



Enhanced Anticancer Effect of Temozolomide through Synergistic Combination with Diltiazem in Neuroblastoma (SH-SY5Y) Cell Line

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ABSTRACT

Objective: This study aimed to assess the enhanced anticancer effect of Temozolomide (TMZ) through a synergistic combination with Diltiazem (DTZ) in the neuroblastoma cell line (SH-SY5Y).

Material and Methods: The SH-SY5Y neuroblastoma cell line was cultured in a DMEM medium. TMZ and DTZ stock solutions were prepared and diluted to obtain different concentrations. The XTT assay was performed to evaluate cell viability. Cells were treated with TMZ alone, DTZ alone and DTZ+ TMZ combination for 24 and 48 hours. XTT-formazan product was measured, and statistical analysis was performed.

Results: The viability of neuroblastoma cells treated with DTZ, TMZ alone and TMZ+DTZ combination was assessed. At 24 hours, TMZ showed no cytotoxic effect, while DTZ showed a low cytotoxic impact. At 48 hours, TMZ and TMZ +DTZ combination exhibited significant cytotoxic effects. The cytotoxic effects were concentration-dependent and time dependent. DTZ alone showed significant cytotoxic effects on neuroblastoma cells at specific concentrations.

Conclusion: The study demonstrated that TMZ alone and combined with DTZ had a significant cytotoxic effect on neuroblastoma cells. The combination of TMZ and DTZ may enhance the anticancer effect of TMZ and overcome drug resistance in neuroblastoma treatment. DTZ, a P-glycoprotein inhibitor, could prevent the efflux of TMZ from cancer cells, increasing its concentration and effectiveness.

Keywords: Neuroblastoma, Temozolomide, Diltiazem, cytotoxic effect, drug resistance, P-glycoprotein inhibitor.

Temozolomidin Diltiazem ile Sinerjik Kombinasyonunun Nöroblastom (SH-SY5Y) Hücre Hattında Artmış Antikanser Etkisi

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Öz

Amaç: Bu çalışmada Temozolomidin (TMZ) nöroblastom hücre hattında (SH-SY5Y) Diltiazem (DTZ) ile sinerjistik bir kombinasyon yoluyla artmış antikanser etkisinin değerlendirilmesi amaçlandı.

Gereç ve Yöntemler: SH-SY5Y nöroblastom hücre hattı bir DMEM ortamında kültürlendi. Farklı konsantrasyonlar elde etmek için TMZ ve DTZ stok çözeltileri hazırlandı ve seyreltilti. Hücre canlılığını değerlendirmek için XTT tahlili yapıldı. Hücreler 24 ve 48 saat boyunca tek başına TMZ veya DTZ ile tedavi edildi. Canlılık XTT-formazan ürünü kullanılarak ölçüldü ve istatistiksel analiz yapıldı.

Bulgular: TMZ, DTZ ve TMZ+DTZ kombinasyonu ile tedavi edilen nöroblastom hücrelerinin canlılığı değerlendirildi. 24 Saatte TMZ sitotoksik etki göstermezken, DTZ düşük sitotoksik etki gösterdi. 48 Saatte, TMZ ve TMZ + DTZ kombinasyonu önemli sitotoksik etkiler sergiledi. Sitotoksik etkiler konsantrasyona ve zamana bağlıydı. Tek başına DTZ, spesifik konsantrasyonlarda nöroblastom hücreleri üzerinde önemli sitotoksik etkiler gösterdi.

Sonuç: Çalışma, TMZ'nin tek başına ve DTZ ile birlikte nöroblastom hücreleri üzerinde önemli bir sitotoksik etkiye sahip olduğunu göstermiştir. TMZ ve DTZ'nin kombinasyonu, TMZ'nin antikanser etkisini artırabilir ve nöroblastom tedavisinde ilaç direncinin üstesinden gelebilir. Bir P-glikoprotein inhibitörü olan DTZ, TMZ'nin kanser hücrelerinden çıkışını önleyerek konsantrasyonunu ve etkinliğini artırabilir.

Anahtar Kelimeler: Nöroblastom, temozolomid, diltiazem, sitotoksik etki, ilaç direnci, P-glikoprotein inhibitörü.

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Introduction

Neuroblastoma is a type of cancer that develops in childhood from precursor cells of the central nervous system. It commonly affects the tissues of the sympathetic nervous system, primarily found in the adrenal medulla or paraspinal ganglia. Neuroblastoma is most prevalent in children under 17 months old, constituting approximately 15% of all pediatric cancers. However, what sets neuroblastoma apart is its remarkable biological and clinical diversity. In infants, the tumor often displays spontaneous regression, even in cases of metastasis. On the other hand, in children older than one year, neuroblastoma tends to be aggressive and resistant to standard treatments, despite the use of intensive multimodal therapies. While a combination of chemotherapeutic agents is typically employed, essential drugs for treating neuroblastoma include cisplatin, cyclophosphamide, vincristine, doxorubicin, and etoposide ^{1,2}.

P-glycoprotein (P-gp) is in the plasma membranes of barrier and elimination organs. It is crucial in drug absorption and excretion, functioning as an efflux (excretion) protein ³. When drugs interact with P-gp, a carrier protein, their bioavailability can be reduced, and their passage across the blood-brain barrier can be hindered. Immunohistochemical studies have demonstrated that P-gp is prominently expressed in cerebral capillary endothelial cells and tightly linked choroid plexus epithelial cells, responsible for cerebrospinal fluid production ⁴. P-gp, encoded by the ABCB1 gene, also plays a role in developing multidrug resistance in cancer treatment ⁵. It acts as a mechanism for neoplastic cells to resist various chemotherapeutic agents. This understanding has opened avenues for combining P-gp inhibitor drugs with known chemotherapeutics, offering the potential to develop innovative cancer therapies ⁶.

In the cases of neuroblastoma, drug resistance poses a significant challenge in treatment. Once resistance develops, patient survival rates decrease substantially, severely limiting treatment options ⁷. One of the primary mechanisms underlying resistance is the P-gp pump's active functioning, which actively removes drugs from within the cells, effectively reducing their therapeutic efficacy ⁸.

The antineoplastic agent Temozolomide (TMZ) is commonly used to treat glioblastoma and neuroblastoma. It exerts its therapeutic effects by inhibiting the proliferation of glioma cells and promoting apoptosis through DNA alkylation ². On the other hand, Diltiazem (DTZ) is a calcium channel blocker primarily used in cardiology practice to manage arrhythmia and hypertension ⁹. A notable property of DTZ is its ability to inhibit the P-gp pump, which is

involved in drug resistance ¹⁰. Considering this information, the combination of TMZ and DTZ holds promise for enhancing the cytotoxic activity of TMZ in neuroblastoma cells. By inhibiting the P-gp pump, DTZ may prevent the efflux of TMZ from cancer cells, thereby increasing its concentration and effectiveness against the tumor. This approach may help overcome drug resistance and improve the therapeutic outcome in neuroblastoma treatment. Further research and investigation are necessary to validate the potential benefits of combining TMZ with DTZ in the context of neuroblastoma therapy.

This study aimed to assess the impact of TMZ alone and its combination with DTZ on a neuroblastoma cell line SH-SY5Y. We employed the XTT method to evaluate cell viability and determine the cytotoxic effects of TMZ and its combination with DTZ.

Materials and methods

To assess the cytotoxic effects of Temozolomide (TMZ) alone and in combination with Diltiazem (DTZ) on the SH-SY5Y neuroblastoma cell line (ATCC CRL-2266), the following experimental procedures were carried out.

Cell Culture:

The SH-SY5Y cells were cultured in 25cm² polystyrene flasks using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 100 IU/ml penicillin, and 10 mg/ml streptomycin. The cells were incubated in a humidified atmosphere at 37°C with 5% CO₂. Passage of cells was performed when they reached approximately 80% confluence.

Drug Preparation:

TMZ and DTZ stock solutions were prepared in Dimethyl Sulfoxide (DMSO). The stock solutions were sterilized through a 0.2 µm syringe tip filter, ensuring a maximum DMSO concentration of 0.1%. Serial dilutions were then made from these stock solutions to obtain different concentrations of inhibitors for cell treatment. The concentration range included low to high concentrations to determine the IC₅₀ values.

XTT Assay:

The XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxamide) assay was employed to assess the viability of the SH-SY5Y cell line. Cells were seeded in 96-well plates at 1 × 10⁴ cells per well in 100 µl colorless DMEM medium. The cells were treated with TMZ alone (ranging from 100 µM to 10 mM) or in combination with DTZ at a concentration that was determined to have no inhibitory effect on cell viability in this study. The cells were incubated with the drugs for 24 hours and 48 hours.

XTT Labeling and Measurement:

At the end of the 24-hour and 48-hour incubation periods, the XTT labeling mixture was added to each well and incubated at 37°C for 4 hours. The resulting XTT-formazan product was measured at 450 nm using a microplate (ELISA) reader. The absorbance values were compared to the control samples, and cell viability was determined as a percentage relative to the control (considered 100% viability). The experiments were independently repeated three times to ensure the reproducibility and reliability of the results. The absorbance measurements provided information about cell viability and the drug combination's potential synergistic or additive effects.

Statistical Analysis

For the statistical analysis of the obtained data, the SPSS 22 package program was utilized. A significance level of $p < 0.05$ was considered to determine statistical significance. A one-way analysis of variance (ANOVA) was applied for data with a normal distribution. Non-parametric tests were used for data that did not follow a normal distribution. The Kruskal-Wallis test was employed for comparing multiple independent groups, similar to ANOVA but for non-parametric data. It assesses whether there are significant differences among the groups. In addition, the Mann-Whitney U test, another non-parametric test, was used to compare two independent groups when analyzing data without normal distribution. This test evaluates whether there are significant differences between the two groups.

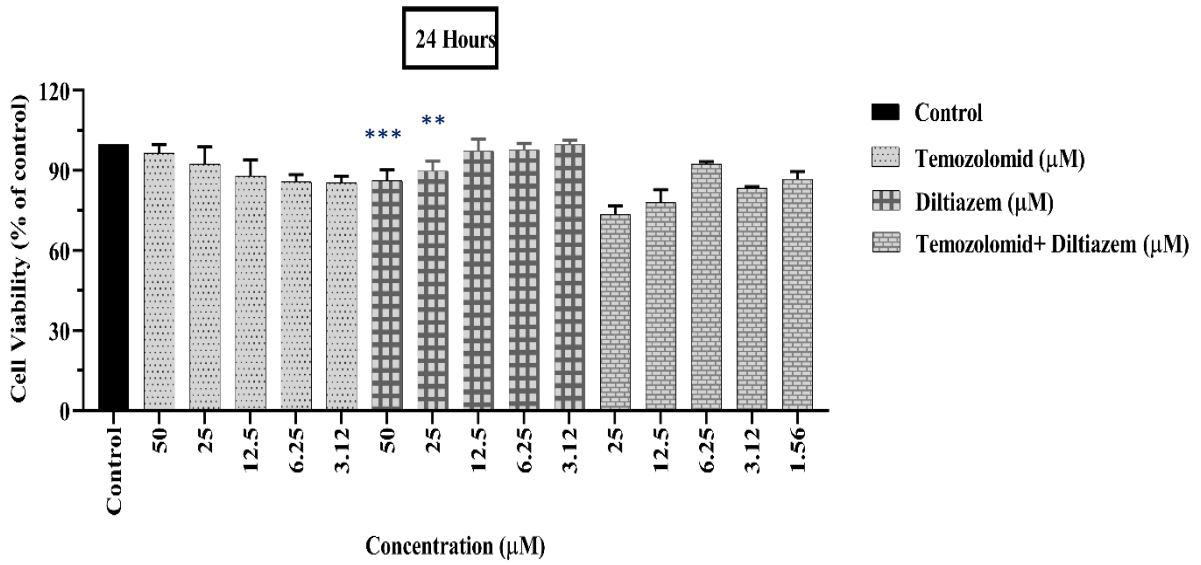
Results

The viability percentages of Neuroblastoma cells exposed to different concentrations of TMZ, Diltiazem, and TMZ + Diltiazem active substances ranged between μM (1.56) and μM (50) over 24 hours. TMZ showed no cytotoxic effect at the 24 hours. The active Diltiazem has exhibited a significant cytotoxic effect on Neuroblastoma cells at a concentration of $\mu\text{M} = 50$ ($p < 0.001$). The active Diltiazem has also shown a significant cytotoxic effect on Neuroblastoma cells at a concentration of $\mu\text{M} = 25$ ($p < 0.01$). (Figure 1)

The viability percentages of Neuroblastoma cells exposed to different concentrations ranging from μM (1.56) to μM (50) of TMZ, Diltiazem, and TMZ + Diltiazem active substances are shown over a 48-hour duration. Diltiazem active substance exhibited a

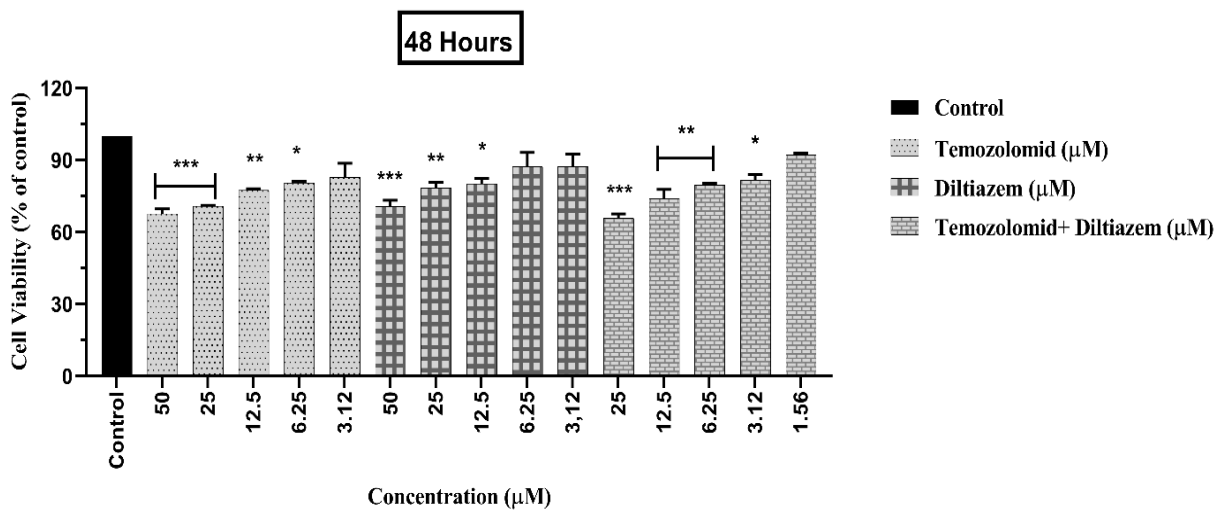
significant cytotoxic effect on Neuroblastoma cells at concentration levels ranging from μM (6.25) to μM (50), with a 1% error margin at the concentration level of μM (3.12) ($p < 0.001$, $p < 0.01$, $p < 0.05$). TMZ active substance induced a significant cytotoxic effect on Neuroblastoma cells at concentration levels of μM (3.12) and μM (6.25) with a 5% error margin, at concentration levels of μM (12.5) and μM (25) with a 1% error margin, and at the concentration level of μM (50) with a 1% error margin ($p < 0.001$, $p < 0.01$, $p < 0.05$). When TMZ + Diltiazem active substances were used together, a significant cytotoxic effect was observed on Neuroblastoma cells at concentration levels of μM (6.25) and μM (12.5) with a 1% error margin and at the concentration level of μM (6.25) with a 1% error margin ($p < 0.001$, $p < 0.01$, $p < 0.05$). (Figure 2)

Figure 1: The effects of TMZ, DTZ, and TMZ + DTZ active substances on the viability of Neuroblastoma cells over 24 hours.



Values are presented as mean + SEM (**p<0.01, ***p<0.001 compared to the control group).

Figure 2: The effects of TMZ, Diltiazem, and TMZ + Diltiazem active substances on the viability of Neuroblastoma cells over 48 hours.



Values are presented as mean + SEM (*p<0.05, **p<0.01, ***p<0.001 compared to the control group).

Discussion

Our study determined that applying TMZ alone and with DTZ had a significant cytotoxic effect on neuroblastoma SH-SY5Y cell lines. After the application at two different time points, 24 hours and 48 hours, we observed that TMZ showed no cytotoxic effect at the 24 hours, and DTZ showed only a low cytotoxic effect. At the end of the 48 hours, we assessed that TMZ and TMZ+DTZ combination has a significant cytotoxic effect. The cytotoxic effect alone TMZ and TMZ+DTZ combination are time-dependent and concentration-dependent. Xu et al., in their study of glioblastoma cells, reported that TMZ and the variety of TMZ+ paclitaxel drugs did not show cytotoxic effects in the 24 hours and, however, showed significant cytotoxic effects in the 48-hour and 72-hour period. Our study is consistent with the literature ¹¹.

Cornwell et al. reported that calcium channel blocker drugs such as verapamil (VER), DTZ, and nitrendipine reduce drug resistance in cancer treatment by their P-gp binding properties ¹². Summers MA et al. stated that a calcium channel blocker VER used as a P-gp inhibitor reduces epileptic seizures by increasing the intracellular concentration of antiepileptic drugs ¹³. El-Mahdy et al. reported that DTZ increased the cytotoxic properties of gemcitabine and 5-fluorouracil in pancreatic cancer cells ¹⁴. Our study, consistent with the literature, found that calcium channel blocker effective DTZ produced a significant cytotoxic effect on the human neuroblastoma cell line both alone and in combination with the known anticancer agent TMZ.

Carrier proteins play a crucial role in the structure of cell membranes and are involved in various biological processes and diseases essential for life. Their impact on the development of drug resistance in cancer and infectious diseases has been established, as these proteins can impede drug entry into cancer cells, earning them the name "excretion proteins." One such protein is P-gp, a plasma membrane protein belonging to the family of ATP-dependent carrier proteins. P-gp functions as an active drug efflux pump, expelling numerous pharmaceutical compounds. Its name, P-gp, derives from its role in regulating the passage of cytotoxic drugs and creating a barrier to their permeability ¹⁵.

Understanding the drug delivery mechanism mediated by P-gp is crucial when developing new drugs. Prior knowledge of P-gp expression levels in tumors has been identified as valuable for personalized cancer treatment ¹⁶. High expression of P-gp often leads to multidrug resistance. Alternatively, researchers have explored inhibitors that specifically target and inhibit the functional expression of P-gp. By doing so, cancer

cells become more sensitive to standard antitumor drugs ¹⁷.

DTZ is one of the potent P-gp inhibitors. So It is not surprising that DTZ prevented cells from reducing the cytotoxic effects of TMZ by simply inhibiting the P-gp pumps. The data we gathered current study supports the inial hypothesis of our research. This approach may be implemented for other medical cancer treatment modalities ¹⁵.

Limitations

This study was conducted in vitro using a specific neuroblastoma cell line. Further research and investigation, including in vivo studies and clinical trials, are necessary to validate the potential benefits of combining TMZ with DTZ in neuroblastoma therapy.

Conclusion

As a result of our study, it was seen that TMZ did not affect the viability level of Neuroblastoma cells during the 24 hours. Still, in the 48-hour examination, TMZ showed a significant cytotoxic effect on the viability level of neuroblastoma cells when used alone and in combination with DTZ at different concentration levels. We believe that DTZ can be used as an effective pharmacological agent in new-generation neoadjuvant cancer therapy applications that can increase the cytotoxicity of anticancer drugs and reduce drug resistance.

Declarations

Author contribution statement

Mustafa Karademir: Conceived and designed the experiments, performed the experiments, wrote the paper, analyzed and interpreted the data, and contributed reagents, materials, and analysis tools.

Saime Surardamar: Conceived and designed the experiments and performed the experiments.

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Data availability statement

Data will be made available on request.

Declaration of Interests Statement

The authors declare no conflict of interest.

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