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INVESTIGATION OF SEIZURE ACTIVITY AFTER CYCLIC NUCLEOTIDE PHOSPHODIESTERASE INHIBITION WITH SECOND MESSENGER AND CALCIUM ION CHANNEL INHIBITION IN MICE

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ABSTRACT: The role of PDE-4 inhibitor etazolate, was evaluated in the presence of PDE-7 inhibitor, BRL-50481, in animal models of epilepsy. Seizures were induced in the animals by subjecting them to injection of chemical convulsants, Pilocarpine, Kainic acid (KA) and maximal electroshock (MES). The combination of etazolate and BRL50481 treated mice showed a significant (P<0.001) quick onset of action, jerky movements and convulsion when compared to gabapentin. The combination of etazolate and sGC inhibitor, methylene blue (MB) treated mice showed a significant (P<0.001) delay in onset of action, jerky movements and convulsion when compare to gabapentin as well as against the combination of etazolate with BRL 50481.The present study mainly highlights the individual effects of etazolate and combination with BRL-50481 potentiates (P<0.001) the onset of seizure activity against all models of convulsion. The study mainly comprises the onset of seizures, mortality/recovery, percentage of prevention of seizures (anti-convulsant) and total duration of convulsive time. The total convulsive time was prolonged significantly (P<0.05 and P<0.01) in combination of methylene blue with etazolate treated (28.59% and 35.15 %) groups, compared to DMSO received group (100%) in the MES model. In the same way, the combination of calcium channel modulator (CCM) and calcium channel blocker (CCB) amiodarone and nifedipine respectively, with etazolate showed a significant (P<0.001) delay in onset of seizures, compared to DMSO and etazolate treated groups in all models of epilepsy. This confirms that both CCM and CCB possess anti-convulsant activity. Finally, the study reveals that identification of new cAMP mediated phosphodiesterases family members offers a potential new therapy for epilepsy management in future.

Key words : Guanylate cyclase, PDE, etazolate, BRL50481, methylene blue, amiodarone, nifedipine, pro-convulsant, anti-convulsant.

INTRODUCTION

Epilepsy is a chronic neurological disorder that consists of recurrent seizures. It is a disorder of brain characterized by unpredictable and periodic occurrence of a transient alteration of behavior due to the disordered, abnormal, hypersynchronous and rhythmic firing of populations of brain cortical neurons (1). Incidence of epilepsy in developed countries is approximately 50 per 100,000 while that of developing country is 100 per 100,000 (2). While research into the mechanisms of epilepsy has centered on electrophysiology over the last couple of decades, progress has recently been made in the fields of biochemical molecular biology. For instance, the functions of biological membranes include the actions of receptors, enzymes, ion channels, etc. Therefore, it is proposed that the disturbances of the membrane functions are possibly associated with the provocation of epileptic seizures. Many receptors, when stimulated by various neurotransmitters and hormones, may stimulate second messengers to elicit biological reactions. It has been observed that the presently available antiepileptic drugs are unable to control seizures effectively in as many as 25% of the patients (3). The conventional anti-epileptic agents like phenytoin, carbamazepine and sodium valporate carry with them several serious side effects notably neurotoxicity (4). As majority of antiepileptic drugs are consumed life long, concomitant administration of other drugs predisposes to the risk of drug interaction. However, newer anti-epileptics like gabapentin, vigabatrin, lamotrigine, etc., are used supplemental to the conventional agents (1).

A common way to explain seizures in a normal individual is that a disruption has occurred in the normal balance of excitation and inhibition. The fact that multiple mechanisms exists is not surprising given the varied ways the normal nervous system controls this balance. In contrast, understanding seizures in the brain of an individual with epilepsy is more difficult because seizures are typically superimposed on an altered nervous system. The different environment includes diverse changes, making mechanistic predictions a challenge. Understanding the mechanisms of seizures in an individual with epilepsy is also more complex than understanding the mechanisms of seizures in a normal individual because epilepsy is not necessarily a static condition but can continue to evolve over the lifespan (1). The cyclic guanosine 3',5'- monophosphate (cGMP) plays a major role in the production of seizure activity. An elevation in cGMP content has been reported in the brain cortex accompanying chemically induced epileptic activity (5, 6 &7). The guanylate cyclase (GC), an important transmembrane enzyme possesses certain activity in the brain, which promotes the intracellular level of cGMP, from guanosine triphosphate (GTP) (7). In epileptic conditions, markedly elevated cGMP concentration was found in the hippocampus, with lesser elevations in striatum and cortex (8, 9). Cyclic GMP plays a key function by controlling a wide variety of cellular processes (10), also which acts as a ubiquitous second messenger and modulator of signal transduction processes (5). This cGMP is generated by the action of guanylate cyclase (10) and degraded by hydrolysis process, which is regulated by a family of cyclic nucleotide phosphodiesterases (PDEs) (11, 12).

PDE enzymes regulate the degradation of cyclic GMP a product of the guanylate cyclase activation and could contribute to the pathophysiology of the seizure mechanisms. PDE enzymes are responsible for the hydrolysis of the cyclic nucleotides and therefore have a critical role in regulating intracellular levels of the second messengers cAMP, cGMP, and hence cell function as well as downstream cell signalling in the various body systems (13). Recent evidence that the cyclic nucleotide phosphodiesterases exist in several molecular forms and that these isozymes are unequally distributed in various tissue (14). Twelve members of the PDE family have been identified and these can be further divided into 50 isoforms of subtypes and splice variants (15). Out of the twelve PDE gene families, PDE 4 is cAMP- specific (16 &17) PDE-5 & 6 belongs to cGMP-specific (18-20), PDE-7&8 are cAMP-specific (21,22), PDE-10&11 related with cGMP-sensitive and dual specificity (23,24). Clinical signs of epilepsy arise from the intermittent, excessively synchronized activity of group of neurons. Different neurotransmitters and neuro- modulators are known to play a significant role in the system of excitation (25). In this study, we used etazolate, is a pyrazolopyridine compound belongs to phosphodiesterase 4 enzyme (PDE4) inhibitor, and $GABA_A$ receptor modulator that also stimulates alpha-secretase activity and neurotrophic soluble amyloid precursor protein (sAPPα) production (26-28).

On the other hand, modifications of ion channels in the brain are being recognized as causes of hyperexcitability in the central nervous system and hence of an ancient phenotype, epilepsy. The bursting of neurons is the cardinal feature of all seizure disorders (29). Calcium channels are important modulators of membrane excitability, transmitter release. This hyper-excitability may result in anti-seizure activity (30).

Thus, it is necessary to conduct research for an antiepileptic agent that is highly efficacious as well as safe in terms of drug related toxicity. The aim of treating an epileptic is not only to abolish the occurrence of seizures but also to lead a self sustained life. The present study will examine the role of cyclic nucleotide phosphodiesterase- 4 inhibitor in the generation of seizure threshold. We used pharmacological tools like BRL-50481 (PDE-7 inhibitor), methylene blue (MB, guanylate cyclase inhibitor), amiodarone (calcium channel modulator) and nifedipine (calcium channel blocker) to block and attenuate the effect of PDE and evaluate the effect on chemical convulsant and maximal electroshock induced seizures in mice.

MATERIALS AND METHODS

Animals Used:

Swiss Albino mice of either sex weighing between 24-26 g were utilized for this study. The animals were placed randomly and allocated to treatment groups in polypropylene cages with paddy husk as bedding. Animals were housed at temperature of 24 ± 2 ^{0}C and relative humidity of 30-70%. A 12:12 dark: light cycle was followed during the experiments. All the animals were allowed free access to water *ad libitum* and fed with standard commercial pelleted rat chaw (M/s. Hindustan Lever Ltd., Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethical Committee and were in accordance with the guidelines of the CPCSEA.

Drugs and Chemicals:

The following drugs and chemicals were used for conducting this study. 10% w/v of dimethyl sulfoxide (DMSO) Sigma, USA, gabapentin (Micro labs Ltd., Bangalore, India), etazolate (Tocris Bioscience, UK), methylene blue (Sigma, USA), Zonisamide (Sun Pharma, Mumbai, India), BRL50481 (Tocris Bioscience, UK), Amiodarone (Zydus Alidac, Ahemedabad, India), Nifedipine (Bayer India Ltd., Mumbai, India) and Except gabapentin, methylene blue and zonisamide, other drugs are soluble in DMSO, rest of others are soluble in sterile water for injection.

I. Chemoshock Method

A. Kainic acid (KA) induced seizure model in mice:

Swice Albino mice were divided into seven groups with six animals (n=6) in each. Treatment protocol and group description is mentioned as follows,

Group - I: Mice served as solvent control, received 10 % w/v of dimethylsulfoxide [DMSO] (5 ml/kg, i.p.),
Group - II:Mice received gabapentin (2.5 mg/kg, i.p.), treated as positive control,
Group - III:Mice received etazolate (7 mg/kg, i.p.), a PDE-4 inhibitor,
Group - IV:Mice received etazolate (7 mg/kg, i.p.) along with BRL-50481(2 mg/kg, i.p.) a PDE-7 inhibitor,
Group - V: Mice received etazolate (7 mg/kg, i.p.) along with methylene blue (50 mg/kg, i.p.) an soluble GC inhibitor,
Group -VI:Mice received etazolate (7 mg/kg, i.p.) along with amiodarone (40 mg/kg, i.p.) a calcium channel modulator,
Group -VII:Mice received etazolate (7 mg/kg, i.p.) along with nifedipine (20 mg/kg, i.p.) a calcium channel blocker,

B. Pilocarpine induced seizure model in mice:

Swice Albino mice were divided into seven groups with six animals (n=6) in each. Treatment protocol and group description is mentioned as follows,

Group - I:Mice served as solvent control, received 10 % w/v of dimethylsulfoxide [DMSO] (5 ml/kg, i.p.),
Group - II:Mice received gabapentin (2.5 mg/kg, i.p.), treated as positive control,
Group - III:Mice received etazolate (7 mg/kg, i.p.), a PDE-4 inhibitor,
Group - IV:Mice received etazolate (7 mg/kg, i.p.) along with BRL-50481 (2 mg/kg, i.p.) a PDE-7 inhibitor,
Group - V:Mice received etazolate (7 mg/kg, i.p.) along with methylene blue (50 mg/kg, i.p.) an soluble GC inhibitor,
Group -VI:Mice received etazolate (7 mg/kg, i.p.) along with amiodarone (40 mg/kg, i.p.) a calcium channel modulator,
Group -VI:Mice received etazolate (7 mg/kg, i.p.) along with nifedipine (20 mg/kg, i.p.) a calcium channel blocker,

All the drugs were administered intraperitoneally 60 min prior to the administration kainic acid (20 mg/kg, i.p.) and pilocarpine (500 mg/kg, i.p.). The animals were observed for 1 hour by placing in a separate cage. The onset time of various phases of convulsions like action, jerky movement, convulsions and recovery / mortality were noted in seconds as per the method shown by Yemitan and Salahdeen, 2005; Salahdeen and Yemiten, 2006 (31,32).

II. Maximal electroshock (MES) method for mice

Swice Albino mice were divided into seven groups with six animals (n=6) in each. Treatment protocol and group description is mentioned as follows,

Group - I:Mice served as solvent control, received 10 % w/v of dimethylsulfoxide [DMSO] (5 ml/kg, i.p.),
Group - II:Mice received zonisamide (35 mg/kg, i.p.), treated as positive control,
Group - III:Mice received etazolate (7 mg/kg, i.p.), a PDE-4 inhibitor,
Group - IV:Mice received etazolate (7 mg/kg, i.p.) along with BRL-50481 (2 mg/kg, i.p.) a PDE-7 inhibitor,
Group - V:Mice received etazolate (7 mg/kg, i.p.) along with methylene blue (50 mg/kg, i.p.) an soluble GC inhibitor,
Group -VI:Mice received etazolate (7 mg/kg, i.p.) along with amiodarone (40 mg/kg, i.p.) a calcium channel modulator,
Group -VII:Mice received etazolate (7 mg/kg, i.p.) along with nifedipine (20 mg/kg, i.p.) a calcium channel blocker,

All the drugs will be administered intraperitoneally 60 min prior to the electroshock. The electroshock was be induced in animals by passing a current of 55 mA for 0.2 sec duration through electroconvulsiometer (Techno India) using corneal electrodes. The incidence of seizures, tonic limb flexion, tonic extensor, clonus, stupor and recovery / mortality of the animals were observed and tabulated as per Achliya *et al*., 2005 (33).

Statistical analysis

All the data were expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey-Kramer Multiple Comparisons Test was applied for the statistical analysis of the data in order to compare the inter group differences and one way analysis of variance (ANOVA) followed by Dunnett's test was also applied to compare with DMSO treated group for estimation of total duration of convulsion time in seconds and percentage of change from control were analysed. P values <0.05 were considered as statistically significant.

RESULTS

Evaluation of onset of seizures

I. Chemoshock Method

A. Kainic acid induced seizure model in mice:

Table 1 summarizes the data attained from experiments conducted with PDE- 4 inhibitor with the presence of PDE-7 inhibitor, sGC inhibitor, a calcium channel modulator and a calcium channel blocker on kainic acid (20 mg/kg, i.p.) induced seizures in mice**.** The PDE-4 inhibitor, etazolate (7 mg/kg, i.p.) alone receiving mice showed a significant (P<0.001) quick onset of seizure threshold when compared to gabapentin. The combination of etazolate (7 mg/kg, i.p.) and BRL50481 (2 mg/kg, i.p.) received mice showed a significant (P<0.001) quick onset of action, jerky movements and convulsion when compare to gabapentin. The combination of etazolate (7 mg/kg, i.p.) and methylene blue (50 mg/kg, i.p.) treated mice group showed a significant (P<0.001) delay in onset of action, jerky movements and convulsion when compare to gabapentin, etazolate as well as combination of etazolate with BRL 50481. Combined effect of etazolate with amiodarone (40 mg/kg, i.p.) showed a significant delay in onset of seizure activity when compared to gabapentin (P<0.001), etazolate with BRL 50481 (P<0.001) received mice. There was no much difference observed in between etazolate with calcium channel blocker and modulator.

Table 1 : Effect of PDE-4 inhibitor along with PDE-7 inhibitor, soluble guanylate cyclase (sGC) inhibitor, calcium channel modulator and blocker on kainic acid (20 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Kainic acid (20 mg/kg, i.p.)		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p) Solvent Control	204.36 ± 9.12	225.17 ± 10.14	252.47 ± 8.12
II	Gabapentin (2.5 mg/kg, i.p.) Positive Control	390.43 ± 12.14***	420.16 ± 17.4***	480.53 ± 16.17***
III	Etazolate (7 mg/kg, i.p.) PDE-4 inhibitor	150.42 ± 8.17*,ΔΔΔ	184.67 ± 7.67ΔΔΔ	214.73 ± 11.67ΔΔΔ
IV	Etazolate (7 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	143.81 ± 6.74**,ΔΔΔ	163.12 ± 8.67*,ΔΔΔ	190.36 ± 15.67*,ΔΔΔ
V	Etazolate (7 mg/kg, i.p.) + Methylene blue (50 mg/kg, i.p.) sGC inhibitor	220.40 ± 14.17ΔΔΔ,ΨΨΨ,♣♣♣	252.53 ± 12.13ΔΔΔ,ΨΨ,♣♣♣	296.74 ± 11.41ΔΔΔ,ΨΨ,♣♣♣
VI	Etazolate (7 mg/kg, i.p.) + Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	201.38 ± 10.14ΔΔΔ,Ψ,♣♣	244.63 ± 14.7ΔΔΔ,Ψ,♣♣♣	281.67 ± 12.35ΔΔΔ,Ψ,♣♣♣
VII	Etazolate (7 mg/kg, i.p.) + Nifedipine (20 mg/kg, i.p.) Calcium channel blocker	215.71 ± 11.72ΔΔΔ,ΨΨ,♣♣♣	235.19 ± 13.8ΔΔΔ,♣♣	259.61 ± 12.67ΔΔΔ,♣♣

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 60 mins prior to chemical -convulsant injection of kainicacid (20 mg/kg, i.p.). *, ** and *** denotes p<0.05, p<0.01 and p<0.001, respectively, compared with DMSO received group, ΔΔΔ denotes p<0.001 compared with gabapentin received group, Ψ, ΨΨ and ΨΨΨ denotes p<0.05, p<0.01 and p<0.001, respectively, compared with etazolate received group, ♣♣ and ♣♣♣ denotes p<0.01 and p<0.001 compared with etazolate and BRL 50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

B. Pilocarpine induced seizure model in mice:

Table 2 summarizes the data obtained from experiments conducted with etazolate, a PDE- 4 inhibitor with the presence of BRL 50481, a PDE-7 inhibitor, methylene blue, a sGC inhibitor, amiodarone, a calcium channel modulator and nifedipine, a calcium channel blocker on pilocarpine (500 mg/kg, i.p.) induced seizures in mice**.** The etazolate (7 mg/kg, i.p.) alone treated mice group showed a significant (P<0.001) quick onset of seizure activity when compared to gabapentin. The combination of etazolate (7 mg/kg, i.p.) and BRL50481 (2 mg/kg, i.p.) received mice group showed a significant (P<0.001) quick onset of action, jerky movements and convulsion when compared to gabapentin received group. The combination of etazolate (7 mg/kg, i.p.) and methylene blue (50 mg/kg, i.p.) treated mice group showed a significant delay in onset of action, jerky movements and convulsion when compare to gabapentin (P<0.01), etazolate alone (P<0.001) as well as combination of etazolate with BRL 50481 (P<0.001). Combined effect of etazolate with amiodarone (40 mg/kg, i.p.) showed a significant (P<0.01) delay in onset of seizure activity when compared to gabapentin (P<0.001), etazolate alone (P<0.05), etazolate with BRL 50481 (P<0.01) treated mice group.

Table 2: Effect of PDE-4 inhibitor along with PDE-7 inhibitor, soluble guanylate cyclase (sGC) inhibitor, calcium channel modulator and blocker on pilocarpine (500 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Pilocarpine (500 mg/kg, i.p.)		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p) Solvent Control	140.71 ± 4.2	164.71 ± 7.87	190.78 ± 8.72
II	Gabapentin (2.5 mg/kg, i.p.) Positive Control	241.43 ± 8.71***	260.79 ± 9.87***	287.91 ± 8.79***
III	Etazolate (7 mg/kg, i.p.) PDE-4 inhibitor	121.47 ± 6.71ΔΔΔ	147.24 ± 8.67ΔΔΔ	169.73 ± 9.73ΔΔΔ
IV	Etazolate (7 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	107.81 ± 4.67ΔΔΔ	135.67 ± 7.45ΔΔΔ	156.31 ± 7.39ΔΔΔ
V	Etazolate (7 mg/kg, i.p.) + Methylene blue (50 mg/kg, i.p.) sGC inhibitor	188.68 ± 9.43**,ΔΔΔ,ΨΨΨ,♣♣♣	235.13 ± 6.12***,ΨΨΨ,♣♣♣	256.71 ± 9.31***,ΔΔΔ,ΨΨΨ,♣♣♣
VI	Etazolate (7 mg/kg, i.p.) + Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	164.57 ± 8.52ΔΔΔ,ΨΨ,♣♣♣	187.32 ± 7.37ΔΔΔ,Ψ,♣♣, ΣΣ	206.47 ± 10.14♣, Σ
VII	Etazolate (7 mg/kg, i.p.) + Nifedipine (20 mg/kg, i.p.) Calcium channel blocker	184.32 ± 9.21**,ΔΔΔ,ΨΨΨ,♣♣♣	228.70 ± 9.87***,ΨΨΨ,♣♣♣,Θ	247.67 ± 12.63**,ΔΔΔ,ΨΨΨ, ♣♣♣

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 60 mins prior to chemical -convulsant injection of pilocarpine (500 mg/kg, i.p.). ** and *** denotes p<0.01 and p<0.001, respectively, compared with DMSO received group, ΔΔΔ denotes p<0.001 compared with gabapentin received group, Ψ, ΨΨ and ΨΨΨ denotes p<0.05, p<0.01 and p<0.001, respectively, compared with etazolate received group, ♣, ♣♣ and ♣♣♣ denotes p<0.05, p<0.01 and p<0.001 compared with etazolate and BRL 50481 received group, Σ and ΣΣ denotes p<0.05 and p<0.01, respectively, compared with etazolate and methylene blue received group, Θ denotes p<0.05 compared with etazolate and amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 4 summarizes the total duration of convulsion, percentage change from control, mortality and protection in incredible levels of percentage. The total convulsive time was prolonged significantly (P<0.05) in combination of etazolate with methylene blue, amiodarone and nifedipine treated (30.55% , 29.55% and 27.98%) group, compared to DMSO received group (100%). The data shows that 83.3% of protections of animals were noticed in etazolate with methylene blue and amiodarone treated groups against pilocarpine induced seizures in mice.

The results show that there was an increase in seizure activity (12.88% and 19.13%) when etazolate alone as well as etazolate with BRL-50481 treated respectively, while compared to DMSO treated group (100%). The data shown in Table 4.also demonstrates that i.p administration of etazolate with methylene blue (50 mg/kg, i.p), amiodarone (40 mg/kg, i.p.) greatly increased the anti-convulsant activity (P<0.05) along with higher protection (83.3%) range. Simultaneously, the combined effect of etazolate with exogenously administered nifedipine (20 mg/kg, i.p.) showed a significant (P<0.05) anti-convulsant activity with moderate protection (66.7%) range respectively (Table 4).

II. Maximal electroshock (MES) method for mice:

Table 5 depicts the data obtained from experiments conducted with etazolate, a PDE- 4 inhibitor with the presence of BRL 50481, a PDE-7 inhibitor, methylene blue, a sGC inhibitor, amiodarone, a calcium channel modulator and nifedipine, a calcium channel blocker on maximal electroshock induced seizures in mice**.** It is evident from the data shown in Table 5 that combination of etazolate and BRL50481 effectively (P<0.001) decreased the tonic limb flexion, tonic extensor, clonus and stupor stage of convulsion, compared to zonisamide treated mice. The overall highlights of Table 5 exhibits the combined effects of etazolate with methylene blue received groups, potentiates the delay onset of seizure activity against in MES induced convulsion compared to DMSO (P<0.001), zonisamide (P<0.001), etazolate (P<0.001) as well as etazolate with BRL 50481 (P<0.001). Also this emphasizes that etazolate with methylene blue delays the onset of seizure activity as well as prolongs the total duration of convulsive time (Table 5). The mice received etazolate along with amiodarone, which potentiates the delay of onset of seizure threshold when compared to DMSO (P<0.001), zonisamide (P<0.001), etazolate alone (P<0.001) and etazolate with BRL 50481 (P<0.001).

Table 3 : Effect of PDE-4 inhibitor along with PDE-7 inhibitor, soluble guanylate cyclase (sGC) inhibitor, calcium channel modulator and blocker on MES (55 mA for 0.2 secs) induced seizures in mice (n=6).

sGroup	Treatment	Onset time (sec) in various phases of convulsion			
		Tonic limb flexion	Tonic extensor	Clonus	Stupor
I	10% w/v DMSO (5 ml/kg, i.p) Solvent Control	9.40 ± 0.21	19.52 ± 1.3	32.34 9 ± 1.47	55.3 ± 1.41
II	Zonisamide (35 mg/kg, i.p.) Positive Control	47.63 ± 0.91***	64.63 ± 3.4***	76.53 ± 4.3***	120.1 ± 5.5***
III	Etazolate (7 mg/kg, i.p.) PDE-4 inhibitor	7.56 ± 0.13ΔΔΔ	13.31 ± 0.17ΔΔΔ	26.47 ± 0.19ΔΔΔ	45.70 ± 1.73ΔΔΔ
IV	Etazolate (7 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p.) PDE-7 inhibitor	6.43 ± 0.13**,ΔΔΔ	10.47 ± 0.12***,ΔΔΔ	20.51 ± 0.23*,ΔΔΔ	39.32 ± 1.63*,ΔΔΔ
V	Etazolate (7 mg/kg, i.p.) + Methylene blue (50 mg/kg, i.p.) sGC inhibitor	40.31 ± 0.41***,ΔΔΔ, ΨΨΨ,♣♣♣	53.71 ± 1.8***,ΔΔΔ, ΨΨΨ,♣♣♣	65.13 ± 2.2***,Δ, ΨΨΨ,♣♣♣	85.40 ± 3.40***,ΔΔΔ, ΨΨΨ,♣♣♣
VI	Etazolate (7 mg/kg, i.p.) + Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	39.41 ± 0.67***,ΔΔΔ, ΨΨΨ,♣♣♣	49.67 ± 0.72***,ΔΔΔ, ΨΨΨ,♣♣♣	61.43 ± 1.72***,ΔΔΔ, ΨΨΨ,♣♣♣	76.47 ± 1.63***,ΔΔΔ,ΨΨΨ, ♣♣♣
VII	Etazolate (7 mg/kg, i.p.) + Nifedipine (20 mg/kg, i.p.) Calcium channel blocker	36.13 ± 0.53***,ΔΔΔ,ΨΨΨ, ♣♣♣, ΣΣΣ, ΘΘ	47.31 ± 1.65***,ΔΔΔ, ΨΨΨ,♣♣♣	57.18 ± 2.84***,ΔΔΔ, ΨΨΨ,♣♣♣	73.47 ± 3.71**,ΔΔΔ, ΨΨΨ,♣♣♣

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 60 mins prior to maximal electroshock (55 mA for 0.2 secs). *, ** and *** denotes p<0.05, p<0.01 and p<0.001, respectively, compared with DMSO received group, ΔΔ and ΔΔΔ denotes p<0.01 and p<0.001, respectively, compared with zonisamide received group, ΨΨΨ denotes p<0.001 compared with etazolate received group, ♣♣♣ denotes p<0.001 compared with etazolate and BRL 50481 received group, ΣΣΣ denotes p<0.001, respectively, compared with etazolate and methylene blue received group, ΘΘ denotes p<0.01 compared with etazolate and amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 4: **Effect of drugs on pilocarpine induced seizures in mice.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	222.0 ± 14.3	100	100	Nil	Nil
II	Gabapentin	304.7 ± 16.7	37.25	16.7	83.3	$P<0.01$
III	Etazolate	193.4 ± 11.3	12.88	66.7	33.3	NS
IV	Etazolate + BRL-50481	179.7 ± 12.3	19.13	83.3	16.7	NS
V	Etazolate + Methylene blue	290.1 ± 14.8	30.55	16.7	83.3	$P<0.05$
VI	Etazolate + Amiodarone	229.4 ± 16.9	29.55	16.7	83.3	$P<0.05$
VII	Etazolate + Nifedipine	264.6 ± 17.7	27.98	33.3	66.7	$P<0.05$

The group of mice (n=6) were injected with 500 mg/kg, i.p. of pilocarpine for induction of convulsion and the total convulsive time was estimated. A value of $P<0.05$ was considered significant Vs DMSO group, NS= $P > 0.05$. All the drugs were administered intraperitoneally. The drugs used were administered in the following doses. DMSO (5 ml/kg, i.p.), gabapentin (2.5 mg/kg, i.p.), etazolate (7 mg/kg, i.p.), methylene blue (50 mg/kg, i.p.), BRL-50481 (2 mg/kg, i.p.), amiodarone (40 mg/kg, i.p.) and nifedipine (20 mg/kg, i.p.). (One way ANOVA followed by Dunnett's test compared with DMSO treated mice)

Table 5 summarizes the total duration of convulsion, percentage change from control, mortality and protection in incredible levels of percentage. The total convulsive time was prolonged significantly ($P<0.01$) in combination of etazolate with methylene blue treated (35.15%) group, compared to DMSO received group (100%). Similarly, the total convulsive time was prolonged significantly ($P<0.05$) in combination of etazolate with amiodarone and nifedipine treated (24.39% and 24.22%) group, compared to DMSO received group (100%). The data shows that 83.3% of protections of animals were noticed in etazolate with methylene blue and amiodarone treated groups against MES induced seizures in mice. The results show that there was an increase in seizure activity (22.30% and 21.96%) when etazolate alone as well as etazolate with BRL-50481 treated respectively, while compare to DMSO received group (100%). The data shown in Table 5 also demonstrates that i.p administration of etazolate with methylene blue (50 mg/kg, i.p.) greatly increased the anti-convulsant activity ($P<0.01$) along with higher protection (83.3%) range. Simultaneously, the combined effect of etazolate with exogenously administered amiodarone (40 mg/kg, i.p.) and nifedipine (20 mg/kg, i.p.) showed a significant ($P<0.05$ and $P<0.05$) anti-convulsant activity with fine protection (83.3% and 66.7%) range respectively (Table 5).

Table 5: **Effect of drugs on maximal electroshock induced seizures in mice.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	231.8 ± 13.1	100	100	Nil	Nil
II	Zonisamide	295.69 ± 14.9	27.15	16.7	83.3	$P<0.05$
III	Etazolate	180.7 ± 13.7	22.30	83.3	16.7	NS
IV	Etazolate + BRL-50481	181.5 ± 11.2	21.96	83.3	16.7	NS
V	Etazolate + Methylene blue	314.3 ± 17.3	35.15	16.7	83.3	$P<0.01$
VI	Etazolate + Amiodarone	289.29 ± 16.7	24.39	16.7	83.3	$P<0.05$
VII	Etazolate + Nifedipine	288.89 ± 14.8	24.22	33.3	66.7	$P<0.05$

The group of mice (n=6) were subjected to 55 mA (0.2 sec) electroshock and the total convulsive time was estimated. A value of $P<0.05$ was considered significant Vs DMSO group, NS= $P > 0.05$. All the drugs were administered intraperitoneally. The drugs used were administered in the following doses. DMSO (5 ml/kg, i.p.), zonisamide (35 mg/kg, i.p.), etazolate (7 mg/kg, i.p.), methylene blue (50 mg/kg, i.p.), BRL-50481 (2 mg/kg, i.p.), amiodarone (40 mg/kg, i.p.) and nifedipine (20 mg/kg, i.p.). (One way ANOVA followed by Dunnett's test compared with DMSO treated mice).

DISCUSSION

According to our study and based on our results it can be stated that Table 1, 2, 3 & 4 revealed that etazolate, a PDE- 4 inhibitor with the presence of BRL 50481, a PDE-7 inhibitor, methylene blue, a sGC inhibitor, amiodarone, a calcium channel modulator and nifedipine, a calcium channel blocker on kainic acid, pilocarpine and MES induced seizures in mice**.** Methylene blue, is a guanylate cyclase inhibitor belongs to thaizine dye, the combination of sGC inhibitor, methylene blue with etazolate showed significant (P<0.001) delay in onset of seizures, compared to control, standard received groups. This confirms that sGC inhibitor having anti-convulsant activity. NO, a soluble gas with a very short half life is produced form L-ARG by three different nitric oxide synthase (NOS) isoenzymes (34). In the CNS, NO is mostly produced by the neuronal NOS (nNOS), a Ca-dependent enzyme, acting as neurotransmitter and an intracellular messenger in many physiological and pathological reactions (35). Inducible NOS (iNOS), a Ca-independent enzyme, is involved in various inflammation and pathophysiological processes including ischemia, stroke, trauma, infection, and autoimmunity (36). NO is also a major stimulator of cGMP generation via guanylate cyclase, which is assumed to play a major role in seizure (37). The study also demonstrates that etazolate alone and etazolate with BRL50481 greatly enhances quick onset of seizures. On the other hand, this study strongly suggests that etazolate having pro-convulsant activity.

Etazolate is a pyrazolopyridine compound (38), which selectively modulates GABA receptor. However, it should be noted that etazolate is also known to have a phosphodiesterase 4 enzyme (PDE4) inhibitor activity (61). Therefore, we cannot exclude a convergence of the two pathways and the possibility that some of the effects shown here by etazolate are also mediated through elevated cAMP levels in cortical neurons and not the $GABA_A$ receptor (39-41). In our study the etazolate showed (Table 1-4) proconvulsant activity, so as enhances the mortality range upto 66.7% while it is treated with alone as well as combination with BRL-50481. The effect of etazolate demonstrates, which exerts a neuroprotective effect against Aβ via the $GABA_A$ receptor. The neuroprotective effects of etazolate were fully blocked by $GABA_A$ receptor antagonists indicating that this neuroprotection was due to $GABA_A$ receptor signalling.

In general, Glutamate is the principal excitatory, amino acid based neurotransmitter in the brain. Glutamate receptor over stimulation increases intracellular calcium by directly opening ion channels and secondarily affecting calcium homeostatic mechanisms. The initial glutamate receptor opening of the sodium/calcium channels not only allows the influx of calcium but also causes membrane depolarization. The depolarization would in turn activate the voltage-dependent calcium channels. Glutamate excitotoxicity is the final common pathway resulting in neuronal injury for many seemingly unrelated disorders, including ischemia, trauma, seizures, hypoglycaemia, hypoxia and even some neural degenerative disorders (42). Both neuronal calcium currents and neurotransmitter release play pivotal roles in the generation and propagation of seizures. (43-46). Several studies have demonstrated that dihydropyridine (DHP) calcium channel antagonists possess anti-seizure/anti-convulsant activity (43, 44).

In our study we used nifedipine, is DHPs calcium channel antagonist. Nifedipine, in combination with etazolate exerts a very good remarkable change in delay onset of seizures in both models, when compared to DMSO as well as etazolate alone treated group (Table 1-4). Table 4-5 summarizes, the protection range of animals were significantly (P<0.05) good, when compared to DMSO against etazolate with nifedipine. This indicates, nifedipine possess a strong anti-convulsant activity. A study described the low doses of structurally different DHPs calcium channel antagonist and its enatiomer such as nifedipine and amlodipine possess anti-convulsant activity (47).

Amiodarone is a multiple ion-channel blocker drug, inhibiting sodium and calcium inward currents and potassium outward current, and having noncompetitive adrenergic blocking effect (48-50). As like nifedpine, amiodarone also promotes significant (P<0.01) anti-seizure activity and showed good percentage of protection of animals in both models (Table 1-3). Inhibition or excitation of a neuron depends on concentrations of intracellular Ca^{2+} and Na^{+} and extracellular K^{+}, and also on balance between GABAergic and adenosinergic inhibitory transmissions and glutamatergic excitatory transmission. In epilepsy and sleeping, ion channels and neurotransmitters have important roles. Since amiodarone has multiple ion-channel blocker properties and increases the inhibitory neurotransmitters, in this study we utilized amiodarone has possible anticonvulsant effects in various epilepsy models.

It has been reported that PTZ has induced seizures by inhibiting GABA pathway in CNS (51,52), acting as an antagonist at GABA-A receptor complex (53), increasing the central noradrenergic activity (51, 54) and increasing the intracellular calcium and extracellular potassium ion concentrations (55-57). Turovaya *et al*. (58) reported that amiodarone had increased the concentrations of inhibitory GABA and glycine and had decreased those of excitatory aspartate and glutamate in rat medulla oblongata. In our study, the anticonvulsant effect of amiodarone may be partially due to blockage of ion channels (Na+, K+ and Ca2+) and/or its involvement in noradrenergic pathways and/or in GABAergic pathway and/or its anti-oxidant effect. Some anticonvulsant drugs act by means of ion channels. Anticonvulsant activities of calcium channel blockers had been shown in in vivo and in vitro experiments (59, 60).

Thus, in conclusion the study reflects, (i) the combination of sGC inhibitor, methylene blue with etazolate showed a delay onset of seizures, compared to other groups. This confirms that sGC inhibitor having anti-convulsant activity, (ii) similarly, the combination of CCM and CCB amiodarone and nifedipine respectively, with etazolate showed a delay onset of seizures, compared to DMSO and etazolate received groups. This confirms that both CCM and CCB possess anti-convulsant activity, (iii) the study also demonstrates that etazolate alone and etazolate in combination with BRL50481 greatly enhances quick onset of seizures. So, this study strongly suggests that etazolate having proconvulsant activity, (iv) the occurrence of high percentage of mortality is associated with quick onset of seizures resembles proconvulsant action in etazolate alone as well as combination with BRL-50481 treated groups in both animal models of epilepsy, (v) This study also reflects the identification and investigation of new cAMP mediated phosphodiesterases family members which may offer a new strategy for improvement of brain function and possible new therapy for epilepsy in future.

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