

ORIGINAL RESEARCH

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Na⁺ channel blocker enhances metformin effects on neuroblastoma cell line**Ali Taghizadehghalehjoughi^{1,2}, Ahmet Hacimuftuoglu², Aysegul Yilmaz²**¹*Ataturk University Faculty of Veterinary, , Department of Toxicology and Pharmacology, Erzurum, Turkey*²*Ataturk University, Faculty of Medicine, Department of Medical Pharmacology, Erzurum, Turkey*

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Abstract

The aim of the current study is the evaluation of valproic acid and Metformin antitumor effect on neuroblastoma (SH-SY5Y) and answer the question, is valproic acid (VPA) increase Metformin (MET) antitumor effect on the SH-SY5Y cancer cell line or not. The SH-SY5Y cell line was grown in culture medium. A different dose of MET and VPA were applied to SH-SY5Y cancer cell lines for 24 hrs. 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell viability, Annexin-V-FITC apoptosis, Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS) tests were done 24 hours after drug administration. As a result of the tests, the combination of MET and VPA reduced cancer cell viability compared with the control group ($p<0.001$). According to our result, valproic acid increased metformin antitumor effects more than metformin alone.

Keywords: Flowcytometry, metformin, neuroblastoma, valproic acid, TAC, TOS**Introduction**

Neuroblastoma commonly occurs in the embryonic stage and appears during early post-natal or fetal life from sympathetic cells originated from the neural crest. Neuroblastoma is the most common malignant tumor in newborn [1] and accounts for 15% of all cancers in children [2]. The neuroblastoma tumor shows diversity in morphological and biochemical grade (heterogeneous populations) [3]. These heterogeneous tumors may return automatically, mature or return more aggressively and may display a treatment-resistant phenotype [1].

VPA causes a significant decrease in tumor volume in vivo, which is an histone deacetylase inhibitor (HDACI) and an antiepileptic agent [4,5]. There are several ways in which VPA contributes to the antitumor effects, such as aging, angiogenesis, apoptosis and cell cycle arrest [6]. The recent studies shows, VPA strongly can block the voltage-dependent Na⁺ channels

channel [7]. VPA is a type of inhibitors that present as histone acetylases (HDACs) and DNA methyltransferases (DNMTs) [8]. In addition, HDACs that are in an abnormally active state in many cancer cells cause cell cycle inhibitors to be suppressed [9]. On the other hand, it has been reported that HDAC inhibition induces apoptosis, tumor cell differentiation or growth arrest and sensitizes the cells to radiation therapy or chemotherapy [10,11].

MET is oral biguanide, which has been clinically used since the 1950s due to hypoglycemic activity [12]. Some studies have underlined the positive effects of MET to decrease the cancer rate and mortality [13,14]. MET has shown antitumor effects in various forms of cancer, but the mechanisms of anti-neoplastic properties of MET are still doubtful [15]. The anticancer effect of MET is thought to have appeared in two ways: direct and indirect; the direct effect is partly through the activation of 5'-adenosine monophosphate-active protein kinase (AMPK) on cancer cells, while the indirect effect is by decreasing blood sugar and insulin levels [16]. Activation of AMPK represses the mammalian target of rapamycin (mTOR), which leads to decreased protein synthesis and cell reproduction [17]. In addition, the mTOR / AMPK pathway is the principal mechanism of MET [18].

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Although VPA and MET have been studied separately for neuroblastoma, there is not yet a study in which these drugs are studied together. The aim of this study is to clarify the effects of these drugs on neuroblastoma by co-administration and compare the results with previous studies. For this aim, we used MTT, Annexin V-FITC, Total Antioxidant Capacity (TAC) and Total Oxidant Status (TOS) tests for 24 hours.

Material and Methods

Chemicals

MET was obtained from Sandoz, Ltd (Istanbul, Turkey). VPA, Dulbecco Modified Eagles Medium (DMEM), Fetal calf serum (FCS), neurobasal medium (NBM), phosphate buffer solution (PBS), antibiotic antimetabolic solution (100×), L glutamine and trypsin-EDTA were obtained from Sigma Aldrich (St. Louis, MO, USA).

Cell Culture

Neuroblastoma cell cultures were taken from medical pharmacology department of Ataturk University (Erzurum, Turkey). Summarily, the cells in 25 cm² flask were processed with trypsin-EDTA and then the cells were seeded in 24 well plate (Corning, USA) by fresh medium (DMEM, antibiotic 1%, FBS 15%) and store at incubator (5% CO₂; 37°C) [19].

Drug Administration

After gain, 85% confluency in 24 well plates the drugs were added. For this aim, VPA 5mM and MET (10, 20 and 40 µgr) were added to the well plate and incubate for 24 hours (incubate in 5% CO₂, 95% moisture and 37 °C).

MTT Assay

MTT assay was applied by a commercially available kit (Sigma Aldrich, USA). Briefly, the plate was incubated (5% CO₂; 37 °C) for 4h after adding the MTT reagent (10 µL of 5 mg/ml concentration) to each well. At the end of the applications, the medium was removed and 100 µL dimethylsulphoxide (DMSO) (Sigma, USA) was added to each well to dissolve the formazan crystals. Cell viability (%) was calculated by optical density read at 570 nm using a Multiskan™ GO Microplate Spectrophotometer reader (Thermo Scientific, Canada, USA) [20].

TAC Assay

Antioxidant capacity was determined by inhibiting 2-2'-azinobis (3-ethylbenzothiazoline 6-sulfonate = ABTS⁺) radical cation in the TAS assay. The commercial kits which Rel Assay Diagnostics® Company (Gaziantep, Turkey) were used in the analysis process. For this purpose, the cell medium of every group was gathered at the end of the test and collected at -20 °C until the TAC test was performed.

The elements in the kit were Standard 1 solution, Standard 2 Solution Reactive 1 Solution and Reactive 2 Solution. To determine the level of TAC, 500 µL of Reactive 1 solution was added to wells containing 30 µL sample, and the initial absorbance value was read at 660 nm and then 75 µL of Reactive 2 solution was added to the same well. After waiting at room temperature for 10 minutes, the second absorbance value was

read at 660 nm. While Standard 2 solvent in the kit was used for Standard 2, distilled water was used for Standard 1. The absorbance worth have according to the following formula were placed and the TAC levels in the Trolox Equiv/mmol L-1 were determined [21].

TOS Assay

The evaluation of the intensity of the color by spectrophotometric properties, depending on the number of oxidants in the sample, is called the TOS assay. In this study, TOS kits were purchased from Rel Assay Diagnostics® Company (Gaziantep, Turkey). For this purpose, the cell medium of every group was gathered at the end of the test and collected at -20 °C until the TOS test was performed.

The elements in the kit were Standard 1 solution, Standard 2 Solution Reactive 1 Solution and Reactive 2 Solution. To determine the level of TOS, 500 µL of Reactive 1 solution was added to wells containing 75 µL plasma sample, and the initial absorbance value was read at 530 nm and then 25 µL of Reactive 2 solution was added to the same well. After waiting at room temperature for 10 minutes, the second absorbance value was read at 530 nm. Standard 2 solution in the kit was used for Standard 2. Using the absorbance values obtained and the formula below, the TOS levels are calculated as H₂O₂ Equiv/mmol L-1 [20,21].

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

The cells were collected, washed and stained with MET, VPA, and combinations thereof according to the manufacturer's protocol (Biovision, USA). Shortly, the cells were cleaned with PBS and then 500 µL binding buffers, Annexin V-FITC, and PI stains, respectively, were added for 10 minutes in the dark room. The stained samples analyzed on a CytoFLEX flow cytometer as instructed by the manufacturer. Stained samples were examined on the CytoFLEX flow cytometer and as specified by the manufacturer. (Beckman Coulter, USA) [21,22].

Statistically analysis

Statistical calculations were carried out using SPSS 22.0 software. In order to determine the statistical significance of the results, the one-way ANOVA test was applied. The differences between the groups were considered significant at P< 0.05.

Results

MTT Assay

Survival rates of cancer cells were measured by MTT after cells were exposed for 24 hrs. The result of cell viability after treated by MET and VPA acid were shown in figure 1. As we can see in Figure 1, our control group is equal to 100. The survival percentages of the other groups were also calculated by comparison with the control group. When compared with the control group, it was observed that the pure MET and VPA decreased neuroblastoma

viability dose-dependently ($P>0.05$). When we compared the combination groups with the control and other pure groups, a significant decrease in viability ratio was observed, depending on the dose. There was a statistically significant difference between the control group and VPA + MET 40 μ g group ($P<0.001$). At the same time, VPA + MET 20 μ g group was statistically significant when compared to the control group ($P<0.05$).

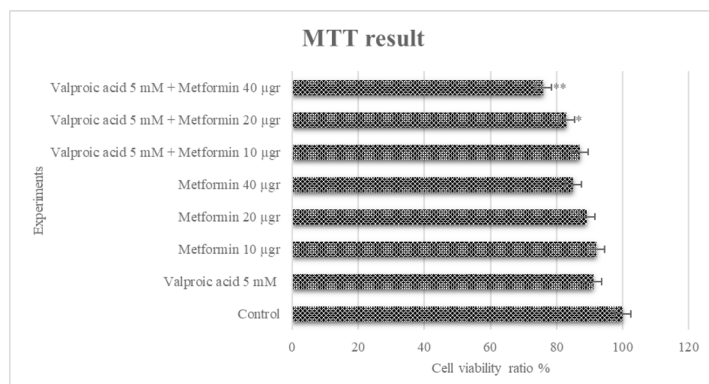


Figure 1. % Viability rates for neuroblastoma cells - MTT test chart

Cytotoxicity was measured by MTT assay after neuroblastoma cells were exposed to VPA 5 mM and MET (10, 20 and 40 μ g) and combinations of these for 24 hours. According to MTT results, the highest survival rate is seen at the highest dose combination of VPA + MET. (** $P<0.001$) (* $P<0.05$).

TAC Results

We showed our TAC test results in Figure 2 based on Trolox equiv/mmol L-1. As seen in our graph, our negative control group is 6.5 Trolox equiv/mmol L-1. There was a decrease in the antioxidant levels of the purely applied VPA 5.9, and MET (6.1, 5.9 and 5.4 respectively). However, no statistically significant difference was observed when comparing these groups to controls. There was a decrease in antioxidant levels in the combination groups (5.6, 5.1 and 4.7 respectively) when compared with control and pure groups. Statistically, significant differences were observed in VPA + MET 40 μ g compared to the control group ($P<0.05$).

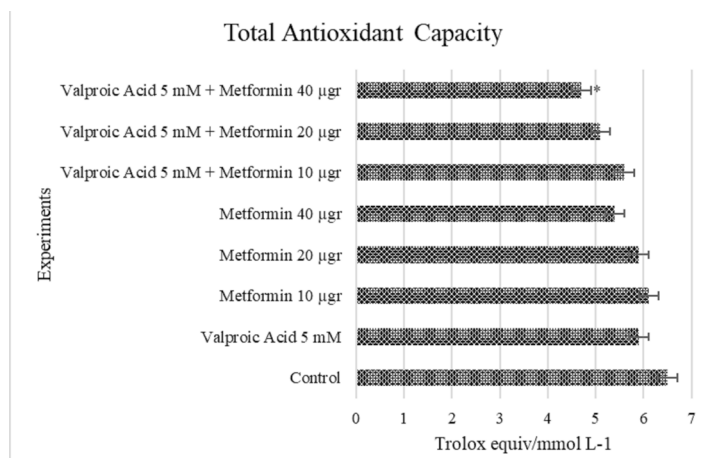


Figure 2. Total antioxidant capacity test values read spectrophotometrically at 660 nm in neuroblastoma cell culture fluid. Total antioxidant capacity levels of SH-SY5Y cells affected by VPA 5 mM and MET (10, 20 and 40 μ g) and combinations (* $P<0.05$).

TOS Results

We evaluated our TOS test results according to H₂O₂ equiv/mmol L-1 values as shown in figure 3. As seen in our graph, the value of the negative control group is 3.1 H₂O₂ equiv/mmol L-1. The oxidant levels of the purely applied MET and VPA groups were increased compared to the control group (VPA 3.5, MET (3.4, 3.7 and 4.2 respectively)). The oxidant capacity of VPA + MET 10 μ g from the combination groups is higher than all other pure groups except the pure MET 40 μ g group. The oxidant levels of the VPA + MET 20 μ g and VPA + MET 40 μ g combination groups were increased compared to the control group and other groups (4.5, 5.1 respectively). It was observed that these combination groups had a statistically significant difference when compared to the control group (for VPA + MET 20 μ g ($P<0.05$) and for VPA + MET 40 μ g ($P<0.001$)).

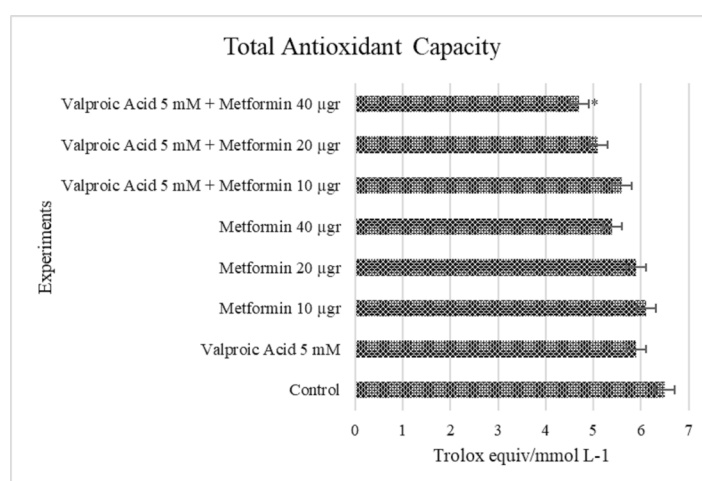


Figure 3. Total oxidant status test values read spectrophotometrically at 530 nm in neuroblastoma cell culture fluid. Total oxidant status levels of neuroblastoma cells affected by VPA 5 mM and MET (10, 20 and 40 μ g) and combinations (** $P<0.001$) (* $P<0.05$).

Flowcytometry Analysis

As shown in Figure 4, apoptosis development of VPA, MET and VPA + MET combination groups applied to the cell line was investigated. According to our data control group, viability, necrosis, early apoptosis, and late apoptosis rates were 89.31%, 1.52%, 6.72%, and 2.66% respectively. The viability (86.31%) and necrosis (1.13%) values of pure VPA were observed to be lower than the control group. However, on the contrary, early apoptosis (6.84%) and late apoptosis (5.71%) values were also increased compared to the control group. Pure MET 10 μ g early and late apoptosis, viability and necrosis were 9.85%, 3.20% 86.51% and 0.44% respectively. Compared with the control group of pure MET 20 μ g, there is a decrease in viability (86.27%) but an increase was seen in necrosis (3.83%), early apoptosis (7.01%) and late apoptosis (2.89%) ratio. Similarly, in MET 40 μ g there was a decrease in viability (81.45%) when compared to the control group, but necrosis (2.71%), early apoptosis (12.13%), and late apoptosis (3.71%) were increased.

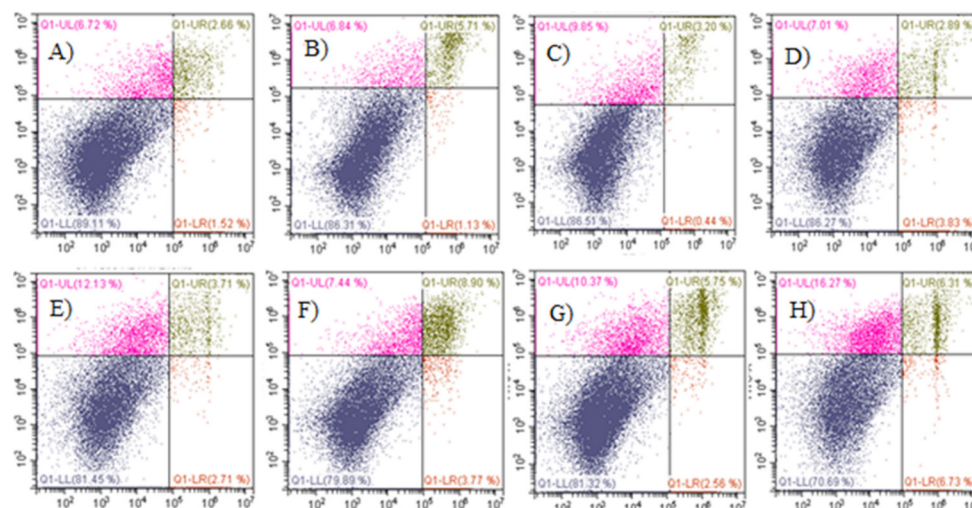


Figure 4. Neuroblastoma cells after VPA and MET treatment for 24 hours. Apoptosis induction in neuroblastoma cells was exposed to VPA 5 mM and MET (10, 20 and 40 μ gr) and combinations. Control groups were incubated in without drug. Apoptosis was measured using Annexin V and PI

Morphological Determination

Morphological determination result was shown in Figure 5. According to the result, the blue arrow shows viable cells and control group shows maximum cell viability among the groups. VPA and metformin low dose slightly affect the neuroblastoma cells, empty and dead cells number are small. Metformin and combination dose-dependently kill the cells, the empty area is large, killed cells number are many and survived cells size are limited. Combination groups are more effective than pure groups. Among the pure groups MET 40 μ gr are more effective than others.

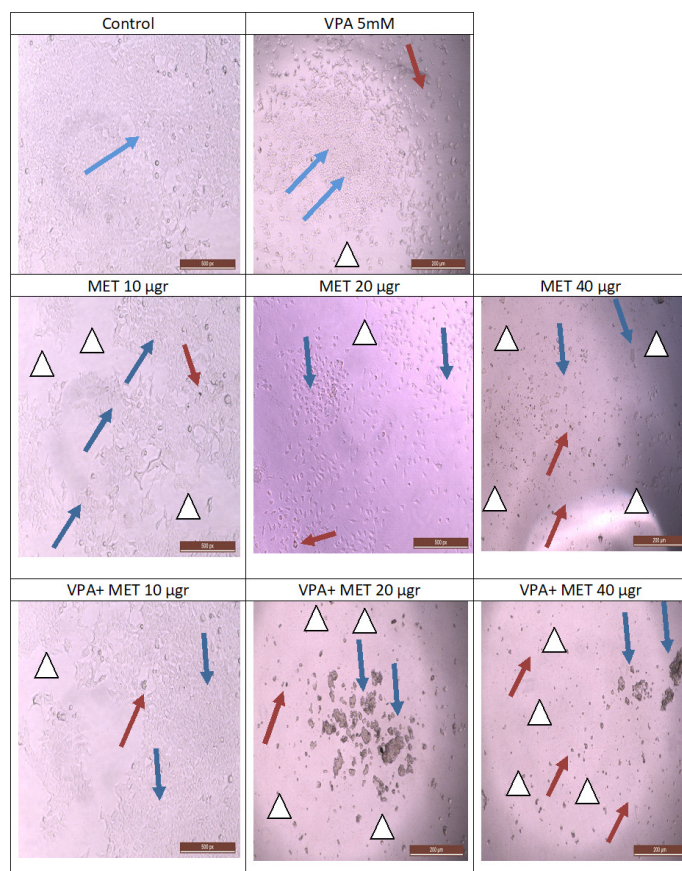


Figure 5. Microscopic view of each group after 24 hrs treatment (20 $\times$) Blue arrow: live cells, Red arrow: died cells, Triangle: Empty space

Discussion

Neuroblastoma cells cultured in 24 well platelets were exposed to VPA, MET and combinations thereof 24 hours after reaching a concentration of 85%. After the completion of the time, viability, oxidant, and antioxidant values were measured by MTT, TAC, TOS and flow cytometry tests.

Delfina Costa et al. for the first time evaluate metformin antiproliferative effect on two different neuroblastoma cell lines (SKNBE2 and SH-SY5Y), and they show MET reduced the proliferation rate of neuroblastoma cell lines [23]. We also observed similar results in our study and showed in a figure 5 and figure 1 that the empty area on culture can be seen and also cell viability was reduced in pure MET group.

Ying-Ka Ingar Lau et al. studied the combination of MET and erlotinib in the human breast cancer cell line. They observed that co-administration of MET and erlotinib-induced cell death. However, when separate administration was performed, it was observed that each agent inhibited proliferation but did not induce apoptosis [15]. In contrast, Akimasa Takahashi et al. found that our results similarly showed that the proportion of apoptotic cells was higher in groups treated with MET compared to control [24]. According to the results of Figure 2; The VPA + MET combination group reduced antioxidant levels in a dose-dependent. This increased the early and late apoptosis rates of the cells (figure 4). According to Figure 3, the VPA + MET combination increased the amount and value of cellular stress. The rise of free radicals caused the cells to be exposed to more stress. This is reflected in our graph as an increase in mortality compared to control in neuroblastoma cells. in addition, flow cytometry result showed an increase in apoptosis and necrosis in the experimental group, especially in a combined group.

Histone deacetylase inhibitors with ant proliferative and pro-differentiation properties are a promising new class of cancer therapy [25]. Zhao Shan et al. found that the SKOV3 cell line inhibited the proliferation of cancer cells by the HDAC inhibitor VPA [26]. Similar results were observed in our study and VPA was found to reduce viability in the neuroblastoma cell line. The pure Valproic acid decrease cell viability and antioxidant capacity in

neuroblastoma culture but this reduction did not show statically difference with the control group in 5 mM dosage. Whereas increase MET effect (figure 1) and effective dose nearly to half (the viability of Metformin 40 µgr are nearly the same with VPA + MET 20 µgr).

The antitumor effects of MET and VPA have been revealed in various studies as mentioned above. However, we have seen that using MET and VPA together increases antitumor efficacy in our study.

Conclusion

In conclusion, we found that we could prevent neuroblastoma proliferation by VPA + MET combination. The combination group shows quite impressive result in compared to the pure drugs.

Competing interests

The authors declare that there are no conflicts of interests.

Financial Disclosure

All authors declare no financial support.

Ethical approval

Consent of ethics was approved by the local ethics committee.

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