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In Vitro Determination of Valproic Acid and Cisplatin Combination Antitumor Effect on Neuroblastoma Tumors Viability

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Abstract: Neuroblastoma (SH-SY5Y) is one of the most common solid tumors in children, with aggressive and resistant features. Valproic acid (VPA) is a histone deacetylase inhibitor (HDAC) and is thought to have antitumoral activity. The aim of the current study is the evaluation of valproic acid and cisplatin (CSP) combination antitumor effects on SH-SY5Y. For this aim, the different doses of cisplatin (5, 10, and 15 µg/ml), VPA (5mM), and CSP (5, 10 and 15 µg/ml) + VPA (5 mM) were applied on SH-SY5Y tumor cell culture for 24 hours. For evaluation of cell viability, apoptosis, antioxidant and oxidant status, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT), Anexin-V-FITC apoptosis, Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS) tests were done 24 hours after drug administration. According to our results, the combination of 15 µg/ml cisplatin and 5 mM VPA reduced cell viability and increased apoptosis status, compared with the negative control group ($P<0.05$). Our studies showed valproic acid increased the cisplatin effect and reduced the viability of tumor cells. The combination of VPA + CSP is more effective than only using cisplatin. In the light of these data, it is thought that the efficacy of VPA + CSP combined therapy should be investigated more comprehensively with in vivo studies.

Keywords: Annexin V, Cisplatin, Neuroblastoma, TAC, TOS.

Valproik Asit ve Cisplatin Kombinasyonunun Nöroblastom Tümörlerinde Canlılık Üzerine Antitümör Etkisinin İn Vitro Olarak Belirlenmesi

Öz: Nöroblastoma (SH-SY5Y), agresif ve dirençli özellikleri ile çocuklarda en sık görülen solid tümörlerden biridir. Valproik asit (VPA) bir histon deasetilaz inhibitörüdür (HDAC) ve antitümoral aktiviteye sahip olduğu düşünülmektedir. Bu çalışmanın amacı valproik asit ve sisplatin (CSP) kombinasyonun SH-SY5Y üzerindeki antitümör etkilerinin değerlendirilmesidir. Bu amaçla farklı dozlarda sisplatin (5, 10 ve 15 µg / ml), VPA (5mM) ve CSP (5, 10 ve 15 µg / ml) + VPA (5 mM) SH-SY5Y'ye hücre hattına 24 saat boyunca uygulanmıştır. Hücre canlılığı, apoptoz, antioksidan ve oksidan durumunun değerlendirilmesi için, 3- (4,5-Dimetiltiyazol-2-Yl) -2,5-Difeniltetrazolyum Bromür (MTT), Anexin-V-FITC apoptoz, Toplam Antioksidan Kapasite (TAC) ve Toplam Oksidan Durum (TOS) testleri ilaç uygulamasından 24 saat sonra yapılmıştır. Sonuçlarımıza göre, 15 µg / ml sisplatin ve 5 mM VPA kombinasyonu, negatif kontrol grubuna kıyasla hücre canlılığını azalttığı ve apoptoz durumunu arttırdığı tespit edilmiştir ($P<0.05$). Çalışmamızın sonuçları, valproik asidin cisplatin etkisini artırdığını ve kanser hücrelerinin canlılığını azalttığını ve VPA + CSP kombinasyonunun tek başına sispilatin uygulamasına kıyasla daha etkili olduğunu göstermektedir. Bu veriler ışığında VPA + CSP kombine tedavi etkinliği in vivo çalışmalar ile daha geniş kapsamlı araştırılması gerektiği düşünülmektedir.

Anahtar Kelimeler: Annexin V, Cisplatin, Neuroblastoma, TAC, TOS.

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INTRODUCTION

Neuroblastoma (SH-SY5Y, NBL) is a type of tumor showing aggressive and resistant features(1,2). Nearly 40% of patients with SH-SY5Y did not respond to chemotherapeutic drugs (3). The SH-SY5Y is an embryonal tumor originating (undifferentiated tumor type) from the precursor cells of the sympathetic nervous system (4-7). Clinically, any effort to improve the treatment and diagnosis is very important and it emerges to design new agents or adjuvant therapy to reduce antitumor drugs dose (and, reduce side effects) but increase efficacy (8).

It was found SH-SY5Y human neuroblastoma cells were included in several Na^+ (v) isoforms like Na (v)1.2 and Na^+ (v)1.7 (9). Valproic acid (VPA) ($C_8H_{16}O_2$) acts on g-aminobutyric acid (GABA) levels in the brain, blocks voltage-gated ion channels, and also acts as a histone deacetylase inhibitor (HDAC) (10). VPA has been searched intensively as an adjunctive for the treatment of various animal models, from severe injuries to tumors (11,12). VPA induces tumor differentiation and apoptosis as well as suppresses tumor growth and metastatic processes (13).

Cisplatin (CSP) or cis-diamminedichloroplatinum is a platinum-based chemotherapeutic compound and is one of the most commonly used drugs in the treatment of SH-SY5Y. Cisplatin-induced death by activating mitochondrial apoptosis pathways (14). CSP mechanism of action is represented by the formation of DNA adducts by modifying the structure of DNA molecule stopping basilar processes such as transcription and replication, leading the cell to death (15). Furthermore, the long-term use of the drug leads to the development of resistance because of 1- high glutathione levels in the cytoplasm of the cell, 2-efficient DNA repair activity (16).

Despite the fact that there are many separate studies in the literature (7,10) regarding Cisplatin and Valproic acid, there is limited research on the combined treatment (17,18). In this study, for the first time, we evaluate the antitumor effects of VPA and cisplatin combination therapy on neuroblastoma. For this aim, we designed the current study in 9 separate groups and the antitumor study was done by using MTT, TAC, TOS, flow cytometry and morphological determination method for 24 hrs.

MATERIALS and METHODS

Chemicals and Reagents

Cisplatin was obtained from Kocak Pharma LTD (Tekirdag, Turkey). Valproic acid, Dulbecco Modified Eagles Medium (DMEM), phosphate buffer solution (PBS), fetal calf serum (FCS), antibiotic antimitotic solution (100×), L glutamine and trypsin–EDTA were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell Cultures

For our study, neuroblastoma cell cultures were obtained from the department of medical pharmacology of Ataturk University (Erzurum, Turkey). Briefly, the cells were resuspended by fresh medium (Dulbecco's modified eagle medium (DMEM), Fetal bovine serum (FBS) 10% and antibiotic 1% (penicillin, streptomycin, and amphotericin B). Then the cells were seeded in 24 well plates (Corning, USA) and stored at an incubator (5% CO_2; 37 $^{\circ}$C) (19).

Drug Administration

After gain, 85% confluency in 24 well plates VPA (5mM) and Cisplatin (5, 10 and 15 µg) were added to the well plate and incubate for 24 hours (incubate in 5% CO_2, 95% moisture and 37 $^{\circ}$C).

MTT Assay

At the end of the experiment (after 24 hours of treatment), 10 µL of MTT solution is added to each well plate. After 4 hours of incubation time, 100 µL of DMSO solution was incorporated into all wells. The density of the Formazan crystals was read at a

wavelength of 570 nm by the Multiskan ™ GO Microplate Spectrophotometer reader (20).

Total Oxidant Status (TOS) and Total Antioxidant Capacity (TAC)

TOS and TAC (Rel Assay Diagnostics ® Company, Gaziantep, Turkey) evaluations were done by measuring spectrophotometrically (Multiskan ™ GO Microplate Spectrophotometer reader).

In order to determine the TOS level; 500 µl Reactive solutions were added to the wells in which the 75 µl plasma sample was present and after reading the initial absorbance value at 530 nm (20). TOS =Δ example/Δ ST2×20

In order to determine the TAC level; 500 µl Reactive solutions were added in the wells containing 30 µl sample, and the first absorbance was read at 660 nm (20).

TAC = (Δ ST1-Δ example)/(ΔST1-ΔST2)

Morphologic Determination

The morphological determination was done by Leica microscope (USA). All the application groups image was taken after 24 hours of exposure time. For this aim, all images were taken at 20× magnitude.

Flow cytometry analysis

Annexin V assay kit was obtained from BioVision Company (USA). The staining with the Annexin V-FITC was applied according to the manufacturing methods. Briefly, reagent solution (250 µl), Annexin V (5 µl) and PI (5 µl) were added for 5 min in a dark place and then the samples were read by using flow cytometry device (CyFlow® Cube Flow Cytometer, Sysmex) (21).

Statistical Analyses

Statistical analysis was performed using one-way analysis of variance (ANOVA) with Tukey's HSD for posthoc comparisons using SPSS 22.0 software. The differences between the groups were considered significant at $P<0.05$.

RESULTS

MTT Analysis Results

The MTT assay results are shown in Figure 1. The survival percentages of all other groups were compared with the control group. It was determined that the cell viability of the control group was 100% and the viability of the positive control group was 96%. The highest viability ratio among treatment groups was seen in VPA (5 mM) (92%, $P>0.05$). In addition, the survival rate in pure CSP (5, 10 and 15 µg) groups dose-dependently decrease from 91 to 82%. According to our data, a combination of VPA and CSP kill neuroblastoma cell more effectively than pure CPS. That means the combination of VPA + CSP 5 µg had nearly the same cell viability rate with CSP 10 µg. The combination of VPA + CSP 15 µg reduced cell viability by 21% ($P<0.05$).

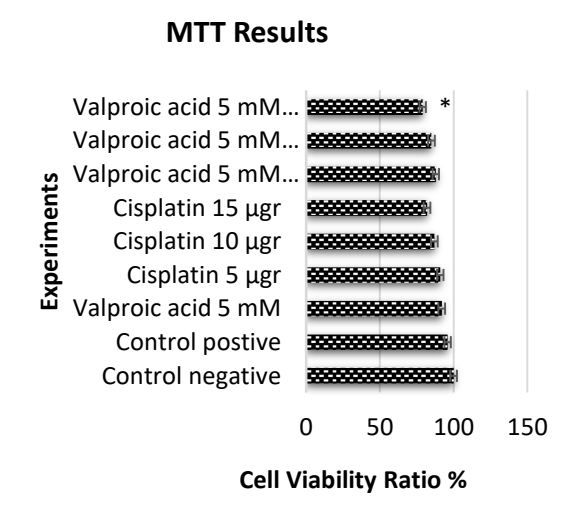

Figure 1. % Viability rates for SH-SY5Y cells - MTT test chart.
Cytotoxicity was measured by MTT assay after SH-SY5Y cells were exposed to CSP, VPA, and combinations for 24 hours.
Şekil 1. SH-SY5Y hücreleri için% Canlılık oranları - MTT test tablosu.
Sitotoksisite, SH-SY5Y hücreleri 24 saat boyunca CSP, VPA ve kombinasyonlara maruz bırakıldıktan sonra MTT testi ile ölçüldü.

TOS and TAC Analysis Results

We evaluated the TOS test based on H_2O_2 equiv/mmol L^{-1} (Figure 2). As a result of this test, the

oxidant level of the negative control group was 2.4 and the positive control group was 2.6 H_2O_2 equiv/mmol L^{-1}. Oxidant level of pure drugs slightly increased compared to the control group. Lowest oxidant ratio among the treatments was seen in VPA. Oxidant status increased in pure CSP and combination groups. According to these results, VPA combination with different CPS dose increased the total oxidant ratio in tumor cells, and the highest oxidant level was seen in VPA + CSP 15 µg/ml group 3.8 H_2O_2 equiv/mmol L^{-1} ($P<0.05$).

We evaluated the TAC test based on Trolox equiv/mmol L^{-1} (Figure 2). According to this test, the TAC level of the negative and positive control groups were 5.6 and 5.4 Trolox equiv/mmol L^{-1} respectively. A decrease in antioxidant levels of pure drugs (VPA 5.2, CSP 5 µg/ml 4.9, CSP 10 µg/ml 4.8 and CSP 15 µg/ml 3.4) was observed. In addition, the level of antioxidants also shows the reduction in all combination groups dose-dependently (low to high doses were 4.6, 3.5, and 3.1, respectively). In the VPA + CSP 15 µg/ml group was found statistically significant compared to the control group ($P<0.05$). Furthermore, no statistically significant difference was observed between the other groups ($P>0.05$).

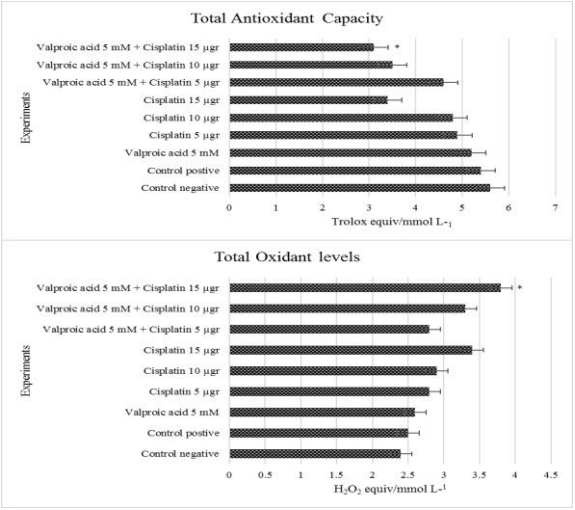

Figure 2. TOS values read spectrophotometrically at 530 nm in the fluid of cell culture and TAC values read spectrophotometrically at 660 nm in the fluid of cell culture.
TOS levels and TAC status of SH-SY5Y cells were affected by 5, 10 and 15 µg/ml CSP, 5mM combination of VPA.

Şekil 2. TOS değerleri hücre kültürü sıvısında 530 nm'de spektrofotometrik olarak okunur ve TAC değerleri hücre kültürü sıvısında 660 nm'de spektrofotometrik olarak okunur.
SH-SY5Y hücrelerinin TOS ve TAC seviyeleri 5, 10 ve 15 µg / ml CSP, 5mM VPA kombinasyonlarından etkilenmiştir.

Morphologic Determination

The morphological determination was done by Leica microscope (USA). After 24 hours, all groups images were taken at 20× magnitude. The blue arrow, red arrow, and the triangle are shown respectively live cells, dead cells, and empty space. The control group had the largest number of live cells, whereas the combination of VPA + CSP 15 µg combination had the least number of live cells (Figure 3). These results have a correlation with MTT results.

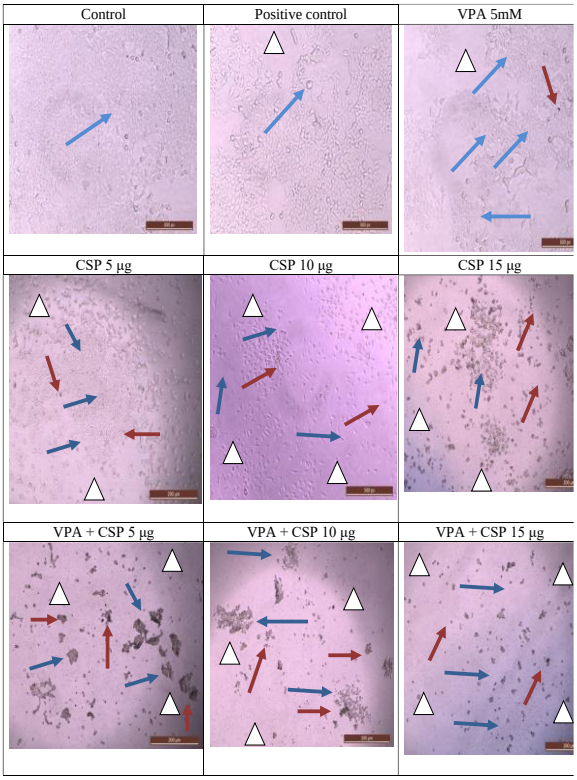

Figure 3. Microscopic view of each group after 24 hours of treatment (20×).
Blue arrow: live cells, Red arrow: died cells, Triangle: Empty space.
Şekil 3. 24 saatlik tedaviden sonra her grubun mikroskobik görünümü (20 ×).
Mavi ok: canlı hücreler, Kırmızı ok: ölü hücreler, Üçgen: Boş alan.

Flow Cytometry Analysis

We examined the development of apoptosis in the SH-SY5Y cell line (Figure 4). The viability rate of the negative control group was 99.42% and the positive control group was 97.42%. The apoptosis and necrosis ratio of the negative group and positive control group were (0.12% and 0.32%) (1.12% and 1.10%) respectively. In addition, an increase in early (0.08%, 1.24% and 0.04% respectively) and late apoptosis levels (0.28%, 6.98% and 12.02% respectively) dose-dependently were seen. The combination groups show late apoptosis, early apoptosis and necrosis levels increased dramatically in comparison with pure drugs and both control group. It was found, VPA + CSP 15 µg/ml and VPA + CSP 10 µg/ml groups combination group are induced apoptosis more effective than each dose of pure CSP groups.

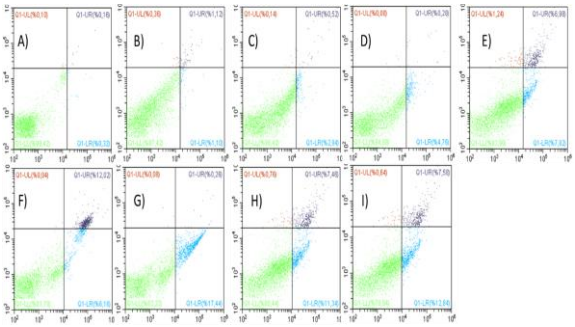

Figure 4. SH-SY5Y cells after treatment with VPA, CSP and a combination of them for 24 hours.
Apoptosis induction in SH-SY5Y cells was exposed to 5 mM VPA, 5, 10 and 15 µg/ml CSP, and combinations of them. Control cells are incubated in the absence of the drug. Apoptosis was measured using Annexin V and propidium iodide.
Şekil 4. SH-SY5Y hücreleri, VPA, CSP ve bunların bir kombinasyonu ile 24 saat süreyle tedaviden sonra.
SH-SY5Y hücrelerinde apoptoz indüksiyonu 5 mM VPA, 5, 10 ve 15 µg / ml CSP kombinasyonlarına maruz bırakıldı. İlaç yokken kontrol hücreleri inkübe edildi. Apoptoz Annexin V ve propidium iyodür kullanılarak ölçüldü.

DISCUSSION and CONCLUSION

In the present study, the antitumor effect of VPA and CSP and combination therapy of these drugs were studied on the SH-SY5Y cell line by using MTT test, TOS, TAC and flow cytometry. Various studies have shown that HDAC inhibitor combination with chemotherapeutic drugs such as doxorubicin, etoposide, or CSP, has given a new insight into the patient's lifespan (14,22). Valproic acid is Na^+ channel blocker and can effectively inhibit neuroblastoma Na^+ voltage-dependent channels. Therefore, we investigate the effects of the HDAC inhibitor VPA combined with the DNA-damaging drug CSP in the NBL cell line.

Tang and colleagues (22) demonstrated that VPA inhibits proliferation and induces apoptosis in acute myeloid leukemia cells. For this purpose, after preparation of neuroblastoma culture, Bcl2 and Bax gene were evaluated. According to this study, VPA 1 mM strongly induced cell arrest and apoptosis to tumor cells. This study is very important because of the VPA dose. The author declared that the minimum VPA dose has to be 1mM. In the current study, we used 5 mM dose of VPA to gain high efficacy (23).

Cerna et al. (2018) evaluated the antitumor and synergic effect of VPA combination with ellipticine on SH-SY5Y for 24 and 48 hrs. According to this study VPA + ellipticine combination are more effective than pure drugs. At the base of this study, to gain the synergic effect, ellipticine has to use at the same time or prior to VPA (24). This data shows a correlation with our study. According to our data, VPA increased CSP affects more than pure drugs. In addition, this combination may reduce the dose and side effects of CSP.

In a recent study, VPA has been used to enhance efficacy and decrease drug resistance in different tumors, including medulloblastoma (MB) (17). In this study, Cordeiro and his colleagues investigated the role of VPA + CSP in cell viability and gene expression of MB cell lines by using the quantitative PCR technique. Their result shows that using 3 mM VPA increased the antitumor effect in the different cultures. Similarly, we use 5 mM VPA and gain nearly the same results.

VPA inhibited adhesion of neuroblastoma cells, resistance to Vincristine (VCR) and CSP, to the endothelium (25). That study from the experimental design has a difference to our study but VPA showed the same effects as our study.

Similarly, Schuchmann et al. (25) found out that VPA induced down-regulation of cellular FLICE-

inhibitory protein (c-FLIP/CASH, also known as Casper/iFLICE/FLAME-1/ CLARP/MRIT/usurpin), this provides a possible molecular mechanism underlying the increased sensitivity towards cell death-mediated apoptosis. These data have a correlation with our study. According to our data, the CSP antitumor effect increased when VPA was added to the culture. In the other hand, VPA increased CSP uptake by neuroblastoma cells (26). A combination group of VPA + CSP 15 µg/ml is induced late apoptosis, early apoptosis, and necrosis levels more effectively than the pure drug group.

Recently, TOS and TAC levels of NB cells were investigated after VCR and VPA treatment. Depending on the dose level, the VCR antitumor effect is enhanced by VPA. The highest TOS status and lowest TAC capacity were found in 2 µg/ml VCR + VPA group respectively (21). Similar findings were obtained in the current study and we found highest TOS status and lowest TAC capacity were seen in VPA + CSP 15 µg/ml group 3.8 (H_2O_2 equiv/mmol L^{-1}) and 3.1 (Trolox equiv/mmol L^{-1}) respectively.

In conclusion, a combination of the VPA + CSP may inhibit the proliferation of NB tumor cells. The combination of CSP + VPA may be a new agent for the treatment of NB as a single agent. The future study needs to focus on the resistance mechanism of the chemotherapeutic and reveal the exact mechanism of VPA relation on this resistance.

Conflict of interest

The authors declare that they have no conflict of interest.

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