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Effects of All-Trans-Retinoic Acid on Endometrium Cancer Cell Culture (Ishikawa) Alone and Combined with Classic Chemotherapeutics

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Abstract

Background: Endometrial cancer is the most common type of gynecological cancer encountered in developed countries. Combination therapy is a treatment method that combines more than one agent used in treatment. Today it is one of the most important weapons in cancer treatment. In this study, the aim was to examine the sole and combined effects of classical chemotherapeutics drugs and ATRA on Ishikawa cells in vitro.

Methods: To determine the effects of classical chemotherapeutics and ATRA on Ishikawa cells, MTT, DAPI staining, caspase-3 and real-time PCR analyses was performed.

Results: It was observed that the combination of classical chemotherapeutics+ATRA significantly decreased cell viability at all doses. DAPI staining showed apoptosis. In apoptotic cells, it was observed that the nuclei strangulated, divided into small pieces, and condensed. Caspase 3 activity increased in parallel with the increase in the doses of ATRA and other agents and these increases were statistically significant. It was observed that combined applications do not have a reduction effect on gene activations as much as single applications.

Conclusion: In this study, ATRA and other agents sole and in combination showed significant anticancer effects in MTT, DAPI Staining and Caspase-3 analyses in Ishikawa cells. In Real-Time PCR analysis, ATRA and other agents showed similar anticancer effects when applied alone. However, the same effect was not observed when agents combined and this is different from some results in the literature. Researching this subject with new studies will contribute to the literature.

Keywords: ATRA; Cancer therapy; Combination therapy; Ishikawa cell lines; Broad-spectrum

Introduction

Cancer is a disease that claims the lives of approximately ten million people a year worldwide. The biggest challenge we face in cancer treatment today is the lack of anticancer drugs with high efficacy, broad-spectrum and low side effects. In this respect, innovative approaches in drug development are extremely important. In the light of advances in molecular biology, drug development studies against cancer have gained momentum all over the world, although the discovery and development of anticancer drugs are progressing slowly. It is aimed to create clinical paradigms around the world in anti-cancer drug development. Global participation and cooperation in this area are supported [1].

Cancer is a serious problem with significant impacts on quality of life and healthcare systems. Despite advances in diagnosis, prevention methods and treatments, this disease still affects millions of patients all over the world. Endometrial cancer is the most common type of gynecological cancer encountered in developed countries. It is the second most common cancer in developing countries. The five-year survival rate of patients with endometrial cancer diagnosed in the late period is approximately 30%. Therefore, it is very important to develop an appropriate chemotherapeutic regimen for late-stage endometrial cancer. The primary treatment for endometrial cancer is surgery, followed by radiotherapy and/or chemotherapy. However, the response rate is low, and the survival rate is short. The most commonly used chemotherapeutic agents are adriablastin and cisplatin, carboplatin or paclitaxel [2-5].

Today, it has been demonstrated that the results obtained from cell culture tests are more realistic and specific. Cell culture is a versatile tool in the investigation of fundamental scientific issues. The advantage of using cell culture in scientific research is its homogeneity and reproducibility in the data produced. Substances examined in cancer research are expected to activate the apoptosis mechanism in cancerous cells and cause these cells to die selectively [6,7].

Retinoic acid is an important metabolite of vitamin A. Nowadays, various studies are carried out on the effects of retinoic acid on cell development as well as on cancer treatment. It has been shown that lung, prostate; bladder, breast, and ovarian cancers are suppressed by retinoic acid. Retinoic acid stops the cell cycle in cancer cells and induces apoptosis. In addition, the protein p27, which inhibits the cell cycle, and the new cell cycle regulator Cdk5 play a role in the effects of retinoic acid. These results show that the molecular mechanisms of retinoic acid can control the fate of cancer cells. [8,9].

It is necessary to find drugs effective in the treatment of gynecological cancers that lead to death. Studies conducted for this purpose show that All trans-retinoic acid (ATRA), a natural retinoid, stops the growing ovarian cancer cells in the G0-G1 phase. It is known that IRS-1 (insulin receptor substrate-1) factor is involved in proliferation in various tumor types. Some studies have revealed a potential role of ATRA in suppressing gynecological cancer cells, decreasing the level of IRS-1 protein in cells treated with ATRA. ATRA suppresses IRS-1 as a potential molecular target [10,11].

This study aimed to investigate the combined effects of classical chemotherapy drugs (cisplatin, adriablastin) with ATRA, by performing *in vitro* tests on Ishikawa cells. For this purpose, cytotoxic and apoptotic effects of combined therapy were examined. The cytotoxic effects of the agents were measured by the Tetrazolium Test (MTT assay) based on the measurement of mitochondrial activity. Apoptotic effects were examined using DAPI staining and Caspase-3 ELISA colorimetric kit test, which showed changes in nuclear DNA.

Materials and Methods

Preparation of materials

Glass and plastic materials and liquid solutions used in the studies were kept in an autoclave at 121°C for 20 minutes at 1.5 atm/Hg pressures, and at 180°C for 2 hours in a sterilizer. Some liquid chemicals used were passed through a 0.2 mm spaced cellulose nitrate filter.

Preparation of drug dosages: Drug doses were prepared by dissolving ATRA, Paclitaxel, and Carboplatin in Dimethyl sulfoxide (DMSO) (1:40 ratio). Doses were used as soon as they were prepared. The doses to be used were determined using the information obtained from literature reviews.

Cells

Ishikawa cells were obtained from Istanbul University Çapa Medical Faculty. Ishikawa cells were grown by culturing in DMEM/F12 (1:1) medium containing 10% Fetal Bovine Serum, penicillin-streptomycin, and sodium bicarbonate in an environment with 5% CO_2 at 37°C. As a result of the DAPI staining applied after the cells were harvested, mycoplasma was detected in the cells examined under the fluorescent microscope. Ciprofloxacin and Tylosin were added to the medium of the cells, and the cells were cleared from mycoplasma, grown, and stocked in liquid nitrogen (-196°C).

MTT assay

MTT [3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide] analysis was performed to determine the cytotoxic effects of carboplatin, paclitaxel and ATRA on Ishikawa cells. After determining the viability of the cells by Trypan Blue staining, the cells were counted with Thoma slide and cultured in 96-well plates at 5×10^4 cells per well for 24 hours. At the end of this process, the medium in the wells was emptied and the media containing different concentrations of test substances were placed in the plates. At the end of the incubation period, the media were removed from the treated cells for the periods determined by the test substances (24 hours, 24, and 48 hours). The cells were incubated with 5 mg/ml-1 MTT solution for two hours to convert the MTT dye to the water-insoluble formazan salt. At the end of this period, MTT dye was removed from the cells. 0.1 ml DMSO was added to each well to dissolve the formazan salts formed by living cells. Optical densities of the cells in the plates were read on an ELISA device (Spectra max 340 PC Molecular Devices, LLC USA) at a wavelength of 570 nm. The viability rates of the test cells are expressed as a percentage, assuming the control cell viability rate not treated with the test substance as 100%. Experiments in which cells were planted in parallel for each dose of test substance in experiment sets were repeated three times independently of each other. IC50 doses determined by applying the agents alone were used in combinations. SPSS program was used in the statistical evaluation of the results of MTT experiments and the significance levels of the obtained data were determined by applying one-way ANOVA and Tukey test as a post-hoc. The significance limit was determined as $p < 0.05$.

Apoptosis assay

Morphological examination with fluorescent staining (DAPI staining): DAPI staining was performed to determine the apoptotic effects of Carboplatin, paclitaxel, and ATRA doses determined as a result of the MTT assays on Ishikawa cells. Cells were seeded into six-well plates with sterile round lamellas and cultured in a 24-hour CO2 incubator. At the end of this period, the medium in the wells was removed. The effective doses of the agents determined as a result of the cytotoxicity tests were applied to the cells adhered on the lamellae for 12 hours. The medium was then removed from the wells, lamellas were washed with sterile phosphate buffer solution (PBS: 137 mM NaCl, 2.7 mM KCl, 15 mM KH2PO4, 8 mM NaHPO4; PH 7.3), detected for 15 minutes at 37°C in 3.7% paraformaldehyde solution dissolved in PBS. After detection, lamellas were washed 3 times with PBS and incubated for 30 minutes at 37°C with 1 mg/ml DAPI (4'6-diamidino-2 phenylindole) in a dark environment. The lamellas were then washed with PBS and capped and photographed under a fluorescent microscope.

Caspase 3 analysis: The cells were dissected according to the method of the company from which the Caspase-3 kit was purchased to collect the cell contents and analysis was performed. As a result of the analysis, the plates were read with an ELISA microplate reader at a wavelength of 405 nm (R&D systems, Inc. 1-800-343-7475). In the statistical evaluation of the results, the SPSS program was used and the significance levels of the obtained data were determined by applying one-way ANOVA and post-hoc Tukey test. The significance limit was accepted as $p < 0.05$.

Real-time PCR

RNA isolation: Ishikawa cells were cultivated in 75 cm2 flasks as 1 × 104 cells and incubated for 48 hours. Following the incubation, determined concentrations of the agents were added to the cells based on the data obtained from the results of the MTT analysis. After the cells were treated with the substances for 24 hours, the cells were collected into the centrifuge tube with the help of PBS, PBS-EDTA, trypsin together with the supernatant. By using Thoma slide, 4 × 106 cells were taken and the supernatant was centrifuged at 1250 rpm for six minutes. Buffer RTL plus+Beta mercaptoethanol mixture was placed on the cells and placed in columns holding genomic DNA and centrifuged at 10,000 rpm for 1.5 minutes. In this way, genomic DNA has been ensured to remain in the column. 350 µl of 70% ethanol was added onto the lysate where the genomic DNA was not found below and it was centrifuged at 10,000 rpm for one minute by placing it in

pink-colored columns. The underlying liquid was removed, 700 µl RW1 was added to the column, centrifuged at 10,000 rpm for 15 seconds and the liquid was removed. 500 µl Buffer RPE was placed on the pink columns and centrifuged at 14 000 rpm for one minute. Then the pink columns were placed on a new collection tube and the same process was repeated by centrifuging at 14 000 rpm for three minutes. The column was placed in a new collection tube to dry without adding anything, and centrifuged for one minute at 14 000 rpm. After the column was placed in a new clean Eppendorf tube, 40 µl RNAse Free Water was added on it and centrifuged at 10000 rpm for 1.5 minutes. By throwing the upper column, the underlying RNAse were measured and stored at (-80)°C until the process was performed for real-time PCR. Real-time PCR analysis was performed on BioRad, Hercules, California, USA.

RNA concentration measurement: To determine the amount and purity of the RNA, the isolated RNA was measured in a nanodrop device; DNAse was diluted with RNAse free water.

cDNA synthesis: For cDNA synthesis, the reaction was prepared with a total volume of 20 µl and cDNA synthesis was performed on a palm cycler device.

Relative quantification: The concentration of the target is expressed as the ratio of the target to a certain reference. To determine the concentrations of target and reference genes, the use of standard curves of both is necessary when using this method. In our study, while interpreting our real-time PCR results, the concentration value of our target genes was proportioned to the concentration value of the reference gene, and how much the results obtained differed compared to the control group were examined. It is a "housekeeping" gene that is constantly expressed in all cells. The housekeeping gene encodes proteins involved in cell functioning and is therefore always expressed. The 18S rRNA gene is one of the frequently preferred internal control genes in Real Time-PCR studies related to cancer studies. In relative gene expression comparison studies, the best result is obtained by including the internal control in the sample. An unchanged endogenous control is used for the quantification of different target mRNAs in each reaction. The 18S rRNA gene was used for this purpose in our study [12].

Results and Discussion

MTT assay results

The effect of cisplatin on Ishikawa cells was examined by the MTT assay. According to the assay, while cell viability increased on the first day at a dose of 2,5 µM of cisplatin, a decrease was observed at the same dose on the second day. At all other doses, it was determined that with increasing the dose of cisplatin, cell viability decreased. Except for the increase recorded at the 2.5 µM dose on the first day, a significant difference was detected in terms of cell proliferation at all doses (Figure 1).

The effect of adriablastin on Ishikawa cells was examined by the MTT assay. According to the assay, it was determined that the cell viability decreased as the dose of adriablastin increased. On the first day, it was determined that the effect of adriablastin on cell viability at 0.625, 1.25, 2.5, 5, 10 µM doses was similar, and a significant decrease in cell viability was observed after the dose of 20 µM (Figure 1).

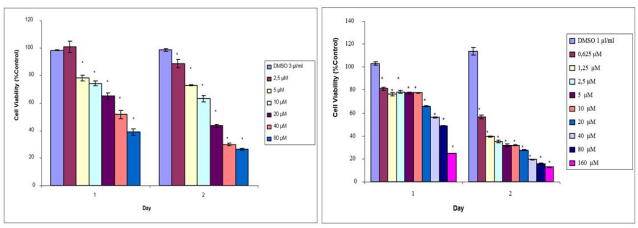

Figure 1: The evaluation of the effects of cisplatin and adriablastin on Ishikawa cells by the MTT Assay significantly varies on (*) icon control groups.

In some studies, it has been observed that low doses (10-90 µM) are used in the combined applications of ATRA. Therefore, the cytotoxic effect of low doses of ATRA on cells has been investigated [13].

The experiments were then repeated by exposing the cells to high doses of ATRA (140-360 µM). According to the results of the MTT experiment, 10-70 µM doses of ATRA did not cause a decrease in cell viability at the end of 24 hours. Similarly, no decrease in cell viability was observed after 48 hours at doses of 10 µM, 30 µM, and 50 µM. A statistically significant decrease in cell viability was detected at all doses starting from 70 µM with the increase in dose, both at the end of 24 and 48 hours (Figure 2).

A dose-dependent effect on cell viability was observed in Ishikawa cells where the combination of cisplatin and adriablastin was applied for 24 hours. Cell viability decreased statistically and significantly with an increase in the dose (Figure 2).

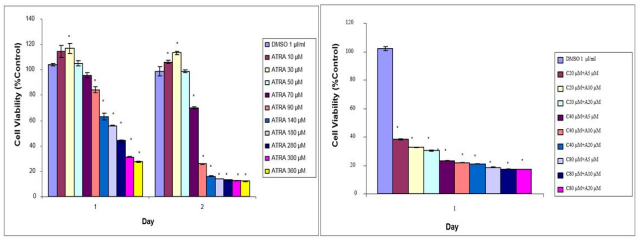

Figure 2: Investigation of the effects of ATRA and cisplatin +adriablastin combination on Ishikawa cells by MTT assay. The (*) sign indicates significant differences compared to the control group.

A dose-dependent effect on cell viability was observed in Ishikawa cells, where cisplatin and ATRA were administered together for 24 hours. Cell viability decreased statistically significantly due to the increase in dose. It was observed that this effect was close to the results obtained when cisplatin was applied alone (Figure 3).

When adriablastin and ATRA were administered together for 24 hours, a statistically significant decrease in cell viability was observed due to the dose increase. This decrease in cell viability is greater than the reduction recorded in the combination of cisplatin+ATRA. It was observed that the decrease in cell viability determined when adriablastin and ATRA were applied together became more pronounced with the increase of ATRA dose (Figure 3).

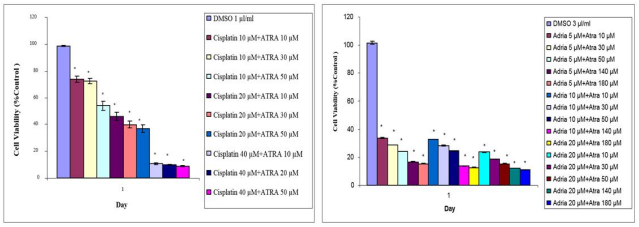

Figure 3: Evaluation of the effects of cisplatin+ATRA and adriablastin+ATRA combinations on Ishikawa cells by MTT assay. The (*) sign indicates significant differences compared to the control group.

DAPI staining results

DAPI staining was carried out to morphologically determine the apoptotic effects of cisplatin, adriablastin and ATRA on Ishikawa cells alone and in combination. Arrows in photos show apoptotic cells. It has been observed that the apoptotic cell nuclei are fragmented into small pieces, the nuclei are strangulated and condensed (Figure 4).

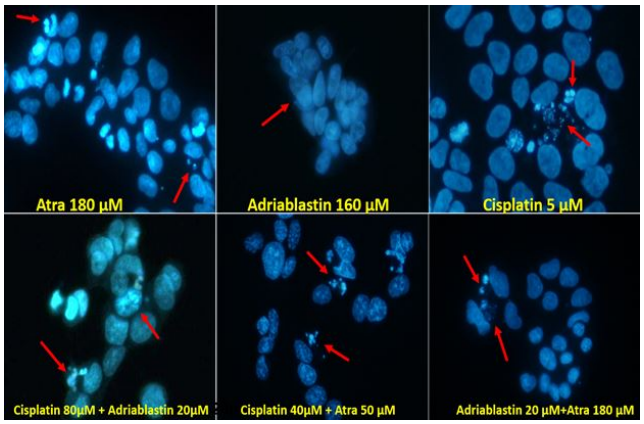

Figure 4: DAPI staining of Ishikawa cells.

Caspase-3 results

The effects of cisplatin, adriablastin and ATRA on the caspase 3 activities of Ishikawa cells alone and in combination are presented. ATRA was observed to increase caspase 3 activity significantly and statistically when applied either alone or in combination with other agents. This increase is similar at 12 hour and 24-hour incubation. The highest increase in caspase 3 activity was observed at 360 µM dose of ATRA and 20 µM cisplatin+140 µM ATRA combination. It was determined that caspase 3 activity increased in parallel with the increase in the doses of ATRA and other agents and these increases were statistically significant.

Real-time PCR results

When cisplatin was applied with 20 µM, it was observed that IRS-1 gene expression in cells decreased 5 times compared to control. When cisplatin 40 µM, adriablastin 10 and 20 µM were applied, the decrease in IRS-1 gene expression was 236-fold. ATRA also affected the IRS-1 gene level from low doses. It was determined that especially 360 µM ATRA caused a 236-fold decrease in IRS-1 expression. Cisplatin and adriablastin combinations decreased the expression level. However, it was determined that adriablastin+ATRA and cisplatin+adriablastin +ATRA combinations did not cause a significant decrease on the IRS-1 gene, contrary to the effects of the agents alone.

It was determined that Ki-67 expression decreased around 90 times with the application of 20 µM cisplatin. adriablastin and ATRA, on the other hand, completely blocked ki-67 expression at almost all doses. However, no significant difference was found in combinations of 2 and 3.

The IC50 value of cisplatin reduced COX-2 expression by 1-1.5-fold. Similarly, the IC50 value of adriablastin inhibited the expression of the gene at a rate of 2-2.5 times. However, it was observed that ATRA alone and in combinations of 2 and 3 did not change the COX-2 gene expression.

VEGF gene expression was significantly suppressed when all the agents we used during the experiments were applied individually to Ishikawa cells for 24 hours. However, as in other genes, no significant suppression was found in VEGF gene in combination of 2 and 3.

Cell culture studies are used by the pharmaceutical industry in drug research and development studies because of their rapid and reproducible nature. The effectiveness of newly developed drug candidates against cancer is generally examined in this way. Findings from research on cancer treatment show that apoptosis and cytotoxicity are closely related to chemotherapy. Cancer cells die as a result of apoptosis that develops through chemotherapy and radiotherapy [14].

Mitochondria are the crossroads in many cellular pathways. It plays an important role in programmed cell death known as apoptosis. Since the continuous development of tumor cells is dependent on high energy production, drugs that disrupt the functional integrity of mitochondria are important in cancer treatments. Mitochondria are the organelle that plays the most important role in the regulation of caspase chain and apoptosis. Cytochrome c release from mitochondria provides activation of caspase-3 and caspase-9. Therefore, in this study, the MTT assay that detects mitochondrial activity and apoptotic effects were investigated in determining cytotoxic effects. DAPI staining was performed to determine the apoptotic effect morphologically [15].

Cisplatin is a commonly used chemotherapeutic agent. It is effective against various types of cancer such as carcinomas, germ cell tumors, lymphomas and sarcomas. It is used in the treatment of many cancers, especially bladder, head and neck, lung, ovarian and testicular cancers. It acts by cross-linking with purine bases on DNA. In this way, DNA repair mechanisms are disrupted, DNA damage occurs and apoptosis is induced in cancer cells. Apoptosis is a type of energy-dependent controlled cell death that leads to the activation of a family of cysteine proteases called caspases. Apoptosis occurs with the activation of caspases such as caspases 3 and 7. Cisplatin is included in the endometrial cancer treatment protocol [16,17].

In our study, cisplatin reduced cell viability on Ishikawa cells at all doses. Cell viability was reduced on the first and second days. Cisplatin dose with the highest reduction is 80 µg. In a study similar to our study, Ishikawa cells were treated with 10, 20, 40 and 80 µg/M cisplatin for 12, 24, 48 and 72 hours. Then the cell was subjected to proliferation assays. Autophagosome formation was observed using transmission electron microscopy to determine the effect of cisplatin on Ishikawa cell autophagy. The results showed that cisplatin

treatment promotes cell autophagy in Ishikawa cells. In addition, cisplatin has been shown to inactivate the PI3K/AKT/mTOR signaling pathway. In our study, cisplatin was used at a dose of 20 μM in PCR examinations. At this dose, it was determined that IRS-1 gene expression decreased five-fold. However, when the dose was doubled and 40 μM was administered, it was observed that the decrease in its expression reached 236 times. In addition, cispaltin suppressed the expression of Ki-67, COX-2 and VEGF genes. However, cispaltin, which suppresses gene expression when used alone, did not show the same effect when used in combination with other agents. In these combinations, cisplatin adriablastin and ATRA were added [18,19].

It has been determined that cisplatin increases caspase-3 activity alone or in combination. It has been observed that it is more effective on caspase-3 activity compared to other applications, especially when combined with ATRA. This situation is the same for both 12-and 24-hour incubations.

In the literature, there are studies showing that cispaltin is effective in cancer treatment by triggering caspase-3 activity and thus apoptosis. Some of these are endometrial cancer, hepatocellular carcinoma and breast cancer. However, the higher effect of cisplatin when applied together with ATRA compared to that applied alone is not a common issue in the literature. There are few studies on this subject in the literature. In our study, it is thought that examining the effects of cisplatin+ATRA combination on Caspase-3 activity at 12-and 24-hour incubation periods and at different doses will contribute to the literature [20-23].

Adriablastin is an anthracycline antibiotic with antineoplastic activity. Adriablastin prevents DNA replication by intervening between base pairs in the DNA helix and inhibits protein synthesis. It also inhibits topoisomerase II and prevents the ligation of the nucleotide strand. Adriablastin is part of the therapy used for recurrent or metastatic endometrial tumors. In this treatment, systemic cisplatine-adriablastin-endoxane is used as an adjuvant treatment after surgery in high-risk endometrial cancer patients [24].

In our study, adriablastin reduced cell viability on Ishikawa cells at all doses. Cell viability was reduced on the first and second days. On the first day, it was observed that low doses (0.625 μM-10 μM) of adriablastin affected the cell viability at similar levels, and the effect increased after 20 μM. The dose with the highest decrease in cell viability is 160 μg. In similar studies, it has been determined that adriablastin decreases cell viability similar to our study. In a similar study evaluating the cytotoxic effect of adriablastin using the MTT assay, Hoechst 33258 staining was used to confirm the induction of apoptosis. Immunoprecipitation and Western blot analysis was performed to examine the effects of adriablastin on Bcl-2 antagonist cell death and phosphorylation of protein kinase B. Adriablastin induced the death of all cell lines in a dose-dependent manner. Low dose adriablastin (0.1 μM) did not affect apoptosis. In high doses, it induced apoptosis in all cell lines [25].

In another study, the effects of adriablastin, cisplatin and resrevatrol on Ishikawa cells at different doses were investigated using MTT. According to the results of the study, when used alone, adriablastin and cisplatin reduced the viability of Ishikawa cells. In the second stage, the experiments were repeated by combining adriablastin and cisplatin with resrevatrol. In the combined application of the drugs, the decrease in the vitality of the cells nearly doubled. Findings obtained from this study are similar to those obtained from our study. In our study too, when used alone, adriablastin and cisplatin reduced the viability of Ishikawa cells. When used in combination with ATRA, the decrease in vitality is more pronounced [26].

In our study, it was observed that when adriablastin was applied alone or in combination with other agents, it increased the caspase 3 activity in Ishikawa cells. The increase recorded in adrablastin is less than the increase seen in cisplatin and high doses of ATRA. This increase is similar at 12 hours and 24 hours of incubation. The results obtained from the studies conducted support those obtained from this study. In a study conducted on breast cancer cell lines, the effect of adriablastin on apoptosis and oxidative stress was examined. Adriablastin has been shown to reduce anti-apoptotic Bcl-2 protein expression, and affect oxidative stress by increasing hydrogen peroxide production and decreasing NF-κB gene and protein expression. The results also showed that adriablastin induced apoptosis by increasing the activation of caspase-8 and caspase-3 [27].

In another study, the effects of simvastatin on adriablastin activity in the MCF-7 (breast cancer) cell line and the mechanisms of this effect were investigated. The effect of simvastatin and adriablastin treatment alone and in combination on the growth of MCF-7 cells was studied. For this purpose, reactive oxygen species (ROS) production, caspase 3 activity, gene expression by quantitative polymerase chain reaction and apoptotic and proliferative protein levels by western blot were investigated. As a result, simvastatin and adriablastin alone and in combination suppressed MCF-7 cell proliferation. Simvastatin 10-50 μM doses increased the effect of adriablastin depending on the dose. Simvastatin when used in combination with adriablastin significantly increased ROS levels and expression of cytochrome c and caspase 3. It was concluded that simvastatin induces apoptosis and exerts a synergistic effect with adriablastin against MCF-7 cells. Although additional studies are required, it has been reported that the combination of simvastatin and adriablastin can be recommended for breast cancer treatment. In our study, results parallel to the results obtained from this study were obtained for endometrial cancer. It was found that adriablastin and ATRA, which increase caspase-3 expression alone, increase the said expression even more when used in combination. Differently from this study, in our study conducted on Ishikawa cells, it was found that adriablastin and ATRA decreased IRS-1, Ki-67, COX-2 and VEGF gene expression compared to control [28].

In our study, it was concluded that ATRA is a potential agent that can be used in the treatment of the disease due to its anticancer effect. It has been observed that it can reduce cell viability more than routinely used cancer drugs, especially at high doses. In various studies, it has been shown that ATRA, strengthens cancer chemotherapy in various ways. ATRA has been shown to increase the effects of different anti-cancer agents with its synergistic effect. The mechanisms of their synergistic effects depend on the tumor and cell type. Reduction of PCK, c-myc, E2F and Bcl-2 expression is examples of these mechanisms. The results suggest that combinations of ATRA and anti-tumor agents are candidates for the development and improvement of anti-carcinogenic therapies [29].

In a similar study on the subject, the effects of ATRA on multiple human endometrial cancer cell lines were examined. The results showed that ATRA may have more than one antitumor effect and can be used in the treatment of endometrial cancer [30].

In another study, the relationship between Oct4 expression caused by chemotherapeutics in bladder cancer cells and the development of drug resistance and tumor recurrence was investigated. In particular, it

has been determined that treatment with cisplatin increases bladder cancer cells by expressing Oct4. When ATRA was used in the same cells, it was observed that Oct4 was inhibited, and sensitivity to cisplatin increased synergistically in bladder cancer cells. In addition, it has been noted that the combination of cisplatin and ATRA is more effective than cisplatin alone in suppressing tumor growth. These results are consistent with those obtained in our study [31].

It is known that angiogenesis induced by VEGF plays a key role in cancer development, especially growth and metastasis processes. The family of insulin receptor substrates (IRS) are proteins involved in the insulin-like growth factor I receptor. IRS-1 supports tumor growth, but the mechanism is not fully understood. Based on data from a large cohort of clinical cancer registries, it has been shown that Ki-67 expression is associated with common histopathological parameters and is an additional independent prognostic parameter for overall and disease-free survival in some cancers. COX-2, catalyzes the first step in the formation of prostaglandins. Nowadays, its role in carcinogenesis has become evident. It affects apoptosis and angiogenesis, and there is a high level of COX-2 expression in cancer cells [32-35].

Conclusion

In our study, ATRA and other agents alone and in combination showed significant anticancer effects in MTT, DAPI Staining and Caspase-3 analyses in Ishikawa cancer cell lines. In Real-Time PCR analysis, ATRA and other agents showed similar anticancer effects when applied alone. However, the same effect was not observed when agents combined and this is different from some results in the literature. Researching this subject with new studies will contribute to the literature.

The biggest challenge we face in cancer treatment today is the lack of anticancer drugs with high efficacy, broad-spectrum and low side effects. It is accepted that this problem can be solved with combination therapies. It is considered that the findings obtained in our study support this point.

Note: This article is based on the data obtained from the scientific research project at Osmangazi University (Eskişehir/Turkey). The data obtained from the project is in large volume. Therefore, the data has been shared in different articles. Other articles are also under publication.

References

1. Moro F, Leombroni M, Pasciuto T, Trivellizzi IN, Mascilini F, et al. (2019) Synchronous primary cancers of endometrium and ovary vs. endometrial cancer with ovarian metastasis: An observational study. Ultrasound Obstet Gynecol 53: 827-835.
2. Devis-Jauregui L, Eritja N, Davis ML, Matias-Guiu X, Llobet-Navàs D (2020) Autophagy in the physiological endometrium and cancer. In: Autophagy: 1-19.
3. Jemal A, Bray F, Center MM, Ferlay J, Ward E, et al. (2011) Global cancer statistics. CA Cancer J Clin 61: 69-90.
4. Kumar BS, Maria S, Shejila CH, Udaykumar P (2018) Drug utilization review and cost analysis of anticancer drugs used in a tertiary care teaching hospital. Indian Journal of Pharmaceutical Sciences 80: 686-693.
5. Kendirlinan R, Gümüşburun R, Çerçi P, Özbek E, Altıner S, et al. (2019) Rapid drug desensitization with chemotherapeutics (platins, taxanes, and others): A single-center retrospective study. Int Arch Allergy Immunol 179: 114-122.
6. Segeritz CP, Vallier L (2017) Cell culture: Growing cells as model systems in vitro. In: Basic Science Methods for Clinical Researchers: 151-172.
7. Cotter TG (2009) Apoptosis and cancer: The genesis of a research field. Nat Rev Cancer 9: 501-507.
8. Chen MC, Hsu SL, Lin H, Yang TY (2014) Retinoic acid and cancer treatment. BioMedicine 4: 22.
9. Alizadeh F, Bolhassani A, Khavari A, Bathaie SZ, Naji T, et al. (2014) Retinoids and their biological effects against cancer. Int Immunopharmacol 18: 43-49.
10. Giuli MV, Hanieh PN, Giuliani E, Rinaldi F, Marianecci C, et al. (2020) Current trends in ATRA delivery for cancer therapy. Pharmaceutics 12: 707.
11. Machado-Neto J, Favaro P, Lazarini M, Duarte ASS, Archangelo LF, et al. (2011) Downregulation of IRS2 in myelodysplastic syndrome (MDS) may be related to impaired cell differentiation. Blood: 3814.
12. Kuchipudi SV, Tellabati M, Nelli RK, White GA, Perez BB, et al. (2012) 18S rRNA is a reliable normalisation gene for real time PCR based on influenza virus infected cells. Virol J 9: 1-7.
13. Saidi SA, Holland CM, Charnock-Jones DS, Smith SK (2006) In vitro and in vivo effects of the PPAR-alpha agonists fenofibrate and retinoic acid in endometrial cancer. Mol Cancer 5: 1-14.
14. Ghanemi A (2015) Cell cultures in drug development: Applications, challenges and limitations. Saudi Pharm J SPJ 23: 453.
15. Kumar N, Afjei R, Massoud TF, Paulmurugan R (2018) Comparison of cell-based assays to quantify treatment effects of anticancer drugs identifies a new application for Bodipy-L-cystine to measure apoptosis. Sci Rep 8: 1-11.
16. Fleming GF, Brunetto VL, Cella D, Look KY, Reid GC, et al. (2004) Phase III trial of doxorubicin plus cisplatin with or without paclitaxel plus filgrastim in advanced endometrial carcinoma: A gynecologic oncology group study. J Clin Oncol 22: 2159-2166.
17. Thigpen JT, Blessing JA, Homesley H, Creasman WT, Sutton G (1989) Phase II trial of clsplatin as first-line chemotherapy in patients with advanced or recurrent endometrial carcinoma: A gynecologic oncology group study. Gynecol Oncol 33: 68-70.
18. Lin Q, Wang Y, Chen D, Sheng X, Liu J, et al. (2017) Cisplatin regulates cell autophagy in endometrial cancer cells via the PI3K/AKT/mTOR signalling pathway. Oncol Lett 13: 3567-3571.
19. Gottwald L, Pluta P, Piekarski J, Spych M, Hendzel K, et al. (2010) Long-term survival of endometrioid endometrial cancer patients. Arch Med Sci AMS 6: 937.
20. Fukuda T, Oda K, Wada-Hiraike O, Sone K, Inaba K, et al. (2015) The anti-malarial chloroquine suppresses proliferation and overcomes cisplatin resistance of endometrial cancer cells via autophagy inhibition. Gynecol Oncol 137: 538-545.
21. Dąbruś D, Kiełbasiński R, Grabarek BO, Boroń D (2020) Evaluation of the impact of cisplatin on variances in the expression pattern of leptin-related genes in endometrial cancer cells. Int J Mol Sci 21: 4135.
22. Blanc C, Deveraux QL, Krajewski S, Jänicke RU, Porter AG, et al. (2000) Caspase-3 is essential for procaspase-9 processing and cisplatin-induced apoptosis of MCF-7 breast cancer cells. Cancer Res 60: 4386-4390.
23. Yin W, Nie Y, Zhang Z, Xie L, He X (2015) miR-193b acts as a cisplatin sensitizer via the caspase-3-dependent pathway in HCC chemotherapy. Oncol Rep 34: 368-374.
24. PubChem Compound Summary for CID 31703, Doxorubicin. National Center for Biotechnology Information.
25. Wan X, Li J, Xie X, Lu W (2007) PTEN augments doxorubicin induced apoptosis in PTEN null Ishikawa cells. Int J Gynecol Cancer 17: 802-812.
26. Rezk YA, Balulad SS, Keller RS, Bennett JA (2006) Use of resveratrol to improve the effectiveness of cisplatin and doxorubicin: Study in human gynecologic cancer cell lines and in rodent heart. Am J Obstet Gynecol 194: e23-26.

27. Pilco-Ferreto N, Calaf GM (2016) Influence of doxorubicin on apoptosis and oxidative stress in breast cancer cell lines. Int J Oncol 49: 753-762.
28. Buranrat B, Suwannaloet W, Naowaboot J (2017) Simvastatin potentiates doxorubicin activity against MCF-7 breast cancer cells. Oncol Lett 14: 6243-6250.
29. Schultze E, Collares T, Lucas CG, Seixas FK (2018) Synergistic and additive effects of ATRA in combination with different anti-tumor compounds. Chem Biol Interact 285: 69-75.
30. Tsuji K, Utsunomiya H, Miki Y, Hanihara M, Fue M, et al. (2017) Retinoic acid receptor β: A potential therapeutic target in retinoic acid treatment of endometrial cancer. Int J Gynecol Cancer 27: 643-650.
31. Lu CS, Shieh GS, Wang CT, Su BH, Su YC, Chen YC, et al. (2017) Chemotherapeutics-induced Oct4 expression contributes to drug resistance and tumor recurrence in bladder cancer. Oncotarget 8: 30844.
32. Li X, Gao Y, Li J, Zhang K, Han J, et al. (2018) FOXP3 inhibits angiogenesis by downregulating VEGF in breast cancer. Cell Death Dis 9: 1-12.
33. Inwald EC, Klinkhammer-Schalke M, Hofstädter F, Zeman F, Koller M, et al. (2013) Ki-67 is a prognostic parameter in breast cancer patients: Results of a large population-based cohort of a cancer registry. Breast Cancer Res Treat 139: 539-552.
34. Ghosh N, Chaki R, Mandal V, Mandal SC (2010) COX-2 as a target for cancer chemotherapy. Pharmacol reports 62: 233-244.
35. Reiss K, Del Valle L, Lassak A, Trojanek J (2012) Nuclear IRS 1 and cancer. J Cell Physiol 227: 2992-3000.