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Exploring the Protective Role of Everolimus in Preventing Pentylenetetrazole-Triggered Injury in Hippocampal Neurons Using the HT-22 Cell Line

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Research Article

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ABSTRACT

This study aimed to explore the neuroprotective properties of everolimus and its association with oxidative stress. The HT-22 hippocampal neuronal cell line was utilized as the in vitro model. To assess the protective effects of everolimus, four experimental groups were designed. The control group received no treatment. The pentylenetetrazol (PTZ) group was exposed to 60 mM PTZ for 1 hour. In the everolimus group, cells were treated with increasing concentrations of everolimus (0.1, 0.5, 1, 5, and 10 nM) for 2 hours. In the combination group (everolimus + PTZ), cells were pre-treated with everolimus at the same concentrations for 1 hour, followed by 60 mM PTZ exposure for an additional hour. Cell viability was assessed via the XTT assay, while oxidative stress was evaluated by measuring total antioxidant status (TAS) and total oxidant status (TOS) using commercial assay kits. Treatment with everolimus significantly enhanced cell viability at concentrations of 5 and 10 nM ($p < 0.01$). Additionally, TAS levels were markedly elevated, and TOS levels were significantly reduced in the PTZ + everolimus (10 nM) group compared to the PTZ-only group ($p < 0.01$). These findings suggest that everolimus confers neuroprotection by enhancing the antioxidant defense system and mitigating PTZ-induced cytotoxicity

Keywords: *Everolimus, HT-22 Cell Line, Neuroprotective Effect, Pentylenetetrazol.*

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1. Introduction

Epilepsy is a prevalent neurological disorder characterized by abnormal, excessive synchronization of neuronal activity, which disrupts normal brain function. Recurrent seizures can result in cognitive impairments, behavioral disturbances, and may ultimately lead to irreversible neural damage.

Pentylenetetrazol (PTZ) is a bicyclic tetrazole derivative known for its ability to induce epileptiform activity by interfering with γ-aminobutyric acid (GABA)-mediated neurotransmission. It exerts its proconvulsant effect through widespread distribution in the brain and suppression of inhibitory signaling pathways [1,2]. The seizure model induced by PTZ has been widely utilized for screening potential antiepileptic drugs. Biochemical and electrophysiological evidence indicates that the pharmacological actions of PTZ are primarily mediated through inhibition of the GABA-A receptor complex, specifically by blocking the benzodiazepine (BZ) binding sites [3-5]. Experimental findings suggest that PTZ acts as an antagonist at benzodiazepine binding sites, thereby facilitating the onset of seizure activity. In contrast, agonists targeting these sites exhibit significant anticonvulsant effects against PTZ-induced tonic-clonic seizures in rats [6,7].

The inhibitory function of GABA-A receptors is primarily associated with the formation of a chloride-permeable ion channel within the neuronal membrane. Korda et al. [8] investigated the relationship between PTZ-induced seizures and chloride uptake in the cerebral cortex. Their findings demonstrated that repeated PTZ injections may significantly reduce GABA-stimulated chloride influx. Furthermore, they concluded that PTZ treatment impairs the functionality of the chloride channel associated with GABA-A receptors. These observations support the hypothesis that PTZ induces seizures by suppressing GABA-A and BZ receptor activity, as well as the GABA-evoked chloride flow across cortical neuronal membranes [9].

In humans, the hippocampus plays a critical role in both the initiation and propagation of seizures. Following PTZ administration, activation has been observed particularly in the CA3 region of the hippocampus [10]. Studies have demonstrated that PTZ administration leads to a reduction in the number of neurons within the CA1 region of the hippocampus [11]. In the seizure model induced by PTZ, it has been demonstrated that the epilepsy-related gene c-Fos is markedly overexpressed in hippocampal tissues [12]. In addition to excitotoxic mechanisms, accumulating evidence indicates that oxidative stress plays a pivotal role in hippocampal vulnerability during epileptic seizures. Excessive production of reactive oxygen species (ROS) during PTZ-

induced seizures leads to lipid peroxidation, protein oxidation, and DNA damage in hippocampal neurons [13]. The hippocampus is particularly vulnerable to oxidative damage due to its high metabolic activity, abundant excitatory synaptic connections, and relatively limited antioxidant defense capacity. Accumulation of oxidative damage in hippocampal neurons contributes to membrane instability, impaired ion homeostasis, and activation of apoptotic pathways. These alterations not only exacerbate acute neuronal injury but also promote long-term structural and functional remodeling associated with epileptogenesis. Therefore, oxidative stress in the hippocampus is considered a central pathological mechanism underlying seizure-induced neuronal degeneration and disease progression in epilepsy.

The PI3K/Akt/mTOR pathway is essential for regulating multiple processes in the central nervous system. Upon activation, PI3K catalyzes the production of the secondary messenger PIP3 at the plasma membrane, which subsequently recruits Akt. Akt becomes activated through phosphorylation. Once activated, Akt can phosphorylate multiple substrates in both the cytoplasm and nucleus, with mTOR being one of the most significant targets [14]. It is important to distinguish between physiological and pathological activation of the PI3K/Akt/mTOR pathway. As part of the PI3K/Akt pathway, mTOR acts as a regulatory kinase influencing cell proliferation, survival, metabolic activity, autophagic processes, and cellular dynamics including migration [14]. While physiological activation of the PI3K/Akt/mTOR pathway supports cellular homeostasis, pathological overactivation of this signaling cascade under conditions of oxidative stress and recurrent seizures has been associated with neuronal dysfunction and epileptogenesis [15]. Experimental evidence suggests that oxidative stress can dysregulate mTOR signaling, thereby linking redox imbalance to seizure progression and neuronal injury [16]. Therefore, while basal pathway activation supports cell survival, pathological overactivation may exacerbate neuronal damage. In this context, pharmacological inhibition of mTOR may restore cellular homeostasis and attenuate seizure-related injury.

Oxidative stress and the PI3K/Akt/mTOR pathways interactively regulate various cellular stress responses, including apoptosis [17], autophagy [18], and endoplasmic reticulum (ER) stress [19]. The PI3K/Akt/mTOR pathway has the capacity to regulate oxidative stress. Phosphatase and tensin homolog (PTEN) proteins act as inhibitors of PI3K signaling [18]. Consequently, in prostate cancer cells lacking PTEN, Akt becomes hyperactivated to strongly induce reactive oxygen species (ROS) production attributed to Oxphos induction [20]. Growth factors stimulate Akt activation, leading to increased ROS generation, which contributes to uncontrolled proliferation of cancer cells [21].

Everolimus is an mTOR inhibitor capable of reducing secondary damage following focal ischemia, which is produced by activated microglia [22]. Studies have demonstrated that mTOR activation contributes to neuronal excitability and seizure generation in various hereditary and acquired animal models of epilepsy [23]. Given the interplay between oxidative stress and pathological mTOR activation in seizure-related neuronal injury, pharmacological inhibition of mTOR has emerged as a potential strategy to attenuate oxidative damage and neuronal degeneration. Everolimus has been shown to inhibit mTOR activity, resulting in a reduction in both the frequency and severity of seizures, suggesting a potentially critical link between mTOR signaling and seizure progression [24]. As a result, everolimus is thought to regulate neuronal activity and may provide beneficial effects in the treatment of seizures.

We hypothesized that oxidative stress plays a central role in PTZ-induced hippocampal neuronal injury and that pharmacological inhibition of mTOR by everolimus may attenuate this damage by restoring oxidant/antioxidant balance.

2. Materials and Methods

2.1. Cultivation of Cells

The HT-22 hippocampal neuronal cell line (Merck Millipore, USA) was cultured in a growth medium composed of Dulbecco's Modified Eagle Medium (DMEM;Sigma-Aldrich, USA) supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Prior to treatment, everolimus and PTZ (Sigma-Aldrich, USA) were dissolved in DMEM to prepare stock solutions. Cell morphology and confluency were monitored using an inverted phase-contrast microscope (ZEISS AXIO Vert. A1, Germany).

2.2. Viability Assessment of Cells

To assess cell viability, the XTT assay (Roche Diagnostics, USA) was employed. HT-22 cells were seeded into 96-well plates at a density of 10^4 cells per well and incubated for 24 hours [25,26]. On the following day, four distinct cell groups were established to investigate the neuroprotective effect of everolimus on hippocampal damage. No treatment was administered to the control group. The PTZ group was exposed to 60 mM PTZ for 2 hours [27]. In the everolimus group, cells were treated with varying concentrations of everolimus (ranging from 0.1 to 10 nM). Everolimus concentrations (0.1–10 nM) were selected according to preliminary dose–response experiments to identify non-toxic concentrations with potential neuroprotective efficacy. For the everolimus + PTZ group, cells were pretreated with everolimus at different concentrations (0.1–10 nM) for 1 hour, followed by exposure to 60 mM PTZ for 2 hours. After incubation, the wells were washed three times with phosphate-buffered saline (PBS). Then, 50 µL of XTT reagent and 100 µL of clear DMEM were added to each well. Cells were maintained at 37°C for the following four hours. Absorbance was measured at 450 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, UK). Cell viability was assessed

by performing the experiment in triplicate and calculating the ratio of viable cells relative to the control group.

2.3. Cell Lysate Preparation

Cells from all experimental groups (control, 60 mM PTZ, 10 nM everolimus, and 10 nM everolimus + 60 mM PTZ) were harvested in sterile tubes and centrifuged at 2000 RPM for about 10 minutes. After discarding the supernatants, the remaining cell pellets were resuspended in phosphate-buffered saline (PBS, pH 7.4) to achieve a final cell density of approximately 1×10^6 cells/mL. To lyse the cells and extract intracellular contents, multiple freeze–thaw cycles were applied. The lysates were then centrifuged again at 4000 RPM for 10 minutes at 4°C, and the resulting supernatants were collected for further biochemical evaluation. Total protein concentration was measured using a Bradford assay kit (Merck Millipore, USA).

2.4. Quantification of TAS and TOS

To assess the oxidative and antioxidant balance in the samples, commercially available Total Antioxidant Status (TAS) and Total Oxidant Status (TOS) kits (Rel Assay Diagnostics, Gaziantep, Turkey) were employed. Trolox was used as the reference compound in TAS measurements, while hydrogen peroxide was utilized as the standard for TOS quantification [28,29]. Both assays were performed using a colorimetric method according to the manufacturer's instructions. Absorbance values were measured at 660 nm for TAS and 530 nm for TOS using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific, UK). The results were reported as micromoles of Trolox equivalent and hydrogen peroxide equivalent per milligram of protein (µmol Trolox Eq/mg protein and µmol H_2O_2 Eq/mg protein, respectively).

2.5. Analysis of Data

Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using SPSS software version 23.0 for Windows. Differences between groups were evaluated by one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. A p-value below 0.05 was regarded as statistically significant.

3. Results

4.1. Role of Everolimus in Protecting HT-22 Cells Against PTZ-Mediated Hippocampal Injury

An XTT cell proliferation assay was performed to assess the neuroprotective effects of everolimus against PTZ-induced damage in HT-22 cells. Compared to the control group, treatment with 60 mM PTZ significantly reduced cell viability (*$p<0.01$, Figure 1). Furthermore, pretreatment with everolimus at concentrations of 5 and 10 nM significantly attenuated PTZ-induced cytotoxicity compared to the PTZ group (#$p<0.01$, Figure 1). Everolimus alone did not cause any toxicity in HT-22 cells compared to the control ($p>0.05$, Figure 1). Microscopic (ZEISS AXIO Vert. A1, Germany) images demonstrated that everolimus pretreatment preserved cellular morphology and reduced PTZ-induced structural damage in HT-22 cells (Figure 2).

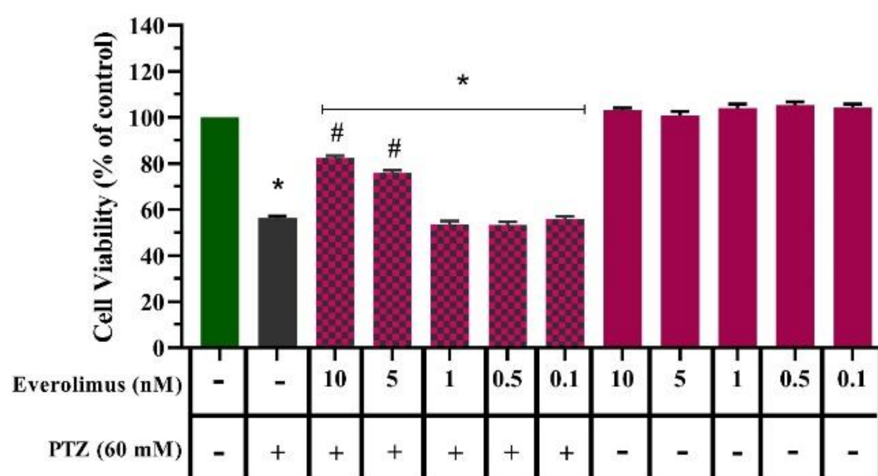

Figure 1. Effect of Everolimus pretreatment on cell viability in HT-22 cells exposed to PTZ. . Results are presented as mean ± SEM (*p<0.01, #p<0.01).

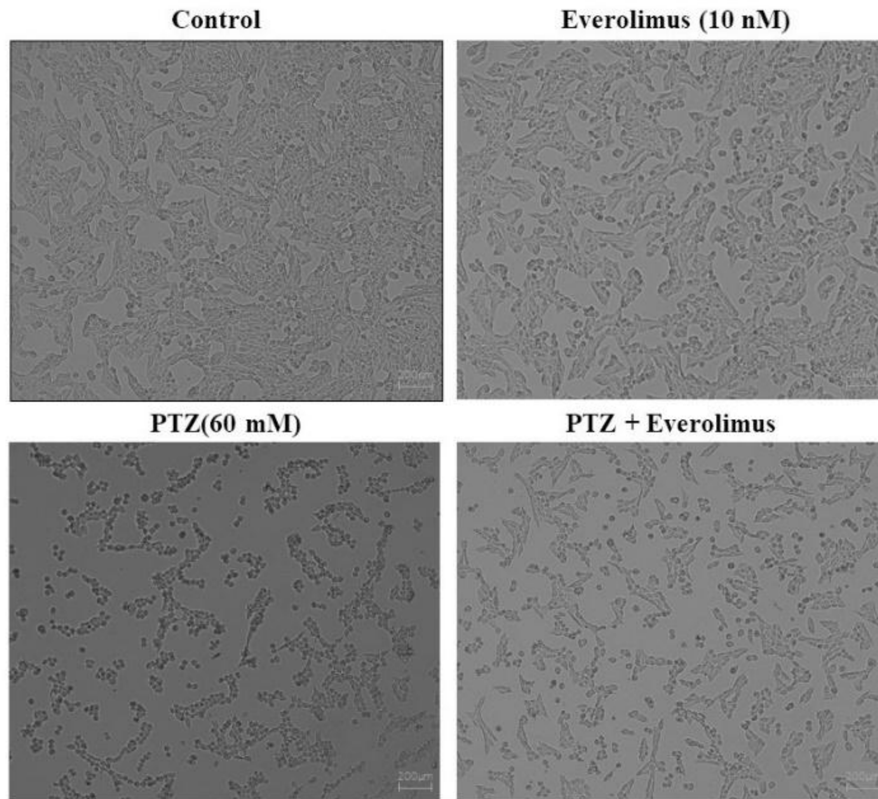

Figure 2. Representative inverted microscopic images demonstrating the protective effect of everolimus pretreatment against PTZ-induced morphological damage in HT-22 cells. (ZEISS AXIO Vert. A1, Germany).

4.2. Influence of Everolimus on Cellular TAS and TOS Concentrations in HT-22 Cells Subjected to PTZ-Induced Damage

Cellular levels of TAS and TOS were measured using commercial kits. TAS levels were significantly lower in the PTZ group compared to the control group (*p<0.01, Figure 3A). Upon everolimus treatment, TAS levels in the everolimus + PTZ group were significantly elevated compared to the PTZ group (#p<0.01; Figure 3A). Additionally, TOS levels were markedly higher in the PTZ group than in controls (*p<0.01, Figure 3B). Treatment with everolimus led to a significant decrease in TOS levels in the everolimus + PTZ group relative to the PTZ group (#p<0.01; Figure 3B).

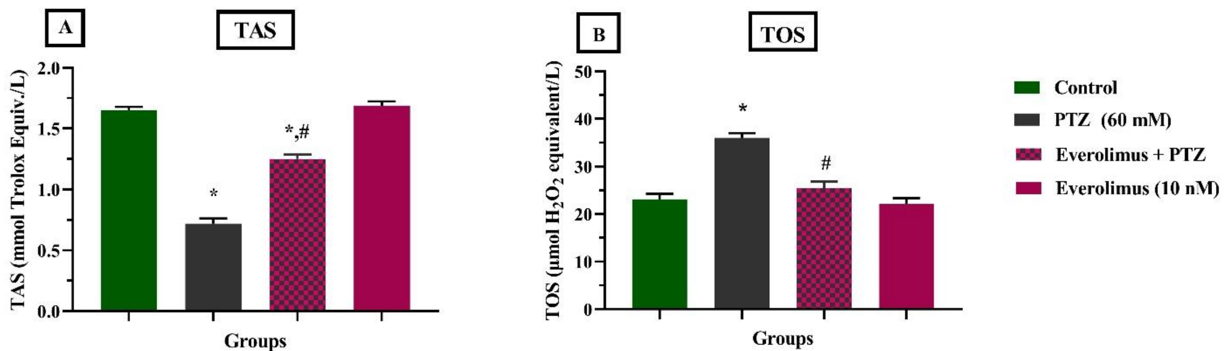

Figure 3. Effect of everolimus on TAS and TOS levels in HT-22 cells following PTZ-induced hippocampal damage. Results are presented as mean ± SEM (*p<0.01, #p<0.01).

4. Discussion

Everolimus, an mTOR inhibitor that targets the PI3K/Akt/mTOR signaling pathway, exhibited protective effects against PTZ-induced damage by alleviating hippocampal injury and degeneration. Additionally, it modulated oxidative stress by lowering total oxidant levels and enhancing antioxidant capacity, thereby demonstrating neuroprotective properties. These findings suggest that everolimus plays a neuroprotective role, potentially through mechanisms involving inhibition of the PI3K/Akt/mTOR signaling pathway.

Oxidative stress plays a critical role in both the onset of epileptic seizures and the progression of epileptogenesis due to an imbalance between oxidant production and antioxidant defense systems [30]. TAS and

TOS were selected as global indicators of redox status, as they provide an integrated assessment of oxidative and antioxidant balance rather than individual biomarkers. A clinical study demonstrated that lipid peroxidation levels are significantly elevated in individuals with epilepsy compared to healthy controls [31]. Another investigation reported that antioxidant markers such as glutathione reductase and vitamins C, E, and A are markedly reduced in epileptic patients relative to non-epileptic individuals [32]. Furthermore, experimental research has shown that PTZ-induced seizures promote the generation of free radicals, resulting in oxidative damage to proteins, lipids, and cellular DNA [33].

In a study investigating the role of the PI3K/Akt/mTOR pathway inhibitor, chrysophanol, it was shown to improve neurological deficits in a rat model of intracerebral hemorrhage by reducing oxidative stress, demonstrating neuroprotective effects similar to those observed in our research [34]. Similarly, another study reported that oxymatrine exerted neuroprotective effects through the PI3K/Akt/mTOR pathway following hypoxic-ischemic brain injury, aligning with our findings [35]. The study concluded that activation of this pathway plays a role in neuroprotection. Additionally, Huang et al. demonstrated that everolimus protects against kainic acid-induced brain damage resulting from seizures, supporting results consistent with our work [24]. Although the PI3K/Akt/mTOR pathway was not directly assessed at the molecular level in the present study, the use of everolimus—a well-established mTOR inhibitor—and the observed neuroprotective and antioxidative effects strongly suggest the involvement of this signaling pathway. Nevertheless, further studies incorporating direct molecular analyses are warranted to confirm this mechanistic link.

In conclusion, the findings of this study suggest that Everolimus exerts neuroprotective effects by modulating oxidative stress and may exert these effects, at least in part, through mechanisms related to mTOR inhibition. Considering the significant contribution of oxidative stress to epileptogenesis and the intricate interaction between redox balance and PI3K/Akt/mTOR signaling, targeting this pathway may present a promising therapeutic strategy. Similar neuroprotective outcomes have been reported with other inhibitors acting on the same pathway in different experimental models. These parallels reinforce the idea that modulation of PI3K/Akt/mTOR signaling could play a critical role in mitigating neuronal damage associated with seizures. Thus, our results provide novel insights into the potential mechanisms underlying Everolimus's protective effects and support further investigation into this pathway as a target in epilepsy treatment.

This study has certain limitations, including the lack of direct molecular assessment of PI3K/Akt/mTOR signaling components and the use of a single experimental seizure model. Future studies employing multiple epilepsy models and pathway-specific analyses are needed to validate and extend these findings.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this study.

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Declaration of Generative AI

The authors used [ChatGPT] for language editing during the preparation of this manuscript. Each AI-generated output was carefully reviewed and verified by the authors to ensure scientific integrity and accuracy. Consequently, the authors take full responsibility for the final content and conclusions of the study.

The authors did not use any generative AI or AI-assisted technologies in the preparation of this manuscript, including the data analysis and writing stages.

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