimus, C: Metformin, D: Lithium, E: Everolimus+ Metformin, F: Everolimus +Lithium, G: Everolimus + Metformin + Lithium. UL: Necrotic cell, LL; Live cell, UR: Late apoptotic cell, LR: early apoptotic cell

Table 2. The mRNA expression changes of cell cycle and apoptosis genes in Ishikawa cell line treated with everolimus (Eve), metformin (Met) and lithium (Lit), compared with the control group cells

Gene Symbol	Fold regulation (comparing to the control grup)					
	Eve		Lit		Met	
	Fold regulation	*p*-value	Fold regulation	*p*-value	Fold regulation	*p*-value
ACTB	1.00	nan	1.00	nan	1.00	nan
CASP-8	1.13	0.64	-4.92	0.01	-1.16	0.40
FASL	2.11	0.02	-1.38	0.47	1.87	0.04
FADD	6.88	0.23	23 75	0.10	32.90	0.05
TNF-Alpha	10.51	0.09	5.64	0.19	2.60	0.35
TRADD	-1.71	0.90	3.54	0.15	13.83	0.00
CASP-9	-1.63	0.56	4.75	0.13	2.30	0.00
BAX	-1.45	0.31	3.12	0.14	1.98	0.19
CASP-3	1.65	0.04	1.39	0.46	1.38	0.06
TP53	1.21	0.79	-1.94	0.91	5.75	0.27
PTEN	-1.46	0.05	1.02	0.90	346	0.00
PIK3CA	2.72	0.66	6.93	0.42	40.13	0.13
PIK3CB	-54.07	0.24	-1.64	0.35	2.41	0.76
AKT1	-1.39	0.19	2.73	0.11	2.05	0.20
MTOR	-8.67	0.38	1.50	0.87	1.46	0.42

Table 3. The mRNA expression changes of cell cycle and apoptosis genes in Ishikawa cell line treated with everolimus+metformin (Eve+Met), everolimus+lithium (Eve+Lit), and everolimus+metformin+lithium (Eve+Met+Lit) compared with the control group cells

Gene Symbol	Fold regulation (comparing to the control grup)					
	Eve+Met		Eve+Lit		Eve+Met+Lit	
	Fold regulation	*p*-value	Fold regulation	*p*-value	Fold regulation	*p*-value
ACTB	1.00	nan	1.00	nan	1.00	nan
CASP8	-1.57	0.39	2.80	0.16	-1.13	0.47
FASL	-1.77	0.41	-2.14	0.76	1.47	0.18
FADD	18 55	0.00	15.21	0.03	-2.43	0.00
TNF	7.24	0.31	34.70	0.00	8.42	0.02
TRADD	66.72	0.33	33.59	0.37	-2.12	0.06
CASP9	7.43	0.01	7.91	0.06	-1.31	0.41
BAX	4.41	0.02	2.35	0.25	1.17	0.64
CASP3	2.44	0.21	1.32	0.42	-1.10	0.57
TP53	100.66	0.37	12.35	0.10	4.34	0.03
PTEN	2.79	0.24	1.78	0.11	-2.76	0.00
PIK3CA	21,51	0.37	15.93	0.17	-2.47	0.41
PIK3CB	4.52	0.50	-3.18	0.28	-9.49	0.26
AKT1	2.08	0.19	3.42	0.15	5.51	0.36
MTOR	6.56	0.62	4.77	0.41	1.40	0.42

Discussion

This is the first study to investigate the effects of single and combined forms of Eve, Met and Lit on cell viability, colony formation, cell invasion, spheroid formation, apoptosis, PI3K/Akt/mTOR signaling pathway in cultures of Ishikawa cells. In our study, colony formation and invasion capacity decreased significantly in the single and combined treatment groups compared with the control group. In particular, the combination of Met and Eve and Eve+Met+Lit was significantly more effective than either monotherapy or the other combined therapies in inhibiting cell invasion and the ability of Ishikawa cells to form colonies, a crucial event involved in tumorigenesis. These results were consistent with previously reported findings that the combined use of Met and Eve synergistically augmented anticancer activity in the treatment of breast and cervical cancers [21-25].

3D spheroids geometrically and molecularly mimic tumors *in vivo* compared to monolayer 2D cells [26]. 3D cell culture has become an effective method for drug screening [27]. In our study, the effects of Met, Eve, and Lit alone and in combination on the growth of Ishikawa 3D cells were examined. Interestingly, Eve, Met and Lit alone and in combination blocked the

growth rate of Ishikawa spheroids. However, Eve alone was quite potent in reducing the growth of spheroids compared to other groups, except for the Eve+Met+Lit group. The results in the triple combination group were similar to those in the Eve group.

We examined whether the antineoplastic effects of Met, Eve, and Lit single and combined administration were mediated by the induction of apoptosis. Apoptosis was higher in all treatment groups than that in the control group. However, while early apoptosis was highest in the Eve treatment group, late apoptosis was slightly higher in all combined groups than in the single treatment groups.

When the molecules involved in the extrinsic and intrinsic apoptotic pathways were examined by RT-PCR analysis to elucidate the mechanism of apoptosis, it was determined that both the extrinsic and intrinsic pathways were effective in the single and combined treatments. In addition, another apoptosis-related gene, *TP53,* which plays an important role in the regulation of cell proliferation, DNA repair, apoptosis, was significantly upregulated in the Eve+Met+Lit treatment group.

The PI3K/AKT/mTOR is closely related to various common malignancies and plays a major role in resistance to anticancer drugs in response to treatment [28-32]. In a wide range of tumours, the components of this pathway support irregular and limitless cancer cell growth and proliferation and contribute to the tumor by allowing the avoidance of apoptosis [33, 34]. It is known that the increased mTOR activity supports the PI3K/AKT/mTOR pathway. Supported by preclinical data the important role of mTOR in cancer, resulting in mTOR inhibiting drugs being developed as a therapeutic target [35]. Eve is an inhibitor of rapamycin (mTOR), which has an antitumor effect on cancers and reduces the proliferation of endometrial cancers through mTOR inhibition [36]. According to our PCR results, a decrease in *mTOR* mRNA levels was observed in all the groups.

The *PTEN* gene is a negative regulator of the PI3K/AKT/mTOR pathway, which is the most frequent alteration in endometrioid tumors [14, 15]. In preclinical studies, mutations in various genes have been identified in the molecular analysis of endometrial cancers. *PTEN* and *PI3CA* were the most common genes. *PTEN* loss has been detected in 80% of endometrial tumors [14]. Thus, abnormal cell growth and escape from apoptosis due to loss of PTEN activity [13]. We observed that single-met treatment increased *PTEN* mRNA levels. These results are in line with those reported by other studies that Met increased *PTEN* mRNA levels in Ishikawa cells and advanced and recurrent endometrial cancers [37, 38]. According to the PCR results, the lowest *PTEN* levels were determined in the Eve alone and Eve+Met+Lit combined groups. These results suggest that Eve suppresses the efficacy of Met, and that Eve and Eve+Met+Lit may use different pathways to inhibit mTOR in endometrial cancers.

Increased *PI3K* activity via gain of function has also been shown in several human cancers: *PIK3CA,* with a frequency of up to 33% in Type I endometrial tumors and 15-20% in Type II endometrial tumors, is the second most mutated gene [39-42]. Previous studies have shown that *PIK3CB*, the other catalytic component of PIK, is highly expressed in endometrial cancer cell lines and clinical samples taken at the initial stage of endometrial carcinogenesis. *PIK3CB* mutations are less frequent than *PIK3CA* mutations [43, 36]. Both *PIK3CA and PIK3CB* mRNA levels were low in the Eve+Met+Lit group, indicating that the triple combined treatment was effective against *PIK3CA* and *PIK3CB* mutations. *AKT* is known to regulate signaling events that promote cell survival, proliferation, angiogenesis, and invasion [44]. In our study, no significant change was observed in *AKT* mRNA level in any groups.

This study has some limitations. These include not demonstrating the effects of the tested substances on non-cancerous cell lines by using any healthy normal cells in the study, and also evaluating the study using only one endometrial cancer cell line. It is recommended to improve the study by using additional cell lines and healthy cells.

In conclusion, we determined that the single and combination of Eve, Met, and Lit were effective in inhibiting Ishikawa cell proliferation, promoting apoptosis, and preventing colony formation and invasion capacity, probably acting via different pathways in mTOR inhibition.

Our study showed that single and combined therapies, especially Eve alone and Eve+Met+Lit, were more effective than other therapies in the treatment of endometrial cancer. We believe that Eve+Met+Lit therapy will be an alternative in the treatment of endometrial cancer because of the possibility of resistance in a single application, the high probability of combined treatments to prevent this condition, and the ability to treat it as an anti-carcinogen at lower doses; however, we believe that further preclinical and clinical studies are required on this matter.

Conflict of interest: No conflict of interest was declared by the authors.

References

1. Ferlay J, Soerjomataram I, Dikshit R, et al. Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015;136:359-386. https://doi.org/10.1002/ijc.29210
2. Colombo N, Creutzberg C, Amant F, et al. 2 ESMO-ESGO-ESTRO consensus conference on endometrial cancer: diagnosis, treatment and follow-up. Ann Oncol 2016;27:16-41. https://doi.org/10.1093/annonc/mdv484
3. Nyen TV, Moiola CP, Colas E, Annibali D, Amant F. Modeling endometrial cancer: past, present, and future. Int J Mol Sci 2018;19:2348. https://doi.org/10.3390/ijms19082348
4. Bokhman JV. Two pathogenetic types of endometrial carcinoma. Gynecol Oncol 1983;15:10-17. https://doi.org/10.1016/0090-8258(83)90111-7
5. Piulats JM, Guerra E, Gil Martín M, et al. Molecular approaches for classifying endometrial carcinoma. Gynecol Oncol 2017;145:200-207. https://doi.org/10.1016/j.ygyno.2016.12.015
6. Korch C, Spillman MA, Jackson TA, et al. DNA profiling analysis of endometrial and ovarian cell lines reveals misidentification, redundancy and contamination. Gynecol Oncol 2012;127:241-248. https://doi.org/10.1016/j.ygyno.2012.06.017
7. Mendivil A, Schuler KM, Gehrig PA. Non-endometrioid adenocarcinoma of the uterine corpus: A review of selected histological subtypes. Cancer Control 2009;16:46-52. https://doi.org/10.1177/107327480901600107
8. Alzahrani AS. PI3K/Akt/mTOR inhibitors in cancer: at the bench and bedside Semin Cancer Biol 2019;59:125-132. https://doi.org/10.1016/j.semcancer.2019.07.009
9. Peng Y, Wang Y, Zhou C, Mei W, Zenget C. PI3K/Akt/mTOR pathway and its role in cancer therapeutics: are we making headway? Front Oncol 2022;12:818128(e-1-17). https://doi.org/10.3389/fonc.2022.819128
10. Rubinstein M, Shen S, Monk BJ, et al. Looking beyond carboplatin and paclitaxel for the treatment of advanced/recurrent endometrial cancer. Gynecol Oncol 2022;167:540-546. https://doi.org/10.1016/j.ygyno.2022.10.012
11. Soliman PT, Westin SN, Iglesias DA, et al. Everolimus, letrozole, and metformin in women with advanced or recurrent endometrioid endometrial cancer: a multi-center, single Arm, Phase II Study. Clin Cancer Res 2020;26:581-587. https://doi.org/10.1158/1078-0432.CCR-19-0471
12. Oza AM, Elit L, Tsao MS, et al. Phase II study of temsirolimus in women with recurrent or metastatic endometrial cancer: a trial of the NCIC clinical trials group. J Clin Oncol 2011;29:3278-3285. https://doi.org/10.1200/JCO.2010.34.1578
13. Hasskarl J. Everolimus. Recent Results Cancer Res 2018;211:101-123 https://doi.org/10.1007/978-3-319-91442-8_8
14. Sarbassov DD, Guertin DA, Sabatini DM, et al. Phosphorylation and regulation of Akt/PKB by the rictormTOR complex. Science 2005;307:1098-1101. https://doi.org/10.1126/science.1106148
15. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt Cancer Res 2006;66:1500-1508. https://doi.org/10.1158/0008-5472.CAN-05-2925
16. Wang X, Yue P, Chan CB, et al. Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. Mol Cell Biol 2007;27:7405-7413. https://doi.org/10.1128/MCB.00760-07
17. Vidal F, Araujo WM, Cruz AL, et al. Lithium reduces tumorigenic potential in response to EGF signaling in human colorectal cancer cells. Int J Oncol 2011;38:1365-1373. https://doi.org/10.3892/ijo.2011.955
18. Cho YJ, Kim JH, Yoon J, et al. Constitutive activation of glycogen synthase kinase-3beta correlates with better prognosis and cyclin-dependent kinase inhibitors in human gastric cancer. BMC Gastroenterol 2010;10:91. https://doi.org/10.1186/1471-230X-10-91
19. Bilir A, Ergüven M, Yazihan N, Aktas E, Gulperi Oktem G, Akin Sabanci A. Enhancement of vinorelbine-induced cytotoxicity and apoptosis by clomipramine and lithium chloride in human neuroblastoma cancer cell line SH-SY5Y. J Neurooncol 2010;100:385-395. https://doi.org/10.1007/s11060-010-0209-6
20. Bilir A, Erguven M, Ermis E, Sencan M, Yazihan N. Combination of imatinib mesylate with lithium chloride and medroxyprogesterone acetate is highly active in Ishikawa endometrial carcinoma in vitro. J Gynecol Oncol 2011;22:225-232. https://doi.org/10.3802/jgo.2011.22.4.225

21. Liu H, Scholz C, Zang C, et al. Metformin and the mTOR inhibitor everolimus (RAD001) sensitize breast cancer cells to the cytotoxic effect of chemotherapeutic drugs in vitro. Anticancer Res 2012;32:1627-1637.
22. Chen YH, Wu JX, Yang SF, et al. Metformin potentiates the anticancer effect of everolimus on cervical cancer in vitro and in vivo. Cancers 2021;13:4612. https://doi.org/10.3390/cancers13184612
23. Wang Y, Wei J, Li L, et al. Combined use of metformin and everolimus is synergistic in the treatment of breast cancer cells. Oncol Res 2015;22:193-201. https://doi.org/10.3727/096504015X14348950540999
24. Wong C, Vosburgh E, Levine AJ, Cong L, Xu EY. Human neuroendocrine tumor cell lines as a three-dimensional model for the study of human neuroendocrine tumor therapy. J Vis Exp 2012:e4218. https://doi.org/10.3791/4218
25. Friedrich J, Seidel C, Ebner R, Kunz Schughart LA. Spheroid-based drug screen: considerations and practical approach. Nat Protoc 2009;4:309-324.
26. Zhu H, Han B, Pan X, Qi H, Xu L. Thiazolidenediones induce tumour-cell apoptosis through the Akt-GSK3β pathway. J Clin Pharm Ther 2012;37:65-70. https://doi.org/10.1111/j.1365-2710.2011.01251.x
27. Slomovitz BM, Coleman RL. The PI3K/AKT/mTOR pathway as a therapeutic target in endometrial cancer. Clin Cancer Res 2012;18:856-864. https://doi.org/10.1158/1078-0432.CCR-12-0662
28. Barra F, Evangelisti G, Desideri LF, et al. Investigational PI3K/AKT/mTOR inhibitors in development for endometrial cancer. Expert Opin Investig Drugs 2019;28:131-142. https://doi.org/10.1080/13543784.2018.1558202
29. Stringer EM, Fleming GF. Hormone therapy plus mTOR inhibitors in the treatment of endometrial carcinoma. Eur Endocrinol 2013;9:18-21. https://doi.org/10.17925/EE.2013.09.01.18
30. Leslie R, Downes CP. PTEN function: how normal cells control it and tumour cells lose it. Biochem J 2004;382:1-11. https://doi.org/10.1042/BJ20040825
31. De Melo AC, Paulino E, Garces ÁHI. A review of mTOR pathway Inhibitors in gynecologic cancer. Oxid Med Cell Longev 2017;2017:4809751. https://doi.org/10.1155/2017/4809751
32. Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer 2004;4:335-348. https://doi.org/10.1038/nrc1362
33. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev 2000;18:1926-1945. https://doi.org/10.1101/gad.1212704
34. Faivre S, Kroemer G, Raymond E. Current development of mTOR inhibitors as anticancer agents. Nat Rev Drug Discov 2006;5:671-688. https://doi.org/10.1038/nrd2062
35. Hollander MC, Blumenthal GM, Dennis PA. PTEN loss in the continuum of common cancers, rare syndromes and mouse models. Nat Rev Cancer 2011;11:289-301. https://doi.org/10.1038/nrc3037
36. Mutter GL. PTEN, a protean tumor suppressor. Am J Pathol 2001;158:1895-1898. https://doi.org/10.1016/S0002-9440(10)64656-1
37. Pabona JMP, Burnett AF, Brown DM, et al. Metformin promotes anti-tumor biomarkers in human endometrial cancer cells. Reprod Sci 2020;27:267-277. https://doi.org/10.1007/s43032-019-00019-2
38. Roncolato F, Lindemann K, Willson ML, et al. PI3K/AKT/mTOR inhibitors for advanced or recurrent endometrial cancer. Cochrane Database Syst Rev 2019;10,CD012160. https://doi.org/10.1002/14651858.CD012160.pub2
39. Shoji K, Oda K, Kashiyama T, et al. Genotype-dependent efficacy of a dual PI3K/mTOR inhibitor, NVP-BEZ235, and an mTOR inhibitor, RAD001, in endometrial carcinomas. PLoS One 2012;7:e37431. https://doi.org/10.1371/journal.pone.0037431
40. Wu H, Goel V, Haluska FG, et al. PTEN signaling pathways in melanoma. Oncogene 2003;22:3113-3122. https://doi.org/10.1038/sj.onc.1206451
41. Janku F, Wheler JJ, Westin SN, et al. PI3K/AKT/mTOR inhibitors in patients with breast and gynecologic malignancies harboring PIK3CA mutations. J Clin Oncol 2012;30,777-782. https://doi.org/10.1200/JCO.2011.36.1196
42. Sun H, Enomoto T, Fujita M, et al. Mutational analysis of the PTEN gene in endometrial carcinoma and hyperplasia. Am J Clin Pathol 2001;115:32-38. https://doi.org/10.1309/7JX6-B9U9-3P0R-EQNY
43. Kong D, Suzuki A, Zou TT, et al. PTEN1 is frequently mutated in primary endometrial carcinomas. Nat Genet 1997;17:143-144. https://doi.org/10.1038/ng1097-143
44. Hayes MP, Douglas W, Ellenson LH. Molecular alterations of EGFR and PIK3CA in uterine serous carcinoma. Gynecol Oncol 2009;113:370-373. https://doi.org/10.1016/j.ygyno.2008.12.021

Acknowledgment: This work was supported by The Pamukkale University Scientific Research Projects Coordination Unit (grant 2019TIPF015).

Ethics committee disclosure: In our study “The Effects of Lithium, Metformin and Everolimus Substances on Cell Growth in 2D and 3D Ishikawa Endometrial Carcinoma Cell Culture”, human Endometrial Carcinoma Cell (Ishikawa Cell Line) that we have in stock were used. Cells are cultured in medium. Cells that become confluent between 2 and 3 days are multiplied by changing the medium, and experimental groups of cells that have reached sufficient density are formed. Ethics committee approval is not required for cell culture studies and cells are in our stock.

Contributions of the authors to the article

E.T. and G.A.M. constructed the main idea and hypothesis of the study. E.T. and G.A.M. formulated the research hypothesis, designed the methodology, literature review. N.C. developed the theory and arranged/edited the material and method section. M.S. designed and implemened a 2D cell culture experiment. H.D. designed the 3D cell culture involved in the research. A.B. contributed to the formulation of the research hypothesis. E.K. provided space and personnel for the 3D culture of work. The article was written by E.T., N.C., and G.A.M. reviewed the article and made the necessary corrections and approved it. In addition, all authors discussed the entire study and approved the final version.