Experimental and Applied Medical Science, 3, 1: 284-297, 2022. DOI 10.46871/eams.2022.33

In Vitro Investigation of the Effects of Caffeic Asid Phenethyl Ester Contribution to Chemotherapeutic Drug Treatment on the Neuroblastoma Cell Line (SH SY- 5Y) on the Inflammatory Process

Çiğdem Karaca[1*], Hilal Susam Şen[2], Fatma Fırat[3], Esra Aslan[3]

[1]*Gaziantep Islam Science and Technology University, Faculty of Medicine, Department of Histology & Embryology, Gaziantep, Turkey.*
[2]*Afyonkarahisar Health Science University, Faculty of Medicine, Department of Pediatrics, Afyonkarahisar, Turkey.*
[3]*Afyonkarahisar Health Science University, Faculty of Medicine, Department of Histology & Embryology, Afyonkarahisar, Turkey.*

Abstract

Neuroblastoma is a sympathetic nervous system tumor with a 5-year survival rate of less than 50% in high-risk patients that are unresponsive to conventional multimodal treatments. Although chemotherapy, radiotherapy, and surgery are used according to the stages in the treatment protocols, the complications and toxic effects of these treatments are also among the causes that increase mortality. Topotecan is an agent that is actively used in the treatment of neuroblastoma, but its use is limited due to its toxic effects. For these reasons, neuroblastoma is a disease that needs new treatment approaches and agents to reduce the complications of existing treatments. Caffeic Acid Phenethyl Ester (CAPE) is a natural compound that is known for its anti-inflammatory, immunomodulatory, antioxidant, and anticancer properties and is particularly preferred because of its selective effects on cancer cells. Regulation of the inflammatory microenvironment during cancer treatment is an important factor in the treatment plan. According to this information, we aimed to compare the effects of Topotecan and CAPE treatments on the inflammatory process in the SHSY-5Y neuroblastoma cell line with IL-1α, IL-6, IL-18, and VEGF antibodies on the apoptotic process using the TUNEL method. After the cells have been cultured, four groups were formed. Topotecan and CAPE were given separately to the 1st and 2nd groups, Topotecan and CAPE were given together to the 3rd group for 24 hours. A control group was formed by not giving any substance. Then, staining was done with ICC and TUNEL methods. In the results, IL-1α, IL-6, IL-18, and VEGF staining intensities were found to be increased in the CAPE and Topotecan applied groups, especially in the CAPE+Topotecan co-administered

[*]**Corresponding Author:** Çiğdem Karaca, E-mail: drc_karaca@hotmail.com. Tel: +90 505 891 3238. ORCID ID: 0000-0003-2106-2422.

group at the end of 24 hours. In the evaluation made with TUNEL staining, although the most apoptotic cells were seen in the CAPE applied group, an increase in apoptosis was also detected in the groups that were administered Topotecan and CAPE+Topotecan together The results of our study showed that CAPE and Topotecan triggered apoptosis by exacerbating inflammation in SH-SY5Y neuroblastoma cells, more apoptotic cells in the CAPE group were more cytotoxic than Topotecan, and when used together, CAPE had a synergistic effect with Topotecan.

Key words: *Apoptosis, CAPE, Mediators of inflammation, SHSY-5Y, Topotecan.*

Introduction

Neuroblastoma (NB) is a pediatric sympathetic nervous system tumour arising from neural crest cells. It can also be found in sympathetic nerve ganglia located throughout the sympathetic nervous system in the neck, chest, abdomen, and pelvis (1). The molecular basis of NB is not fully understood. The heterogeneity of subspecies of NB variations creates difficulties in the identification and classification of the disease (2). Tumors with heterogeneity are rare, but very aggressive and often with a severe prognosis. The clinical and biological features of NB also differ according to the primary tumour sites (3). According to the World Cancer Report, more than 15% of child deaths from cancer are composed of patients with NB (4-6). Pediatric cancers are treated with a combination of therapies (surgery, radiation, chemotherapy and targeted therapy) selected according to the type and stage of the cancer (7). Although clinical diagnosis and treatment for NB are continually improving, the 5-year survival rate for children with high-risk NB remains less than 50% (8, 9). Low-risk NBs are mainly seen in children younger than 12 months, and surgical treatment is usually sufficient. In other cases, chemotherapy is used as the first-line treatment. Chemotherapy, radiation therapy and stem cell transplantation can be used for high-risk NB treatment. A 5-year survival rate approaching 70% has been achieved with different and combined treatment approaches (10). But current treatment for high-risk patients includes intensive and toxic chemotherapy. In stage 4 patients, chemotherapy causes more treatment-related deaths than surgery, accounting for 15.5% of all deaths. An important side effect of chemotherapy in patients with NB is the development of secondary myelodysplastic syndromes and transformed acute myeloid leukaemia (11). For these reasons, the pursuit of new treatments in NB continues. Topotecan is a semi-synthetic derivative of camptothecin and is an antineoplastic agent used in the current treatment of NB, as it activates the topoisomerase I-inhibitor (12). It triggers cell death by creating DNA breaks (13). It causes haematological toxicities, especially severe myelosuppression (14). New and supportive treatment agents are needed in the treatment of NB because of its cytotoxic effects in other tissues and many undesirable side effects.

Caffeic acid phenethyl ester (CAPE), a natural phenol in honeybee propolis, has been reported to have anti-inflammatory, antifungal, antimicrobial,

immunomodulatory, antimutagenic and antioxidant properties (15-19). As an antioxidant agent, CAPE completely blocks the production of reactive oxygen species in human neutrophils and xanthine/xanthine oxidase systems at a concentration of 10 μM and demonstrates adequate antioxidant capacity (20). CAPE has been shown to increase cancer cell death by apoptotic mechanisms in many cancer cell lines (21-24). Macroscopic studies have also been conducted and it has been suggested that treatment with CAPE causes a significant dose-dependent reduction in tumour growth by assessing volume and weight of tumour in the xenograft model (25). On the other hand, in a study conducted in Burkitt lymphoma cells, it has been stated that CAPE causes cell death with pyknotic nuclei devoid of nuclear or nucleosomal fragmentation, which shows the characteristic features of necrosis rather than apoptosis, and it has been emphasised that CAPE, which is an anti-inflammatory agent, causes necrotic cell death in cancer cells (26). CAPE has also been shown to be an effective agent in preventing metastases (27). It has also been suggested that CAPE has a specific antiproliferative effect on cancer cells, and also exerts homoeostatic control in multicellular organisms for cell proliferation, differentiation, wound healing, and regulation of adaptive responses of differentiated cells (28).

Cytokines are cellular regulatory proteins that are classified as chemokines, interferons, interleukins, lymphokines, and are involved in these pathophysiological mechanisms by directing the inflammatory response. Cytokines that increase non-specific immunity and inflammation are known as proinflammatory cytokines. IL-1, IL-5, IL-6, IL-8, TNF-α, IFN-ɣ are proinflammatory cytokines. IL-18 is a cytokine formerly known as interferon (IFN) gamma inducing factor (IGIF). Although it is similar to the IL-1 family in structure and function, it is also considered as a new member of the IL-1 family (29-31). The inflammatory micro-environment of a tumour is a complex network of multiple cells, cytokines, enzymes, and signalling pathways. Inflammatory cytokines are the most effective targets for modulating the inflammatory micro environment during cancer treatment. Inflammation is a regulatory system with both pro- and anti-tumour properties. As part of the immune response, inflammation can activate immune cells and induce the production of inflammatory proteins such as cytokines and enzymes to inhibit the growth of tumours. Therefore, appropriately manipulating inflammation is considered a promising strategy for cancer therapy (32, 33).

VEGF (Vascular Endothelial Growth Factor) is a highly specific mitogen for vascular endothelial cells. Expression of VEGF is increased by activated oncogenes and various cytokines in response to hypoxia. It is known that VEGF accelerates tumour development by inducing endothelial cell proliferation, promoting cell migration and inhibiting apoptosis and is a metastatic marker (34, 35). In addition, it has been emphasised in recent studies that the increase in VEGF expression in SHSY-5Y cells is not associated with tumour progression and metastasis (36, 37).

SH-SY5Y was first subcloned in 1978 from the SK-N-SH cell line isolated from a bone marrow biopsy of a four-year-old girl with NB in 1973. It has a morphologically similar phenotype to neuroblast cells in vitro and is used in neurodegenerative

disease studies and NB modelling due to its neuronal cell-like characteristics (38, 39).

In this study, it has been aimed to evaluate the IL-1α, IL-6, IL-18 and VEGF immunoreactivities of Topotecan and CAPE in the SH-SY5Y NB cell line and to observe their effects on the inflammation and angiogenesis process, and on the apoptotic process with the TUNEL method.

Material and Method

Cell Passage

In our study, commercially purchased human SH-SY5Y NB cell line was used. Cells were incubated in RPMI medium containing 10% (v/v) heat-inactivated fetal calf serum (FCS) and 5 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin in an atmosphere of 37 °C, containing 5% CO2 and 95% air. The cells were placed in a chamber slide (150-mm flask) containing 12 ml of medium in 75 cm² flasks and incubated for 2 days. Cells with 80% confluency were removed by adding 0.25% trypsin. Trypsin activity was terminated by adding a medium twice the volume of trypsin to the resulting suspension. The suspension was taken into a 15 ml Falcon tube and centrifuged at 400xg and 25 °C for 5 minutes to obtain cell debris (pellet). After removing the supernatant, 2400 µl of fresh medium was placed on the pellet and mixed with a pipette. Cell culture was performed in a 12-well (2 pcs.) chamber slide with 200 µl suspension in each well. The wells were divided into CAPE (5 µM, 24h), Topotecan (2 µM, 24h), CAPE (5 µM)+Topotecan(2 µM) and Control groups, and drugs were applied at the given doses. The procedures were repeated 3 times.

Immunocytochemical Staining Protocol

Cells forming the control group and treated with CAPE, Topotekan and CAPE+Topotekan groups were cultured in the 12-well chamber slide. The medium was removed with a sterile pipette at the end of the 24th hour and 4% paraformaldehyde was used for fixation of cells. After washing with PBS, they were kept on ice for 15 minutes in 0.1% Triton-X100 solution. The cells were incubated with 3% H_2O_2 for endogenous peroxidase inactivation. After washing with PBS three times for 5 min, it was treated with blocking solution for 10 minutes. Cells were incubated with primary antibodies VEGF (1/250, ab76055- abcam), IL-1α (1/250, ab9614-abcam), Il-6 (1/250, ab233706, abcam), IL-18 (1/250, ab191152-abcam) for 1 hour at room temperature. Then washed 3 times with PBS. After treatment with secondary antibody, it was colored using DAB Substrate. Mayer's hematoxylin was used for counter-staining and covered with a water-based sealer.

Cells on stained slides were counted under light microscopy with Image Analysis Program (NIS elements, Japan). 500 different cells were counted at x20 objective magnification. While scoring, the area with the highest score was determined by scoring the staining degree. Scoring was done by a semi-quantitative method (H-Score). Stained cells were evaluated in terms of percentage and staining intensity was taken as a second criteria. There is no, slight, moderate and severe staining were assessed as 0,1,2 and 3, respectively.

Tunel Staining Protocol

After removing the media in the wells with a sterile pipette, applications were made according to the ApoTag Plus Peroxidase In Situ Apoptosis Detection Kit (S7101)

protocol. After washing the wells with PBS, they were fixed with 4% paraformaldehyde for 30 minutes. Then, they were permeabilised with Triton X 100 for 15 minutes on ice and washed with distilled water. After the endogenous preoxidase blockade was applied for 5 minutes at room temperature with 3% hydrogen peroxide ($H_2 O_2$), they were washed again with PBS and incubated for at least 10 minutes by applying 75 µL of Equilibration Buffer. 55 µL of TdT ENZYME was dropped, Stop Wash Buffer was applied, and then they were incubated with Anti-Digoxigenin Conjugate for 30 minutes at room temperature in a humid environment. DAB substrate was applied and waited for 3 to 6 minutes, and they were treated with water-based blocking solution by counterstaining with Mayer's Hemotoxylin. Evaluation of apoptosis in SHSY-5Y cells was calculated using the Apoptotic index (AI) formula, considering cells with brown staining nuclei as TUNEL positive.

AI = Number of TUNEL Positive Cells x 100 / Total number of cells (40).

Statistical Analysis

The data obtained as a result of the Apoptotic Index and H-Score evaluation were prepared as mean ± standard error. Shapiro Wilk test was used to evaluate the normality of the data. T test was applied in dependent groups. Statistical comparisons were made using one-way ANOVA followed by post-hoc test (Tukey). Comparisons where P-values are less than 0.05 were considered statistically significant. Analyses were made in the SPSS 20.0 package program.

Results

VEGF Staining Intensities

The most intense staining was seen in the CAPE+Topotecan applied group. There was no significant difference in staining between the groups in which CAPE and Topotecan were given separately (p= 0.928). In the CAPE+Topotecan applied group, the staining intensities were increased compared to the CAPE and Topotecan administered group (p=0.011, p=0.025). There was also a significant increase in staining between the groups administered CAPE+Topotecan, CAPE and Topotecan and the Control group (p<0.001, p<0.001, p<0.001) (Figure 1) (Graphic 1).

IL-1α Staining Intensities

The most intense IL-1α staining was seen in the CAPE+Topotecan applied group. There was no significant difference in staining between the groups administered CAPE and CAPE+Topotecan and Topotecan (p=0.177, p= 0.294). In the CAPE+Topotecan administered group, the intensity of staining increased compared to the Topotecan administered group (p=0.012). There was also a significant increase in staining between the groups administered CAPE+Topotecan, CAPE and Topotecan and the Control group (p<0.001, p<0.001, p<0.001) (Figure 1) (Graphic 1).

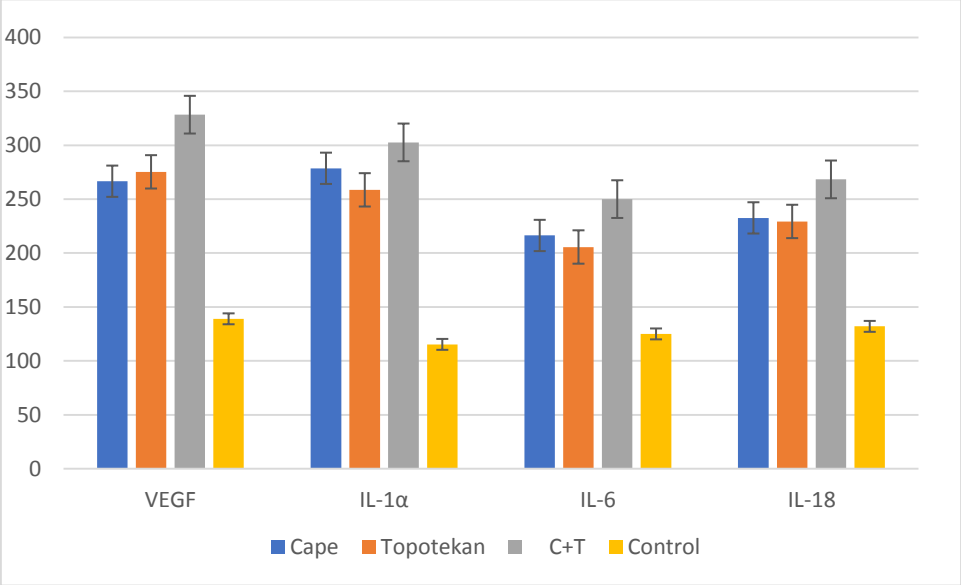
Il-6 Staining Intensities

The most intense IL-6 staining was seen in the CAPE+Topotecan applied group. There was no significant difference in staining between the groups administered CAPE and Topotecan (p= 0.570). In the CAPE+Topotecan administered group, the intensity of staining increased compared to the Topotecan and CAPE administered group (= 0.002, p=0.013). There was also a significant increase in staining between the groups administered CAPE+Topotecan, CAPE and Topotecan and the Control

group ($p<0.001$, $p<0.001$, $p<0.001$) (Figure 1) (Graphic 1).

Il-18 Staining Intensities

While the staining intensity of IL-18 was increased in the CAPE+Topotecan applied group compared to the CAPE and Topotecan applied groups, this increase was not considered statistically significant ($p= 0.117$, $p=0.083$). There was also no significant difference in staining between the groups administered CAPE and Topotecan ($p= 0.995$). There was also a significant increase in staining between the groups administered CAPE+Topotecan, CAPE and Topotecan and the Control group ($p<0.001$, $p<0.001$, $p<0.001$) (Figure 1) (Graphic 1).



Graphic 1: H-Score evaluations of staining intensity with VEGF, IL-1α, IL-6, IL-18 antibodies after CAPE, Topotecan and CAPE+Topotecan application for 24 hours.

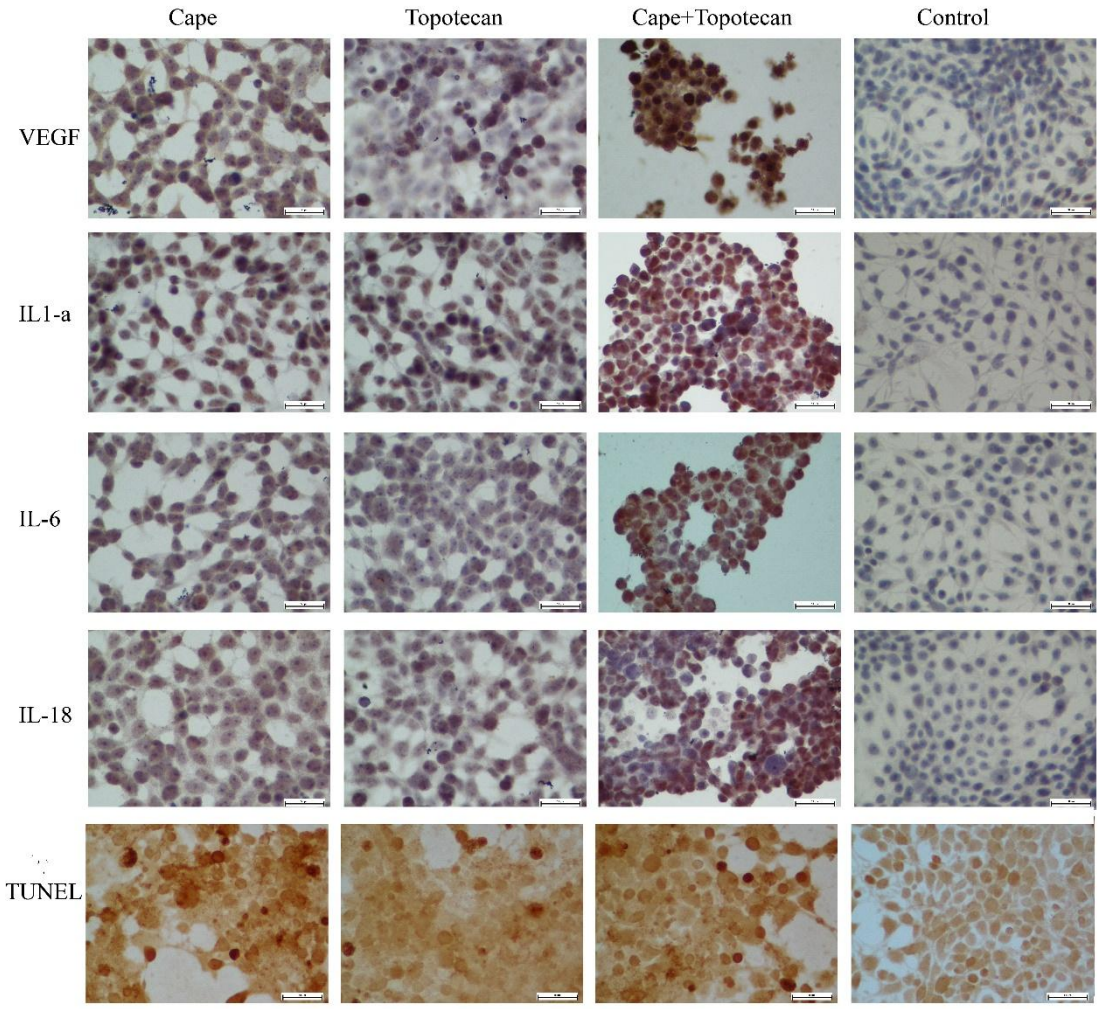
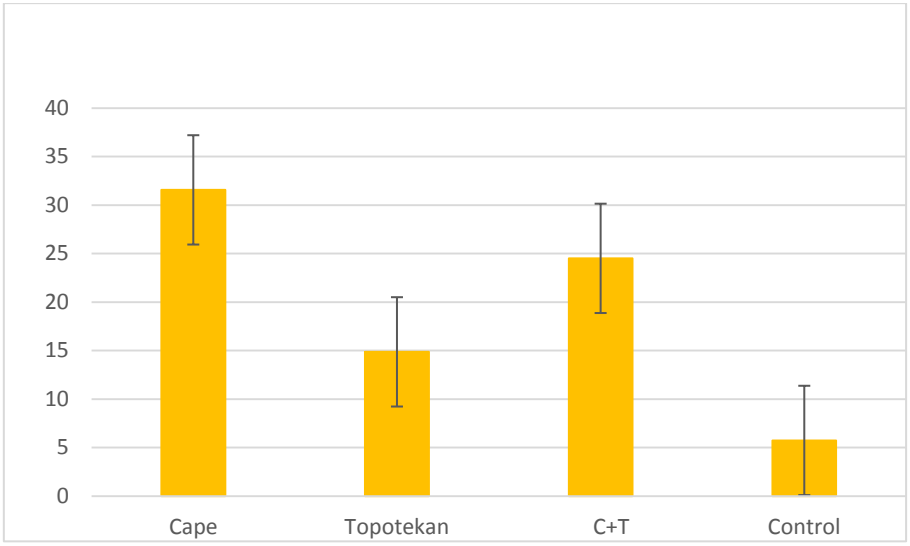


Figure 1: ICC staining and TUNEL staining with VEGF, IL-1α, IL-6, IL-18 antibodies after CAPE, Topotecan and CAPE+Topotecan application for 24 hours. 20X objective, scale bar 50µm.

TUNEL Staining

The most apoptotic cells were seen in the CAPE applied group. Apoptotic index was found to be increased in CAPE applied group compared to Topotecan and Control groups ($p=0.001$, $p<0.001$). Compared to the CAPE+Topotecan group, this increase was not considered significant ($p=0.120$). Apoptotic index in the topotecan administered group was found to be decreased compared to CAPE and CAPE+Topotecan groups ($p=0.001$, $p=0.032$) and increased compared to the control group ($p=0.041$). Apoptosis was increased in the CAPE+Topotecan applied group compared to the Control group and Topotecan applied group ($p=0.001$, $p=0.032$) (Figure 1) (Graphic 2).



Graphic 2: Apoptotic Index evaluation by TUNEL staining after CAPE, Topotecan and CAPE+Topotecan administration for 24 hours.

Discussion

NB is the most common extracranial solid tumour in childhood. While NB, which may arise from the sympathetic ganglia and/or adrenal medulla, may spontaneously transform or regress to a benign form within the first year, the 5-year survival rate of patients in the high-risk group is below 50% and the pursuit of new treatments is still ongoing. In this process, there is a need for agents that can specifically treat cancer cells while reducing the side effects of current treatments. Topotecan is a chemotherapeutic agent currently used in the treatment of NB. In our study, in addition to topotecan treatment, CAPE, which is known to have strong antioxidant effects and also has anti-inflammatory, antitumour, neuroprotective and immunostimulatory effects, was applied to SHSY-5Y NB cell lines. Guowen W. et al. reported that topotecan inhibited cell proliferation, cell migration and invasion in a time- and dose-dependent manner in the SHSY-5Y NB cell line (41). In another study, Beppu K. et al. reported that cell viability decreased and cell death increased in topotecan applied SHSY-5Y cells (21). In a study with CAPE, Tomiyama R. et al. stated that endoplasmic reticulum stress and autophagy increased in SHSY-5Y cells after CAPE application (42). In our study, in the TUNEL evaluation, apoptosis was found to be increased in SHSY-5Y cells treated with Topotecan, CAPE and CAPE+Topotecan compared to the control group. The most cell death was seen in the CAPE applied group, while cell death was found to be lower in the Topotecan applied group than in the CAPE and CAPE+Topotecan applied groups. It has been reported that CAPE is being effective by reducing the intercellular connection proteins such as β-catenin, claudin, and nectin, preventing the adhesion of cancer cells and weakening their intercellular connections (43-46). In our study, cell shedding was higher in the CAPE applied group compared to the other groups. This made us think that CAPE would have anticancer effects by both increasing cell death and weakening the adhesion strength and intercellular connections of cells.

VEGF-A is known as a key regulator of physiological and pathological

angiogenesis (47). It can be expressed by activated oncogenes and various cytokines in response to hypoxia. Deregulated VEGF expression contributes to the development of solid tumours by promoting tumour angiogenesis and to the ethiology of some diseases characterised by abnormal angiogenesis (48). Many studies have shown that VEGF-A plays an important role in the progression and metastasis of cancer cells. VEGF-A expression has been demonstrated in both human and NB cell lines (49-52). There are studies showing that VEGF expressions are decreased in SHSY-5Y cells treated with Topotecan and CAPE (21, 53). In our study, we observed an increase in VEGF staining intensity in SHSY-5Y cells in the Topotecan, CAPE and Topotecan+CAPE applied groups compared to the control group. Although this result we found contradicts the literature studies made with CAPE and Topotecan, it has been shown by recent literature studies that the increase in VEGF expression in SHSY-5Y cells is not associated with tumour progression and metastasis, and the roles of VEGF in NB tumourigenesis remain unclear (36, 37). However, increased VEGF expression has been evaluated in favour of increased inflammation in some studies (54-56). The fact that other inflammation markers were found to be increased in the treatment groups in our study supports this situation.

Cytokines are molecules produced by immune system cells. They have an important role in the production and activity of immune system cells and blood cells. Although there are many different types, the most commonly used ones are interleukins, interferons and GM-CSF (Granulocytemacrophage colony-stimulating factor) (57). The importance of cytokines in cancer biology was understood at the end of the 1800s with the demonstration that some malignant tumours regressed after bacterial infections. Cytokines, while causing tumour regression by directly inhibiting growth on cancer cells, or acting by supporting other antitumour effects in the body, they can also be growth factors for malignant cells on the contrary, and provide therapeutic support by inhibiting the effects of these cytokines (58). These bidirectional behaviours of cytokines change the approach in different tissues and different tumours and different antineoplastic drugs. In general, proinflammatory cytokines are mediators that are detected to be increased in the blood during cancer treatment. Tsavaris N. et al. found increased IFN-ɣ, IL-2, IL-6, GM-CSF cytokine levels in the peripheral blood of breast cancer patients they treated with taxane group antineoplastic drugs and stated that taxanes are also an effective treatment agent by increasing cytokines in patients with terminal breast cancer (59). Wood L. J. et al. administered rats an antineoplastic agent, etoposide, and observed that IL-6 serum levels increased, resulting in symptoms such as fatigue, weakness, loss of interest in social activities, and difficulty concentrating (60). However, in most studies, it has been stated that the increase in serum cytokines observed after the administration of many antineoplastic drugs, including topotecan, is responsible for chronic fatigue in patients (61). There are also studies showing that cytokines are decreased in the tumour micro-environment and tumour cells. In their study, Lasitiotaki I. et al. showed that IL-1β and IL-18 were increased in the plasmas of patients with lung cancer, while IL-6 and TNF-α were decreased in the tumour micro-

environment. As a result, they stated that tumour-induced immunosuppression occurred in the lung micro-environment and that this could provide new targets for cancer immunotherapy (62). Antineoplastic therapy promotes cell death by increasing cytokine expression and inflammation in the cancer micro-environment in most cancer cells. Tsang P. S. et al. reported that the NFkB pathway was activated in NB cells to which they applied Topotecan treatment, and as a result, inflammation in the cells increased (63). In our study, we observed an increase in staining in IL-6 and IL-18 compared to the control group, although it was more pronounced in IL-1α in Topotecan applied SHSY-5Y cells. This increase suggests that Topotecan triggers cell death by increasing inflammation in cancer cells.

CAPE is a well-known anti-inflammatory compound with antioxidant, antineoplastic and immunomodulatory properties. CAPE suppresses the inflammatory process by inhibiting cytokine and chemokine production, T-cell proliferation and lymphokine production, as well as reducing prostaglandin and leukotriene synthesis. It has also been suggested that the anti-inflammatory effect exhibited by CAPE is a result of inhibition of arachidonic acid release from the cell membrane (64, 65). CAPE is a compound with anticancer properties due to its cytotoxic effect directly against cancer cells by preventing chronic inflammation. Since it is selectively cytotoxic for cancer cells, it has been considered as an advantageous agent that can be used alongside antineoplastic agents. Studies in which CAPE acts by reducing inflammatory mediators in normal cell lines are frequently encountered in the literature. There is information that it results in a decrease in anti-inflammatory cytokines, especially in inflammatory disease models such as Alzheimer's Disease and Helicobacter Pylori (66, 67). In cancer cell models, however, the effects of CAPE on cytotoxicity, cell death and metastasis have often been evaluated. There are also articles in the literature reporting that CAPE has an antiproliferative effect on cancer cell lines (68, 69). In contrast, CAPE has been reported to ameliorate brain atrophy after neurological injury, including ischaemia and epilepsy, by inhibiting neuronal apoptosis and astrocyte proliferation in animal models (70, 71). These findings reveal that CAPE is a potent bioactive compound and can initiate multiple molecular responses in treated cell/animal models (72). In our study, the proinflammatory cytokines IL-1α, IL-6 and IL-18 were found to be increased in SHSY-5Y cells to which we applied CAPE, which has a dominant anti-inflammatory feature, compared to the control group. This increase was greater than the group to which only topotecan was administered. The fact that the increase was higher in the CAPE+Topotecan applied group reveals the synergistic effect of these two molecules. In our study, we found that CAPE and Topotecan increased inflammation and cell death in SHSY-5Y cancer cell line, suggesting that these agents trigger cell death by increasing inflammation in NB cells.

Conclusion

In our study, SHSY-5Y NB cell lines were immunostained with IL-1α, IL-6, IL-18 and VEGF antibodies by applying CAPE, Topotecan and CAPE+Topotecan for 24 hours, and TUNEL method was applied for

cell death evaluation. Inflammation and cell death were found to be increased in CAPE, Topotecan and CAPE+Topotecan applied groups. An abnormal VEGF increase was observed again after drug administration. This abnormal VEGF increase was considered as a pathway contributing to the inflammatory process rather than neovascularisation. Although the fact that CAPE causes an increase in inflammation mediators in the cancer cell line seems to be inverse to its general anti-inflammatory effect, it shows the selective effect of CAPE on neoplastic cells, which is a desired condition in cancer treatment and also shows that it is an agent that can be preferred in the treatment because it protects normal cells. In our study, it has been shown that CAPE and Topotecan increase inflammation and apoptotic cell death in the NB cell line. In this context, we believe that CAPE is an agent that can be used in addition to the others in the treatment of NB, and there is a need for in vivo and in vitro studies in this area.

Conflict of interest:

There is no conflict of interest between the authors.

Acknowledgement:

This project was supported by Afyonkarahisar Health Sciences University of Scıentıfıc Research Projects Unit with project number 20.GENEL.009.

References

1. Pastor ER, Mousa SA. Current management of neuroblastoma and future direction. Critical reviews in oncology/hematology. 2019;138:38-43.
2. Peifer M, Hertwig F, Roels F, et al. Telomerase activation by genomic rearrangements Effects of CAPE on Chemotherapeutic Intervention in high-risk neuroblastoma. Nature. 2015;526(7575):700-4.
3. Jin Z, Lu Y, Wu Y, et al. Development of differentiation modulators and targeted agents for treating neuroblastoma. European Journal of Medicinal Chemistry. 2020:112818.
4. Ackermann S, Cartolano M, Hero B, et al. A mechanistic classification of clinical phenotypes in neuroblastoma. Science. 2018;362(6419):1165-70.
5. Brodeur GM. Spontaneous regression of neuroblastoma. Cell and tissue research. 2018;372(2):277-86.
6. Joshi S. Targeting the tumor microenvironment in neuroblastoma: recent advances and future directions. Cancers. 2020;12(8):2057.
7. Miller KD, Siegel RL, Lin CC, et al. Cancer treatment and survivorship statistics, 2016. CA: a cancer journal for clinicians. 2016;66(4):271-89.
8. Smith V, Foster J. High-risk neuroblastoma treatment review. Children. 2018;5(9):114.
9. Tolbert VP, Matthay KK. Neuroblastoma: clinical and biological approach to risk stratification and treatment. Cell and tissue research. 2018;372(2):195-209.
10. Geurten C, Geurten M, Hoyoux C, et al. Endocrine consequences of neuroblastoma treatment in children: 20 years' experience of a single center. Journal of Pediatric Endocrinology and Metabolism. 2019;32(4):347-54.
11. Berthold F, Hero B. Neuroblastoma. Drugs. 2000;59(6):1261-77.
12. Koster DA, Palle K, Bot ES, et al. Antitumour drugs impede DNA uncoiling by topoisomerase I. Nature. 2007;448(7150):213-7.
13. Beker B. Çocukluk çağı kanserlerinde kemoterapi. http://www.klinikgelisim.org.tr/eskisayi/cilt20sayi2/baharbeker.pdf
14. Längler A, Christaras A, Abshagen K, et al. Topotecan in the treatment of refractory neuroblastoma and other malignant tumors in childhood-a phase-II-study. Klinische Pädiatrie. 2002;214(04):153-6.
15. Dobrowolski JW, Vohora S, Sharma K, et al. Antibacterial, antifungal, antiamoebic, antiinflammatory and antipyretic studies on propolis bee products. Journal of ethnopharmacology. 1991;35(1):77-82.
16. Pascual C, Gonzalez R, Torricella R. Scavenging action of propolis extract against oxygen radicals. Journal of Ethnopharmacology. 1994;41(1-2):9-13.
17. Dimov V, Ivanovska N, Bankova V, et al. Immunomodulatory action of propolis: IV. prophylactic activity against gram-negative

infections and adjuvant effect of the water-soluble derivative. Vaccine. 1992;10(12):817-23.
18. Edenharder Rv, Von Petersdorff I, Rauscher R. Antimutagenic effects of flavoniods, chalcones and structurally related compounds on the activity of 2-amino-3-methylinidazo [4, 5-*f*] quinoline (IQ) and other heterocyclic amine mutagens from cooked food. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis. 1993;287(2):261-74.
19. Krol W, Czuba Z, Scheller S, et al. Anti-oxidant property of ethanolic extract of propolis (EEP) as evaluated by inhibiting the chemiluminescence oxidation of luminol. Biochemistry International. 1990;21(4):593-7.
20. Sud'Ina G, Mirzoeva O, Pushkareva M, et al. Caffeic acid phenethyl ester as a lipoxygenase inhibitor with antioxidant properties. FEBS letters. 1993;329(1-2):21-4.
21. Beppu K, Nakamura K, Linehan WM, et al. Topotecan blocks hypoxia-inducible factor-1α and vascular endothelial growth factor expression induced by insulin-like growth factor-I in neuroblastoma cells. Cancer research. 2005;65(11):4775-81.
22. Lee Y-J, Kuo H-C, Chu C-Y, et al. Involvement of tumor suppressor protein p53 and p38 MAPK in caffeic acid phenethyl ester-induced apoptosis of C6 glioma cells. Biochemical pharmacology. 2003;66(12):2281-9.
23. Yu H-J, Shin J-A, Yang I-H, et al. Apoptosis induced by caffeic acid phenethyl ester in human oral cancer cell lines: Involvement of Puma and Bax activation. Archives of oral biology. 2017;84:94-9.
24. Beauregard A-P, Harquail J, Lassalle-Claux G, et al. CAPE analogs induce growth arrest and apoptosis in breast cancer cells. Molecules. 2015;20(7):12576-89.
25. Tseng T-H, Lee Y-J. Evaluation of natural and synthetic compounds from East Asiatic folk medicinal plants on the mediation of cancer. Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents). 2006;6(4):347-65.
26. Berger N, Ben Bassat H, Klein BY, et al. Cytotoxicity of NF-kappaB inhibitors Bay 11-7085 and caffeic acid phenethyl ester to Ramos and other human B-lymphoma cell lines. Exp Hematol. 2007;35(10):1495-509.
27. Chung TW, Moon SK, Chang YC, et al. Novel and therapeutic effect of caffeic acid and caffeic acid phenyl ester on hepatocarcinoma cells: complete regression of hepatoma growth and metastasis by dual mechanism. Faseb j. 2004;18(14):1670-81.
28. Akyol S, Öztürk G, Ginis Z, et al. In vivo and in vitro antıneoplastic actions of caffeic acid phenethyl ester (CAPE): therapeutic perspectives. Nutrition and Cancer. 2013;65(4):515-26.

29. Dinarello CA. Interleukin-18. Methods. 1999;19(1):121-32.
30. Borish LC, Steinke JW. 2. Cytokines and chemokines. J Allergy Clin Immunol. 2003;111(2 Suppl):S460-75.
31. Commins SP, Borish L, Steinke JW. Immunologic messenger molecules: cytokines, interferons, and chemokines. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S53-72.
32. Salem ML, Attia ZI, Galal SM. Acute inflammation induces immunomodulatory effects on myeloid cells associated with anti-tumor responses in a tumor mouse model. Journal of advanced research. 2016;7(2):243-53.
33. Greten FR, Grivennikov SI. Inflammation and cancer: triggers, mechanisms, and consequences. Immunity. 2019;51(1):27-41.
34. Carmeliet P. VEGF as a key mediator of angiogenesis in cancer. Oncology. 2005;69 Suppl 3:4-10.
35. Zachary I, Gliki G. Signaling transduction mechanisms mediating biological actions of the vascular endothelial growth factor family. Cardiovascular research. 2001;49(3):568-81.
36. Weng WC, Lin KH, Wu PY, et al. Calreticulin Regulates VEGF-A in Neuroblastoma Cells. Mol Neurobiol. 2015;52(1):758-70.
37. Becker J, Pavlakovic H, Ludewig F, et al. Neuroblastoma progression correlates with downregulation of the lymphangiogenesis inhibitor sVEGFR-2. Clin Cancer Res. 2010;16(5):1431-41.
38. Xie H-r, Hu L-s, Li G-y. SH-SY5Y human neuroblastoma cell line: in vitrocell model of dopaminergic neurons in Parkinson's disease. Chinese medical journal. 2010;123(8):1086-92.
39. Påhlman S, Mamaeva S, Meyerson G, et al. Human neuroblastoma cells in culture: a model for neuronal cell differentiation and function. Acta physiologica scandinavica Supplementum. 1990;592:25-37.
40. Sun H, Yang Y, Gu M, et al. The role of Fas-FasL-FADD signaling pathway in arsenic-mediated neuronal apoptosis in vivo and in vitro. Toxicology Letters. 2022;356:143-50.
41. Guowen W, Fei C, Dehui X, et al. The effects of topotecan on neuroblastoma cell line SH-SY5Y proliferation, invasion and migration.
42. Tomiyama R, Takakura K, Takatou S, et al. 3,4-dihydroxybenzalacetone and caffeic acid phenethyl ester induce preconditioning ER stress and autophagy in SH-SY5Y cells. J Cell Physiol. 2018;233(2):1671-84.
43. Sonoki H, Tanimae A, Furuta T, et al. Caffeic acid phenethyl ester down-regulates claudin-2 expression at the transcriptional and post-translational levels and enhances chemosensitivity to doxorubicin in lung adenocarcinoma A549 cells. J Nutr Biochem. 2018;56:205-14.
44. He YJ, Liu BH, Xiang DB, et al. Inhibitory effect of caffeic acid phenethyl ester on the growth

of SW480 colorectal tumor cells involves beta-catenin associated signaling pathway down-regulation. World J Gastroenterol. 2006;12(31):4981-5.
45. Wang D, Xiang D-B, He Y-J, et al. Effect of caffeic acid phenethyl ester on proliferation and apoptosis of colorectal cancer cells in vitro. World journal of gastroenterology: WJG. 2005;11(26):4008.
46. Tseng J-C, Lin C-Y, Su L-C, et al. CAPE suppresses migration and invasion of prostate cancer cells via activation of non-canonical Wnt signaling. Oncotarget. 2016;7(25):38010.
47. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9(6):669-76.
48. Ferrara N, Gerber H-P, LeCouter J. The biology of VEGF and its receptors. Nature medicine. 2003;9(6):669-76.
49. Bäckman U, Svensson A, Christofferson R. Importance of vascular endothelial growth factor A in the progression of experimental neuroblastoma. Angiogenesis. 2002;5(4):267-74.
50. Fakhari M, Pullirsch D, Paya K, et al. Upregulation of vascular endothelial growth factor receptors is associated with advanced neuroblastoma. Journal of pediatric surgery. 2002;37(4):582-7.
51. George ML, Tutton MG, Janssen F, et al. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. Neoplasia. 2001;3(5):420-7.
52. Meister B, Grünebach F, Bautz F, et al. Expression of vascular endothelial growth factor (VEGF) and its receptors in human neuroblastoma. European Journal of Cancer. 1999;35(3):445-9.
53. Mirzaei S, Gholami MH, Zabolian A, et al. Caffeic acid and its derivatives as potential modulators of oncogenic molecular pathways: New hope in the fight against cancer. Pharmacological Research. 2021;171:105759.
54. Scaldaferri F, Vetrano S, Sans M, et al. VEGF-A links angiogenesis and inflammation in inflammatory bowel disease pathogenesis. Gastroenterology. 2009;136(2):585-95. e5.
55. Proescholdt MA, Heiss JD, Walbridge S, et al. Vascular endothelial growth factor (VEGF) modulates vascular permeability and inflammation in rat brain. Journal of neuropathology and experimental neurology. 1999;58(6):613-27.
56. Waldner MJ, Wirtz S, Jefremow A, et al. VEGF receptor signaling links inflammation and tumorigenesis in colitis-associated cancer. Journal of Experimental Medicine. 2010;207(13):2855-68.
57. Barbaros MB, Dikmen M. Kanser immünoterapisi. Erciyes Üniversitesi Fen Bilimleri Enstitüsü Fen Bilimleri Dergisi. 2015;31(4):177-82.
58. Akdoğan M, Yöntem M. Sitokinler. Online Türk Sağlık Bilimleri Dergisi. 2018;3(1):36-45.
59. Tsavaris N, Kosmas C, Vadiaka M, Kanelopoulos P, Boulamatsis D. Immune changes Effects of CAPE on Chemotherapeutic Intervention in patients with advanced breast cancer undergoing chemotherapy with taxanes. Br J Cancer. 2002;87(1):21-7.
60. Wood LJ, Nail LM, Perrin NA, et al. The cancer chemotherapy drug etoposide (VP-16) induces proinflammatory cytokine production and sickness behavior-like symptoms in a mouse model of cancer chemotherapy-related symptoms. Biol Res Nurs. 2006;8(2):157-69.
61. Eyob T, Ng T, Chan R, et al. Impact of chemotherapy on cancer-related fatigue and cytokines in 1312 patients: a systematic review of quantitative studies. Curr Opin Support Palliat Care. 2016;10(2):165-79.
62. Lasithiotaki I, Tsitoura E, Samara KD, et al. NLRP3/Caspase-1 inflammasome activation is decreased in alveolar macrophages in patients with lung cancer. PLoS One. 2018;13(10):e0205242.
63. Tsang PS, Cheuk AT, Chen QR, et al. Synthetic lethal screen identifies NF-κB as a target for combination therapy with topotecan for patients with neuroblastoma. BMC Cancer. 2012;12:101.
64. Jung WK, Choi I, Lee DY, et al. Caffeic acid phenethyl ester protects mice from lethal endotoxin shock and inhibits lipopolysaccharide-induced cyclooxygenase-2 and inducible nitric oxide synthase expression in RAW 264.7 macrophages via the p38/ERK and NF-kappaB pathways. Int J Biochem Cell Biol. 2008;40(11):2572-82.
65. Lee KW, Chun KS, Lee JS, et al. Inhibition of cyclooxygenase-2 expression and restoration of gap junction intercellular communication in H-ras-transformed rat liver epithelial cells by caffeic acid phenethyl ester. Ann N Y Acad Sci. 2004;1030:501-7.
66. Toyoda T, Tsukamoto T, Takasu S, et al. Anti-inflammatory effects of caffeic acid phenethyl ester (CAPE), a nuclear factor-kappaB inhibitor, on Helicobacter pylori-induced gastritis in Mongolian gerbils. Int J Cancer. 2009;125(8):1786-95.
67. Cao Q, Kaur C, Wu CY, et al. Nuclear factor-kappa β regulates Notch signaling in production of proinflammatory cytokines and nitric oxide in murine BV-2 microglial cells. Neuroscience. 2011;192:140-54.
68. Sawicka D, Car H, Borawska MH, et al. The anticancer activity of propolis. Folia Histochem Cytobiol. 2012;50(1):25-37.
69. Hernandez J, Goycoolea FM, Quintero J, et al. Sonoran propolis: chemical composition and antiproliferative activity on cancer cell lines. Planta Med. 2007;73(14):1469-74.
70. Zhang L, Zhang WP, Chen KD, et al. Caffeic acid attenuates neuronal damage, astrogliosis and glial scar formation in mouse brain with cryoinjury. Life Sci. 2007;80(6):530-7.
71. Yiş U, Topçu Y, Özbal S, et al. Caffeic acid phenethyl ester prevents apoptotic cell death in the developing rat brain after pentylenetetrazole-

induced status epilepticus. Epilepsy Behav. 2013;29(2):275-80.
72. Konar A, Kalra RS, Chaudhary A, et al. Identification of Caffeic Acid Phenethyl Ester (CAPE) as a Potent Neurodifferentiating Natural Compound That Improves Cognitive and Physiological Functions in Animal Models of Neurodegenerative Diseases. Front Aging Neurosci. 2020;12:561925.