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Antitumor Effects of Dihydromyricetin and Bevacizumab on SKOV-3 Ovarian Cancer Cell Line

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Abstract: Introduction: Ovarian cancer is the deadliest gynecological malignancy, usually not detected until the late stages. In vitro cell culture used for studies such as the study of interactions of drugs. Dihydromyricetin is a key ingredient in many healthy foods or beverages. It has an excellent safety profile for human. **Material-Method**: SKOV-3 cells were cultured in RPMI medium with 100 units/mL-100 µg/mL of penicillin-streptomycin and 10% fetal bovine serum in a CO_2 incubator. After incubation for 3 h at 37°C with 5% CO2, the media was removed and 0.1 mL of DMSO was added. The plates were read at 570 nm in the spectrophotometer. The MTT assay was repeated at least three times independently. Following this procedure, RT-PCR was performed. **Results**: SKOV-3 cells were treated with different concentrations of DHM. DHM was found to be cytotoxic to the SKOV-3 cell line in a time- and concentration-dependent manner. SKOV-3 cells were treated with different concentrations of BEV. Cell viability was decreased at the different concentrations. To evaluate the cytotoxic effects of DHM and BEV together on the SKOV-3 cell line, cells were treated with a combination of IC_{50} concentrations of DHM and BEV. According to combination results, DHM + BEV treatment was found to be more cytotoxic to SKOV-3 cells than treatment with DHM or BEV alone. **Conclusion**: In our study, the combined effects of two anti-cancer agents on ovarian cancer cell lines were investigated. It has been determined that the effect of the agents in the combined treatment is greater than that of the agents alone.

Keywords: Dihydromyricetin, Bevacizumab, SKOV-3, Anti-cancer effects, MTT, RT-PCR Analysis.

INTRODUCTION

Cancer is a serious problem with significant impacts on health systems. Despite advances in diagnosis and treatment, it still affects millions of people around the world. Ovarian cancer is the deadliest gynecological malignancy. Ovarian cancer is usually not detected until the late stages because its early symptoms are not specific. 2/3 of ovarian cancer patients have late-stage disease (stages III-IV) at the time of diagnosis. The current standard of care is debulking surgery to remove tumor masses followed by first-line Platinum and Taxol chemotherapy [Boettcher, A.N. *et al*., 2019].

In vitro cell culture is a method used to study the behavior of animal cells in a controlled environment free of systemic variations. It is used for studies such as the study of basic cell biology, interactions of drugs and other chemicals with cells, production of vaccines and proteins. Cell culture was developed to study the behavior of animal cells in an environment without the systemic changes that can be found in an animal during normal homeostasis. At the beginning of cell culture applications, the negative effects of growth factors and growth-promoting substances, cytotoxic compounds or the negative effects of xenobiotics, and events related to programmed cell death (apoptosis) should be examined. In addition, cell culture applications are also used in studies on the positive effects of cell proliferation, cell activation, cell signaling, or any other cellular process. The main advantage of in vitro cell culture assays is the consistency and reproducibility of results. In cell cultures, the physicochemical environment (pH, temperature, pressure, etc.) and physiological conditions can be controlled very precisely [Hynds, R.E. *et al*., 2018; Mirabelli, P. *et al*., 2019].

MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. It is a colorimetric analysis. It is based on the reduction of the yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to purple formazan crystals by metabolically active cells. Living cells contain NADPH-dependent oxidoreductase enzymes that reduce MTT to formazan. MTT is used in the measurement of cell growth and viability, cell proliferation, cytotoxicity, and cell activation [Tada, H. *et al*., 1986; Hansen, M.B. *et al*., 1989].

Bevacizumab (BEV) is a monoclonal antibody that targets VEGF-A. It is the first angiogenesis inhibitor approved for use. VEGF not only controls angiogenesis but also regulates tumor-induced immunosuppression. These recently identified immune-modulatory roles of VEGF

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have made BEV an important alternative for combination therapies. Recently, the clinical benefit of combined therapies involving BEV has been observed in the treatment of non-small cell lung cancer and hepatocellular carcinoma. The immunomodulatory properties of BEV have developed new perspectives in combination therapy research [Costantini, L. *et al*., 2020; Garcia, J. *et al*., 2020].

Dihydromyricetin (DHM) is found in large quantities in some commonly consumed fruits and vegetables. DHM is commonly found in some plants such as grapes, bayberry, ampelopsis, ginkgo, and tea. It is listed as a health product in Europe and approved by the FDA as a food and health product in the USA. DHM is a key ingredient in many healthy foods or beverages. It has an excellent safety profile for humans.

Reverse transcription-polymerase chain reaction (RT-PCR) is a relatively simple and inexpensive technique for determining the expression level of target genes and is widely used in biomedical science research for semi-quantitative analysis. Real-time PCR allows detection of PCR amplification at the exponential growth stage of the reaction and is much more quantitative than conventional RT-PCR. This technique became possible after the addition of an oligonucleotide probe designed to hybridize within the target sequence. Due to the 5' nuclease activity of Taq polymerase, probe cleavage during PCR can be used to detect amplification of the target-specific product. The basic principles for RT-PCR and real-time PCR are the same [Mo, Y. *et al*., 2012].

This study aims to examine the anticancer effects of DHM and Bevacizumab in a cell culture medium.

MATERIAL AND METHODS

Cell Culture

SKOV-3 cell line was purchased from American Cell Culture Collection (ATCC- HTB77). Cells were grown in RPMI-1640 HEPES media (Gibco, #22400097) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and incubated at 37°C with 5% CO_2.

Cytotoxicity Assay

Half maximal inhibitory concentrations (IC_{50}) of DHM (Sigma-Aldrich, St. Louis, MO, USA) and BEV were established by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cells were seeded at 5×10^3 per well in 96-well plates and incubated at 37°C with 5% CO_2 for adhesion. After 24 h, media was replaced with the media containing different concentrations of DHM, BEV, or the combinations of them. The plates were incubated at 37°C with 5% CO_2 for 24 and 48 h. At the end of the incubation period, the media was discarded and the new media containing 0.5 mg/mL MTT was added to each well. After incubation for 3 h at 37°C with 5% CO_2, the media was removed and 0.1 mL of DMSO was added. The plates were read at 570 nm in the spectrophotometer. Untreated cell viability rate was assumed as 100%, and the cell viability rate of the test cells was expressed as a percentage. The MTT assay was repeated at least three times independently.

RT-PCR Analysis

RNA Isolation

SKOV-3 cells were plated at T25 flasks and incubated at 37°C with 5% CO_2 in a humidified incubator. After reaching %70 confluence, the media was discarded and new media containing IC_{50} concentrations of DHM, BEV, or DHM and BEV combined. The flasks were incubated at 37°C with 5% CO_2 for 48 hours. Afterward, the cells were collected in a centrifuge tube with the help of PBS, PBS-EDTA, and trypsin, together with the supernatant. Using a Thoma slide, $4x10^6$ cells were taken and the supernatant was removed, which was centrifuged at 1250 rpm. Buffer RTL plus + Beta mercaptoethanol mixture was placed on the cells and centrifuged by placing them on the columns holding the genomic DNA. In this way, genomic DNA is ensured to remain in the colon. 350 µl of 70% ethanol was added to the lysate without genomic DNA and centrifuged by placing it on colored columns. The remaining liquid was removed and 700 µl of RW1 was added to the column, centrifuged again and the liquid was removed. 500 µl Buffer RPE was placed on the pink columns and centrifuged. The same procedure was then repeated by placing the pink columns on a new collection tube. The column was placed in a new collection tube to dry and was centrifuged at 14 000 rpm for one minute. 40µl of RNAse Free Water was added to the column and centrifuged again. The upper column was discarded, the lower RNAs were measured and stored at -80 °C until processed for real-time PCR. Real-time PCR analysis was performed on a BioRad, Hercules, California, USA instrument.

Calculation of RNA Concentration

In order to determine the amount and purity of RNA, the isolated RNA was measured in a

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nanodrop device and diluted with DNAse, RNAse free water.

CDNA Synthesis
For cDNA synthesis, the reaction was prepared with a total volume of 20 µl and cDNA synthesis was carried out in a palm cycler device.

Evaluation of the Results
The Cp value refers to the point at which PCR amplification of a sample is assumed to show the amount of PCR product for each curve in the PCR.

Relative Quantification
The concentration of the target is expressed as the ratio of the target to a particular reference. To determine the concentrations of the target and reference genes with this method, the use of standard curves of both genes is required. In our study, while interpreting our real-time PCR results, the concentration value of our target genes was proportional to the concentration value of the reference gene, and how much the results obtained changed compared to the control group was examined. It is a housekeeping gene that is constantly expressed in all cells. The housekeeping gene is the one that encodes the 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 on cancer studies. The best results in relative gene expression comparison studies are obtained when the internal control is present in the sample. An invariant endogenous control is used for the quantification of different target mRNAs in each reaction. In our study, the 18S rRNA gene was used for this purpose [Zhang, Z. *et al*., 2017].

Statistical Analysis
SPSS software (Statistical Package for the Social Sciences, version 23.0, New York, USA) was used for the determination of statistically significant differences. One-way ANOVA followed by Tukey's test was used. Results are expressed as means ± SEM. Results with a p-value of less than 0.05 were considered statistically significant.

Availability of Data and Materials
All data and materials of this study can be accessed from the authors' contact addresses.

RESULTS
Cytotoxicity Assay Results

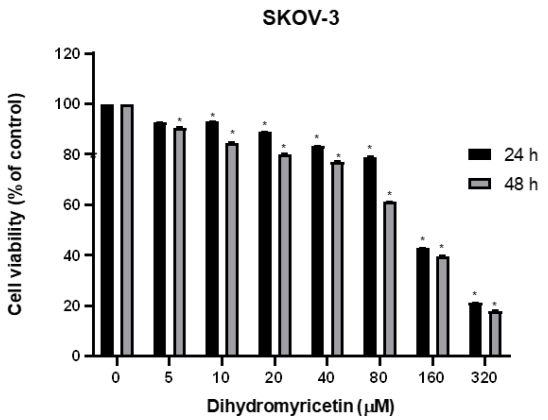

Figure 1: Effects of dihydromyricetin on SKOV-3 cell viability. The cells were treated with different concentrations of BEV for 24 and 48 h (5, 10, 20, 40, 80, 160 and 320 µM). At least three independent experiments were performed. The data is expressed as means ± SEM. The results were compared to non-treated control cell group. *p<0.001

SKOV-3 cells were treated with different concentrations of DHM for 24 and 48 h. At 24 h, cell viability was decreased by 7.29%, 6.96%, 11.08%, 16.68%, 20.95%, 57.12% and 78.76% at the concentrations of 5, 10, 20, 40, 80, 160 and 320 µM, respectively when compared to non-treated control group. At 48 h, decrease in cell viability rate was 9.52%, 15.22%, 19.78%, 22.72%, 38.85%, 60.35% and 82.16% at the concentrations of 5, 10, 20, 40, 80, 160 and 320 µM, respectively when compared to non-treated control group. IC_{50} of DHM was found to be 143.0 µM and 101.8 µM at 24 and 48 h respectively. According to these findings, DHM was found to be cytotoxic to the SKOV-3 cell line in a time- and concentration-dependent manner.

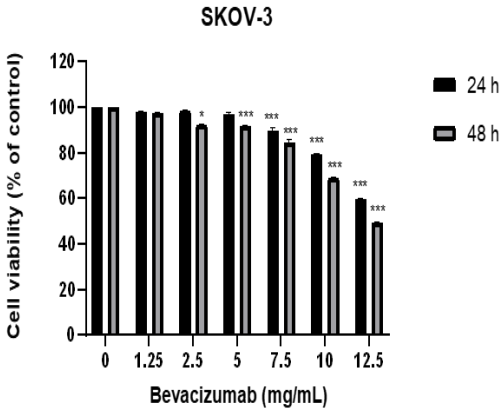

Figure 2: Effects of BEV on SKOV-3 cell viability. The cells were treated with different concentrations of BEV for 24 and 48 h (1.25, 2.5,

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5, 7.5, 10 and 12.5 mg/mL). At least three independent experiments were performed. The data is expressed as means ± SEM. The results were compared to non-treated control cell group. *p<0.05, ***p<0.001

SKOV-3 cells were treated with different concentrations of BEV for 24 and 48 h. At 24 h, cell viability was decreased by 2.2%, 1.70%, 3.17%, 10.36%, 20.64% and 40.44% at the concentrations of 1.25, 2.5, 5, 7.5, 10 and 12.5 mg/mL, respectively when compared to non-treated control group. At 48 h, decrease in cell viability rate was 2.99%, 7.75%, 8.47%, 15.86%, 31.39% and 50.81% at the concentrations of 1.25, 2.5, 5, 7.5, 10 and 12.5 mg/mL, respectively when compared to non-treated control group. IC_{50} of BEV was found to be 10.43 and 10.04 mg/mL at 24 and 48 h respectively.

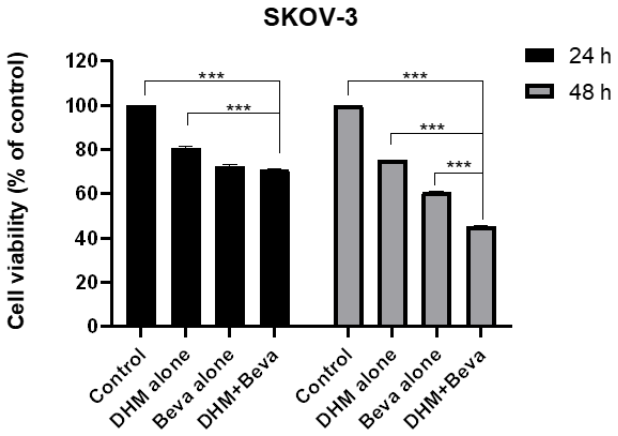

Figure 3: Results of RT-PCR analysis Effects of BEV + DHM on SKOV-3 cell viability. The cells were treated with IC_{50} concentrations of DHM, BEV and DHM+BEV for 24 and 48 h. At least three independent experiments were performed. The data is expressed as means ± SEM. DHM+ BEV combination results were compared to cell groups that are non-treated and treated with DHM or BEV alone. ***p<0.001

To evaluate the cytotoxic effects of DHM and BEV together on the SKOV-3 cell line, cells were treated with a combination of IC_{50} concentrations of DHM and BEV. The results were compared to the non-treated control group, and the groups treated with DHM or BEV alone. According to combination results, DHM + BEV treatment was found to be more cytotoxic to SKOV-3 cells than treatment with DHM or BEV alone. However, while the combined treatment at 24 h significantly reduced cell viability compared to the group treated with DHM alone, there was no statistically significant difference between the group treated with BEV alone. At 48 h, BEV + DHM treatment significantly decreased cell viability compared to treatment with both BEV alone and DHM alone (p<0.001).

DISCUSSION

It was determined that DHM was cytotoxic to the SKOV-3 cell line in a time and concentration-dependent manner and decreased cell viability at the end of 24 and 48 hours. Similar to DHM, BEV showed a cytotoxic effect on the SKOV-3 cell line in a time- and concentration-dependent manner. According to the RT-PCR results, BEV+DHM treatment significantly reduced cell viability at 24 hours compared to the group treated with DHM alone. There was also a decrease in viability in the group treated with BEV alone, but no statistically significant difference was found. BEV+DHM treatment significantly reduced cell viability after 48 hours compared to treatment with BEV and DHM alone (p<0.001).

DHM, a natural flavonoid, shows a wide range of pharmacological effects. In addition to the general features of flavonoids, it has effects such as anti-diabetes, cardioprotective, hepatoprotective, nephroprotective and anti-tumor. The mechanisms for these effects of DHM are associated with several different molecules involved in cellular apoptosis, oxidative stress, and inflammation. Some of the molecules with which it is associated are AMP-activated protein kinase, mitogen-activated protein kinase, protein kinase B, nuclear factor-kB.

Hepatocellular carcinoma is a type with high mortality due to its late diagnosis. Studies have shown that DHM can restrict the growth of HCC cells and activate apoptosis in hepatocarcinoma HepG2 cells. DHM suppresses Bcl-2 expression and increases caspase-3 cleavage and activation. DHM induces mitochondria-mediated apoptosis in HepG2 cells. It has also been reported that DHM alters the expression of cell cycle proteins. Data obtained from studies show that DHM can be a promising therapeutic drug for hepatocellular carcinoma growth and metastasis [Liu, B. *et al.*, 2015; Kao, S.J. *et al.*, 2017].

Studies on human non-small cell lung cancer have revealed that DHM exerts a selective cytotoxic effect on NSCLC cells. The mechanism of this effect is that DHM induces cell apoptosis by depolarization of the mitochondrial membrane. In addition, DHM increases intracellular peroxide

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and activates signaling pathways reversed by N-acetylcysteine. It is stated that combined treatment of DHM with extracellular signal-regulated kinase or c-Jun N-terminal kinase inhibitors may be ideal strategies for non-small cell lung cancer [Wang, Y. *et al*., 2017].

Osteosarcoma is the most common primary malignant bone tumor in childhood and adolescence. DHM increases p21 expression and inhibits the G2-M cell cycle that causes DNA damage in osteosarcoma cells. The effect of DHM on hydrogen peroxide-induced oxidative stress in osteosarcoma cells was investigated. DHM has been found to induce hydrogen peroxide-induced cell death and apoptosis through caspase inhibition and Bcl-2 activation. These data suggest that DHM may be a therapeutic candidate for the treatment of osteosarcoma [He, M.H. *et al*., 2018].

All-trans retinoic acid (ATRA) is an intermediate of vitamin A metabolism with a wide variety of physiological and pharmacological activities. It is successful in patients with acute promyelocytic leukemia. One study found that combined treatment of ATRA and DHM produced strong synergy in acute promyelocytic leukemia. It has been reported that this synergy is due to the p38-STAT1 activation signaling pathway. It has been emphasized that the joint use of DHM and ATRA is a promising approach in treatment [Zhang, Z. *et al*., 2015].

DHM has been used with this drug on human leukemia cells to counteract the cardiotoxic effects of adriamycin. According to the data obtained from the study, DHM not only prevented cardiotoxicity but also increased the anticancer activity of adriamycin in human leukemia cells. It shows that DHM is a suitable combination therapy candidate to potentiate anticancer activities and potentiate the therapeutic effects of adriamycin [Zhu, H. *et al*., 2015].

Ovarian cancer is one of the most common types of cancer in the female genital organs and has the highest mortality rate among gynecological cancers. Results from a conducted study showed that DHM treatment induced ovarian cancer cell cycle arrest in G0/G1 and S phase. DHM also effectively induced Apoptosis. In the same study, it was determined that DHM significantly reversed paclitaxel and doxorubicin resistance in ovarian cancer cells. It is thought that DHM may be a candidate chemotherapeutic agent for the treatment of ovarian cancer [Xu, Y. *et al*., 2017].

DHM has also been shown in studies to inhibit proliferation in human gastric cancer cells. The mechanism of this effect is to induce cell cytotoxicity, promote apoptosis with p53 upregulation and Bcl-2 downregulation [Ji, F.J. *et al*., 2015].

The first available anti-angiogenic therapy is BEV, a monoclonal antibody that binds to all circulating soluble VEGF-A isoforms. It binds to VEGF-A, prevents its interaction with VEGFR, thus inhibits the activation of VEGF signaling pathways that promote neovascularization. In vivo studies have shown that BEV inhibits vascular growth, induces regression of newly formed vessels, and has direct effects on tumor cells [Ferrara, N. *et al*., 2004].

Colorectal cancer is one of the most common cancers and patients often present with metastases. Prior to the discovery of targeted therapies, treatment options for these patients were limited. In the study evaluating BEV in the first-line treatment of colorectal cancer, BEV was added to chemotherapy and it was shown that the survival of patients was significantly longer than chemotherapy alone. With these results, BEV is approved as the first targeted therapy for these patients. This was followed by several randomized studies using BEV in colorectal cancer, and it was shown that BEV can be used in primary care in combination with new chemotherapy regimens [Saltz, L.B. *et al*., 2008; Bennouna, J. *et al*., 2013].

Lung cancer is among the most common cancers in both men and women, and the majority of patients present with advanced disease. Prior to the discovery of targeted therapies, median survival for patients with advanced non-squamous non-small cell lung cancer, the most common form of lung cancer, was limited to seven to eight months despite aggressive platinum-containing chemotherapy. BEV is one of the first targeted therapies for this disease. It is the first agent to help these patients live longer than one year when added to chemotherapy. Addition of BEV to carboplatin + paclitaxel has been shown to reduce the risk of death by 21% compared to carboplatin [Barlesi, F. *et al*., 2014; Sandler, A. *et al*., 2006].

In ovarian cancer, patients often present with advanced disease. The recurrence rate is high. Prior to the discovery of targeted therapies, treatment was limited to platinum-based chemotherapy. BEV is the first targeted therapy approved for ovarian cancer. It is a new therapeutic option that can delay tumor

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progression compared to chemotherapy. In the group of ovarian cancer patients with stage IV disease, BEV used in combination with chemotherapy followed by maintenance therapy provided a better recovery compared to chemotherapy alone. BEV is currently the only anti-angiogenic agent approved for the treatment of ovarian cancer [Coleman, R.L. *et al*., 2018; Tewari, K.S. *et al*., 2019].

Although the anti-tumoral effects of BEV and VEGF inhibition have been demonstrated on a variety of solid tumors, BEV did not show a significant therapeutic effect in some solid tumors such as pancreatic, gastric and prostate cancer. Potential causes for unresponsiveness to anti-angiogenic therapy include dense tumor stroma, excess of angiogenic factors, and the interaction of the angiogenic signaling pathway and other signaling pathways [Nienhüser, H. *et al*., 2017; Zhang, Z. *et al*., 2018].

Combination therapy can be defined as the combined use of two or more therapeutic agents. It is the cornerstone of cancer treatment today. Combined therapy increases the effectiveness of cancer drugs through synergy or similar ways. In addition, it reduces drug resistance, stops tumor growth and metastatic effect, and induces apoptosis. Five-year survival for metastatic cancers is still low, and developing new cancer drugs is costly and time-consuming. For this reason, the development of agents to be used in cancer treatment by in vitro methods has been one of the issues that have been emphasized in recent years [Mokhtari, R.B. *et al*., 2017].

CONCLUSION

In our study, the combined effects of two anti-cancer agents on ovarian cancer cell lines were investigated. It has been determined that the effect of the agents in the combined treatment is greater than that of the agents alone. This data is considered important. There is no finding in the literature to investigate the effects of this combination in ovarian cancer. It is expected that our study will be a source for new studies to be planned with this aspect.

In studies on the subject, it is stated that BEV and DHM induce apoptosis. The lack of an analysis of apoptosis is a limitation of our study. In addition, it is thought that planning gene-level analyzes and analyzing the effects of the combination of BEV and DHM will increase the power of the studies to be planned.

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