Adjuvant effects of chemotherapeutics and Metformin on MFE-319 endometrial carcinoma cell line

ISIL AYDEMIR1), ELGIN TURKOZ ULUER2), OYA KORKMAZ3), MEHMET IBRAHIM TUGLU2), SEVINC INAN4)

1)Department of Histology and Embryology, Faculty of Medicine, Nigde Omer Halisdemir University, Turkey
2)Department of Histology and Embryology, Faculty of Medicine, Manisa Celal Bayar University, Turkey
3)Department of Histology and Embryology, Faculty of Medicine, Istanbul Medipol University, Turkey
4)Department of Histology and Embryology, Faculty of Medicine, Izmır University of Economics, Turkey

Abstract
We aimed to investigate the cytotoxicity of Metformin, Cisplatin, and Paclitaxel on MFE-319 endometrial carcinoma cell line using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and immunocytochemistry assays. Half maximal inhibitory concentration (IC50) doses of three drugs alone and in the dual combinations were applied to the cells. Immunocytochemical method was performed for the cell survival and for phosphatidylinositol 3-kinase (PI3K), phosphorylated extracellular regulated kinases (pErk)-1/2, Akt-1, phosphorylated Akt (pAkt)-1/2/3 cell growth markers and angiogenic vascular endothelial growth factor (VEGF). Immunoreactivities were evaluated using H-score and analyzed using the one-way analysis of variance (ANOVA) test for statistics. It was found that these drugs caused a decrease in the immunoreactivities of these markers. Particularly, dual combination of Paclitaxel and Cisplatin decreased the immunoreactivities of PI3K, pErk-1/2, Akt-1, and pAkt-1/2/3. Cisplatin and Paclitaxel were more effective than Metformin; on the other hand, Metformin has been shown to enhance the efficacy of these two drugs. In vitro or in vivo further studies are needed to investigate the efficacy of these three drugs via PI3K/Akt signal pathway.

Keywords: Cisplatin, endometrial carcinoma, Metformin, PI3K/Akt, Paclitaxel.

Introduction
Endometrial carcinoma (EC) is one of the most common cancers and the sixth leading cause of cancer incidence in women worldwide [1, 2]. It is reported that EC has different histological subtypes, such as adenocarcinoma and sarcoma. Type 1 endometrial tumors are estrogen receptor and progesterone receptor (ER/PR) positive, whereas type 2 is negative for these receptors. Because of negativities for ER/PR, type 2 is an aggressive form of EC and has poor prognosis. The risk factors for EC are atypical endometrial hyperplasia, hypertension, diabetes, and obesity. The control of these diseases plays an important role in delaying the progression of cancer and in the treatment [3].

The treatment of EC contains hysterectomy, chemotherapy, or radiation. The different chemotherapeutic agents are used in the treatment of EC. Especially the combinations of Doxorubicin, Cisplatin, Cyclophosphamide, Paclitaxel, Carboplatin are used clinically [4–6]. However, it has been indicated that Metformin, an antidiabetic oral agent, has antiproliferative effect on tumor cells in vitro and in vivo conditions. Previous studies have been drawing attention to the relationship between the Metformin and prevention of cancer in diabetic patients; it has also been shown to be effective in many diseases, such as polycystic ovarian syndrome, steatohepatitis, cardiovascular diseases, and human immunodeficiency virus (HIV)-associated metabolic abnormalities [7–11]. The preclinical and in vitro studies reported that Metformin has an antitumor effect on many types of cancer, breast, prostate, hepatocellular and endometrial cancers [7]. Therefore, it is important to define the effective dose for the treatment strategy, because the responses to the chemotherapeutic agents are short duration and differs among women [12]. The status of estrogen dependence and molecular factors ascertain the response to the treatment.

The pathway of phosphatidylinositol 3-kinase (PI3K)/Akt/mechanistic target of rapamycin (mTOR) plays a substantial role in cell survival, proliferation, differentiation, metastasis, and apoptosis [13]. Overexpression of PI3K causes cells to have cancer characteristics and uncontrolled proliferation. In this pathway, Akt inhibits the apoptosis by inactivating the antiapoptotic proteins, such as Bad, and increasing the phosphorylation of the proapoptotic protein Bcl-2 associated X (Bax). mTOR triggers the Akt/mTOR signaling pathway by raising the translation of protein through Akt activation [14, 15]. Also, the extracellular regulated kinases (Erk) pathway is important in cancer progress, via cell proliferation, differentiation, apoptosis, migration, and invasion. Previous experiments showed that the proliferation and invasion of tumor cells were decreased by blocking of Erk/mitogen-activated protein kinase (MAPK) signaling in colorectal and endometrial cancer [16, 17]. Besides the cell proliferation and invasion, the angiogenesis requires some regulatory factors, such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and transforming growth factor (TGF). VEGF enhances the angiogenesis by activating vascular endothelial cells and circulating monocytes. It also induces
the formation of antiapoptotic proteins, Bcl-2 and A1 in cells and provides long-term survival of endothelial cells. VEGF prevents differentiation of monocytes to endothelium-like cells [18]. It is stated that VEGF is expressed in most of the EC cases and it is involved in deep myometrial invasion [19].

Aim

The suppression of angiogenesis and cell proliferation is an important goal in the EC treatment. However, determination of the signaling molecules that play a role in EC will be useful in the management of the cure protocol. The aim of the present study was to investigate the effects of Cisplatin, Paclitaxel, and Metformin on the MFE-319 EC cell line (ER+/PR+) [20] as single agents and in dual combinations with each other in vitro condition via PI3K/Akt signal pathway and VEGF.

Materials and Methods

Cell culture

The MFE-319 EC cell line was grown in 40% Roswell Park Memorial Institute (RPMI)-1640 medium, 40% minimum essential medium (MEM, with Earle’s salts) and 20% fetal bovine serum (FBS, heat inactivated) at 37°C in 5% CO2 in a humidified atmosphere. The medium was changed every two days. The cells were morphologically examined using an inverted microscope and photographs of the cells were taken.

Cytotoxicity assay

The cytotoxicity of Paclitaxel, Cisplatin and Metformin was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. MFE-319 cells were passaged into 96-well (2.5×10⁵ cells/well) plates and seeded for four days before drug treatment. The doubling time of MFE-319 cells was 100 hours. After incubation, the cells were treated with an increasing concentration of Paclitaxel (0, 0.1, 1, 10, 30, 100 μg/mL), Cisplatin (0, 1, 10, 100, 500, 1000 μM) and Metformin (0, 0.1, 1, 10, 30, 100 mM) drugs for 24 hours. Then, the medium containing drug was discharged and fresh medium (100 μL with 100 mM) drugs for 24 hours. Then, the medium containing MTT was removed and 100 μl MTT (5 mg/mL in distilled water) was added. The incubation with MTT performed at 37ºC for four hours. The medium containing MTT was removed and 100 μL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance was measured at a wavelength of 570 nm using an ultraviolet–visible (UV–VIS) spectrophotometer multplate reader [21]. The half maximal inhibitory concentration (IC₅₀) of agents were calculated using absorbance values by GraphPad Software.

Drug treatments

MFE-319 cells were placed in 12-well plates at a density of 2.5×10⁵ cells/well and allowed to grow under routine conditions for four days. The cells were randomly divided into seven groups. These were the control (no drug treatment), Paclitaxel (IC₅₀ dose, 1.5 μg/mL), Cisplatin (IC₅₀ dose, 350 μM), Metformin (IC₅₀ dose, 20 mM) and Paclitaxel + Cisplatin, Paclitaxel + Metformin and Cisplatin + Metformin. The cells were exposed to these drugs for 24 hours.

Immunocytochemistry

After drug treatments, the medium was removed, and cells were rinsed with sterilized phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS at +4°C for 30 minutes. Then, the cells were washed in PBS three times for 5 minutes. Permeabilization was performed with 0.1% Triton X-100 in PBS at +4°C for 15 minutes. Endogenous peroxidase activity was inhibited using 3% hydrogen peroxide for 5 minutes at room temperature. Cells were then washed with PBS and incubated with primary antibodies: anti-Akt-1, anti-pAkt-1/2/3, anti-pErk-1/2, anti-PI3K and anti-VEGF at +4°C overnight. After removal of the primary antibodies, cells were washed with PBS and treated with biotinylated secondary antibodies and peroxidase-conjugated Streptavidin. Cells were then incubated with 3,3′-Diaminobenzidine (DAB)/hydrogen peroxide and counterstaining was performed using Mayer’s Hematoxylin. Stained cells were mounted with mounting medium and were photographed by a light microscope (inverted fluorescence phase microscope). Control samples were processed in an identical manner, but the primary antibody was omitted. Three observers blinded to experiments evaluated the staining scores independently. The immunocytochemical staining was evaluated using H-score method. For this purpose, the intensities of immunocytochemical staining were evaluated as weak (+), moderate (+++) and strong (++++) respectively, and the cells were counted in three different microscopic areas for each intensity. The respective score was then calculated using the formula $H$-score = $\sum P_i (intensity of staining + 1)$. $P_i$ is the percentage of stained cells for intensity, varying from 0% to 100%. The staining procedure was repeated three times and the H-score was evaluated by at least two investigators independently [22].

Statistical analysis

The data was statistically expressed as mean values (mean ± standard deviation) and were analyzed using repeated measures of the one-way analysis of variance (ANOVA) test. The Tukey–Kramer multiple comparisons test was used to determine differences amongst the mean values. Values for $p<0.05$ were considered significant [22].

Results

Cytotoxicity assay

To confirm the cytotoxic effects of Paclitaxel, Cisplatin and Metformin, MTT assay was performed with Metformin (0, 0.1, 1, 10, 30, 100 μM), Paclitaxel (0, 0.1, 1, 10, 30, 100 μg/mL) and Cisplatin (0, 1, 10, 500, 1000 μM) and for 24 hours. The cell proliferation was quantified with increasing concentrations of agents. With the administration of Metformin, the cell survival was 85±7.1% at 0.1 mM, 83±8.0% at 1 mM, 70±6.0% at 10 mM, 51±5.5% at 30 mM and 42±5.1% at 100 mM, whereas the survival was 96±9.5% in the control group (0 mM). The viability rates in the presence of Paclitaxel were 82±7.5% at 0.1 μg/mL, 65±7.0% at 1 μg/mL, 60±6.7% at 10 μg/mL, 51±6.0% at 30 μg/mL and 42±6.2% at 100 μg/mL. It was determined that Cisplatin had a more toxic effect at 500 mM (42±6.0%) and 1000 mM (39±6.1%) doses compared to other doses (0, 1, 10 and
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100 mM (**p<0.001). IC50 doses of agents were calculated as 1.5 μg/mL Paclitaxel, 350 μM Cisplatin and 20 mM Metformin (Figure 1, A–C).

**Figure 1 –** The survival of MFE-319 endometrial carcinoma cells with Metformin (A), Paclitaxel (B) and Cisplatin (C) for 24 hours was analyzed using MTT assay. Percentage of cell survival was analyzed using the one-way ANOVA test with Tukey–Kramer multiple comparisons test. ANOVA: Analysis of variance; MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SD: Standard deviation.

Immunocytochemistry

For the immunocytochemical detection of Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF in MFE-319 EC cells, single and dual combinations of Paclitaxel, Cisplatin and Metformin were administered to the cells. The H-score evaluations were shown in Figures 2 and 4. In the control and Metformin groups, the immunoreactivities of Akt-1, pAkt-1/2/3, PI3K and VEGF were greater than the Paclitaxel and Cisplatin groups (Figure 2), whereas they were significantly decreased in the Paclitaxel and Cisplatin groups (**p<0.001). The distribution of Akt-1 was reduced by administration of Paclitaxel (136±12.4) and Cisplatin (128±12.0) compared to control (215±11.0) and Metformin (221±12.0) groups (Figure 2).

Cisplatin especially decreased the immunoreactivities of PI3K, pErk-1/2, Akt-1, and pAkt-1/2/3 (**p<0.001). VEGF immunostaining was the highest in the control group (295±13.0) and decreased with Cisplatin (160±12.1) application. The applications of Paclitaxel and Cisplatin caused a decrease in the markers of cell survival and angiogenesis (Figure 3). It was ascertained that these drugs caused a decrease in the immunoreactivities in the following order of potency: Cisplatin > Paclitaxel > Metformin.

With the dual combinations of drugs, Paclitaxel + Cisplatin prominently reduced the immunoreactivities of Akt-1 (122±12.3), pAkt-1/2/3 (133±13.0), pErk-1/2 (124±12.4), PI3K (118±11.8) and VEGF (160±12.1) when compared to the other combinations (**p<0.001). Also, Metformin and Paclitaxel were found to have synergistic effects on MFE-319 cells (Figure 4). The combination of Metformin and Paclitaxel diminished the distributions of Akt-1 (136±12.4), pAkt-1/2/3 (145±12.8), pErk-1/2 (135±12.2), PI3K (128±12.0) and VEGF (230±13.5). The combination of Metformin and Cisplatin has no statistically significant effect on these markers (p>0.05) in comparison with the other combinations. The dual combinations of these drugs have the inhibitor effects in the immunoreactivities in the following order of potency: Paclitaxel + Cisplatin > Metformin + Paclitaxel > Metformin + Cisplatin (Figure 4).

** Discussions**

We ascertained that there was a significant difference in the immunocytochemical reactivities of Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF in MFE-319 cells in the control group and Cisplatin + Paclitaxel group compared to the other groups. There was a prominent decrease in these markers in the presence of dual combination of Paclitaxel and Cisplatin whereas there was no statistical significance between the control and Metformin groups. The current study claimed that Metformin caused cytoxoticity in MFE-319 cells, but it has no inhibition effect on Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF in MFE-319 cells in vitro conditions.

Paclitaxel is an anti-microtubule agent. It blocks the formation of microtubules consisting of tubulin dimers and stabilizes the microtubules by preventing depolymerization and thus mitosis is inhibited. This characteristic of Paclitaxel is important in terms of the preventing cell proliferation in cancer cases. In vivo and in vitro studies showed the antiproliferative effect of Paclitaxel in EC cells. In UT-EC-1 endometrial cells, Paclitaxel caused 72% of growth inhibition. The 73% of growth inhibition occurred with the combination of Paclitaxel and Carboplatin, whereas Carboplatin caused 54% of growth inhibition. Paclitaxel was found to show synergistic effect with Carboplatin [12]. Similarly, Paclitaxel has a cytotoxic effect on Ishikawa cells, by increasing the expression of caspase-3 and decreasing the levels of VEGF and proliferating cell nuclear antigen (PCNA) [23].
Figure 2 – The H-score analysis of immunocytochemical staining of Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF in MFE-319 endometrial carcinoma cells after application with Paclitaxel, Cisplatin and Metformin for 24 hours. pAkt-1/2/3: Phosphorylated Akt-1/2/3; pErk-1/2: Phosphorylated extracellular regulated kinases-1/2; PI3K: Phosphatidylinositol 3-kinase; SD: Standard deviation; VEGF: Vascular endothelial growth factor.

Figure 3 – The immunocytochemical staining of Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF in MFE-319 endometrial carcinoma cells after application with Paclitaxel (Pac), Cisplatin (Cisp), Metformin (Met), and dual combinations of drugs for 24 hours. Arrows: Immunopositive cells. Scale bars: 10 μm. pAkt-1/2/3: Phosphorylated Akt-1/2/3; pErk-1/2: Phosphorylated extracellular regulated kinases-1/2; PI3K: Phosphatidylinositol 3-kinase; VEGF: Vascular endothelial growth factor.
In patients with EC, increased expression of angiogenic markers, such as VEGF, are related to the progression or metastasis of carcinoma cells. According to data from M. D. Anderson, overexpression of VEGF was detected in 56% of endometrial tumors [24]. Besides VEGF, PI3K/Akt/mTOR pathway plays an important role in tumor cell survival, angiogenesis, and metastasis mechanism; therewithal, the increase in PI3K expression stimulates proliferation in tumor cells. Akt plays a role in the suppression of apoptosis [14, 15]. In the present study, the proliferation of MFE-319 endometrial cells were suppressed markedly by Paclitaxel, and we determined that there was a significant diminished expression of Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF. Dinkic et al. (2017) stated a similar cytotoxic effect of Paclitaxel in HEC-1A and AN3CA EC cells [25].

Recent studies have reported that Metformin, an anti-diabetic drug used in the cure of type 2 diabetes mellitus, has an antiproliferative effect on several cancer types. It has been reported that Metformin blocks the cell cycle and triggers apoptosis in tumor cells [26–29]. Also, it has been indicated that Metformin exerts its antitumorigenic effect by inhibiting gluconeogenesis in the liver and by down-regulating signal pathways such as Erk and mTOR [30–32]. Therefore, signaling pathways that play a role in cancer progression and proliferation are emphasized. In a previous study, biopsy samples were taken from women diagnosed with EC and who were treated with or without Metformin. The biopsy samples were immunohistochemically stained. In the group of Metformin treatment, the immunoreactivities of Ki-67, PI3K, pAkt, phosphorylated ribosomal S6 kinase 1 (p-S6K1), and phosphorylated 4E-binding protein (p-4E-BP1) significantly decreased compared to non-treatment of Metformin [29]. It has been observed that meta-analysis studies support these findings. The effect of Metformin usage in women with EC has been indicated that Metformin exerts its antitumorigenic effect by inhibiting the phosphorylated ribosomal protein S6 (p-S6), p Akt, p-4E-BP1 were reduced in women with EC who intake Metformin [33]. In a similar study, 50% reduction in cell proliferation markers was detected when women diagnosed with EC were given an increasing dose of Metformin (750, 1500 and 2250 mg/day) for four to six weeks. In tumor samples from patients, it was determined that the levels of phospho-ribosomal protein S6, pErk-1/2, cyclin D1 decreased after Metformin uptake [34]. Metformin was found to be toxic for ECC-1 and Ishikawa EC cell lines. The IC50 doses were approximately 1 mM for both cells for 72 hours. By the Western immunoblotting assay, it has been shown that AMPK was increased and p-S6 was decreased in the presence of Metformin. Based on these findings, researchers concluded that Metformin has an antiproliferative effect by inhibiting the mTOR signaling pathway [26]. In another study, the 5 mM Metformin dose caused cell death in both HEC-1-A and Ishikawa cell lines by suppressing the mTOR signaling. It was suggested that the inhibitor effect occurred by inhibiting the phosphorylated ribosomal protein S6 kinase β-1 (p70S6K) and activating the AMPK molecule [35]. In our experiment, we used MFE-319 EC cell line, and found that Metformin was toxic on MFE-319 cells via MTT assay (IC50 dose, 20 mM). But there was no inhibitory effect on the PI3K/Akt/mTOR signal pathway. This finding suggested that Metformin may have exerted its toxic effect through another signaling pathway in MFE-319 cells.

Cisplatin, another chemotherapeutic agent, is used clinically for the cancer treatments. It targets deoxyribonucleic acid (DNA) replication and interferes the cell proliferation in tumor cells, such as cervical, ovarian, testicular, lung, bladder head and neck cancers [36, 37]. It was also found that it accumulates in the cell organelles and cytosol, such as lysosomes, mitochondria, endoplasmic reticulum, and nucleus, and leads the cell to death in tumor cells. It is claimed that Cisplatin disrupts the structure and function of organelles, at the same time it alters the protein structures; thus, it causes the cell death [37]. Like other two agents, Paclitaxel and Metformin, Cisplatin suppresses EC cells in vivo and in vitro conditions. In vitro conditions,
Cisplatin has been shown to induce cell death through autophagy in EC cells. It has been stated that the increased doses of Cisplatin (10, 20, 40, 80 μg/mL) caused the suppression of cell proliferation in EC cells. Also, at 20 μg/mL, the expression levels of pAkt-1, PI3K, phosphorylated mTOR (p-mTOR) was reduced notably [38]. It was indicated that 1 mM of Cisplatin inhibited the cell proliferation in HeLa cervical cells and Ishikawa EC cells by 50% in vitro conditions. Beside the inhibition of cell proliferation, it was determined that it caused changes in both cell membranes by using atomic force microscopy quantitatively. After 24 hours of Cisplatin application, the size of both cells was decreased, whereas the membranes of HeLa cells were found to be rougher than the Ishikawa cells [39]. In our study, we found that cisplatin caused cell death in MFE-319 cells by inhibiting the PI3K/Akt/mTOR signaling.

In addition to these studies which the drugs were administered alone, there were also combination dual drug trials in both clinical and experimental studies. In a phase I study, for 28 days, different types of cancer patients (colorectal, sarcoma, endometrial, ovarian, and other types) were given 25 mg of weekly Temsirolimus and Metformin in increasing doses (500, 1000, 1500 and 2000 mg/day), and the synergistic effect between these two drugs was evaluated. It was observed that Metformin enhanced the efficacy of Temsirolimus, another chemotherapy agent that have targeted mTOR [40]. In HO-8910 human ovarian cancer cells treated with Metformin, the cell proliferation was reduced dose dependent manner (0.5, 1, 5 mM), whereas Metformin combined with Cisplatin (5 μM) markedly inhibited the cell proliferation and also the expressions of pErk-1/2, VEGF and VEGF receptor 2 (VEGFR2) messenger ribonucleic acid (mRNA). These two drugs showed a synergistic effect, and their toxic efficiencies were increased [41]. Asik et al. reported that the dual administration of Metformin (4 μM) and Paclitaxel (34 nM) caused an alteration in the 86 genes that have a role in leukemia in HL-60 human promyelocytic leukemia cell line. It has been stated that Metformin and Paclitaxel decreased the cancer cell proliferation markers, such as Akt-1, MAPK14, cluster of differentiation 40 ligand (CD40LG), while there was an increase in the molecules, apoptotic protease activating factor 1 (APAF1), caspase-1, -4, -5, -8 and -14, that played role in apoptosis [42]. In MFE-319 cells, there was no significant difference immunocytochemically in the group of Metformin and Cisplatin. This finding suggested that MFE-319 cells showed resistance to these two drugs. Concurrently, Metformin enhanced the antitumor activity of Paclitaxel [43, 44]. We also obtained similar results with this finding in our study.

In summary, our experiment demonstrate that the combination of Paclitaxel and Cisplatin has diminished the distributions of Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF, according to immunocytochemical staining. In order to understand the pathways through which these drugs exert their cytotoxic effects, the other signaling molecules should also be investigated.

Conclusions
In our experiment, when drugs were administered alone to MFE-319 cells, the toxicities of Cisplatin and Paclitaxel were higher than Metformin. In the dual combinations, Metformin increased the cytotoxicity of Paclitaxel on the MFE-319 cells. Also, Paclitaxel showed a synergistic effect by improving the activity of Cisplatin. The inhibition effect of Paclitaxel + Cisplatin administration on Akt-1, pAkt-1/2/3, pErk-1/2, PI3K and VEGF was higher than Metformin + Paclitaxel and Metformin + Cisplatin groups. These findings belong to our experiment of cell culture conditions and therefore further studies of in vivo cancer model are needed to determine the efficacy of drugs.

Conflict of interests
The authors declare no conflict of interests.

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Corresponding author
Isil Aydemir, Assistant Professor, MD, PhD, Department of Histology and Embryology, Faculty of Medicine, Nigde Omer Halisdemir University, 51200 Nigde, Turkey; Phone +90 542 473 95 56, e-mail: isil_aydemir@yahoo.com

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