Archives of Epilepsy

Original Article

Investigation of the Effects of Antiepileptics On Mitotic Proliferation in Glioblastoma and Neuroblastoma in Vitro Cell Cultures

Şeyda Çevik Güneri[1], Ahmet Altun[2], Mehmet Celalettin Güneri[3], Ertuğrul Bolayır[4]

[1]Department of Clinic Neurology, Dr. Ersin Arslan Training and Research Hospital, Gaziantep, Turkey
[2]Department of Pharmacology, Cumhuriyet University, Faculty of Medicine, Sivas, Turkey
[3]Department of Clinic Internal Diseases, Dr. Ersin Arslan Training and Research Hospital, Gaziantep, Turkey
[4]Department of Neurology, Cumhuriyet University, Faculty of Medicine, Sivas, Turkey

Cite this article as: Çevik Güneri Ş, Altun A, Güneri MC, Bolayır E. Investigation of the effects of antiepileptics on mitotic proliferation in glioblastoma and neuroblastoma in vitro cell cultures. *Arch Epilepsy.* 2021;28(1):1-7.

Corresponding Author: Şeyda Çevik Güneri E-mail: drseydacevik@hotmail.com

Received: June 7, 2021 **Accepted:** December 10, 2021

DOI: 10.54614/ArchEpilepsy.2022.47135

Abstract

Objective: The frequency of epileptic seizures is high in patients with brain tumors and its treatment is important. It is atypical mitotic proliferation that is effective in the proliferation of malignant tumor cells. It is known that antiepileptics have direct or indirect effects on mitotic proliferation. In our study, we aimed to maximize the use of both the antiepileptic effect and the cytoreductive effect by suppressing tumor growth while choosing the drug to stop the seizure.

Methods: In our study, anti-tumoral activities of antiepileptic agents containing gabapentin, pregabalin, valproic acid, levetiracetam, zonisamide, phenytoin, carbamazepine in in vitro glioblastoma (c6) and neuroblastoma cell cultures (NA/An1) were evaluated with a real-time cell analysis system. Statistically, the difference between groups was investigated by analysis of variance, followed by post hoc Tukey's test Statistical Package for the Social Sciences software 16.0 (IBM Inc, Chicago, IL, USA).

Results: In our study, it was observed that all drugs except gabapentin had antimitotic effects in glioblastoma cell cultures, among which phenytoin, levetiracetam, and valproic acid had dose-dependent antimitotic effects, while carbamazepine had reduced antimitotic effects at concentrations above 25 µg/mL. In in vitro neuroblastoma cell cultures, it was found that only valproic acid and zonisamide had antimitotic effects, and other drugs studied did not have antimitotic effects.

Conclusions: In our study, it was observed that the preference of antiepileptics with a high antimitotic effect on tumoral cells was important when arranging seizure treatment in patients with brain tumors.

Keywords: Antiepileptics, cell culture, cytotoxicity, glioblastoma, mitotic proliferation, neuroblastoma, xcelligence

INTRODUCTION

Although the frequency of epileptic seizures in patients with brain tumors varies according to the type of tumor, studies have shown that this rate is high.[1] Although intracranial pathologies account for only 4% of the etiology in patients with epilepsy, it is known in clinical practice that the frequency of seizures increases in patients with brain tumors. More than one-third of patients with brain tumors have seizures. It is also known that slow-growing tumors are more epileptogenic than high-grade malignant tumors. It is known that neuronal tumors are more epileptogenic than lymphomas and glial malignancies and lymphomas, and younger patients with long survival are more prone to seizures.[2]

There is an atypical mitotic proliferation in tumor cells, especially in malignant brain tumors. There is not enough information in the literature about the response of cancer cells, whether non-operated or residual cancer cells after the operation, to the antiepileptics used. In some studies, it has been shown that carbamazepine stops mitosis by blocking the metaphase-anaphase transformation, and it has been shown that when the drug is removed, it continues to proliferate rapidly from the phase where mitosis is left.[3] Gabapentin has been found to cause an increase in pancreatic acinar cell tumors in rats through non-genotoxic mechanisms.[4] In the literature, it has been shown that valproate increases tumor differentiation and suppresses metastasis and tumor growth in in vivo and in vitro studies.[5]

In our study, the effects of the most commonly used antiepileptics on in vitro tumor cells were compared in order to both minimize the frequency of epileptic seizures and suppress mitotic proliferation and prevent tumor recurrence or growth when starting antiepileptic seizures in patients with glioblastoma and neuroblastoma, with or without surgery.

METHODS

Our study was carried out at Sivas Cumhuriyet University Faculty of Medicine Research Center. In our study, the NA/An1 Mouse neuroblastoma cell line obtained from the Foot and Mouth Institute of the Ministry of Agriculture and Rural Affairs, tumor necrosis foci developed from Wistar rats, LN-229 Glioma cell line (C6 glioma) having the same histological features as human glioblastoma multiforme such as nuclear polymorphism and high mitotic index, 7 different antiepileptic drugs (valproate, levetiracetam, phenytoin, gabapentin, carbamazepine, zonisamide, and

pregabalin) which are clinically commonly used were used. To determine the mitotic proliferation effects of the test materials, preparation and sterilization of test samples, preparation of cell culture, and administration of xcelligence test were followed respectively.

In order to ensure standardization in the tests to be applied, all samples were prepared in accordance with the ISO 10993-5 protocol, which includes standard procedures for in vitro cell culture studies.

Drugs

The drugs used were levetiracetam (UCB Pharma, Brussels, Belgium), phenytoin (Actavis Italy SpA, Nerviano – Milan/Italy), carbamazepine (Novartis Pharma, Basel, Switzerland), valproic acid (VPA) (Sanofi Winthrop Industrie, France), zonisamide (Sumitomo Dainippon Pharma, Osaka, Japan), gabapentin, and pregabalin (Pfizer, Freiburg, Germany). The concentrations of the drugs were prepared by dissolving them in ethanol at varying concentrations (1/1 and 1/2 dilutions) after examining the methodology in in vitro studies in which these drugs were used. After thawing, the drugs were studied in cell culture after dilution with 1000-fold medium.

CELL CULTURE

Cells were propagated in 25 cm^2 and 75 cm^2 flasks in an incubator with 5% CO_2 and 95% humidity in a carbon dioxide incubator. Dulbecco's modified Eagle medium (DMEM) was used as the medium. In addition to DMEM, 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin were used. When the cells reached 70% density and covered the surface of the flask, they were multiplied by passaging at least twice a week. All processes were carried out in a laminar flow cabinet sterilized by ultraviolet light. Cells were separated from the flask with 0.05% trypsin and 0.53 mM EDTA solution. The effect of trypsin was neutralized by adding DMEM to the cell suspension, and the cell suspension was divided among the flasks. To calculate the number of cells per milliliter of the total cell suspension, a hemocytometer with an area of 1 mm^2 and a depth of 0.1 mm, consisting of 4 regions containing 16 small squares, was used. The total number of cells per milliliter of suspension was calculated by the following formula.

Total cell count/mL = hemocytometer count result × 104 × Medium amount (mL)

The falcon tube containing the cells, the medium-containing falcon tube with 90% FBS, and 10% dimethyl sulfoxyl (DMSO) were placed in ice. The cold-resistant storage tubes (cryotube) to be used were also placed in ice to cool them. The cells taken from the single-cell suspension in the tube into serum medium with DMSO were added dropwise to the cryotube. After the storage, the tube was kept at −20°C for 1 night and also at −70°C for 1 night, and it was taken to liquid nitrogen vapor. All these applications were made so that the final concentration in the tube was 2.5 × 10^6 cells/mL.

Xcelligence System

Xcelligence (RocheDiagnostics GmbH Türkiye) consists of 4 main components: Real Time Cell Analysis (RTCA) analyzer, RTCA DP

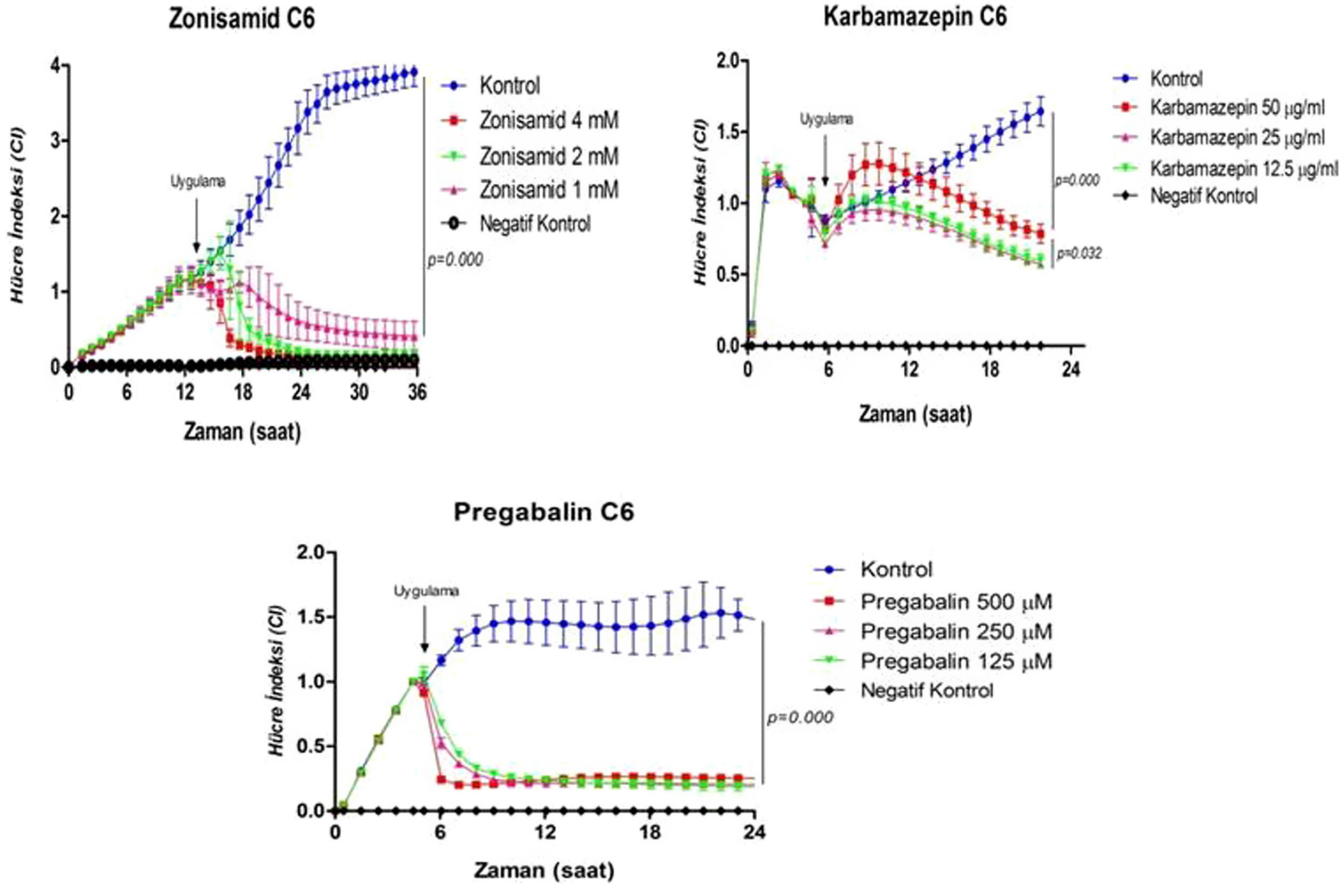

Figure 1. Mitotic proliferation findings of antiepileptics in C6 glioma cells.

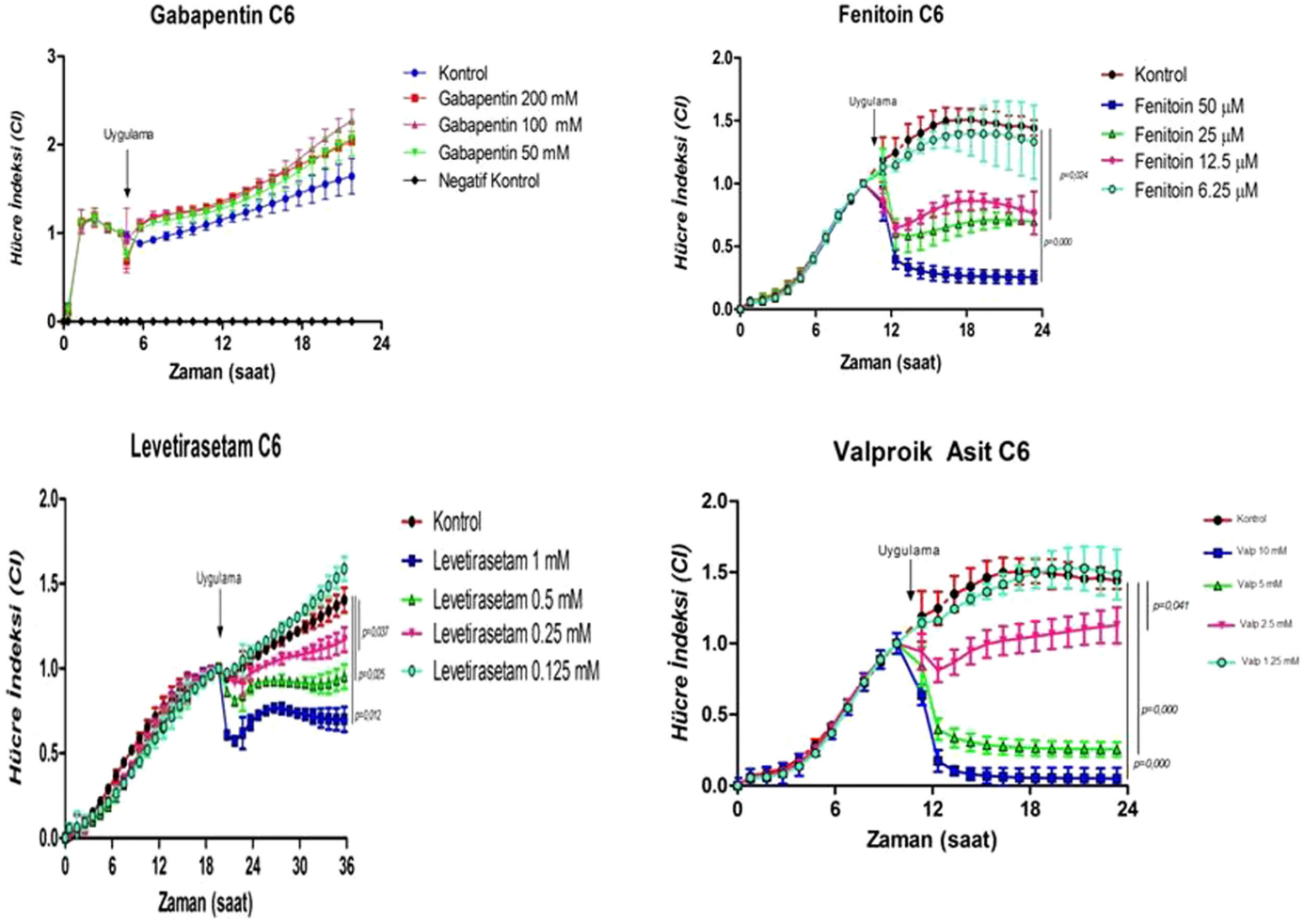

Figure 2. Mitotic proliferation findings of antiepileptics in C6 glioma cells.

station, RTCA computer with integrated software, and disposable e-plate 16. RTCA software allows users to obtain parameters such as mean value, maximum and minimum values, standard deviation, concentration that produces half the maximum effect (EC_{50}), concentration that produces half the maximum inhibition (IC_{50}), cell index, and graph. In the study, plates named e-plate 16, each well with a volume of 250 µL, a base diameter of 5 mm, consisting of 16 wells were used. First, a basal reading was taken on the device by adding 100 µL of cell-free medium mixture to each well. Cells collected by centrifugation (800 rpm, 10 minutes) were then seeded into the wells in

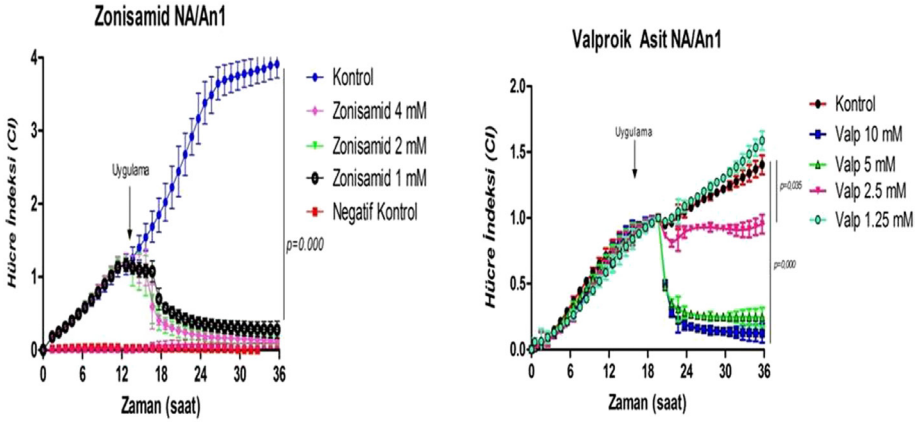

Figure 3. Mitotic proliferation findings in NA/An1 cells of antiepileptics.

a volume of 100 µL. After the C6 and NA/An1 cells were seeded into the wells, the plate was placed in the incubator and cell growth was followed at 1-hour intervals. C6 and NA/An1 cells seeded in the wells adhered to the bottom of the well, where they continued to grow in the appropriate nutrient and O_2/CO_2 environment. The gold plate placed at the bottom of the well measured the electric charge impedances of the dividing cells using electric current and allowed us to determine the growth index of the cells. After the cells enter the rapid growth phase (log phase), the agents whose cytotoxic activity will be evaluated in the wells determined as the experimental group were applied in a volume of 10 µL in varying concentrations by examining the methodologies in in vitro studies in which these drugs were used.[6-8]

The control group, which did not contain antiepileptic but contained only medium and cells, was added to the same experimental set. In addition, the negative control group without cells was also included in the study. Following this application, the plate was placed back into the incubator. After the extraction fluid application, 1-hour measurements were continued. Follow-up was continued for 24-72 hours. The cytotoxic effect of the materials on C6 and NA/An1 cells was evaluated with the Xcelligence test, and the results and the values obtained by calculating the average cell proliferation percentages of each material were compared statistically.

Statistical evaluation of the data obtained from this study was performed using the Statistical Package for the Social Sciences software 16.0 (IBM Inc, Chicago, IL, USA). The mean and standard error of all data were calculated and presented as mean ± standard error of the mean (mean ± SEM). The difference between the groups was investigated by post hoc Tukey test, following analysis of variance. The difference was considered significant if the *P* value was less than .05. The study was approved by the Ethics Committee of Cumhuriyet University (Date: December 27, 2011, Decision no: 2011/043).

RESULTS

Results on C6 Cell Lines

All applied concentrations of zonisamide showed a statistically significantly stronger cytotoxic effect compared to the control group ($P < .05$) (Figure 1). The inhibitory concentration 50 (IC50) value was calculated as 5.32×10^{-5} M, and the R^2 value was calculated as 9993×10^{-1} (Figure 5).

All applied concentrations of carbamazepine showed a statistically significantly strong cytotoxic effect compared to the control group ($P < .05$). It was observed that the 12.5 and 25 µg/mL concentrations of carbamazepine had a statistically significantly more cytotoxic effect than the 50 µg/mL concentration (Figure 4). The IC_{50} value was calculated as 4.56×10^{-5} M and the R^2 value as 8.601×10^{-1} (Figure 5).

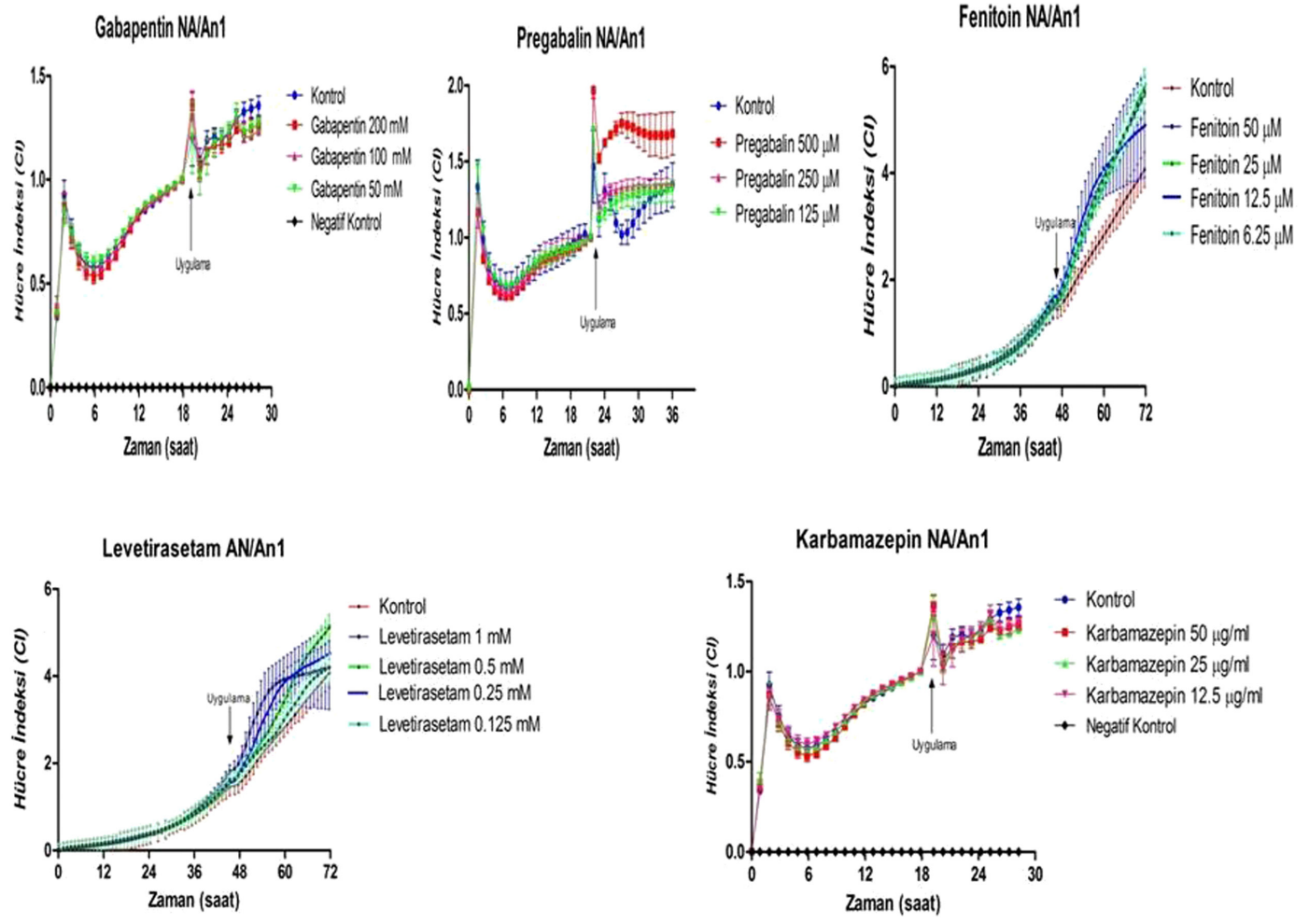

Figure 4. Mitotic proliferation findings in NA/An1 cells of antiepileptics.

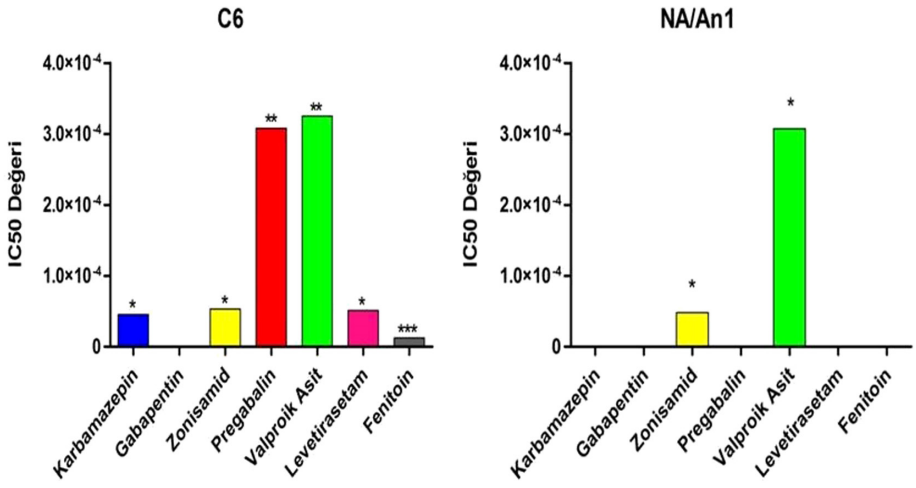

Figure 5. Mitotic proliferation findings of antiepileptics in C6 glioma cells.

All pregabalin applied concentrations showed a statistically significantly stronger cytotoxic effect compared to the control group ($P < .05$) (Figure 4). The IC_{50} value was calculated as 3.08×10^{-4} M, and R^2 value was calculated as 1.0 (Figure 5).

Gabapentin did not cause cytotoxicity at all concentrations compared to the control group ($P > .05$) (Figure 2).

Phenytoin 6.25 μM concentration did not show any cytotoxic effect ($P > .05$). Other concentrations of phenytoin showed a statistically significant dose-dependent cytotoxic effect compared to the control group ($P < .05$) (Figure 5). The IC_{50} value was calculated as 1.25×10^{-5} M, and R^2 value was calculated as 9.72×10^{-1} (Figure 5).

Levetiracetam did not show any cytotoxic effect at 0.125 mM concentration ($P > .05$). Other concentrations showed a statistically significant dose-related cytotoxic effect compared to the control group ($P < .05$) (Figure 5). The IC_{50} value was calculated as 5.1×10^{-5} M and the R^2 value as 9.05×10^{-1} (Figure 5).

Valproic acid 1.25 mM concentration did not show any cytotoxic effect ($P > .05$). Other concentrations showed a statistically significant dose-related cytotoxic effect compared to the control group ($P < .05$) (Figure 5). The IC_{50} value was calculated as 3.25×10^{-4} M and the R^2 value as 9.25×10^{-1} (Figure 5).

Results on NA/AN1 Cell Lines

All concentrations of zonisamide showed a strong cytotoxic effect compared to the control group ($P < .05$) (Figure 6). The IC_{50} value was calculated as 4.82×10^{-5} M and the R^2 value as 9.173×10^{-1} (Figure 5).

The 1.25 mM concentration of VPA did not show any cytotoxic effect ($P > .05$). It was observed that other concentrations showed a statistically significant cytotoxic effect compared to the control group ($P < .05$) (Figure 6). The IC_{50} value of the applied VPA was calculated as 3.25×10^{-4} M, and the R^2 value was calculated as 9.12×10^{-1} (Figure 5).

Significant cytotoxic effects of other drugs on NA/An1 cell lines were not observed compared to the control group ($P > .05$) (Figure 4).

DISCUSSION

Neuroblastoma and glioblastoma were preferred in our study because of their prevalence, poor prognosis, and higher frequency of seizures. Since the concentration values in which the drugs were used for the study were applied in the log phase, which is the period in which the cell number increases in accordance with the proliferation pattern, there was a difference in the administration times of the drugs. The end times of the experiments were determined when the control cells reached saturation and the curves went down. In addition, the concentration differences of drugs were determined by examining the methodology in in vitro studies in which these drugs were used.

When we compared the IC_{50} levels of antiepileptics with cytotoxic properties on C6 cell lines, pregabalin and VPA were found to be statistically significantly more cytotoxic than carbamazepine, zonisamide, levetiracetam, and phenytoin ($P < .05$). When the values of VPA and pregabalin were compared with each other statistically, no significant difference was found ($P > .05$). When carbamazepine, zonisamide, and levetiracetam were compared with each other, no significant statistical difference was found. However, these 3 drugs were found to be statistically more cytotoxic than phenytoin ($P < .05$). Only zonisamide and VPA showed cytotoxic effects in NA/An1 cell lines. When the IC_{50} values of these 2 drugs were compared, VPA was found to be statistically more cytotoxic ($P < .05$) (Figure 8). In studies with VPA, it has been suggested that besides being an antiepileptic drug, it has anticancer drug properties by causing histone deacetylase inhibition, tumor cell differentiation, apoptosis, and growth arrest.[9] In addition, VPA was found to stimulate the expression of endogenous inhibitors of angiogenesis, including thrombospondin-1 and secreted protein acidic and rich in cysteine).[10] The effects of ABT-510 and VPA on neuroblastoma angiogenesis and tumor growth were evaluated. As expected, both agents inhibited (basic Fibroblast Growth Factor) bFGF-induced endothelial cell migration in vitro. In addition to inhibition of angiogenesis, VPA has also been observed to inhibit neuroblastoma cell proliferation and neuroblastoma-induced apoptosis.[11] In addition, VPA has been shown to be a potent inhibitor of neuroblastoma angiogenesis.[10] In addition, although the mechanism of action is unclear, VPA has been shown to reduce the histone deacetylase-dependent transcriptional repression of many antiangiogenic proteins.[12] Valproic acid has been shown to directly affect the growth of neuroblastoma cells by inhibiting

cell cycle progression and inducing cell differentiation.[13] Valproic acid has been shown to induce growth arrest and differentiation of human neuroectodermal tumors, similar to some other fatty acids. In the same study, it was shown that continuous VPA treatment with interferon-alpha (INF-alpha) synergistically inhibited cell growth on a neuroblastoma cell model. It has been shown that the combination of VPA and INF-alpha inhibits tumor cell growth, induces tumor differentiation, and suppresses malignant biology by reducing angiogenesis and metastatic potentials, thus providing a new therapeutic strategy for neuroblastoma.[14]

In our study, it was shown that VPA had a cytotoxic effect in both C6 and NA/An1 cell cultures, similar to other studies, and this effect increased significantly with increasing dose.

In a study with carbamazepine, which is an old generation antiepileptic, it was observed that carbamazepine showed antiproliferative effects on mammalian vero cells, and in this study, it was determined that carbamazepine showed antiproliferative activity between the normal organization of mitotic spindles[3], and in this mechanism, the mitotic block caused by drugs with antimicrobial properties has been held responsible for the suppression of mitotic spindle dynamics. In another study, mitotic arrest was thought to be associated with inhibition of both mitotic kinases and microtubule motor proteins involved in spindle morphogenesis.[15,16]

After carbamazepine treatment, an increase in apoptotic cells was detected in parallel with the disappearance of the cells blocked in the metaphase, and thus, this mitotic disorder accelerated apoptosis.[17] In another study, cell death due to abnormal mitosis (catastrophic mitosis) was seen only in a few large-shaped, micronucleated cells with delayed apoptosis. It has also been observed that mitotic blockade of more than 9 hours inevitably results in apoptosis.[12]

In our study, similar to other studies, while a decrease in mitotic proliferation was observed with an increase in the dose of carbamazepine up to 50 µg/mL concentration on C6 cell cultures, and unlike other studies, it was determined that the cytotoxic effect of the drug decreased in the application at 50 µg/mL concentration of carbamazepine. In the study performed on NA/An1 cell cultures, it was observed that it did not cause cytotoxicity.

In a study with phenytoin, a kinetic study of mitotic index and cell cycle was performed in short-term cultures of stimulated peripheral blood lymphocytes of untreated epileptics, control group, and epileptics under phenytoin treatment. In this study, it was determined that the mitotic index on blood lymphocyte cells of the patients treated with phenytoin (PHT) was significantly inhibited ($P < .001$) compared to the control group, and the estimated cell cycle delay in the mean proliferation rate index values was found to be higher in lymphocytes treated with phenytoin ($P < .001$).[18]

In our study using C6 cell lines, phenytoin did not show cytotoxic effects at very low doses, while it was observed that it blocked mitotic proliferation significantly as the dose was increased. It did not cause cytotoxicity in NA/AN1 cell lines.

Despite the lack of sufficient data on the mitogenic activity of gabapentin, an in vivo study showed a very low level of mitogenic activity in pancreatic acinar cells that could not be easily detected.[4] In our study, however, gabapentin did not show any cytotoxic effect on either C6 or NA/AN1 cell lines.

For levetiracetam, pregabalin, and zonisamide, there is no study on the mitotic effects and cytotoxic effects yet. In our study, while levetiracetam did not show cytotoxicity at a concentration of 0.125 mM in C6 cell lines, an increase in cytotoxicity was observed in proportion to the increase in concentration at other applied concentrations. Cytotoxicity was not observed in NA/AN1 cell lines.

In all concentrations of pregabalin applied to C6 cell lines, an increase in cytotoxicity was observed in proportion to the increase in concentration. Cytotoxicity was not observed in NA/AN1 cell lines. On the other hand, an increase in cytotoxicity was observed in proportion to the increase in concentration at all concentrations applied in both C6 cell lines and NA/AN1 cell lines.

Study Limitations

Since the drugs we used in our study were not pure active ingredients, they were drugs bought from the pharmacy, other additives in the drugs may have affected the concentrations. In addition, since our study was conducted with a limited number of antiepileptics, the study could not compare all antiepileptics with each other.

In our study, it has been shown that the Xcelligence method is an easy and fast method to determine the mitotic proliferation of antiepileptics and that antiepileptics have different antimitotic effects on tumor cells. More clinical and laboratory studies are needed to understand the antitumoral effect of antiepileptics and to evaluate their effects on patients.

Ethics Committee Approval: The study was approved by the Ethics Committee of Cumhuriyet University (Date: December 27, 2011, Decision no: 2011/43).

Informed Consent: Written informed consent was obtained from patients.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept – S.C.G., E.B.; Design - S.C.G., A.A.; Supervision - S.C.G., E.B.; Resources - S.C.G., A.A.; Materials - S.C.G., A.A.; Data Collection and/or Processing - S.C.G., M.C.G.; Analysis and/or Interpretation - S.C.G., A.A.; Literature Search - S.C.G., A.A.; Writing Manuscript - S.C.G., M.C.G.; Critical Review - S.C.G., M.C.G.; Other - S.C.G., M.C.G.

Declaration of Interests: The authors have no conflicts of interest to declare.

Funding: This study was carried out as Cumhuriyet University Scientific Research Project numbered T-520.

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