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# Therapeutic enhancing potential of piracetam with diethylstilbestrol in prevention of grand-mal seizures in rats: inhibition of PI3K/Akt/mTOR signaling pathway and IL-1β, IL-6, TNF-α cytokines levels

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**Abstract. – OBJECTIVE: Epilepsy, a neurodegenerative disorder, continues to throw challenges in the therapeutic management. The current study sought to ascertain if the therapeutic interactions between piracetam and diethylstilbestrol may prevent grand-mal seizures in rats.**

**MATERIALS AND METHODS: Piracetam (PIR; 10 and 20 mg/kg) and diethylstilbestrol (DES; 10 and 20 mg/kg) alone as a low-dose combination were administered to rats for 14 days. The electroshock (MES; 180 mA, 220 V for 0.20 s) was delivered via auricular electrodes on the last day of treatment and rats were monitored for convulsive behavior. To elucidate the mechanism, hippocampal mechanistic target of rapamycin (mTOR) and interleukin (IL)-1β, IL-6 and tumor necrotic factor-alpha (TNF-α) levels were quantified. Hippocampal histopathology was conducted to study neuroprotective effect of drug/s. *In vitro* studies and *in silico* studies were conducted in parallel.**

**RESULTS: To our surprise, the low dose of the combination regimen of PIR (10 mg/kg) and DES (10 mg/kg) unfolded synergistic anti-seizure potential, with brimming neuroprotective properties. The mechanism could be related to a significant reduction in the levels of hippocampal mTOR and proinflammatory cytokines. The docking scores revealed higher affinities for phosphatidylinositol 3-kinase (PI3K) in co-bound complex, and when docking DES first, while better affinities for protein kinase B (Akt) were revealed when docking PIR first (both drugs bind cooperatively as well). This indicated that the entire PI3K/Akt/mTOR signaling pathway is intercepted by the said combination. In addition, the % of cell viability of HEK-293 cells [pre-exposed to pentylenetetrazol (PTZ)] was increased by 327.29% compared to PTZ-treated cells (toxic control; 85.16%).**

**CONCLUSIONS: We are the first to report the promising efficacy of the combination (PIR 10 mg/kg + DES 10 mg/kg) to restrain seizures and epileptogenic changes induced by electroshock by a novel mechanism involving inhibiting the PI3K/Akt/mTOR signaling.**

*Key Words:*
Piracetam, Diethylstilbestrol, Estrogen, Electroshock, Neurodegeneration, PI3K/Akt/mTOR signaling, Molecular docking, Cooperative binding.

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## Introduction

Epilepsy (recurring seizures) is a chronic neurological condition of the brain[1]. Seizure episodes occur when there is an abnormality in brain nerve cell conduction[2]. The global incidence rate for epilepsy is 61.4 per 100,000 person-years (95% CI 50.7-74.4) with overall lifetime prevalence of 7.60 per 1,000 population (95% CI 6.17-9.38)[3]. Achieving effective control of seizure will minimize the prospect of death and injury and improve the quality of life[4]. There are different types of seizures which are focal seizures, focal aware, focal impaired awareness, focal to bilateral tonic-clonic, generalized tonic clonic and epileptic spasm[5]. 30-35% of epileptic patients fail to achieve seizure control even with a combination of antiepileptic drugs (AEDs)[6,7]. The maximal electroshock (MES) model is a preclinical screening test conducted by passing electrical current through auricular/corneal electrodes. It is a valid test to find drugs effective against grand-mal epilepsy[8,9]. Chemically grand-mal epilepsy in rats is replicated by administering pentylenetetrazol (PTZ)[10].

Piracetam (PIR) is a levetiracetam analogue with wide indications and few adverse events[11]. PIR (2-oxo-1-pyrrolidine-acetamide) is a nootropic drug that shows dose-related efficacy as antiepileptic agent[12]. PIR suppressed spike-and-wave discharge in Genetic Absence Epilepsy Rat from Strasbourg (GAERS) significantly at higher doses (1,000 mg/kg)[13]. Diethylstilbestrol (DES) is a synthetic estrogen that has spasmolytic property on contractions triggered by serotonin, angiotensin II and noradrenaline[14]. The study conducted in $Arx^{(GCG)10+7}$ mouse found that the administration of estrogen in the course of early postnatal development interrupted spasms in infancy and convulsions in adult mutant[15,16]. Neonatal estradiol leads to significant increase in -aminobutyric acid (GABA)ergic neurons in the neocortex of infant rats[15,16].

The mechanistic target of rapamycin (mTOR) is considered as the signaling protein of intracellular pathways[17]. mTOR belongs to the "phosphatidylinositol 3-kinase (PI3K)-related kinase family" and its activity can be initiated by serine-threonine protein kinase[18]. The mTOR pathway carries imperative roles in the brain development, neuronal plasticity and excitability, immunity, seizures and inflammation[19-22]. There are two mTOR multiprotein complexes, which are mammalian target of rapamycin complex 1 (mTORC1) (known as rapamycin sensitive) and mTORC2 (known as rapamycin insensitive)[23]. The presence of mTORC1 in the brain regulates the learning, memory formation, and the excitability of neuron, whereas mTORC2 is involved in the migration of cells and integrity of cytoskeleton[24,25]. The mTOR pathway is implicated in Parkinsonism and Alzheimer disease[26] and has a key role in seizures and epileptogenesis[27-29]. An abnormality of mTOR activity has been found[24,30,31] in all acquired and genetic epilepsies. The epileptogenic property of mTOR pathway can cause mutations in tuberous sclerosis complex 1/2 (*TSC1/2)*, DEP domain-containing protein 5 (*DEPDC5)*, Protein kinase B (*Akt*) and phosphatase and tensin homolog (*PTEN*) genes that result in neuronal hyperactivation[32,33]. So, by the inhibition of mTOR the neuronal alterations and epilepsy could be halted[32]. Molecular docking could also be employed to rationalize the activity of test drug/s towards PI3K/Akt/mTOR signaling pathway and could suitably identify the binding of test drug/s with the protein of interest[34,35].

Many studies in the literature found that inflammation and oxidative stress can aggravate the severity of epilepsy i.e., elevated inflammatory cytokines are critical to the development of seizures[36].The proinflammatory cytokines that can be quickly triggered (and activated by the resident glial cells) are interleukin-1 (IL-1β), interleukin-6 (IL-6) and tumor necrotic factor-alpha (TNF-α)[37-39]. In animal study, researchers[40] found that seizure episodes upregulate IL-6-receptor mRNA in the hippocampus, while interleukin-6 mRNA was rapidly secreted to the cortex, dentate gyrus, hippocampus, meninges and amygdala. The increase of IL-6 expression may be coupled to the depolarization of membrane that leads to the buildup of IL-6 protein/mRNA in neurons[40]. This increase can be limited by inhibiting the channels of L-type $Ca^{2+}$, decreasing the levels of extracellular $Ca^{2+}$, or blocking the protein kinases of $Ca^{2+}$/calmodulin-dependent[40].

This study explored the combination of PIR and DES against maximal electroshock (MES) provoked convulsions in rats. Since existing antiepileptic drugs (AEDs) fail to achieve seizure remission in 35% of patients, despite the use of combinatorial regimens from the marketed AEDs, we hypothesized that additional mechanisms are contributing to seizure generation and epileptogenesis, thus bringing drugs with differ-

ent mechanisms of actions will serve the purpose, and the hypothesis was successful when tested experimentally.

## Materials and Methods

### Animals

This preclinical experimental study utilized 70 Wistar rats (both sexes, 8-10 weeks old, 180-240 g) to test the antiseizure effect of piracetam (PIR; 10 and 20 mg/kg), diethylstilbestrol (DES 10 and 20 mg/kg) and their low dose combination (PIR 10 mg/kg + DES 10 mg/kg) on electroshock induced seizures. The Wistar rats were divided to 7 groups (10/group; 5 male rats + 5 female rats placed in separate plastic cages). Rats were issued from the central animal house at IAU after obtaining approval (Approval No.: IRB-2021-05-126). All rats were kept under regulated settings of the temperature (23 ± 2°C), humidity (40-70%), and light cycles of 12/12 hours light/dark. Rats were handled in accordance with the protocol from IACUC board.

### Chemicals and Kits

The piracetam (PIR), diethylstilbestrol (DES), pentylenetetrazol (PTZ) were obtained from Sigma Aldrich, St. Louis, MO, USA. mTOR and IL-6 ELISA kits were purchased from Aviva Biosystems, San Diego, CA, USA. IL-1β and TNF-α levels were purchased from OriGene, Rockville, MD, USA.

### Drugs and Dosing Scheme

PIR and DES were administered once daily for a total of 14 days at two different doses i.e., PIR, 10 mg/kg and 20 mg/kg and DES, 10 mg/kg and 20 mg/kg. These test drugs were combined at lower doses i.e., PIR 10 mg/kg + DES 10 mg/kg. All test drugs and their combinations were dissolved in tween 20 (2% aqueous solution) and administered for 14 days *via* oral gavage. The electroshocks were induced on the last day of treatment. The doses of test drugs were taken from the literature. PIR 20 mg/kg was reported[41] to increase noradrenaline concentrations in rat brain and act as -aminobutyric acid (GABA)-mimetic as well. Prophylactic treatment with PIR 50 mg/kg reduced the duration of catalepsy (in 59.5% of mice) induced by haloperidol[42]. The Oral $LD_{50}$ or Lowest Lethal Dose value (rat) of PIR is 5,600 mg/kg. The Oral $LD_{50}$ (rat) of DES is 3,000 mg/kg. We used much less dose range for PIR and DES with reference to their respective $LD_{50}$ values[43].

### Treatment Groups

The wistar rats were divided in seven groups subdivided to ten rats per group, and received the treatment as follows: Group-I, Normal control (10 ml/kg of 2% tween 20); Group-II, toxic control (10 ml/kg of 2% tween 20); Group-III, PIR1 10 mg/kg; Group-IV, PIR2 20 mg/kg; Group-V, DES1 10 mg/kg; Group VI, DES2 20 mg/kg; and Group-VII, PIR1 10 mg/kg + DES1 10 mg/kg. The dosing was scheduled for 14 days *via* oral gavage. The electroshocks were applied to rats from all groups, except from Group-I, on the last day of treatment.

### MES Induced Tonic Hind Limb Extension (THLE)

In order to generate electro convulsions in wistar rats by using auricular electrodes, rats were banded by using ear clips and restrained by hand. The shock was applied by the electroconvulsometer, and rats were freed immediately after the stimulation and left for observation during this period to detect seizure behavior[44]. The percentage of rats protected from electroshock induced THLE gave the measure of anticonvulsant property of that compound[45,46].

### Experimental Design

The electric shock to be applied was initially standardized to remove the bias. The standardization was done by applying different current intensities to different groups of rats. The intensity response curve was plotted and current strength (CS)100 i.e., electric shock able to produce THLE in 100% of rats was recorded, which was MES (180 mA, 220 V, 0.20 s). The rats were monitored post electroshock, until they regained their posture or moved freely (i.e., recovery). Immediately following recovery six rats were sacrificed, their skulls were incised for brain removal, which was washed in normal saline followed by skillful extraction of hippocampus (both right and left lobes) by a technician and preserved at -80°C. This preserved hippocampal tissue was later crushed in phosphate buffer saline (PBS) with tissue homogenizer and centrifuged to obtain the supernatant. This supernatant was used for the assessment of mTOR, IL-1β, IL-6, TNF-α levels by ELISA kits. The remaining four rats were kept under observation for one day, thereafter sacrificed, and skull carefully incised for extraction

of full brain, which was stored in formalin, to be processed for hippocampal histopathology.

### MES Induced Neuronal Damage

To retrospect the neuronal injury evoked by electroshock, histopathological assessment of hippocampus was done 24 h after the induction of MES (180 mA, 220 V, 0.20 s). The following procedure was followed: after the rats were sacrificed, the brains were removed, preserved in formalin 10%. The preserved tissue was dehydrated with different concentrations of ethanol, followed by clearing with xylene. Paraffin wax was used for the formation of blocks. Then, using microtome, 8-µm sections were cut and mounted on glass slides. Which were processed for staining with hematoxylin and eosin (H/E). The photographs were clicked from the hippocampal sections; DG, CA3, CA2 and CA1 at 40X with the light microscope (Leica Biosystems, Wetzlar, Germany). The electroshock was inculcated with unprecedented neuronal loss and severe pkynosis.

### Assessment of Hippocampal mTOR Levels

Enzyme-linked immunosorbent assay (ELISA) kits from Aviva Biosystems, San Diego, CA, USA were used to estimate the mTOR levels in rat hippocampus. The manufacturer instructions were followed for the assay.

### Assessment of Proinflammatory Cytokines in the Hippocampus

Interleukin-6 (IL-6) was quantified using ELISA kit from Aviva Biosystems, San Diego, CA, USA. IL-1β and TNF-α levels were quantified using ELISA kits from OriGene, Rockville, MD, USA. In both cases manufacturer instructions were followed.

### In Vitro Studies

#### Cell culture

The HEK-293 cells were cultured in order to test the neuroprotective potential of PIR, DES alone and in combination, by measuring the % of cell viability. HEK-293 cells have many neuron-specific genes bearing an unexpected relationship to neurons[47,48]. HEK-293 cells express neurofilament, neuroreceptors, and neuron-specific metabolic enzymes[49]. The HEK-293 cells were cultured in 96-microwell plates prepoured with Dulbecco's Modified Eagle Medium (DMEM), streptomycin, fetal bovine serum (FBS), selenium chloride, L-glutamine and penicillin[50,51]. The incubation of cells continued for 48 h at 37°C in the (5%) $CO_2$ incubator in order to achieve 70-80% of cell density.

### In Vitro Model of Cytotoxicity

Cytotoxicity was induced by treating HEK-293 cells with neurotoxin i.e., pentylenetetrazol (PTZ), a tetrazol derivate ($GABA_A$ receptor antagonist) at 0.6 µg/mL for 24 h. Thereafter, morphology and anatomy of cells was observed by inverted wide-field microscope. In the Normal Control (NC) group, the PTZ was not added. This method has been described in previous studies[52].

### Treatment with Test Drugs

The PTZ treated HEK-293 cells were then exposed to PIR, DES, PIR + DES at various concentrations (0.2 µg/mL-0.90 µg/mL) for 24 h. In the Toxic Control (TC) group (PTZ treated only), the test drugs were not added.

### MTT Assay

The MTT (5 mg/mL) was poured into pretreated HEK-293 cell plate and incubated for four hours. Then, cells were rinsed with 100 µl of Dimethyl sulfoxide (DMSO). The cell viability (%) was estimated at 570 nmλ by ELISA Plate Reader (BioTek Instruments, Winooski, Vermont, USA).

### Molecular Modeling

The molecular modelling software (Schrodinger, NY, USA) was used to perform molecular simulations through the molecular operating environment (MOE). This software is capable of generating binding energy calculations, molecular simulations and data visualization. The crystal structure of human Akt (PDB code; 4gv1), and PI3K (PDB code; 1e7v) co-crystallized with inhibitors and were used as targets in the docking studies. The methodology has been described in detail previously by Pottoo et al[52] in 2021.

### Statistical Analysis

The Mean ± SEM from all groups was computed. ANOVA with post hoc Dunnet's test was used to establish significance between different groups. In case of MES induced electro convulsions, the difference in THLE:NO-THLE between toxic control and other groups was calculated *via* one tailed (Fischer's exact test). GraphPad-instat software (Dotmatics, San Diego, CA, USA) was used for statistical analysis and the significance levels *** $p < 0.001$ (extremely significant), ** $p$

< 0.01 (highly significant) and * $p < 0.05$ (significant) were considered as such in all cases.

## Results

Influence of Piracetam (PIR), Diethylstilbestrol (DES) Alone, and as Combinational Drug Therapy Against Electroshock Induced Tonic Hind Limb Extension (THLE)

The electroshock (180 mA, 220 V for 0.20 s) induced THLE in all rats (100%) of TC group. Monotherapy with PIR at 10 and 20 mg/kg produced significant ($p < 0.05$) antiseizure effect (the therapeutic effect was statistically the same at both doses). Monotherapy with DES at 10 mg/kg displayed highly significant ($p < 0.01$) antiseizure effect, while significant ($p < 0.05$) effect was observed at the corresponding higher dose of DES i.e., 20 mg/kg. Thus, lower dose of DES exhibited better anti-seizure effect than the corresponding higher dose. However, extremely significant ($p < 0.001$) rise in the therapeutic effectiveness of PIR (10 mg/kg) and DES (10 mg/kg) in combination was observed (Figure 1).

### *Influence of Piracetam (PIR), Diethylstilbestrol (DES) Alone and in Low Dose Combination, on Recovery Time Following Electroshock-Induced Seizures*

The monotherapy with PIR at 10 and 20 mg/kg and DES at 10 and 20 mg/kg exhibited nonsignificant effect on the recovery time post electroshock stimulation of rats. Only the combination of PIR (10 mg/kg) with DES (10 mg/kg) significantly ($p < 0.05$) reduced recovery time (40.12 s *vs.* 75.25 s from TC) (Figure 2).

### *Effect of Piracetam (PIR), Diethylstilbestrol (DES) Alone and as Low Dose Combination Drug Therapy on Hippocampal mTOR Levels*

To explore the signaling through PI3K/Akt/mTOR pathway, the hippocampal levels of mTOR in all rat groups were measured. The seizures lead to the hyperactivation of mTOR pathway, with extremely significant ($p < 0.001$) upsurge in hippocampal mTOR levels in the TC group, related to the NC. Monotherapy with PIR at 10 and 20 mg/kg significantly ($p < 0.05$) reduced mTOR levels. Monotherapy with DES at 10 mg/kg led to highly significant ($p < 0.01$) reduction in mTOR levels, while DES at 20 mg/kg significantly ($p < 0.05$) reduced mTOR levels. The extremely significant ($p < 0.001$) reduction of mTOR levels was only evidenced from the combination of PIR (10 mg/kg) and DES (10 mg/kg). Thus, seizure promulgated hyperactivation of signaling through mTOR pathway was best counteracted by the low dose combinatorial regimen of PIR and DES (Figure 3).

### *Effect of Piracetam (PIR), Diethylstilbestrol (DES) Alone and as Low Dose Drug Combination Therapy on Pro-Inflammatory Cytokines in Hippocampus*

*Effect on IL-1β levels*

The brain pathology after the electroshock induced seizures presented a state of neuronal hyper-inflammation with hippocampal IL-1β levels showing extremely significant ($p < 0.001$) rise in

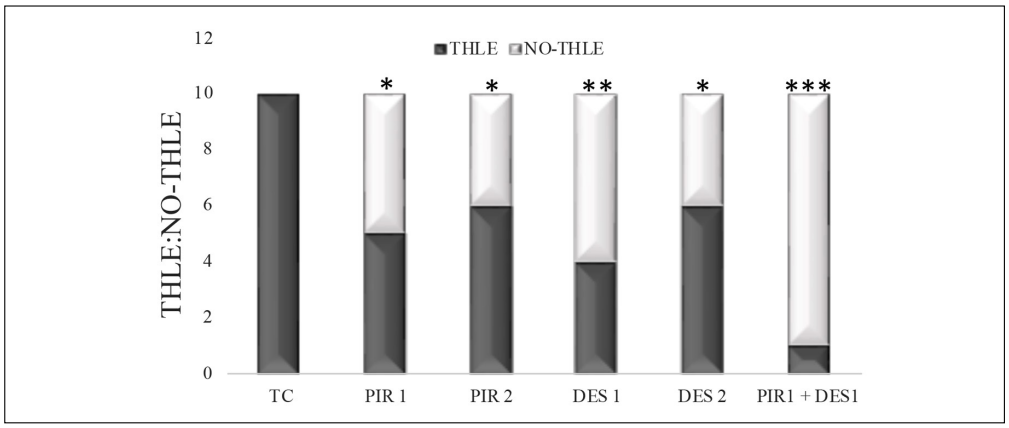


**Figure 1.** Effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on electroshock triggered THLE. Fisher's test (one tailed) was used to calculate *p*-value (< 0.05 *, < 0.01 **, < 0.001 ***). All groups were compared with TC. TC: Toxic control, PIR: Piracetam, DES: Diethylstilbestrol.

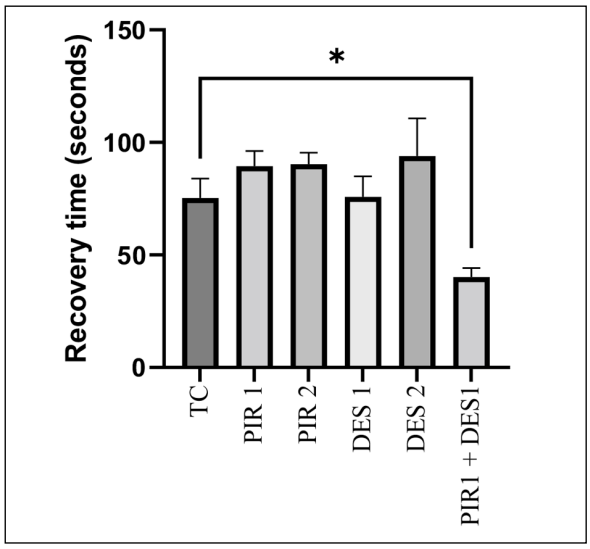


**Figure 2.** Effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on recovery time after electroshock triggered convulsions (in Wistar rats). ANOVA with the post-hoc Dunnet's test calculated the *p*-value (< 0.05 *, < 0.01 **, < 0.001 ***). TC: Toxic control, PIR: Piracetam, DES: Diethylstilbestrol.

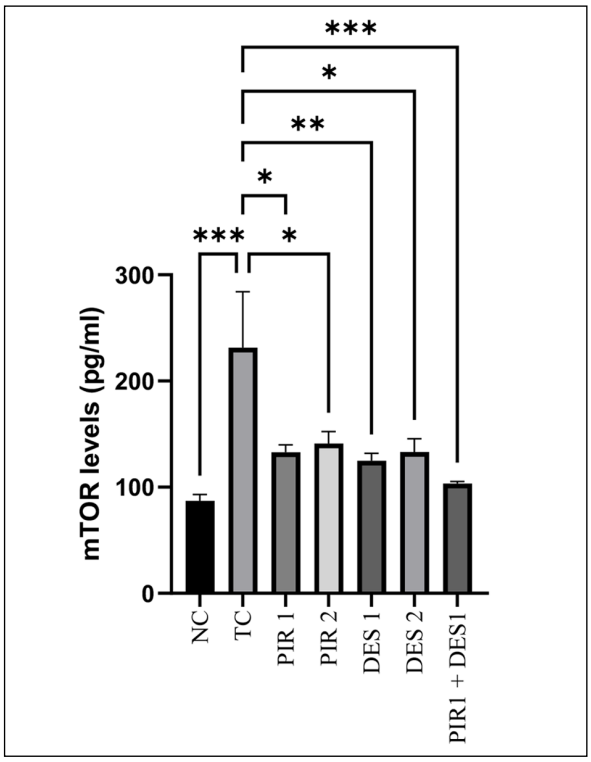


**Figure 3.** Effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on hippocampal mTOR levels after electroshock triggered convulsions (in Wistar rats). ANOVA with the post-hoc Dunnet's test calculated *p*-value (< 0.05 *, < 0.01 **, < 0.001 ***). All groups were compared to TC. TC: Toxic control, PIR: Piracetam, DES: Diethylstilbestrol.

the TC group, compared to the NC. Monotherapy with PIR at 10 and 20 mg/kg, significantly ($p < 0.05$) lowered IL-1β levels. Monotherapy with DES at 10 mg/kg led to highly significant ($p < 0.01$) decrease in IL-1β levels, while DES at 20 mg/kg significantly ($p < 0.05$) lowered IL-1β levels (the lower dose of DES was more effective than the corresponding higher dose). However, the extremely significant ($p < 0.001$) lowering of IL-1β levels was noticed only with the combination of PIR (10 mg/kg) and DES (10 mg/kg). Thus, the combination displays high anti-inflammatory effect (Figure 4).

### *Effect on IL-6 levels*

The brain pathology after electroshock induced seizures revealed a state of neuronal hyper-inflammation with hippocampal IL-6 levels

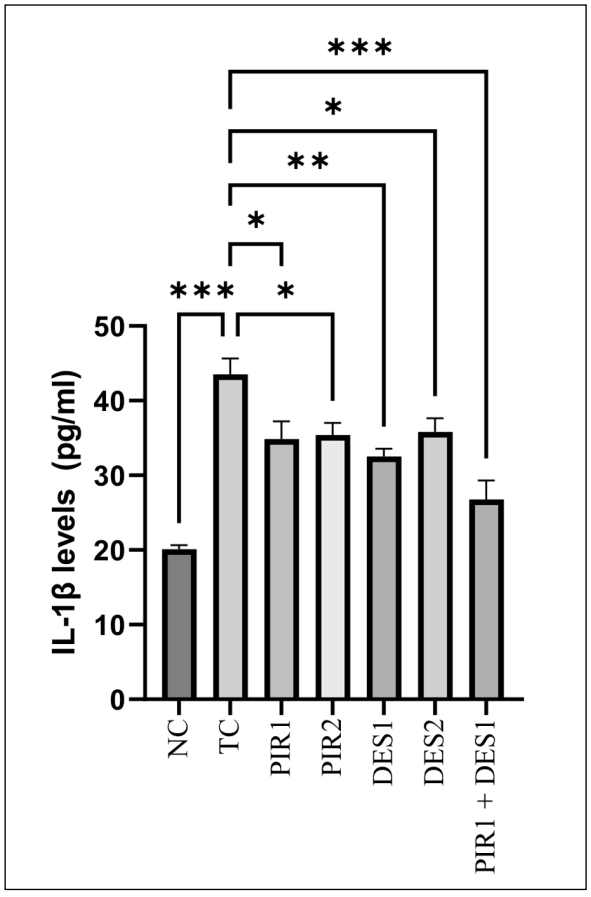


**Figure 4.** Effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on hippocampal IL-1β levels post electroshock induced triggered (in Wistar rats). ANOVA with the post-hoc Dunnet's test calculated *p*-value (< 0.05 *, < 0.01 **, < 0.001 ***). All groups were compared to TC. TC: Toxic control, PIR: Piracetam, DES: Diethylstilbestrol.

showing extremely significant ($p < 0.001$) rise in the TC group, compared to the NC. Monotherapy with PIR at 10 mg/kg significantly ($p < 0.05$) lowered the IL-6 levels (no significant effect was observed from its corresponding higher dose). Monotherapy with DES at 10 mg/kg led to highly significant ($p < 0.01$) lowering of IL-6 levels (no significant effect was observed from its corresponding higher dose). Nonetheless, the extremely significant ($p < 0.001$) reduction of IL-6 levels was observed only with the combination of PIR (10 mg/kg) and DES (10 mg/kg) (Figure 5).

### *Effect on TNF-α levels*

The brain pathology after electroshock induced seizures revealed a state of neuronal hyperinflammation with hippocampal TNF-α levels showing extremely significant ($p < 0.001$) rise in the TC, compared to the NC. Monotherapy with all test drugs, PIR (10 and 20 mg/kg) and DES (10 and 20 mg/kg) significantly reduced ($p < 0.05$) TNF-α levels. Albeit the low dose combination of PIR (10 mg/kg) and DES (10 mg/kg) showed highly significant ($p < 0.01$) reduction in the TNF-α levels. The results confirm the brimming efficacy of the drug combination to halt the inflammatory cascades (Figure 6).

### *Effect of Piracetam (PIR), Diethylstilbestrol (DES) Alone and in Low Dose Combination on Hippocampal Neuronal Damage*

The electroshock inculcated injury was manifested as gross neuronal loss of hippocampal regions; CA1, CA2, CA3 and DG from TC. The monotherapy with test drug, PIR at 10 mg/kg,

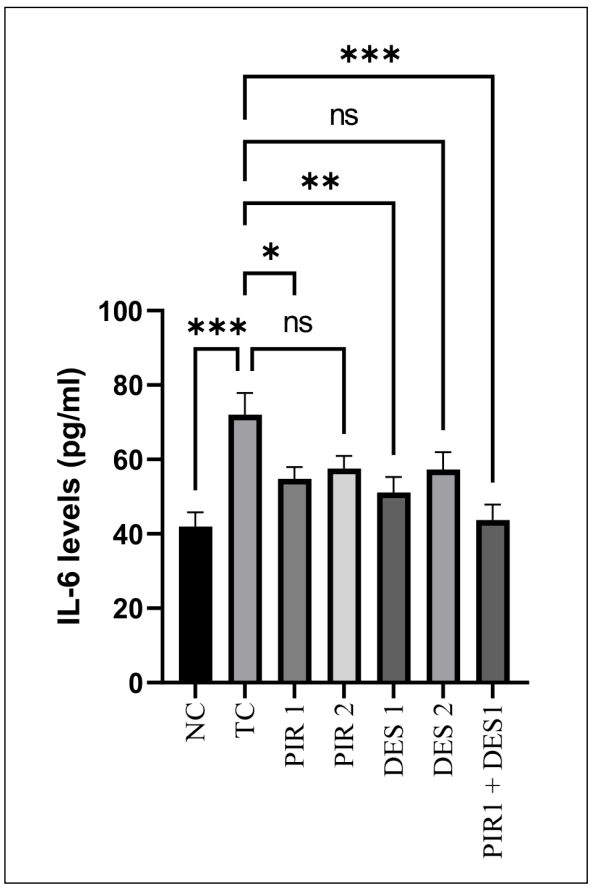


**Figure 5.** Effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on hippocampal IL-6 levels post electroshock convulsions induced (in Wistar rats). ANOVA with the post-hoc Dunnet's test calculated *p*-value (< 0.05 *, < 0.01 **, < 0.001 ***). All groups were compared to TC. TC: Toxic control, PIR: Piracetam, DES: Diethylstilbestrol.

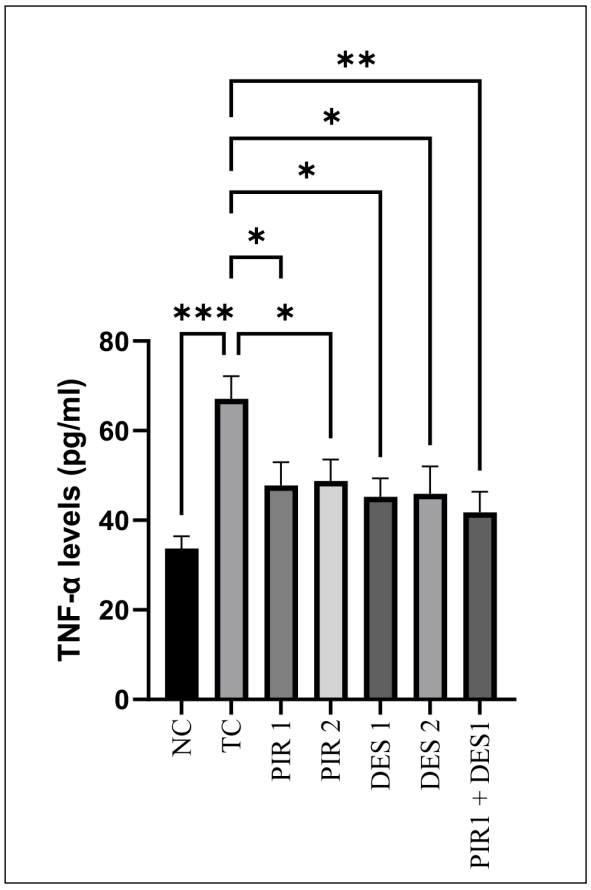


**Figure 6.** Effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on hippocampal TNF-α levels post electroshock induced convulsions (in Wistar rats). ANOVA with the post-hoc Dunnet's test calculated *p*-value (< 0.05 *, < 0.01 **, < 0.001 ***). All groups were compared to TC. TC: Toxic control, PIR: Piracetam, DES: Diethylstilbestrol.

exhibited more neuroprotection than at 20 mg/kg, given that pkynosis is seen in all hippocampal regions from PIR (20 mg/kg). Similarly, the DES at 10 mg/kg exhibited more neuroprotection than at 20 mg/kg, given that heavy pkynosis is seen in DG regions from DES (20 mg/kg). However, the overall neuroprotective effect of DES was better than PIR. The combination of PIR (10 mg/kg) + DES (10 mg/kg) seemed to largely inhibit neurodegenerative signaling cascades, given that neuronal morphology was protected and neuronal loss/pyknosis was minimal in the group, and the neurons seem to be intact (Figure 7).

### Effect of Piracetam (PIR), Diethylstilbestrol (DES) Alone and in Low Dose Combination on Neurotoxin Induced Cytotoxicity

Neuroprotective potential of test drugs alone and in combination was measured as % cell viability with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Pentylenetetrazol was used as neurotoxin. The HEK-293 cells were treated with PTZ (0.6 µg/mL), and cell viability was measured after 24 h. The treatment with PTZ resulted in a reduction in cell viability to 85.16% compared with 100% from normal control. The exposure of these PTZ-treated cells for 24 h with test drugs, PIR (0.25 µg/mL) and DES (0.25 µg/mL), increased the cell viability to 104.40% and 185.18%. However, when cells were treated with combination, PIR + DES (at a ratio of 1:1), the cell viability was increased to 327.29%. The cell viability of treated cells was compared with PTZ-treated cells (85.16%). Our results suggest that the combination of two drugs (PIR + DES) significantly increased the cell growth and cell proliferation compared to cells treated with PIR and DES alone (Figure 8).

### Molecular Docking

Akt and PI3K are upstream targets for mTOR. The molecular docking simulation revealed the affinity of PIR and DES at Akt and PI3K (active sites). The test drugs, PIR and DES, were docked alone and as co-bound complex (in distinct orders) at the active sites of Akt and PI3K for calculating the binding scores. Comparison docking also was performed by comparing the position of co-bound ligand positions to the crystallized reference inhibitor. The docking rating scores indicate better affinity for Akt and PI3K in case of DES. While as co-bound complexes the better affinities were observed for PI3K (when docking DES first), and for Akt when docking PIR first (see Table I). The binding pose analysis for Akt was interesting since the co-bound drugs approximately superpose the reference crystallized ligand at the active site (see Figure 9).

DES and PIR collectively bind the active site, while establishing a great network of intermolecular interactions (Figure 10). Potential hydrogen bonding was noted for DES in the active site with ALA 230, ASN 279, MET 281, and ASP 292. PIR occupies the opposite pocket and binds the active site with potential hydrogen bonding namely with GLY 159, THR 160, and GLY 162. VAL 164 is locking the two bound drugs through van der Waals (vW) interactions.

The interesting poses shown by the DES and PIR at the Akt active site indicate that this co-binding could be further forced by a supramolecular interaction between DES and PIR that renders their co-binding energetically more preferred. This was observed from the VW interactions of DES-phenyl ring and PIR-pyrrole ring. There are also probable hydrogen bonds between the two aforementioned rings (Figure 10).

## Discussion

Seizures are associated with emotional disturbances, odd behaviors, strange movements or sensations in body parts and lapses of consciousness[53]. Seizure episodes are commonly associated[54] with neurological disorders including behavioral and cognitive declines that can impact patient quality of life. The severity of epilepsy induced cognitive declines is highest during childhood, especially in children with epileptic encephalopathies[54]. Moreover, cognitive impairments are found[54] to be a complex interaction of epilepsy, interictal discharges, seizure episodes and antiepileptic medications. Mental impairment, drowsiness and dizziness are found to be the most common central nervous system (CNS) adverse events of AEDs[55]. In epilepsy, if the need arises for combinational drug therapy, the selection of rational polytherapy is superior to random selection[7]. For example, lamotrigine with valproic acid with careful monitoring to the pharmacokinetic interactions and gabapentin with levetiracetam[7]. Other successful combinations are levetiracetam with topiramate and sodium valproate with levetiracetam or carbamazepine[56]. In a double-blind clinical trial[57], the combination of

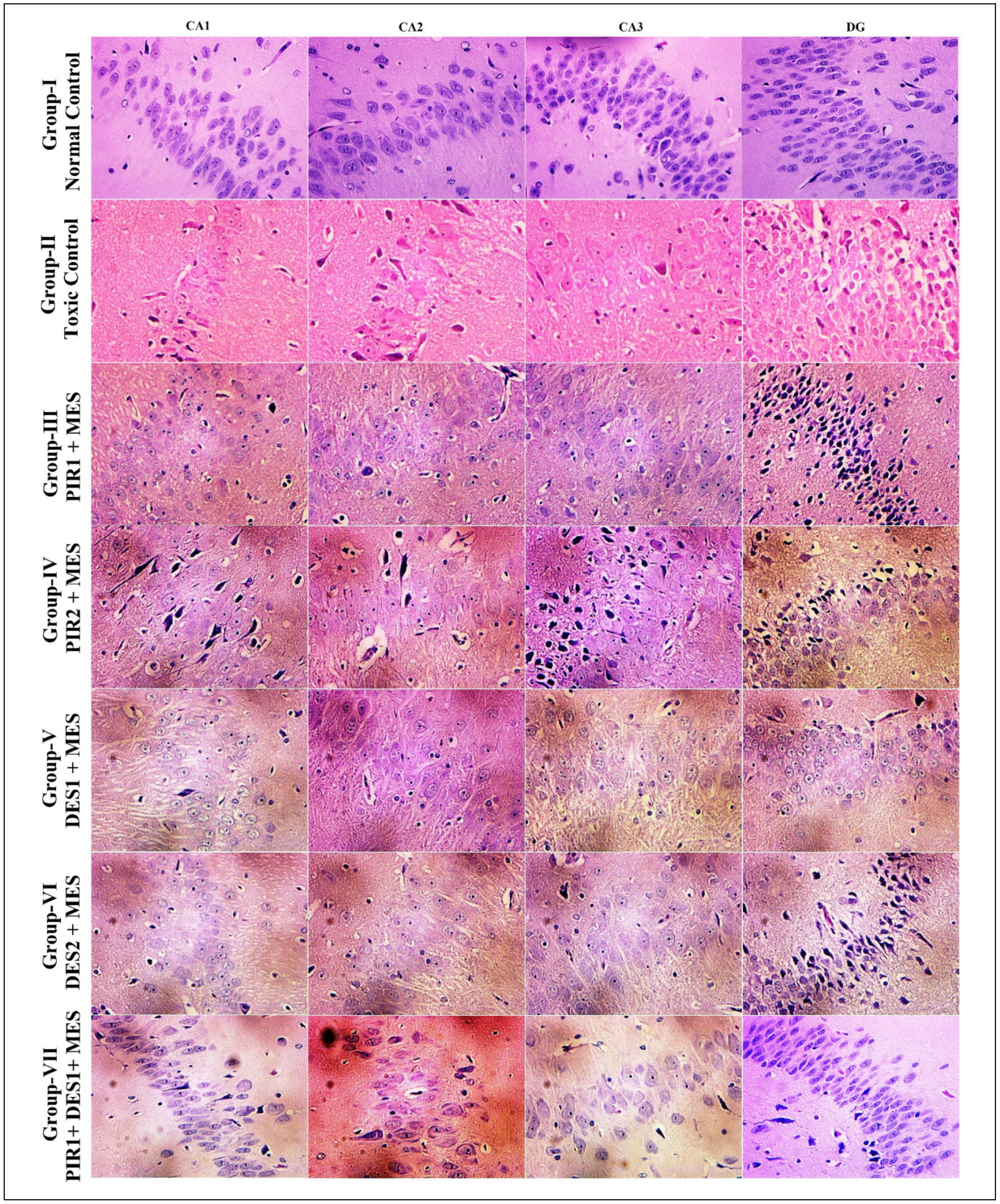


**Figure 7.** Photomicrographs taken from hippocampal slides at 40X magnification, featuring the effect of PIR (10 and 20 mg/kg), DES (10 and 20 mg/kg), PIR (10 mg/kg) + DES (10 mg/kg) on electroshock inculcated neuronal death/changes. The neurons from the normal control present dense intact neuronal layers with normal morphological features. The impact of electroshock is clearly visible in the TC group, which shows a gross loss of neurons in all hippocampal sub-regions, CA1, CA2, CA3 and DG. The monotherapy with PIR and DES, was seen to limit the neuronal damage from the electric shock, the lower doses of both test drugs, restrained damage more than their corresponding higher doses. However, the combinatorial regimen of PIR (10 mg/kg) + DES (10 mg/kg) revealed evident neuroprotection in the form of protection all hippocampal regions from neuronal loss and pyknosis and that the combination group exhibited intact neuronal layers with normal morphological features.

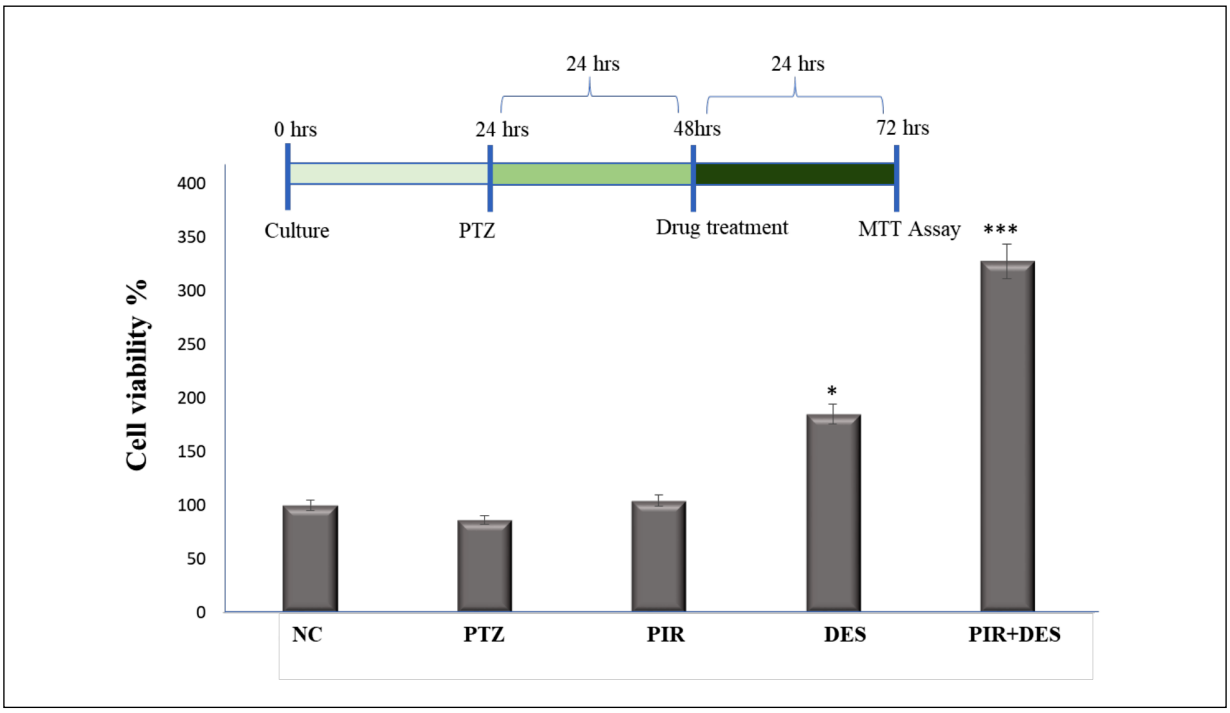


**Figure 8.** Cell viability by MTT assay: Effect of PIR (0.25 µg/mL), DES (0.25 µg/mL), PIR + DES (0.25 µg/mL + 0.25 µg/mL) on PTZ (0.6 µg/mL) treated HEK-293 cells. The cells were first treated with PTZ for 24 h, after that treated with test drug/s for 24 h. The % of cell viability given in the graph is taken from the dose which gave the highest percentage of cell viability. * $p < 0.01$, *** $p < 0.001$ were recognized as significant and extremely significant.

valproic acid (VPA) and carbamazepine (CBZ) (300 mg and 200 mg per day, respectively) *vs.* CBZ monotherapy (400 mg daily) was compared in 130 adult patients. The frequencies of neurotoxicity, seizures or other systemic toxicity failed to reveal any significant difference between the two arms[57]. Thus, combinatorial regimen needs to be carefully selected based on strong scientific evidence. In pursuit of finding novel AED combination regimen with anticonvulsant and neuroprotective properties, we sought to explore PIR (nootropic) with DES (synthetic estrogen). The results revealed that the low dose combination of PIR with DES displayed synergism in abrogation of electroshock incited seizures and neurodegeneration. The electroshock induced seizures were integrated with upregulation of mTOR signaling and upsurge in inflammatory markers, IL-β, IL-6 and TNF-α, which were restrained remarkably with the combinatorial therapy of PIR and DES (at low doses). The synergistic effect of PIR and DES combination featuring the weakening of mTOR signal in animal studies, was further evaluated *in silico* by testing their binding affinities on the active sites of PI3K and Akt (upstream targets of mTOR pathway)[58]. DES and PIR (as co-bound complex) showed better affinities for PI3K (when docking DES first) and for Akt (when docking PIR first). Further the two drugs, DES and PIR, exhibited cooperative bindings, meaning one ligand increasing binding of another ligand. The *in vitro* studies asserted

**Table I.** Docking scores for DES, PIR alone and co-bound in Akt and PI3K.

| | Docking Score | | | |
|---|---|---|---|---|
| | DES 1st | DES/PIR | PIR 1st | PIR/DES |
| Akt | -10.9 | -7.6 | -7.5 | -11.3 |
| PI3K | -12.0 | -9.3 | -6.7 | -9.7 |

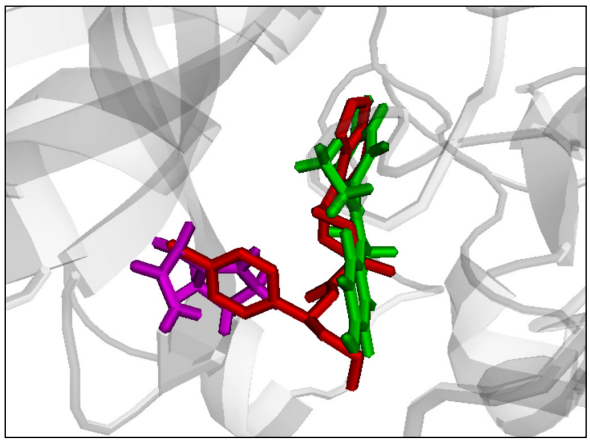

**Figure 9.** Comparative binding positions of co-bound DES (*green*) and PIR (*magenta*) with the reference crystallized ligand (*red*) bound to Akt active site.

the claims, given that the combination of PIR and DES increased cell viability by 327.29% in PTZ pretreated HEK-293 cells compared to PTZ-treated cells (85.16%). Our study undoubtedly features the therapeutic potential of low dose combinatorial regimen of DES and PIR, in restricting electroshock inculcated seizures and neuronal damage by inhibiting the PI3K/Akt/mTOR signaling and proinflammatory cytokines.

PIR is a GABA derivative[59], a nootropic agent and pyrrolidone derivative with anticonvulsant properties[60,61] PIR is available in oral tablet or solution with doses of 800 mg and 1,200 mg or 200 mg/mL and 333.3 mg/mL, respectively[62]. It is well tolerated and efficacious as an adjunctive treatment in the treatment of myoclonus[12]. In the mitigation of progressive myoclonus epilepsy, PIR shows the highest efficacy in the first 12 months of treatment[59]. In clonic and generalized tonic seizures, PIR seems to have null or moderate anticonvulsant action[60]. As a supplement to the standard anticonvulsants, however, it demonstrates higher antiepileptic effectiveness in a number of cases[60]. By using PIR in lethargic mice, significant decreases in the duration and incidence of spike-wave discharges were noticed[60]. Furthermore, repeated or single administration of PIR in the cobalt stimulated focal epilepsy model reduced the frequency of spikes per min[60]. Moreover, PIR can alleviate the cognitive impairment related to epilepsy and chronic therapy with antiepileptic such as sodium valproate, phenytoin, carbamazepine, phenobarbitone and topiramate[61,62]. The use of PIR "memory enhancer" could reverse the cognitive impairment associated with phenytoin without compromising its antiepileptic efficacy[63,64]. PIR has neuroprotective and nootropic effects that prevent cognitive deterioration associated with valproate and topiramate and enhance the cognitive performance[65]. Levetiracetam is an S-enantiomer of PIR, both of them are used in epilepsy and myoclonus in several countries[66]. In agreement to previous studies[66], we found that PIR exhibits anti-convulsant effects, manifested as a decline in the ratio of THLE:NO-THLE.

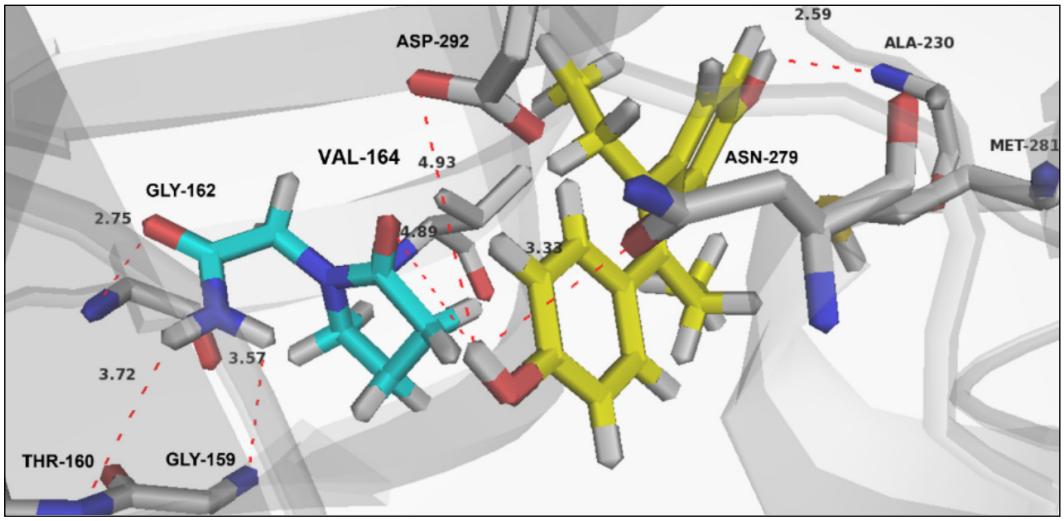


**Figure 10.** Binding interactions of co-bound DES (*carbons in yellow*) and PIR (*carbons in green*) with Akt active site. Distances are represented as red dotted lines and are measured in Å.

DES is a potent synthetic hormone that mimics the action of estrogen[67]. Traditionally, in 1940s-1970s, DES was used to treat pregnancy associated problems and prevent abortion[68,69]. Then, the use of DES became banned during pregnancy as the exposure of prenatal DES is associated with negative effect on reproductive system[69]. DES could increase the risk of breast and uterine cancer in female and lead to stillbirth, preterm delivery, miscarriage, loss of pregnancy in second trimester and neonatal death if used in pregnancy[68,69]. In both animals and humans' studies[65], they found that the exposure to DES in the reproductive tract differentiation period cause permeant alteration of estrogen target tissues and lead to uterine neoplasia. DES is a full agonist at estrogen receptors, but it preferentially binds Estrogen receptor-β ($ER_\beta$) ($EC_{50}$ of 0.06 nM) compared to $ER_\alpha$ ($EC_{50}$ of 0.18 nM)[70]. 17β-estradiol (E2) is the most active estrogen, which acts as a neurotrophic and neuroprotective agent mediated *via* $ER_\alpha$ and $ER_\beta$[71]. Thereby DES could mimic the effect of estrogen. DES inhibited (concentration-dependent) the current evoked by 100 microM kainite[68]. Interestingly, we found that DES exhibits anti-convulsant effect (more at low doses). However, the low dose combination of PIR with DES, fetched a most significant synergistic anticonvulsant effect, which indicates that the combination is able to intercept electroshock inculcated proconvulsive changes in neuronal receptors/channels.

mTOR is inculpated in ageing, autophagy, immune responses, cancer, neurodegenerative diseases etc. AMP-activated protein kinase (AMPK), glycogen synthase kinase 3 (GSK3), insulin-like growth factor 1 (IGF-1), and PI3K/Akt are the upstream regulatory components of mTOR[69]. Inflammation can activate the mTOR pathway[72]. mTOR pathway hyperactivity has been detected[28] in drug resistant epilepsy. In recent clinical trials[46,72], they found that the use of mTOR inhibitors had the ability to reduce seizure frequency, and epileptogenesis. PIR protected rat hippocampal neurons from ethyl alcohol (EtOH) neurotoxicity by increasing the phosphorylation of mTOR[73]. In agreement with literature, we reported the significant activation of mTOR pathway by electroshock, which was reversed with PIR. The DES also restricted increase in mTOR levels following electroshocks. However, the combinatorial therapy (PIR + DES) markedly lowered ($p < 0.001$) mTOR activation, revealing that this combinational therapy has maximum ability to prevent seizures and halt the transition of brain from normal to epileptic.

Recurrent seizures or proconvulsant injuries can induce IL-1β by the activation of microglia and astrocytes, furthermore, IL-1β functional receptor type 1 (IL-1R1) can be induced in astrocytes and neurons[73]. In a recent animal study[74] conducted in rats with cognitive impairments, the PIR and tacrine groups showed a decrease ($p < 0.001$) in the hippocampal level of IL-1β (52.37% with PIR and 61.04% with tacrine)[74]. PIR significantly reduced neuroinflammatory activity by attenuating Lipopolysaccharides (LPS)-stimulated increase in IL-6[75]. The proinflammatory cytokine TNF-alpha works on two receptors which are p55 and p75[76]. In a knockout mice study[77], TNF-α p55 and p75 receptor showed a decrease and increase in the likelihood of seizure, respectively. TNF-α causes elevated transmission of excitatory synaptic activity by modulating the traffic of glutamate receptor through TNF receptor 1 (TNFR1)[78]. The expression of TNF-alpha in the immature rat hippocampi can be modulated by using lipopolysaccharide or myeloid-related protein (MRP8) or inhibiting the usage of lenalidomide on astrocytes[79,80]. In our study, we found that electroshock induced changes include rise of proinflammatory markers levels, IL-1β, IL-6 and Tumor Necrosis Factor alpha (TNF-α), in the hippocampal area. PIR at both doses (10 and 20 mg/kg) reduced hippocampal IL-1β and THF-α, while IL-6 was reduced only at lower dose. This indicates that PIR is more protective at lower doses. The similar pattern of results was superseded with DES, which tended to reduce the levels of hippocampal IL-1β and THF-α at both doses, while IL-6 levels were reduced at lower doses only, which indicates that lower dose is more efficacious than the corresponding higher dose. To our surprise the low-dose combination of PIR and DES bought significant reduction in the levels of all proinflammatory markers in hippocampus, a better effect than monotherapy with these drugs was apparent.

The brain neurodegeneration or pathological alterations in epilepsy most likely include increase in the size and number of astrocyte and loss of neurons[81,82]. Also, it may include an increase in new capillaries formation, blood brain barrier leakage, inflammation of the neurons and sprouting of axon[83]. These alterations are primarily caused by the eventual cascade following seizure that can cause hypoxia and hypoperfu-

sion[84]. Moreover, oxidative stress, excitotoxicity of glutamate and neuroinflammation are considered as common causes of neurodegeneration[85,86]. Neurodegeneration could be prevented by inhibiting glycogen synthase kinase 3 (GSK3) which cause axonal damage[87,88,89]. 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) which inhibits GSK3β pathway reduced kainic acid induced neurodegeneration[87]. We examined the hippocampal sections from normal, toxic and treated groups. The TC revealed the belligerence of electroshock in the form of severe neuronal cell death in all hippocampal regions, CA1, CA2, CA3 and DG. The monotherapy with DES revealed better neuroprotection than PIR. However, the combinatorial therapy with PIR and DES largely preserved the layer organization and cell morphology with bare minimum neuronal loss. The morphology of neuronal cells from the combinatorial regimen resembles that of normal control. We confirmed the neuroprotective effects from *in vitro* studies. After the neurotoxin (PTZ) caused the cells death, PIR, DES and PIR + DES treatments increased the cell proliferation by 104.40%, 185.18%, and 327.29%, respectively, in comparison to the toxic control (85.16%).

### Limitations

This study is based on preclinical *in vitro* and *in vivo* studies, which means that it cannot be accurately related to humans, without valid clinical trials.

## Conclusions

The imbalance between excitatory and inhibitory transmission in epilepsy is an over-simplication of an intricately complex disorder. None of the existing AEDs have been able to achieve seizure remission in 100% patients as mono or combination therapies. However, their brimming adverse effects and potential risks cannot be ignored. The existing therapies are clearly not hitting the pivotal signaling pathways. Neurodegeneration alters the binding of ligands with the receptors/channels, leading to refractoriness. We sought to discover a combination regimen with both anti-convulsant and neuroprotective properties. Fortunately, the low dose combination regimen of PIR and DES exhibited synergism in raising the threshold to electroshock induced convulsions, coupled with anti-inflammatory and neuroprotective properties. The synergism seems to be a result of the inhibition of PI3K/Akt/mTOR signaling at all levels. The electroshock induced seizures replicate what we refer to tonic clonic seizures in the patients, however, any extrapolation of data from this study to humans would need data from clinical trials. Also, safer derivatives of DES or selective estrogen receptor modulators (SERMs) need to be synthesized and tested further in preclinical studies alone and in combination with piracetam.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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### Authors' Contribution

F.H. Pottoo and M. Salah Uddin spearheaded the study, from conceptualization to design, conducting the Invivo study, analyzing the Invivo data, and writing the manuscript. F.A. Khan played a pivotal role in the Invitro study, contributing to its design, conducting the Invitro study, and assisting with data analysis. W.J. Alsaeed and B.T. Albaqshi assisted with data collection and analysis and made significant contributions to the manuscript's writing. J.U. Rahman, on the other hand, performed photomicrography of hippocampal slides, provided critical feedback on the manuscript, and edited it. M.S. Gomaa's & I.A. Salama contribution involved performing molecular docking and writing the methodology and discussion sections pertaining to molecular docking. S. Beigh and M.N. Alomary contributed to data collection and analysis, provided critical feedback on the manuscript, and contributed significantly to the framing and revision of manuscript as per journal guidelines.

### Ethics Approval

Rats were issued from the IAU's primary animal house after receiving permission (Approval No.: IRB-2021-05-126).

### Funding

None.

### Informed Consent

Not applicable.

### Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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