Neuroprotective effect of nilotinib on pentylenetetrazol-induced epilepsy in adult rat hippocampus: involvement of oxidative stress, autophagy, inflammation, and apoptosis

Ghalia Mahfouz Attia 1,2, Rasha Ahmed Elmansy 1,4, Wael M. Elsaed 1,5
1Department of Anatomy, Faculty of Medicine, Taibah University, Madinah, Kingdom of Saudi Arabia, 2Department of Histology and Cell Biology, Faculty of Medicine, Mansoura University, Mansoura, Egypt, 3Department of Anatomy and Embryology, Faculty of Medicine, Ain Shams University, Cairo, Egypt, 4Department of Anatomy, Faculty of Medicine, Unaizah College of Medicine, Al Qassim University, Kingdom of Saudi Arabia, 5Anatomy and Embryology Department, Faculty of Medicine, Mansoura University, Mansoura, Egypt


Abstract

Introduction: Neuronal cell death and glial cell activation are the main pathological findings induced by seizures secondary to oxidative stress. Previous studies have explained neuronal cell death on the basis of cell necrosis and apoptosis. Recent studies have attributed the neuronal loss to autophagy. The proved antioxidant and antifibrotic effect of nilotinib favours its use in the management of epileptic seizures.

Aim of the study was to analyse the neuroprotective and antiepileptic effect of nilotinib and explain its mechanism of action.

Material and methods: Forty adult male rats were divided into four groups: control, pentylenetetrazol (PTZ) group (injected with PTZ 60 mg/kg, s.c.), pregabalin (Pregb)-PTZ group (pretreated with pregabalin daily 30 mg/kg; orally for 1 week) and nilotinib (NIL)-PTZ group (pretreated with nilotinib, 25 mg/kg daily for 1 week) prior to PTZ. Seizure latency was evaluated, the hippocampus tissue level of antioxidant enzymes was assessed. The histopathological changes in the hippocampus were studied using hematoxylin and eosin stain and immunohistochemical stain for brain-derived neurotrophic factor (BDNF), glial fibrillary acidic protein (GFAP), beclin-1, nuclear factor kappa-B (NF-κB) and Bcl-2-like protein 4 (BAX).

Results: Nilotinib induced an increase in the latency of seizures, enhanced the antioxidant levels of the γ-aminobutyric acid and nuclear factor (erythroid-derived 2)-like 2 activities together with the improvement of the hippocampal histology. A reduction was reported for BDNF, GFAP, beclin-1, NF-κB and BAX expression in nerve cells.

Conclusions: Nilotinib may have promising neuroprotective and antiepileptic effects against pentylenetetrazol-induced seizures through promoting the antioxidant, antifibrotic, anti-inflammatory, antiapoptotic pathways and inhibiting autophagy.

Key words: autophagy, apoptosis, hippocampus, epilepsy, nilotinib.

Communicating author
Wael M. Elsaed, Department of Anatomy, Faculty of Medicine, Taibah University, Madinah, Kingdom of Saudi Arabia, postal code: 42221, Madinah, KSA, e-mail: wzaarina@yahoo.com
Introduction

Epilepsy is a common chronic neurological disorder characterized by spontaneous recurrent seizures, affecting millions of people of all ages all over the world [11,20]. Epileptic seizures trigger neuronal death by a variety of mechanisms, of which, oxidative stress is the most common cause [2,32,51,77].

Previous studies reported the main pathological findings following epileptic seizures were neuronal cell death and glial cell activation [22,35]. The neurons of the hippocampus and the hilus of the dentate gyrus were the most affected brain areas [53]. Prevention of neuron loss and the glial cell activation has been a major aim of pharmaceutical research. Previous studies on epilepsy pathogenesis explained neuronal cell death which occurs in epileptic seizures and was based on cell necrosis and apoptosis [25]. Recent studies attributed the neuronal loss to a type of programmed cell death, called autophagy [64,68,86].

Autophagy occurs in all eukaryotic cells as an important process for the maintenance of the intracellular homeostasis. Simply it is the process by which cells break down and reuse their organelles and macronutrients to maintain survival. Unfortunately, autophagy can paradoxically progress to cell death with prolonged stress [6,46]. Many studies proved that modifying autophagy can mitigate the outcome of an epileptic seizure [31]. However, the detailed mechanism of autophagy accompanying epilepsy is still unclear [27]. Epileptic seizures are always associated with the formation of reactive oxygen species (ROS) and oxidative stress in animal models of epilepsy [8]. Antiepileptic drugs having antioxidant properties were proven to have a neuroprotective effect on animals [10,83].

Nilotinib (NIL) has been identified as a recent phenyl amino-pyrimidine derivative with marked selectivity against the Bcr-Abl tyrosine kinase [16]. Some studies proved that NIL has a favourable action in reducing oxidative stress and fibrosis in the rat model for renal disorders and liver injuries [38]. In Alzheimer’s disease, it was found that NIL reduced amyloid accumulation and autophagic activities which was explained by tyrosine kinase inhibition and restoration of parkin-beclin-1 interaction [52].

The antioxidant and antifibrotic effects of NIL favour its use in the management of epileptic seizures. However, studies investigating this effect on experimental models of epilepsy are limited. The possible neuroprotective effect of NIL as a new antiepileptic drug on experimentally-induced acute epilepsy in rats was the aim of this study.

Material and methods

Drugs

Pregabalin (LYRICA CR®-pregabalin tablet, 0071-1027, Pfizer Inc., Giza, Egypt) was suspended in 0.5% carboxymethyl cellulose (CMC).

Pentylenetetrazol (PTZ) (P6500, Millipore-Sigma, St Louis, MO, USA) was dissolved in 0.9% sterile normal saline.

Nilotinib was purchased from (TASIGNA® 200 mg capsule, Novartis Pharmaceuticals UK Ltd Dublin, Ireland). It was ground to form a white to yellowish powder and dissolved in 0.5% (CMC) and given to rats according to the determined dose.

Animals

Forty male adult rats (200 to 250 gm body weight) were obtained from the animal house of the Urology and Nephrology Centre, Mansoura University. Animals were kept under standard conditions of air and temperature throughout the experiment with diet and water obtained ad libitum. All steps of the experiment were in accordance to the regulations of the “Institutional Research Board” for Animal Experimentation, Faculty of Medicine, Mansoura University and the regulations of National Institute of Health Publications No. 80-23, Revised 1978. After one week of acclimatization the animals were divided into 4 groups (10 rats each) as follows:

The control group (n = 10) received 0.5% CMC as a vehicle one week before a subcutaneous (s.c.)
injection with 10 ml/kg body weight (b.w.) sterile normal saline.

The PTZ group (n = 10): the rats received 0.05% CMC one week before an s.c. injection with PTZ (60 mg/kg b.w.), according to de Oliveira et al. [23]. PTZ at a dose of 60 mg/kg b.w. was preferred as higher doses (70 mg and more) can cause more rapid death of rats.

The PTZ group (n = 10): the rats received 0.05% CMC one week before an s.c. injection with PTZ (60 mg/kg b.w.), according to de Oliveira et al. [23]. PTZ at a dose of 60 mg/kg b.w. was preferred as higher doses (70 mg and more) can cause more rapid death of rats.

The Pregb-PTZ group (n = 10): the rats were pretreated with Pregb (30 mg/kg b.w.) orally by a nasogastric tube according to Qureshi et al. [63] daily for one week before the injection with PTZ 60 mg/kg b.w. PTZ was injected 1 hour after last treatment with Pregb.

The NIL-PTZ group (n = 10): the rats were pretreated with NIL (25 mg/kg b.w.); orally by a nasogastric tube according to Karuppagounder et al. [43] daily for one week before the injection with PTZ. 60 mg/kg b.w. PTZ was injected 1 hour after the last treatment with NIL.

After PTZ injection, all rats were placed in a glass observation box and PTZ-induced seizure and latency time were recorded over 45 min. The seizure behaviour was classified according to Watanabe et al. [80] as follows: stage 0, no response; stage 1, ear and facial twitching; stage 2, myoclonic body jerks; stage 3, forelimb clonus, rearing; stage 4, clonic convulsions, turn on the side; and stage 5, generalized clonic convulsions, turn onto the back. The latencies to the onset of myoclonic jerks and generalized tonic-clonic seizures were recorded every minute for a total of 45 minutes [39].

Hippocampus tissue collections

One day after PTZ injection, the animals were humanely euthanized by sodium pentobarbital and then perfusion through the left ventricle was performed with 0.01 mol/l phosphate-buffered saline (PBS) followed by a fixative containing 4% paraformaldehyde, 0.05% glutaraldehyde and 0.2% picric acid in 0.1 mol/l phosphate buffer (pH 7.4). After decapitation of all rats, the brains were quickly removed and immersed into cold 0.05 M PBS to prevent tissue drying and dissected. The hind brains were removed and the two hemispheres were separated by sagittal incisions. Meningeal and any non-cortical forebrain tissues were removed cautiously by forceps to avoid injury or cutting of the hippocampus. Then ten left hemispheres were post-fixed in the same fixative of perfusion, then washed and embedded in paraffin to prepare paraffin blocks which were sectioned into 5-µm-thick sagittal sections. The sections were stained with hematoxylin and eosin stain [9] and immunohistochemical stain for localization of brain-derived neurotrophic factor (BDNF), GFAP, beclin 1, nuclear factor kappa-B (NF-κB) and Bcl-2-like protein 4 (BAX) antibodies immunorepression in the hippocampus tissues using avidin-biotin-peroxidase complex techniques [36].

Hippocampus homogenate preparation for biochemical assays

The other ten right hemispheres per group were rapidly frozen at −80°C before homogenization. Frozen hippocampi were then dissected away and homogenized in 0.05 M phosphate buffer, pH 7.4, (10% w/v) on ice, in a homogenizer (Thomas PA, USA) at 10,000-15,000 rpm for 10 seconds. The tubes with homogenates were kept in ice water for 30 min and then centrifuged at 4°C (3000 g for 10 min). The centrifuged supernatant of each sample was used to measure different biochemical parameters. Activities of hippocampus enzymatic antioxidants (superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GP-x)), non-enzymatic antioxidants (reduced glutathione (GSH) and lipid peroxidation by measuring malondialdehyde (MDA)) levels and nitrate/nitrite (NOx), an indicator of nitric oxide (NO) production were measured using commercially available Elisa kits (Bio-diagnostic Company, Giza, Egypt). Hippocampal contents of gamma-aminobutyric acid (GABA), and nuclear factor erythroid-derived 2-like 2 (Nrf2), were also evaluated using Elisa kits (MyBioSource, Inc., San Diego, CA, USA). The same procedure was used by Dong et al. [26].

Immunohistochemical stain

Paraffin sections were deparaffinized in xylene, rehydrated in descending ethyl alcohol gradient, rinsed in tap water and immersed in 3% Hydrogen peroxide (H₂O₂) in PBS for 10 min to block endogenous peroxidase. Sections were incubated overnight at 4°C with the respective primary antibodies: a rabbit anti-BDNF polyclonal IgG antibody (bs-4989R, concentration: 1 µg/µl, Bioss, Woburn, Massachusetts, USA) diluted at 1 : 200, rabbit anti-GFAP polyclonal IgG antibody (PA5-16291, ThermoFisher Scientific, Rockford, IL, USA), diluted at 1 : 100, rabbit anti-beclin-1 polyclonal IgG antibody (bs-1353R, concentration: 1 µg/µl,
Bioss, Woburn, Massachusetts, USA) diluted at 1 : 200, rabbit anti-p-NF-κB p65 polyclonal IgG antibody (H-286 sc-7151, concentration: 200 µg/ml, Bio-technology, Santa Cruz, CA, USA) diluted at 1 : 100 and mouse anti-BAX monoclonal IgG1 antibody (MA5-14003, clone; 6A7, concentration; 0.2 mg/ml, Thermo Fisher Scientific™, Waltham, MA, USA) diluted at 1 : 50.

Following incubation with the primary antibodies, the sections were then incubated with the appropriate secondary antibodies, Anti-Rabbit IgG F(ab')2, F(ab')2 2 fragment, highly cross absorbed-Biotin antibody produced in goat (SAB3700844, Millipore, Sigma, St Louis, USA) for rabbit all detected primary antibodies and goat anti-Mouse IgG (HRP,31430, Thermo Fisher Scientific™) for detection of BAX antibody. The sections were then incubated for 30 min at room temperature in horseradish peroxidase-avidin-biotin complex (Vectastain Elite, Vector, CA) then 3,3'-diaminobenzidine in H2O2 (DAB kit, Vector, CA) to visualize the reaction as a brown, insoluble product detected in the cytoplasm in BDNF, GFAP and beclin-1 immunostained tissues and in the nuclei in NF-κB and BAX immunostained sections. Sections were then counterstained with hematoxylin and mounted. Negative control sections were obtained following the same steps of staining, but without incubation with the primary antibodies.

**Morphometric study**

Pyramidal layer thickness (µm) in hippocampus Cornu Ammonis (CA1) was measured microscopically at 400× magnification using a H&E section from all rats (n = 40) from all four experimental groups. Ten measurements were taken from the widest and narrowest areas of CA1 pyramidal layer and the mean of these measurements was calculated for each H&E section. Three H&E sections from each block were examined (3 sections × 10 rats = 30). Immunostaining of cells in CA1 immunopositive expressing for BDNF, GFAP, beclin-1, NF-κB, BAX in CA1 were counted microscopically from IHC sections at 400× magnification. A section from each rat hippocampus block had three non-overlapping areas/section used for counting immunopositive cells for a total number of 30 measurements per experimental group (3 areas in a hippocampus section × 10 rats = 30). The sections were photographed using a colour video camera (digital camera CH-9435 DFC 290). Measurement results were analysed using Leica Qwin 500 Imaging Analysis System (Leica Microsystems Image Solutions, Cambridge, UK) within a frame area equal to 293.4288 µm². The morphometric study was done at the Image Analysis Unit, Anatomy Department, Faculty of Medicine, Taibah University, Al Madinah Al Monawarah, KSA.

**Statistical study**

All data were expressed as mean ± SEM. Statistical analysis was performed using IBM SPSS software version 21.00 (Chicago, Illinois, USA) – one-way analysis of variance (ANOVA) (where data are normally distributed and variances of samples are equal) followed by post-hoc and least significant difference (LSD) for inter-group comparison. P > 0.05, P ≤ 0.05 and P ≤ 0.001 were considered nonsignificant, significant and highly significant, respectively.

**Results**

**Effect of NIL versus Pregb pretreatment on the latencies (seizures threshold)/min to the onset of myoclonic and generalized tonic-clonic PTZ-induced convulsion**

Statistically, significant differences between groups were found (Fig. 1). No response was observed in control rats whereas PTZ-treated rats clearly

![Fig. 1. Representative bars show the latencies (seizures threshold)/min to the onset of myoclonic and generalized tonic-clonic in control, PTZ, Pregb-PTZ and NIL-PTZ groups. Data are represented as mean ±SEM. SEM – standard error of mean, P – probability, *significant, **highly significant, ***very highly significant. Tests used: One way ANOVA followed by post-hoc LSD test. P1 – significance when control vs. PTZ groups. P2 – Pregb-PTZ vs. PTZ groups. P3 – NIL-PTZ vs. PTZ groups. P4 – NIL-PTZ vs. Pregb-PTZ groups.](image-url)
exhibited severe convulsions where the onset of the first myoclonic seizures was after 1.38 ±0.6 min and the onset of the first generalized tonic-clonic seizure was after 2.42 ±0.05 min. On the other hand, pretreatment with Pregb and NIL one week before PTZ injection showed a very highly significant increase in the seizure threshold (increased latency for the onset of clonic – 15.39 ±1.24, P2 ≤ 0.0001 and 22 ±0.76, P3 ≤ 0.0001 respectively, and for generalized tonic-clonic phases of convulsion – 19.70 ±0.65, P2 ≤ 0.0001 and 32.60 ±0.79, P3 ≤ 0.0001 respectively) as compared to PTZ-treated animals. It was observed that NIL pretreatment was found to significantly (P4 ≤ 0.0001) delay the onset of seizures as compared to Pregb-treated rats, which indicates the protective effect of NIL against PTZ-induced seizures.

Effect of Pregb and NIL pretreatment on oxidative stress markers and antioxidant status in hippocampus tissue homogenates in PTZ-induced convulsion

A very highly significant elevation in hippocampal lipid peroxidation marker (MDA; 257 ±3.87, P ≤ 0.0001) with a marked decrease in levels of various hippocampal antioxidant markers such as SOD, GSH, GP-x and CAT; were observed in the Pregb-PTZ group (9.40 ±0.40, 114.10 ±3.33, 136 ±1.01 and 42.60 ±1.51, P ≤ 0.0001, respectively) as compared to PTZ-treated rats (59.40 ±1.40). This elevated NOx level was significantly decreased by pretreatment with Pregb-PTZ (99.50 ±2.20, P ≤ 0.0001) and NIL-PTZ (90.80 ±2.81, P3 ≤ 0.0001) as compared to the PTZ only treated group. A nonsignificant difference (P4 = 0.102) in NOx level was observed when the NIL-PTZ group compared to the Pregb-PTZ group (Table I).

Regarding the level of NOx, it was observed that PTZ-induced convulsion in the rats was accompanied by a marked elevation in hippocampal NOx (188.70 ±6.26, P1 ≤ 0.0001) as compared to control rats (59.40 ±1.40). This elevated NOx level was significantly decreased by pretreatment with Pregb-PTZ (99.50 ±2.20, P2 ≤ 0.0001) and NIL-PTZ (90.80 ±2.81, P3 ≤ 0.0001) as compared to the PTZ only treated group. A nonsignificant difference (P4 = 0.102) in NOx level was observed when the NIL-PTZ group compared to the Pregb-PTZ group (Table I).

Effect of Pregb versus NIL pretreatment on GABA and NRf2 in hippocampus tissue homogenates in PTZ-induced convulsion

A very highly significant reduction in the hippocampal tissue homogenates level of GABA and NRf2 (207 ±5.86 and 13.30 ±0.87, respectively, P1 ≤ 0.0001) were observed in the PTZ only treated group when

Table I. Mean concentration of malondialdehyde (MDA), superoxide dismutase (SOD), reduced glutathione (GSH), glutathione peroxidase (GP-x), catalase (CAT) and nitrate/nitrite (NOx) in the hippocampal tissue homogenates of control, PTZ, Pregb-PTZ and NIL-PTZ groups. N = 10/group

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA (nmol/gm tissue)</th>
<th>SOD (U/gm tissue)</th>
<th>GSH (μM/gm tissue)</th>
<th>GP-x (μM/gm tissue)</th>
<th>CAT (U/gm tissue)</th>
<th>NOx (μM/gm tissue)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>114.70 ±3.14</td>
<td>12.20 ±0.55</td>
<td>151.80 ±2.64</td>
<td>177.30 ±3.29</td>
<td>545.70 ±2.95</td>
<td>59.40 ±1.40</td>
</tr>
<tr>
<td>PTZ</td>
<td>257 ±3.87</td>
<td>≤ 0.0001**</td>
<td>72.60 ±1.93</td>
<td>32.30 ±1.01</td>
<td>42.60 ±1.51</td>
<td>188.70 ±6.26</td>
</tr>
<tr>
<td>P1</td>
<td>≤ 0.0001**</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
</tr>
<tr>
<td>Pregb-PTZ</td>
<td>194 ±2.72</td>
<td>9.40 ±0.40</td>
<td>141.70 ±3.33</td>
<td>137 ±2.35</td>
<td>259.10 ±5.19</td>
<td>99.50 ±2.20</td>
</tr>
<tr>
<td>P2</td>
<td>≤ 0.001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
</tr>
<tr>
<td>NIL-PTZ</td>
<td>136 ±2.34</td>
<td>10.60 ±0.48</td>
<td>140.60 ±4.00</td>
<td>152.70 ±4.25</td>
<td>490.90 ±2.16</td>
<td>90.80 ±2.81</td>
</tr>
<tr>
<td>P3</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
<td>≤ 0.0001***</td>
</tr>
<tr>
<td>P4</td>
<td>≤ 0.0001***</td>
<td>0.066</td>
<td>0.0001***</td>
<td>0.0001***</td>
<td>0.0001***</td>
<td>0.102</td>
</tr>
</tbody>
</table>

Data are represented as mean ± SEM. SEM – standard error of mean, P – probability *significant, **highly significant, ***very highly significant.

Tests used: One way ANOVA followed by post-hoc LSD test.

compared to the control group (519 ±14.70 and 33.20 ±1.70, respectively). On the other hand, marked elevation in their level was observed in Pregb-PTZ (393.70 ±11.76 and 24.70 ±1.05, P2 ≤0.0001) and NIL-PTZ groups (417.40 ±18.43 and 26.70 ±1.24, P3 ≤0.0001) when compared to the PTZ group. There was no significant difference in the GABA and NRf2 levels in NIL-PTZ rats as compared to rats in the Pregb treated group (P4 = 0.222 and 0.268, respectively) (Fig. 2).

**Effect of NIL and Pregb on PTZ-associated histological changes in Hippocampus cornu ammonis areas**

H&E sections of the control group showed four different hippocampus cornu ammonis areas: CA1, CA2, CA3 and CA4, and the dentate gyrus (DG) (Fig. 3A). Cornu ammonis (CA1) showed pyramid shaped nerve cell bodies with round vesicular nuclei and prominent nucleoli whereas glial cells were small with small round dark nuclei (Fig. 3A1). In the PTZ group, most nerve cells were smaller, distorted in shape, contained deeply stained nuclei and reduced pyramidal nerve cell layer thickness (Fig. 3B and B1). In Pregb-PTZ (Fig. 3C and C1) and NIL-PTZ (Fig. 3D and D1) sections, the shape for the majority of nerve cells and thickness of the pyramidal nerve cell layer were similar to those seen in the control group.

Statistically, the mean thickness of the pyramidal nerve cell layer of CA1 showed a very highly significant decrease in the PTZ only treated group (22.33 ±0.61, P1 ≤0.0001) as compared to the control group (46.10 ±0.97). Pregb-PTZ and NIL-PTZ groups showed a marked increase in the thickness of the pyramidal layer (32.63 ±1.03, P2 ≤0.0001 and 38.27 ±0.98, P3 ≤0.0001, respectively) as compared to the PTZ group. However, the NIL-PTZ group showed a much higher increase in the thickness of CA1 as compared to the Pregb-PTZ group (P4 ≤0.0001) (Fig. 4).

**Effect of NIL versus Pregb pretreatment on the number of cells expressing BDNF, GFAP, beclin-1, NF-κB and BAX markers in CA1 area with PTZ-induced convulsion**

Immunohistochemical staining on control rat sections showed some nerve cells in the CA1 pyramidal layer and exhibited BDNF expression in the cytoplasm (Fig. 5A). In the PTZ group, a strong BDNF expression was detected in most cells (Fig. 5B). Fewer BDNF positive cells were seen in Pregb-PTZ (Fig. 5C) and NIL-PTZ (Fig. 5D) groups as compared to PTZ treated rats. There were many GFAP positive star-shaped glial cells in control rats (Fig. 5E) with GFAP expressed in the cell body cytoplasm and processes. The PTZ group had numerous GFAP positive cells (Fig. 5F) whereas, in Pregb-PTZ (Fig. 5G) and NIL-PTZ (Fig. 5H) groups, these positive cells were closer in number to the control group. The control group only had a few pyramidal nerve cells expressing beclin-1 in the cytoplasm (Fig. 5I). Nearly all nerve cells in the PTZ group (Fig. 5J) were beclin-1 positive while only some cells were positive in Pregb-PTZ and NIL-PTZ groups (Fig. 5O, 5P) as compared to the PTZ group (Fig. 5N). Nerve cells positive cells in Pregb-PTZ and NIL-PTZ groups (Fig. 5O, 5P) as compared to the PTZ group (Fig. 5N). Nerve cells positive cells in Pregb-PTZ and NIL-PTZ groups (Fig. 5O, 5P) as compared to the PTZ group (Fig. 5N). Nerve cells positive cells in Pregb-PTZ and NIL-PTZ groups (Fig. 5O, 5P) as compared to the PTZ group (Fig. 5N). Nerve cells positive cells in Pregb-PTZ and NIL-PTZ groups (Fig. 5O, 5P) as compared to the PTZ group (Fig. 5N).

In the PTZ only treated group, a very highly significant increase in the mean number of cells expressing...
Fig. 3. A photomicrograph of rat hippocampus cornu ammonis areas CA1, CA2, CA3, and CA4 in the four experimental groups: (A, A1) Control; (B, B1) PTZ; (C, C1) Pregb-PTZ and (D, D1) NIL-PTZ show areas CA1, CA2, CA3. A, B, C, D points out dentate gyrus (DG), upper limb (UL), lower limb (LL). A, B, C show the stem (S) in curved dentate gyrus. A1) Control shows normal shaped pyramidal nerve cells with round vesicular nuclei, prominent nucleoli and many smaller glial cells with small round dark nuclei (long arrows). B1) PTZ shows reduced nerve cell layer thickness. Small cells have signs of degeneration, distorted shape, dark apoptotic nuclei and numerous glial cells. In (C1) Pregb-PTZ CA1 and (D1) NIL-PTZ CA1, most nerve cells have a normal shape (short arrows). The pyramidal nerve cell layer thickness appears preserved and mostly normal (white arrow heads – refer to normal pyramidal nerve cells, red arrow heads – refer to degenerated pyramidal nerve cells, and long arrows – refer to glial cells). H&E stain; A, B, C and D ×100; A1, B1, C1 and D1 ×400, scale bar = 20 µm.
Discussion

PTZ-induced convulsion is a widely accepted model for induction of seizures [85]. PTZ acts by antagonizing the action of GABA with accompanying oxidative stress caused by augmented production of superoxides and free radicals [72].

Pregb is a widely-used antiepileptic. It is a specific modulator of the alpha2 delta subunit of the presynaptic calcium channels with subsequent inhibition of neurotransmitter release [15]. It is used experimentally to compare newly proposed antiepileptics [63]. Nilotinib, a second-generation, selective tyrosine kinase inhibitor which was investigated in the present work to study its neuroprotective and hence its antiepileptic role in the PTZ epileptic rat model.

Although a considerable progress in understanding post-seizure cell death had been achieved, the exact mechanism underlying this process is still unclear [26]. The number and time of onset of seizures is an important marker of the drug activity. Our results showed a highly significant delay of onset of seizures in the NIL-PTZ group in comparison to Pregb-PTZ rats, indicate the protective effect of NIL against PTZ-induced seizures. The decreased latency of seizures was used as a marker of effective antiepileptic action [26,60].

Production of ROS and reduced antioxidant activities are the main causes of pathological changes in nervous tissue [3]. The whole brain is more liable to oxidative stress because of its high metabolic rate and low antioxidant capacity [78]. The hippocampal areas are more sensitive particularly to hypoxia [79]. This fact was explained by its low content of vitamin E which is an important antioxidant [21].

Oxidative damage in the brain induced by status epilepticus (SE) has been studied thoroughly where brain cells antioxidant defence was determined by measuring peroxidation (MDA) antioxidants (SOD, GSH, GP-x, and CAT). The present study showed a marked decrease in antioxidants in the PTZ group, marked improvement after Pregb and more improvement after NIL. Nilotinib was reported to have a similar potent antioxidant effect in renal, pulmonary and hepatic tissues [28,57,69].

The present work demonstrated a marked elevation in hippocampal NOx in PTZ treated rats. Other studies show that NO has a principal role in nervous tissue physiologic and pathological conditions [30]. The NO level was linked to generation and propagation of the epileptiform activity [41].

On the other hand, pretreatment with Pregb and NIL in the hippocampus was found to reverse an elevated NO level to an almost normal control level indicating that these drugs have powerful suppressive oxidant and anti-oxidant defence effects. This action can be further explained on the molecular level by the drug action on NF-κB and Nrf2 pathways [7,33]. It was found that Pregb attenuates the expression of p-NF-κB and restore the activity and diminution of phosphorylation of NOS. The net result will be protection of the neuronal cell from the harms of peroxynitrite generation and nitrosylation of mitochondrial metabolic enzymes [24]. These findings propose that NIL anti-epileptiform occurred through NO-mediated mechanisms.

The demonstrated low level of GABA and Nrf2 in the PTZ treated group and their marked eleva-
Fig. 5. A photomicrograph of CA1 area of rat hippocampus; anti-BDNF immunostained sections (A, B, C and D): A) Control shows cytoplasmic BDNF expression in some nerve cells, B) PTZ group has strong BDNF expression in the majority of nerve cells, C, D) Pregb-PTZ and NIL-PTZ groups show numerous BDNF positive cells. Anti-GFAP immunostained sections (E, F, G and H): E) Control has GFAP positive shaped glial cells with cytoplasmic expression in cell and processes, F) PTZ shows a strong GFAP expression by numerous cells. However, in (G) Pregb-PTZ and (H) NIL-PTZ groups, GFAP expression is similar to number of cells in controls. Anti-beclin-1 immunostained sections (I, J, K and L): I) Control has fewer nerve cells with cytoplasmic beclin-1 expression whereas most nerve cells in (J) PTZ express beclin-1 whereas (K) Pregb-PTZ and (L) NIL-PTZ only a few cells express beclin-1. Anti-NF-κB immunostained sections (M, N, O and P): M) Control nerve cells are nearly NF-κB negative, whereas the (N) PTZ group has strong NF-κB nuclear expression in the majority of nerve cells. In Pregb-PTZ (O) and NIL-PTZ (P) groups, NF-κB immunoreaction is detected in fewer number of cells as compared to PTZ group. In anti-BAX immunostained sections (Q, R, S and T): Q) control group show a negative BAX expression, whereas most of nerve cells in PTZ group (R) show a positive BAX expression in nuclei. In Pregb-PTZ (S) and NIL-PTZ (T) groups, only few cells expressed BAX. Arrow heads refer to nerve cells and arrows refer to glial cells.

Anti-BDNF immunostained sections (A, B, C and D ×400); anti-GFAP immunostained sections (E, F, G and H ×400); anti-beclin-1 immunostained sections (I, J, K and L ×400); anti-NF-κB immunostained sections (M, N, O and P ×400) and anti-BAX immunostained sections (Q, R, S and T ×400), scale bar = 20 µm.
tion observed in Pregb and NIL treated groups in the present work provide the evidence of the neuroprotective role offered by NIL. SE-induced increased ROS have the ability to modify the action of NRF2 and NF-κB transcription factors [17]. Both factors can modify the antioxidant defence and the pro-inflammatory mediator expression [1]. NRf2 was reported as an activator of cell defence against harmful oxidative stress [18]. One of these defences is the suppression of NF-κB in a very complicated unclear mechanism [17]. The normal level of GABA elevates the excitatory threshold of the neurons by inducing hypo polarization of the postsynaptic neurons [48]. PTZ induces seizures by antagonizing the action of GABA. This finding is always supported by the accompanying decrease in GABA concentration in brain tissue. The same finding was reported by previous researchers [48,54].

Degenerated nerve cells with small-size and distorted shape in addition to the decrease in the mean thickness of the pyramidal nerve cell layer of CA1, which were evident in the PTZ group in the current work was supported by similar findings in the hippocampus during the acute phase of seizures [67,82]. Neuronal
a pathologic finding of epileptic foci is associated cell bodies and processes of the astrocytes. It is PTZ was reported to induce astrogliosis [4,50,65]. injury is followed by microglial cell activation [24]. Neuronal of the filament protein system, it is involved in reg- ulation of the cell size and migration [59]. Neuronal formation of the hippocampus as proved in the present work. decrease to BDNF expression by the nerve cells of anti-epileptic effect of NIL could be supported by its bio-chemical result mentioned above. Study results showed that in PTZ-treated rats, nearly all nerve cells in the pyramid layer exhibited strong BDNF expression whereas a fewer cells were positive in Pregb-PTZ and NIL-PTZ groups. BDNF is one of the nerve growth factors that support the survival and the normal functions of nerve cells. The highest concentration of BDNF is known to be in the hippocampus [44]. Its expression was found to increase in various models of epilepsy [12] due to active neo-synthesis resulting from shift of BDNF from inside the neuron to the surrounding neuropil 2-3 hours after seizure [81]. BDNF enhances the presynaptic neurotransmitter release and the postsynaptic receptor response. This action ends in exaggerated impulse transmission and seizures caused by increasing the neuronal excitability and has a pro-epileptic role by potentiating excitatory synapses [75].

Moreover, BDNF inhibits GABA-dependent neurotransmission by downregulation of protein kinases. The enhanced expression of BDNF following PTZ administration was also reported [34]. Therefore the anti-epileptic effect of NIL could be supported by its decrease to BDNF expression by the nerve cells of the hippocampus as proved in the present work.

GFAP is a marker of astrocyte activation following injury or stress of the brain [87]. GFAP enhances the inflammatory response in the nervous tissue through inhibiting neuronal death [49]. Being a part of the filament protein system, it is involved in regulation of the cell size and migration [59]. Neuronal injury is followed by microglial cell activation [24]. PTZ was reported to induce astrogliosis [4,50,65].

Astrogliosis is a condition of hypertrophy of the cell bodies and processes of the astrocytes. It is a pathologic finding of epileptic foci is associated with an increased expression of glial fibrillary protein produced in response to astrocytes reaction to various insults [56]. This was in agreement with the present work which detected reactive astrogliosis in PTZ induced seizures and in turn increased GFAP immunoreactivity. On the other hand, the near normal expression observed in NIL-PTZ groups indicates less stress and tissue damage which in turn raises the probability of the anti-epileptic role of NIL.

A significant increase in beclin-1 expression was detected in the PTZ group indicating an autophagy pathway activation. On the other hand in Pregb-PTZ and NIL-PTZ groups, a fewer number of cells were beclin-1 immunopositive with decreased expression indicating the suppressor effect of Pregb and NIL on autophagy. One of the involved autophagic promoting proteins is beclin-1. Autophagy is a dynamic critical cellular activity that aims to turn over the cytoplasmic components [55]. The process involves the production of auto-phagosome; an intermediate cytoplasmic vacuolar structure that delivers the substrates to the lysosomes [5].

Oxidative stress, a major contributing factor in neurotoxicity, ROS production was shown to induce autophagy in many diseases rather than the nervous tissue but the neurons are particularly sensitive to this process [26]. This is explained by the fact that full differentiated nerve cells have a very specialized structure and will not be able to dilute the damaged organelles and proteins through a next cell division [70]. ROS regulates autophagy by promoting either cell survival or cell death, depending upon the severity of stress occurring with a particular disease [19,61]. Studies showed that in the brain tissue, the cellular autophagic activity may be rapidly increased in response to oxidative stress that occurs during seizures [58,74].

Similarly, autophagy was found to increase in experimental models of PTZ-induced seizures [89]. Pregb and NIL pretreated rats in the present work showed weak beclin-1 expression indicating suppression of autophagy, which agreed with another study that reported inhibited autophagy had a protective response against acute neuronal injury [88].

In the PTZ group, the majority of nerve cells exhibited a strong NF-κB expression whereas few NF-κB positive cells were observed in Pregb-PTZ and NIL-PTZ groups with much better results in NIL. NF-κB is an essential molecule for gene transcription and stability of mRNA [13]. Its expression increases secondary
to oxidative stress. Activation of NF-κB by oxidative stress is under control of NRF2 transcription activation [71]. Previous studies proved the ability of NRF2 activators to inhibit NF-κB [17,45]. Although NF-κB expression in epileptic patients increased, a possibility to increase its expression through activating the NRF2 pathway had been tried in scarce scientific studies [73]. In our results, the elevated NRF2 level in hippocampus after NIL treatment could explain the diminished NF-κB expression. These consequences indicate decreased oxidative stress and hence the antiepileptic effect of NIL.

The significant increase in the number of apoptotic nerve cells marked by BAX expression and the fewer cells in Pregb-PTZ and NIL-PTZ groups was supported by another study where the proapoptotic protein BAX had an important role in neuron death induced by seizures [29]. Although apoptosis and autophagy are different cellular activities, they share some regulatory steps [47]. The autophagic activity in the brain tissue can proceed to apoptosis including acute oxidative stress by an unclear mechanism [26]. The autophagic induced cell death is regulated by beclin-1 [42]. Its upregulation induced by ROS induces cell death [37]. Diminished expression of BAX in Pregb-PTZ and NIL-PTZ groups indicated a decreased cell death oxidative stress. Autophagy is most likely considered as a beneficial cellular response to stress which is the reverse of apoptosis [84].

The current results showed that NIL and/or Pregb treatments induced improvement in the autophagic process evidenced by increased beclin-1 expression and apoptosis as indicated by reduced BAX expression. Although a more or less protective effect of NIL and Pregb was the final result, NIL had better results regarding the increase in the seizure latency and enhance GABA and NRF2 activities, improved pyramidal cell layer histological picture, in addition to decreasing BDNF, beclin-1, NF-κB and BAX expression by nerve cells and GFAP by glial cells in CA1 when compared to Pregb treated rats.

Conclusions

Nilotinib may have a promising role in the management of epilepsy as it provides marked neuroprotective effects through promoting antioxidant, antifibrotic, anti-inflammatory and antiapoptotic pathways and inhibiting autophagy. Further experimental and clinical studies to confirm this beneficial effect are recommended.

Disclosure

The authors report no conflict of interest.

References


47. Koshal P, Kumar P. Neurochemical modulation involved in the beneficial effect of liraglutide, GLP-1 agonist on PTZ kindling...: The combination of art and information can enhance the enjoyment and effectiveness of learning. EMBO Rep 2015; 16: 547-552.


81. Wang L, Huang H, Cai H, Chen Y. Maternal hypoxia increases hippocampal cell susceptibility to ischemia after middle cere
81. Wetmore C, Olson L, Bean AJ. Regulation of brain-derived neurotrophic factor (BDNF) expression and release from hippocampal neurons is mediated by non-NMDA type glutamate receptors. J Neurosci 1994; 14: 1688-1700.