ROLE OF ADENYLATE CYCLASE, GUANYLATE CYCLASE ENZYME AND PHOSPHODIESTERASE ISOZYME MODULATION IN ANIMAL MODELS OF EPILEPSY

Thesis submitted to THE TAMILNADU Dr. M.G.R MEDICAL UNIVERSITY, CHENNAI in partial fulfillment of the requirements for the award of

DOCTOR OF PHILOSOPHY

In Pharmacology

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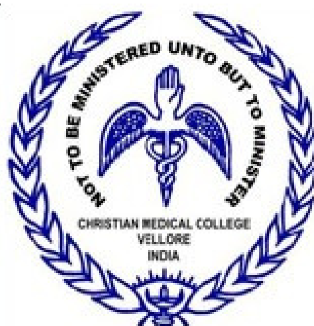

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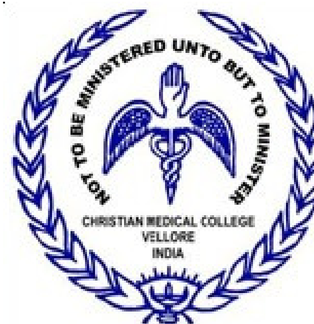

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CERTIFICATE

I certify that Mr. J.NANDHAKUMAR, carried out the research work on **"Role of adenylate cyclase, guanylate cyclase enzyme and phosphodiesterase isozyme modulation in animal models of epilepsy"** for the degree of Doctor of Philosophy in pharmacology of The Tamilnadu Dr. M.G.R Medical University, CHENNAI for the requisite period under the regulation in force and the thesis is a bonafide record of the work done by him under my supervision and guidance. This work is original and has not formed the basis of the award to the candidate of any degree, diploma, associateship, fellowship or other similar title.

I state that the entire research of the thesis represents the independent work of Mr.J.Nandhakumar, and all the experimental techniques employed in this work were actually undertaken by the candidate himself under my guidance.

(Dr. Manoj G Tyagi)
Guide

DECLARATION

I declare that **“Role of adenylate cyclase, guanylate cyclase enzyme and phosphodiesterase isozyme modulation in animal models of epilepsy”** is my own work, that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

J.NANDHAKUMAR October 2011

Signed……………………………..

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ABBREVIATIONS

8-MM-IBMX	-	8-methoxymethyl 3-iso butyl-1-methylxanthine
AC	-	Adenylate cyclase
Ach	-	Acetyl choline
AED	-	Anti-epileptic drug
AMPA	-	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ANOVA	-	One way analysis of variance
ATP	-	Adenosine tri phosphate
CA	-	Cornu ammonis
cAMP	-	Cyclic adenosine mono phosphate
CCA	-	Calcium channel activator
CCB	-	Calcium channel blocker
CCM	-	Calcium channel modulator
cGMP	-	Cyclic guanosine mono phosphate
CNS	-	Central nervous system
CO	-	Carbon Monoxide
DG	-	Dentate Gyrus
DHP	-	Dihydropyridine
DMSO	-	Dimethyl sulfoxide
EEG	-	Electroencephalogram
EHNA	-	Erythro-9-(2-hydroxy-3-nonyl) adenine
GABA	-	ϒ-amino butyric acid
GDP	-	Guanosine diphosphate
GTP	-	Guanosine triphosphate
i.p.	-	Intra peritoneal
ICV	-	Intracerebroventricular
INH	-	Isoniazid

IP_3	-	Inositol-1,4,5-triphosphate
KA	-	Kainic acid
MB	-	Methylene blue
MES	-	Maximal electroshock method
MLCK	-	Myosin light chain kinase
MTZ	-	Metrazol
NHE	-	Sodium-hydrogen exchanger
NMDA	-	N-Methyl-D-Aspartate
NO	-	Nitric Oxide
PDEs	-	Phosphodiesterase
PKA	-	Protein kinase A
PKC	-	Protein kinase C
PKG	-	Protein kinase G
PLA_2	-	Phospholipase A2
MLC	-	Myosin light chain
PLC	-	Phospholipase C
PMCA	-	Plasma membrane Ca^{2+}-ATPase
PTx	-	Picrotoxin
PTZ	-	Pentylenetetrazole
RyR	-	Ryanodine receptors
SCM	-	Sodium channel modulator
sGC	-	Soluble guanylate cyclase

1. INTRODUCTION

There are enormous advances in understanding of the basic biological processes that contribute to human disorders, although a detailed understanding of the processes underlying complex central nervous system (CNS) diseases remains elusive. The various CNS target disorders have been identified and listed below.

C N S Disorders

Neurodegenerative

- Alzheimer’s
- Parkinson’s
- Stroke and head trauma
- Huntington’s
- Amyotrophic lateral sclerosis

Psychiatric

- Depression
- Anxiety
- Schizophrenia

Pain

- Anesthesia
- Neuropathic
- Inflammatory
- Migraine

Epilepsy

Out of these, epilepsy is a common health problem throughout the world. It affects more than 50 million people worldwide, 5 million of them have seizures more than once per month (Porter, 1988). Approximately, 5-10% of the population usually develops seizure at least once during their lifetime, with the highest incidence occurring in early childhood and late adulthood (Lowenstein, 2001). At least 8% of the general population will have at least one seizure in a life time. However, it is possible to have a seizure and not have epilepsy. The rate of recurrence of a first unprovoked seizure within 5 yrs range between

23% and 80%. Children with an idiopathic first seizure and a normal electroencephalogram (EEG) have a particularly favorable prognosis. Some seizures may occur as single events resulting from withdrawal of CNS depressants (e.g., alcohol, barbiturates, and other drugs) or during acute illness (such as meningitis or encephalitis) or toxic conditions (e.g., uremia or eclampsia). Some patients will have seizures only associated with fever. These febrile seizures do not constitute epilepsy (Sander, 2003). The age adjusted incidence of epilepsy is 44 per 100,000 person- years. Each year, about 1,25,000 new epilepsy cases occur; of these, 30% are in people younger than age 18 at the time of diagnosis. There is a bimodal distribution in the occurrence of the first seizure, with one peak occurring in newborn and young children and the second peak occurring in patients older than age 65 yrs. The relatively high frequency of epilepsy in the elderly is now being recognized. At least 10% of patients in long-term care facilities are taking at least one anti-epileptic drug. Varieties of factors have been shown to precipitate seizures in susceptible individuals. Hormonal changes occurring around the time of menses, puberty, or pregnancy have been associated with the onset of or an increased frequency of seizures. A careful history should be obtained from patients presenting with seizures because theophylline, alcohol, high dose phenothiazines, anti-depressants (especially maprotiline or buproprion), and street drug use have been associated with provoking seizures. Also, AEDs in toxic concentrations may cause seizures in certain patients. Immunizations have not been associated with an increased risk of epilepsy.

Epilepsy is defined as a group of disorders of CNS characterized by paroxysmal cerebral dysrhythmia, manifesting as brief episodes (seizures) of loss or disturbances of consciousness with or without characteristic body movement (convulsion), sensory or psychiatric phenomena (Tripathy, 2003). Seizures results from an imbalance between excitatory and inhibitory systems in brain. Some anti-epileptic medications enhance inhibitory influences in the CNS by facilitating ϒ-amino butyric acid (GABA) neurotransmission, where as others reduce excitatory input by inhibitory glutamic acid transmitter activity. Most types of epilepsy are due to a disruption in the balance between these two actions. Although it is likely that virtually every brain neurotransmitter and

neuromodulator is involved in epilepsy, glutamic acid and GABA play prominent roles because they are the major excitatory and inhibitory transmitters in brain, respectively (Enna, 1998).

Anatomy of Hippocampus:

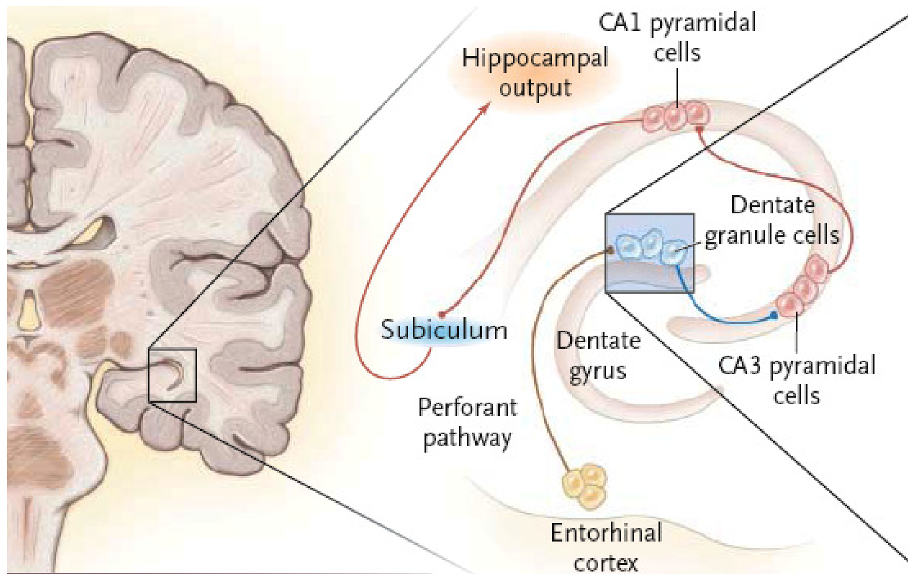


Fig.1: Anatomy structure of hippocampus.

The hippocampus is a major component of the brains of humans and other mammals. It belongs to the limbic system and plays important roles in the consolidation of information from short-term memory to long-term memory and spatial navigation. Like the cerebral cortex, with which it is closely associated, it is a paired structure, with mirror-image halves in the left and right sides of the brain. In humans and other primates, the hippocampus is located inside the medial temporal lobe, beneath the cortical surface.. The hippocampus is composed of multiple subfields. It contains two interlocking parts, termed as dentate gyrus and the cornu ammonis (literally "Amun's horns", abbreviated CA). The dentate gyrus contains the fascia dentata and the hilus, while CA is differentiated into fields CA1, CA2, and CA3.

Cut in cross section, the hippocampus is a C-shaped structure that resembles a ram's horns. The name *cornu ammonis* refers to the Egyptian deity Amun, who has the

head of a ram. The horned appearance of the hippocampus is caused by cell density differentials and the existence of varying degrees of neuronal fibers. In rodents, the hippocampus is positioned so that, roughly, one end is near the top of the head (the dorsal or septal end) and one end near the bottom of the head (the ventral or temporal end). As shown in the figure, the structure itself is curved and subfields or regions are defined along the curve, from CA4 through CA1 (only CA3 and CA1 are labeled). The CA regions are also structured depthwise in clearly defined strata (or layers) such as alveus, Stratum oriens, Stratum pyramidale, Stratum lucidum, Stratum radiatum, Stratum lacunosum, Stratum moleculare, hippocampal sulcus (Chang, 2003)

Basic Mechanisms underlying seizures and epilepsy:

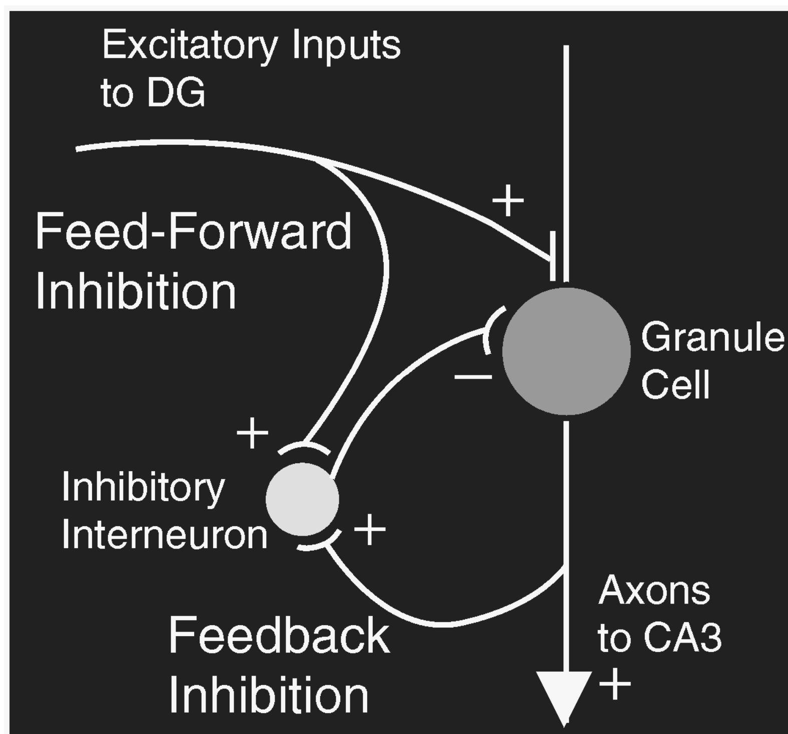


Fig. 2: Illustration of simplified hippocampal circuit.

This fig.2 explains the feedback and feed-forward inhibition, illustrated via cartoon and schematic of simplified hippocampal circuit. Starting at the dentate gyrus and working inward along the S-curve of the hippocampus means traversing a series of

narrow zones. The first of these, the dentate gyrus (DG), is actually a separate structure, a tightly packed layer of small *granule cells* wrapped around the end of the hippocampus proper, forming a pointed wedge in some cross-sections, a semicircle in others. Next come a series of *Cornu Ammonis* areas: first CA4 (which underlies the dentate gyrus), then CA3, then a very small zone called CA2, then CA1. The CA areas are all filled with densely packed pyramidal cells similar to those found in the neocortex. After CA1 comes an area called the subiculum. After this comes a pair of ill-defined areas called the presubiculum and parasubiculum, then a transition to the cortex proper (mostly the entorhinal area of the cortex). Most anatomists use the term "hippocampus proper" to refer to the four CA fields, and "hippocampal formation" to refer to the hippocampus proper plus dentate gyrus and subiculum (Babb, 1987).

Causes of seizures:

Seizures occur because small numbers of neurons discharge abnormally. Anything that disrupts the normal homeostasis of the neuron and disturbs its stability may trigger abnormal activity and seizures. The causes of seizures in the elderly may be multifactorial and include cerebrovascular disease (both ischemic and hemorrhagic stroke), neurodegenerative disorders, tumor, head trauma, metabolic disorders, and CNS infections. In some cases, if an etiology can be found and corrected, the patient will not require chronic AED (Anti- epileptic drug) treatment. In many cases, patients will present with seizures that do not have an identifiable cause and thus have idiopathic epilepsy. The incidence of idiopathic epilepsy is higher in children (Annegeres, 2001)

Brain Neurotransmitters:

The primary process whereby information is conveyed from the presynaptic nerve terminal to the target cell, which may be another neuron, glial cell, or a muscle, is by an endogenous chemical neurotransmitter. The mechanism of action, as well as side effects, of most drugs used to treat CNS disorders are related to their ability to alter neurotransmitter effects in brain (Cooper, 1991). Thus, knowledge of the basic principles of chemical neurotransmission, and of the major classes of neurotransmitters, provides a context for understanding the therapeutic actions of most centrally active agents.

The three major categories of substances that act as neurotransmitters are (1) **amino acids** (primarily glutamic acid, GABA, aspartic acid & glycine), (2) **peptides** (vasopressin, somatostatin, neurotensin, etc.) and (3) **monoamines** (norepinephrine, dopamine & serotonin) plus **acetylcholine**. The major "workhorse" neurotransmitters of the brain are glutamic acid (=glutamate) and GABA. The monoamines & acetylcholine perform specialized modulating functions, often confined to specific structures. The peptides perform specialized functions in the hypothalamus or act as co-factors elsewhere in the brain.

Involvement of Glutamate in Epilepsy:

Glutamate is the most common neurotransmitter in the brain. It is always excitatory, usually due to simple receptors that increase the flow of positive ions by opening ion-channels. Glutamate stimulation is terminated by a (chloride-independent) membrane transport system that is only used for re-absorbing glutamate & aspartate across the pre-synaptic membrane. Glutamate & aspartate re-enter the cell by a transporter driven by the high extracellular concentrations of Na^+ and the high intracellular concentrations of K^+. Soduim enters the cell along with the amino acids and potassium levels the cell, much the way a pulley couples the lifting of a light weight with the fall of a heavier weight. Thus, glutamate/asparate entry is indirectly powered by the ATP-driven Na^+-K^+-ase (sodium pump) which creates the high ion concentration gradients.

Two groups of glutamate receptors are,

- **Ionotropic—fast synaptic transmission**
  - Three subtypes – AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor), kainate, NMDA (N-Methyl-D-Aspartate)
  - Glutamate-gated cation channels
- **Metabotropic—slow synaptic transmission**
  - G-protein coupled, regulation of second messengers (cAMP and phospholipase C)
  - Modulation of synaptic activity

- Modulation of glutamate receptors
    - Glycine, polyamine sites, Zinc, redox site

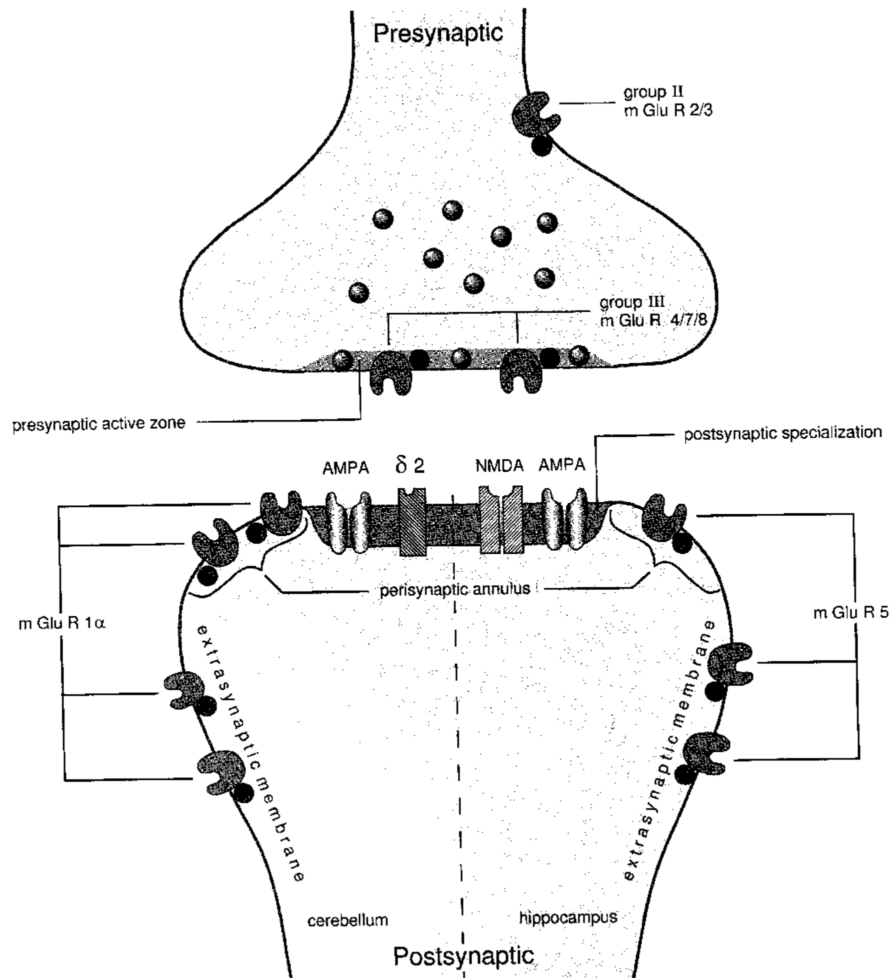


Fig.3: Diagram of the various glutamate receptor subtypes and locations (Takumi *et al,* 1998)

Involvement of GABA in Epilepsy:

GABA is the major inhibitory neurotransmitter of the brain, occurring in 30-40% of all synapses (second only to glutamate as a major brain neurotransmitter). It is most highly concentrated in the substantia nigra & globus pallidus nuclei of the basal ganglia, followed by the hypothalamus, the periaqueductal grey matter ("central grey") and the hippocampus. The GABA concentration in the brain is 200-1000 times greater than that of the monoamines or acetylcholine. GABA is somewhat unique among neurotransmitters insofar as it is commonly inactivated (after release into the synapse) by active transport into the astrocyte glial cells that are closely associated with synapses. Both glutamate and GABA are synthesized in the brain from the Krebs citric acid molecule alpha-keto glutarate — a reaction known as the "GABA shunt". GABA is synthesized from glutamic acid and is catabolized back into the citric acid cycle. The vitamin B6 derivative **pyridoxal phosphate** is a cofactor in the synthesis of GABA, which is why seizures occur in Vitamin B6 deficiency. GABA levels rise when the citric acid cycle activity is low (i.e, when cell energy usage is low), and the resultant generalized GABA inhibitory effect on the brain neurons can be protective during hypoxia or ischemia.

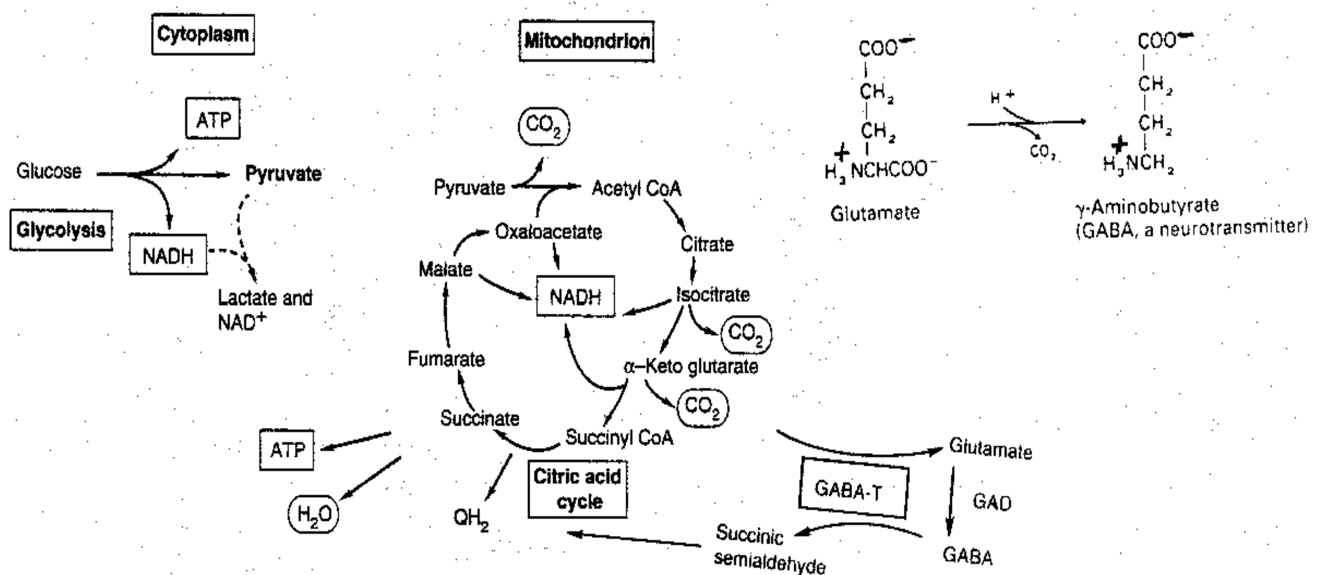


Fig. 4: Illustrates the biochemistry of GABA.

GABA is the major inhibitory neurotransmitter in the CNS.

- Two types of receptors
  - $GABA_A$—post-synaptic, specific recognition sites, linked to CI- channel
  - $GABA_B$ —presynaptic autoreceptors that reduce transmitter release by decreasing calcium influx, postsynaptic coupled to G-proteins to increase K+ current

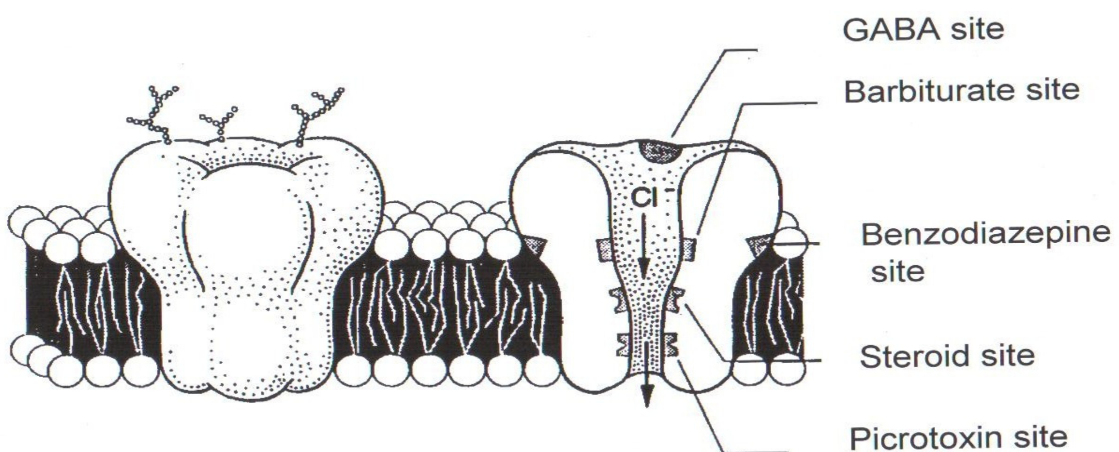


Fig.5: Diagram of the $GABA_A$ receptor (Olsen, 1995)

Pathophysiology of Seizures:

Seizure activity is characterized by paroxysmal discharge occurring synchronously in large population of cortical neurons (Najm, 2001). This is characterized on EEG as a sharp wave or *spike*. The basic physiology of a seizure episode is traceable to an unstable cell membrane or its surrounding supportive cells. The seizure originates from the gray matter of any cortical or perhaps subcortical area. Initially, a small number of neurons fire abnormally. Normal membrane conductances and inhibitory synaptic currents breaks down, and excess excitability spreads, either locally to produce a focal seizure or more widely to produce a generalized seizure. This onset propagates by

physiologic pathways to involve adjacent or remote areas. The clinical manifestations depend on the site the focus, the degree of irritability of the surrounding area of the brain, the intensity of the impulse (Najm, 2001).

An abnormality of potassium conductance, a defect in the voltage-sensitive ion channels, or a deficiency in the membrane ATPases linked to ion transport may result in neuronal membrane instability and a seizure. Selected neurotransmitter (e.g., Glutamate, aspartate, acetylcholine, norepinephrine, histamine, corticotrophin- releasing factor, purines, peptides, cyokines, and steroid hormones) enhances the excitability and propagation of neuronal activity, where as ϒ-aminobutyric acid (GABA) and dopamine inhibit neuronal activity and propagation. A relative deficiency of inhibitory neurotransmitters such as GABA or an increase in excitatory neurotransmitters such as glutamate would promote abnormal neuronal activity.

Normal neuronal activity also depends on an adequate supply of glucose, oxygen, sodium, potassium, chloride, calcium, and amino acids. Systemic pH is also a factor in precipitating seizures. The different kinds of epilepsies probably arise from different neurophysiologic abnormalities (Najm, 2001).

Cellular Mechanisms of Seizure Generation:

Excitation (too much)

- Ionic—inward Na^{+}, Ca^{++} currents
- Neurotransmitter—glutamate, aspartate

Inhibition (too little)

- Ionic—inward Cl^{-}, outward K^{+} currents
- Neurotransmitter—GABA

Normal CNS Function:

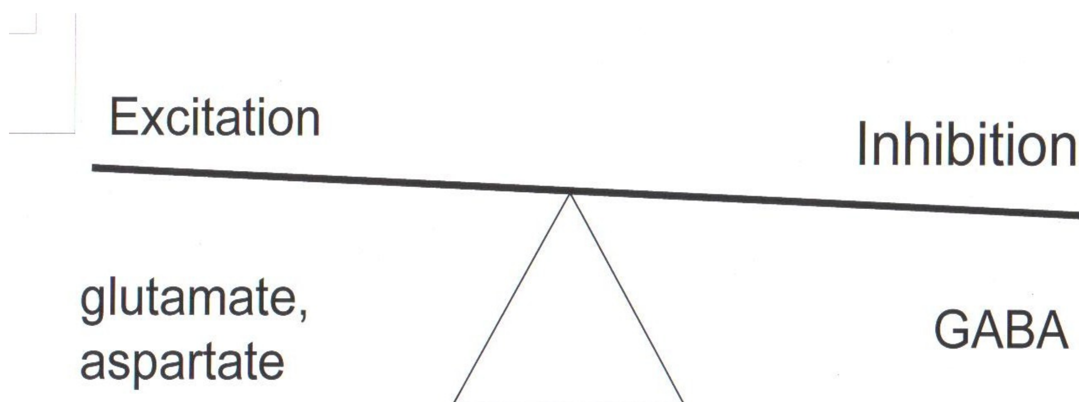


Fig. 6: Illustrates the normal CNS function

Hyperexcitability reflects both increased excitation and decreased inhibition:

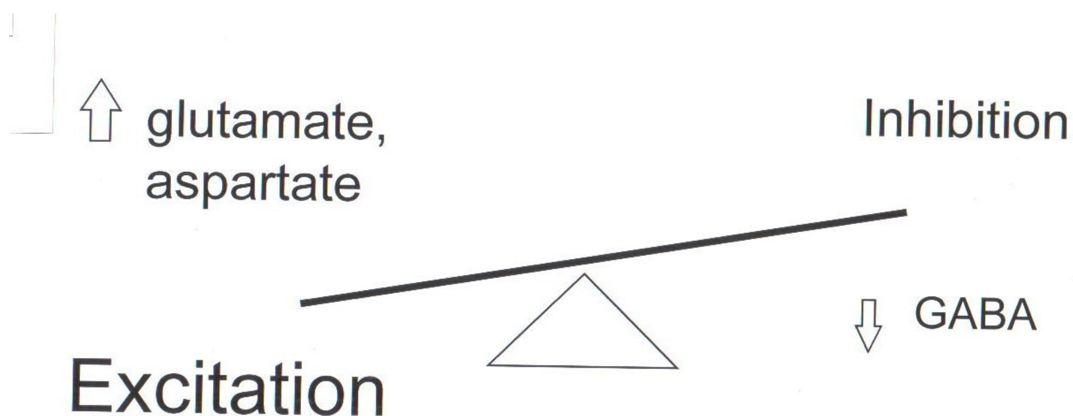


Fig. 7: Illustrates the hyperexcitability state cause epilepsy.

Role of Second Messenger System in Epilepsy (Rang, 2006):

In cell physiology, a secondary or second messenger system is a method of cellular signaling whereby a diffusible signaling molecule is rapidly produced/secreted, which can then go on to activate effector proteins within the cell to exert a cellular response. Secondary messengers are a component of signal transduction cascades.

Secondary messenger system can be activated by diverse means, either by

1. Activation of enzymes that synthesize them, as is the case with the activation of cyclases that synthesize cyclic nucleotides, or
2. By opening of ion channels to allow influx of metal ions, such as in Ca^{2+} signaling. These small molecules may then go on to exert their effect by binding to and activating effector molecules such as
    - protein kinases,
    - ion channels, and
    - a variety of other proteins,

Thus continuing the signaling cascade.

Types of secondary messenger molecules

There are three basic types of secondary messenger molecules:

- **Hydrophobic molecules:** Water-insoluble molecules, like diacylglycerol, and phosphatidylinositols, which are membrane-associated and diffuse from the plasma membrane into the intermembrane space where they can reach and regulate membrane-associated *effector proteins.*
- **Hydrophilic molecules:** Water-soluble molecules, like cAMP, cGMP, IP_3, and Ca^{2+} that are located within the cytosol
- **Gases**: Nitric Oxide (NO) and carbon monoxide (CO), which can diffuse both through cytosol and across cellular membranes.

These intracellular messengers have some properties in common:

- They can be synthesized/released and broken down again in specific reactions by enzymes or ion channels.
- Some (like Ca^{2+}) can be stored in special organelles and quickly released when needed.

- Their production/release and destruction can be *localized*, enabling the cell to limit space and time of signal activity.

Common mechanisms of secondary messenger systems:

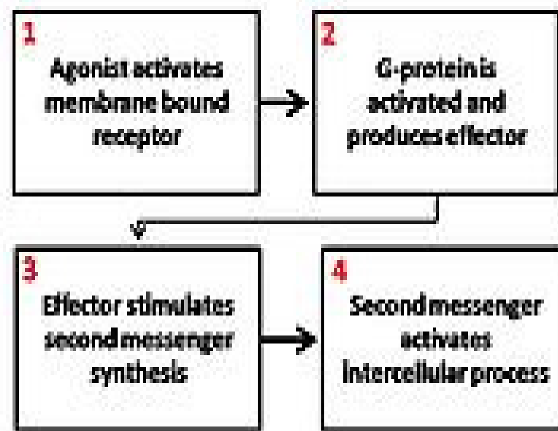


Fig. 8: General schematic of second messenger mechanism

There are several different secondary messenger systems (cAMP system, phosphoinositol system, and arachidonic acid system), but they all are quite similar in overall mechanism, though the substances involved in those mechanisms and effects are different. In all of these cases, a neurotransmitter binds to a membrane-spanning receptor protein molecule. The binding of the neurotransmitter to the receptor changes the receptor and causes it to expose a binding site for a *G-protein*. The G-protein (named for the GDP and GTP molecules that bind to it) is bound to the inner membrane of the cell and consists of three subunits: alpha, beta and gamma. The G-protein is known as the "transducer."

When the G-protein binds to the receptor, it becomes able to exchange a GDP (guanosine diphosphate) molecule on its alpha subunit for a GTP (guanosine triphosphate) molecule. Once this exchange takes place, the alpha subunit of the G-protein transducer breaks free from the beta and gamma subunits, all parts remaining membrane bound. The alpha subunit, now free to move along the inner membrane, eventually contacts another membrane-bound protein - the "primary effector." The primary effector then has an action, which creates a signal that can diffuse within the cell.

This signal is called the "secondary messenger." (The neurotransmitter is the first messenger.) The secondary messenger may then activate a "secondary effector" whose effects depend on the particular secondary messenger system.

Calcium as Second Messenger:

Calcium ions are responsible for many important physiological functions, such as in muscle contraction. It is normally bound to intracellular components, even though a secondary messenger is a plasma membrane receptor. Calcium regulates the protein calmodulin, and, when bound to calmodulin, it produces an alpha helical structure. This is also important in muscle contraction. The enzyme phospholipase C produces diacylglycerol and inositol triphosphate, which increases calcium ion permeability into the membrane. Active G-protein open up calcium channels to let calcium ions enter the plasma membrane. The other product of phospholipase C, diacylglycerol, activates protein kinase C, which assists in the activation of cAMP (another second messenger).

Ca++ ==> very important in regulating cellular and physiological responses

- Extracellular concentrations are 2 mM (EM, blood), and levels in cytoplasmic vesicles and the ER can reach up to 10mM.
- Baseline cytosolic Ca^{2+} concentration is around 100 nM in resting cells.

Conc in mM	ECF	ICF
K+	4.5	160
Na+	144	7
Cl-	114	7
Ca++	2.2	0.0001

- High gradient makes this a very fast and sensitive signaling system: only slight changes in membrane permeability will result in dramatic changes in the concentration of $[Ca^{2+]}i$.
- Low level of $[Ca^{2+]}i$ is also necessary to facilitate a phosphate oriented cellular metabolism
  (high calcium and high phosphate concentrations are incompatible)
  ==> Evolutionary challenge: Maintain calcium gradient

- Evolvement of proteins that bind Ca^{2+} with high affinity, but reject magnesium
- Two classes of Ca-binding proteins:
    - membrane-integrated (unlimited capacity --> transporter systems: Ca-channels, calcium pumps)
    - non-membranous (limited capacity --> not only buffering, but processing of signal through conformational changes that enable interaction with target proteins: Calmodulin, Troponin C).

Sources of Ca^{++}:

- Extracellular compartment: (predominantly in nerve, cardiac and smooth muscle cells)

Three types of plasma-membrane localized calcium channels:

Voltage operated calcium channels:

Action potental depolarizes plasma membrane, which results in the opening of “voltage” dependent calcium channels (channels can be opened by increase in extracellular K^{+}).

Each channel protein has four homologous domains, each containing six membrane spanning α-helices (the fourth one functions as the “voltage” sensor.

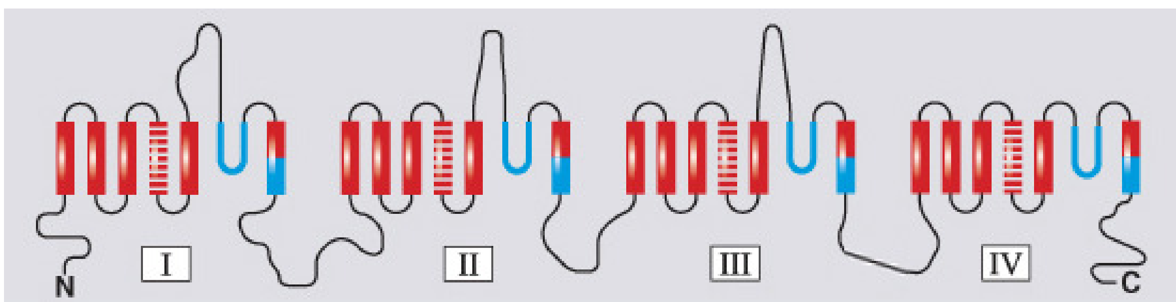


Fig.9: Molecular architecture of ion channels – Voltage-gated sodium and calcium channels.

Three types:

Type	Properties	Location/Function	Blockers
L	High activation threshold; slow inactivation	Plasma membrane of many cells; main Ca^{++} source for contraction in smooth and cardiac muscle	Dihydropyridines; verapamil; diltiazem
N	Low activation threshold; slow inactivation	Main Ca^{++} source for transmitter release by nerve terminals	w-Conotoxin (snail venom)
T	Low activation threshold; fast inactivation	Widely distributed; important in cardiac pacemaker and Purkinje cells	Mibefradil; (verapamil; diltiazem)

Table 1: Types and function of calcium channels

Ligand gated calcium channels:

Calcium channels opened after ligand binding to the receptor (e.g. glutamate/NMDA receptor; ATP receptor; nicotinic ACh receptors (muscarinic ACh receptors signal through G-Proteins --> slower), prostaglandin receptors.

Store operated calcium channels:

Activated by emptying of intracellular stores, exact mechanism unknown

<u>Intracellular compartment</u>: (predominantly in muscle cells)

- Calcium stored in mM concentrations in endo/sarcoplasmatic reticulum bound to *Calsequestrin* . (Previously mitochondria were thought to play an important role as Ca^{++}-stores, but the uptake rate is 10x lower than that of the ER/SR -> not useful)

Calcium release mechanism:

Calcium release from the ER/SR is regulated by two receptors in the ER/SR membrane:

1. Ryanodine receptors (RyR):

- Named after sensitivity to Ryanodine: plant alkaloid, irreversible inhibitor

- Very important in skeletal muscle: direct coupling of RyRs with the dihydropyridine receptors of the T-tubules (dihydropyridine receptors are closely related to the L-type Ca^{++} channels) (see Ca^{++} effects)
- Activity of RyRs in non-muscle cell lacking T-tubules regulated by cyclic ADP ribose
- Caffeine: reversible activator of RYRs

2. IP_3- Receptors (IP_3R):

- Inositol-1,4,5-triphosphate is produced through the activity of receptor activated phospholipases C --> diffuses through cytoplasm and binds IP_3R

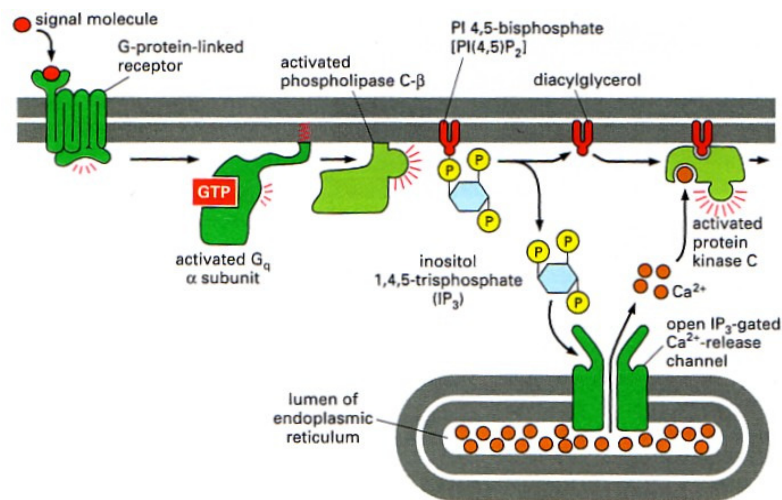


Fig.10: Activation of IP_3 gated calcium release channel.

Removal of Ca^{++} :

- Ca^{++} - pumps:

  Activity of these pumps is induced by increases in cytosolic calcium.

  – Plasma membrane Na^+/Ca^{2+} exchanger (mainly in excitable cells, e.g. cardiac cells) three Na^+- ions are exchanged for one Ca^{++}-ion

  Digitalis alkaloids: Na^+/ K^+-ATPase inhibitors => intracellular Na^+ raises => Na^+/Ca^{2+} exchange less efficient => Ca^{2+} intracellular increases => stronger contractions.

- Plasma membrane Ca^{2+}-ATPase (PMCA)

  two Ca^{++}- ions are transported per ATP molecule hydrolyzed; regulated by CaM, PKA or PKC
- SR/ER Ca^{++}-ATPase (SERCA):

  80% of integral membrane protein of SR

  target of thapsigargin (=> Ca^{++}-release from intracellular stores)

• Ca^{++}- buffers:

  Low affinity (!) but high capacity (50-100 Ca^{++}-ions/molecule)
- Calsequestrin (very acidic, 37% of amino acids are aspartic and glutamic acid),
- Calreticulin, Parvalbumin

Ca^{++} Sensors:

• Annexins:

Family of proteins w/ common feature that they interact w/ membranes in a Ca^{++}- dependent manner. Low affinity for Ca^{++}-ions restricts action to membrane proximity (high local Ca^{++} conc.!); implicated in the regulation of PLA_2, cytoskeletal (re)organization and vesicle movement.

Calmodulin:

Ubiquitous expression; binds 4 Ca^{++}-ions; acts through stimulation of either protein kinases (CaMKs) or protein phosphatases (Calcineurin); also activates cAMP phosphodiesterase.

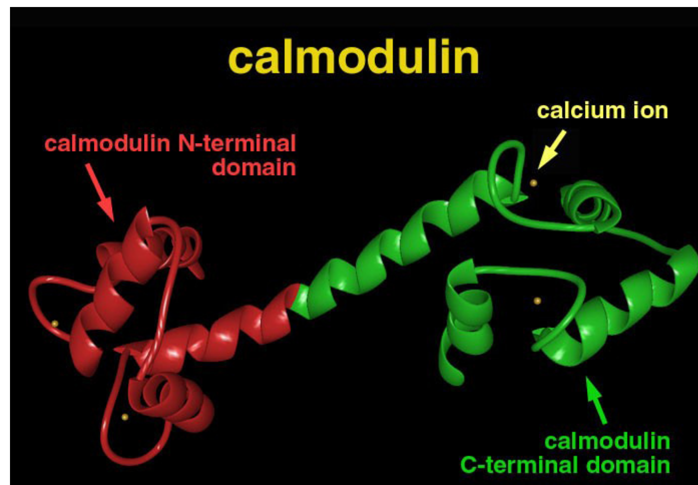


Fig.11: Activation of calmodulin domin via calcium ion.

Troponin C:

Restricted expression, regulates contraction of skeletal and heart muscle.

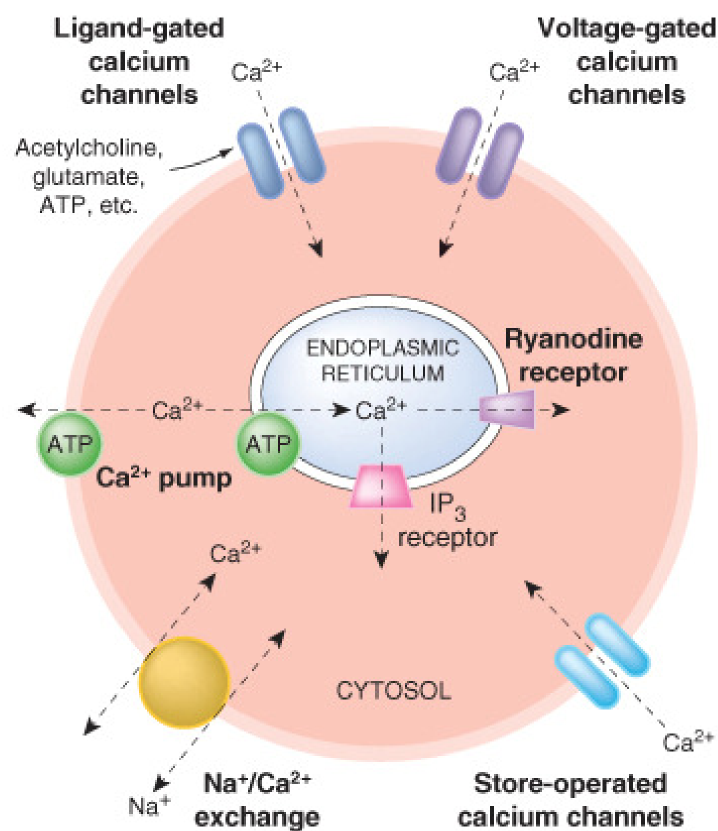


Fig.12: Regulation of intracellular calcium

Function of cytoplasmic free Ca^{++}:

- **Muscle contraction:**
  - **Skeletal and cardiac muscle:**
    - **Contraction** (=actin-myosin interaction) controlled by proteins on actin filaments (tropomyosin w/ troponin)
    - **Troponin I** inhibits formation of *cross-bridges* between actin and myosin => muscle relaxed.
    - **Troponin C** combines with Ca^{2+} ions and blocks the action of Troponin I => muscle contracted.

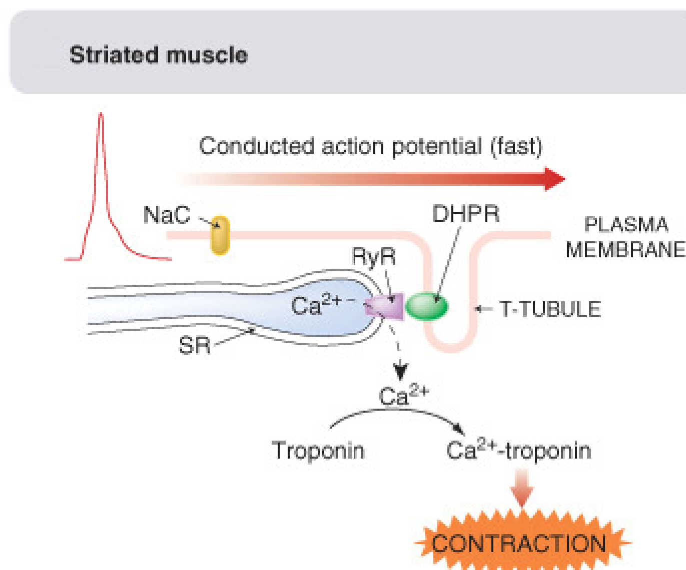
Smooth muscle:

Contraction controlled by proteins acting either on **actin**....

- NO Troponin=>regulation occurs through the CaM binding *Caldesmon* : Low [Ca^{++}]: Caldesmon forms complex with actin and tropomyosin => access of myosin to actin restricted =>muscle relaxed.

or on **myosin**

- Myosin light chains inhibit actin-myosin interaction: phosphorylation of myosin light chain (MLC) by MLC kinase (MLCK) relieves this inhibition => phosphorylated myosin is able to interact w/ actin => contraction



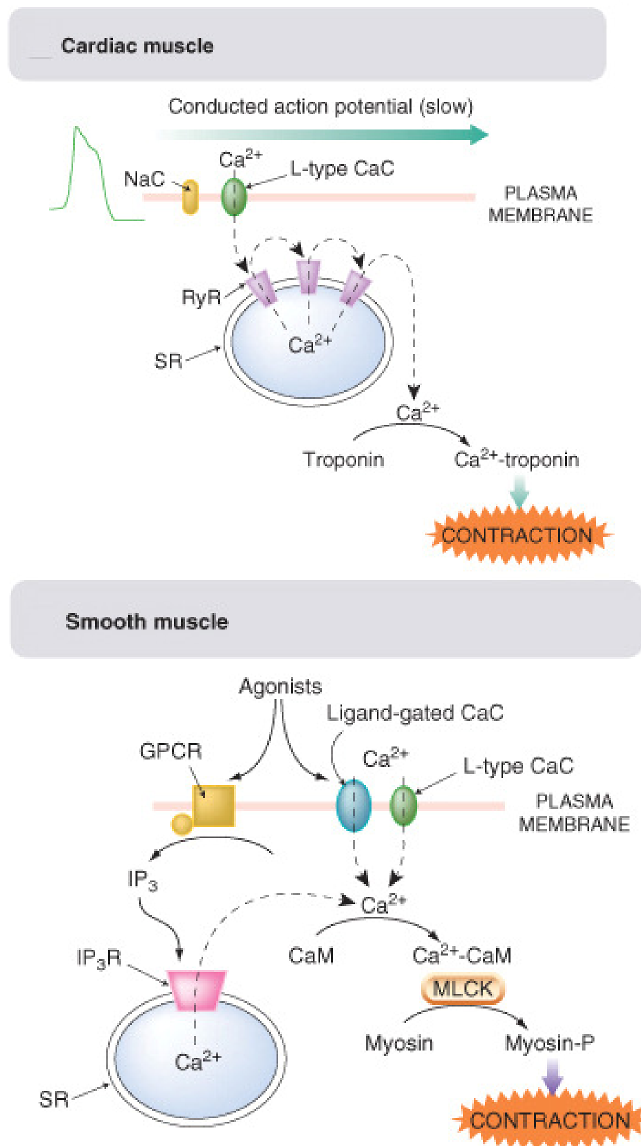


Fig.13: Comparison of excitation-contraction coupling in striated muscle (A),Cardiac muscle(B) and smooth muscle (C).

Neuronal excitibility and secretion:

- Increase of [Ca^{++}] induces fusion of the synaptic vesicles with the plasma membrane => this causes exocytosis of neuro-transmitters into the synaptic cleft.

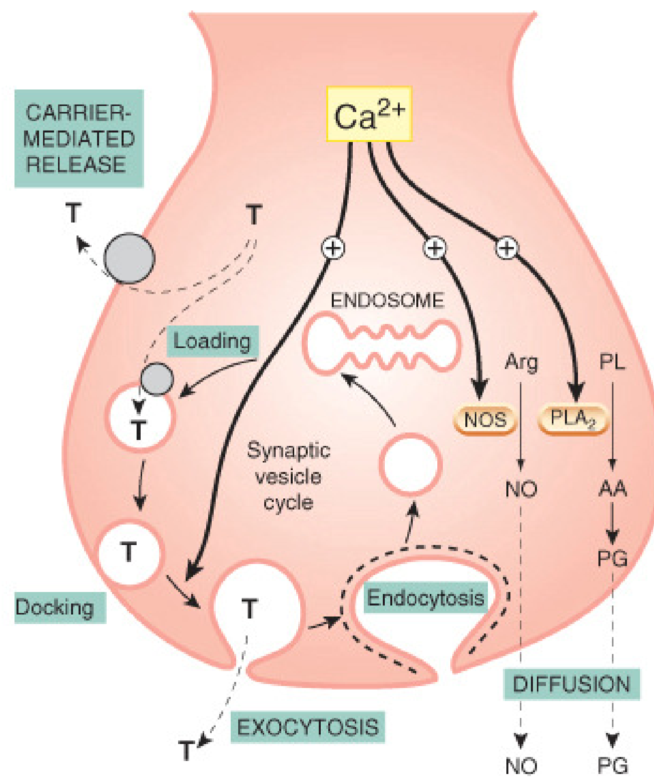


Fig. 14: Role of exocytosis, carrier-mediated transport and diffusion in mediator release.

Cyclic Nucleotides:

- **Guanylate Cyclase:**
  - Yields cyclic GMP
  - Second messenger in only a few cell types (intestinal mucosa, vascular smooth muscle)
  - cGMP stimulates cGMP-dependent protein kinases
  - Action terminated by hydrolysis of cGMP and dephosphorylation of protein kinase substrates
  - **Nitric Oxide** activates cytoplasmic guanylyl cyclase

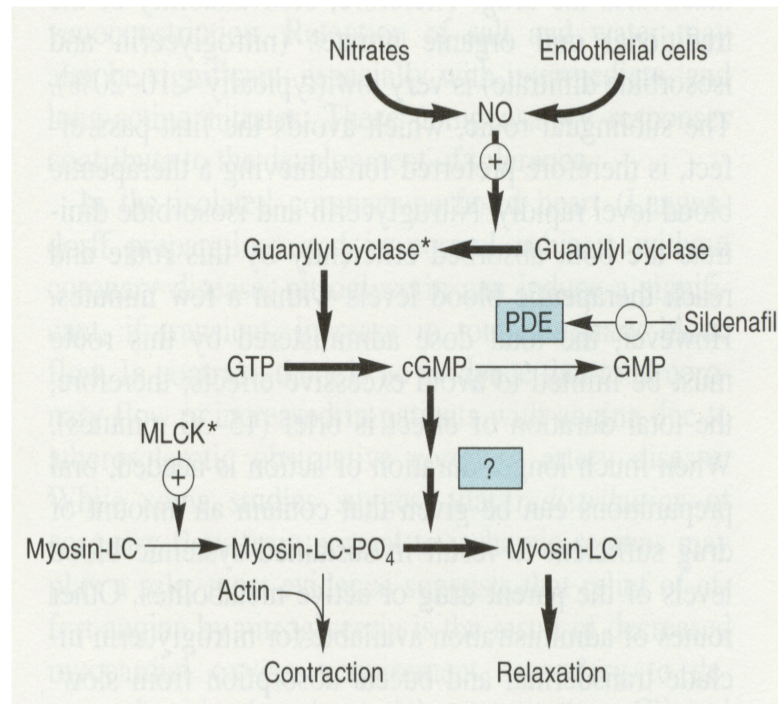


Fig.15: Role of NO in the process of contraction and relaxation via Myocin-LC

- **Adenylate cyclase:**
    - Two repeats of six transmembrane α-helices and two catalytic domains that convert ATP into cAMP.
    - Activated or inhibited by G-proteins (a brain specific isoform is also activated through activated CaM): GTP-bound $G_{\alpha s}$ activates AC, GTP-bound $G_{\alpha i}$ inhibits activity (Forskolin: direct activator of AC => cAMP⇑)

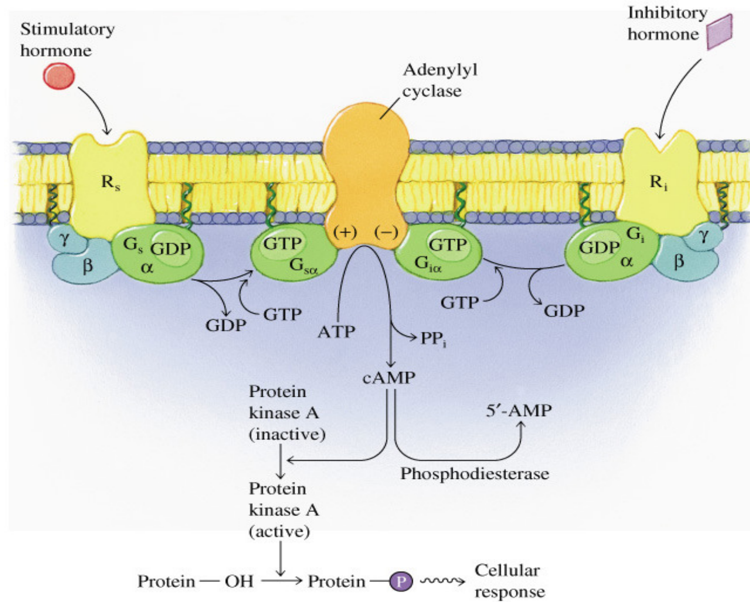


Fig.16: Activation of adenylyl cyclase, stimulation of cellular response.

- **Phosphodiesterases:**
  - Attenuate G-protein/AC coupled-receptor derived signals by converting cAMP to 5'AMP, or cGMP to 5'GMP
  - several families; activation creates feed-back loops

Phosphodiesterase inhibitors (Lerner, 2006):

Methylxanthines: Caffeine, theophylline => enhance and prolong the signals originating from adrenergic receptors.

PDE Family	Type	Genes	Commonly used inhibitors
PDE1	CaM-dependent	1A,1B,1C	Vinpocetine, Nimodipine, 8-MM-IBMX
PDE2	cGMP-stimulated	2A	EHNA
PDE3	cGMP-inhibited	3A/B	Cilostamide, Milrinone,
PDE4	cAMP-specific	4A-D	Rolipram, RO 20-1724,Piclamilast
PDE5	cGMP-specific	5A	Sildenafil, Zaprinast, Dipyridamole
PDE6	Photoreceptor	6A-C	Zaprinast, Dipyridamole,
PDE7	cAMP-specific	7A/B	Dipyridamole
PDE8	cAMP-specific	8A/B	Dipyridamole
PDE9	cGMP-specific	9A	Zaprinast, SCH 51866
PDE10	Dual Substrate (cAMP, cGMP)	10A	Papaverine
PDE11	cAMP, cGMP	11A	Tadalafil, Zaprinast, Dipyridamole

Table 2: Shows the PDE families, the known genes with in them, their affinity constant for cAMP and cGMP, and commonly used pharmacological inhibitors.

ISOENZYME FAMILY	REGULATORY CHARACTERISTICS	KNOWN FUNCTIONAL EFFECTS OF ISOENZYME INHIBITION
I	Ca^{2+}, calmodulin-regulated with different K_m values for cGMP and cAMP hydrolysis	CNS modulation; vasorelaxation
II	cGMP-stimulated cAMP hydrolysis with high K_m for cAMP	
III	cGMP-inhibited cAMP hydrolysis; low K_m for cAMP and cGMP	Positive inotropism; vascular and airway dilation; inhibition of platelet aggregation; stimulation of lipolysis; inhibition of cytokine production*
IV	Low K_m for cAMP hydrolysis	Airway smooth muscle relaxation; inhibition of inflammatory mediator release; CNS modulation; gastric acid secretion
V	High and low K_m isoforms for cGMP specific hydrolysis	Platelet aggregation inhibition
VI	Activity regulated by interaction with transducin	Photoreceptor phosphodiesterase
VII	Low K_m for cAMP hydrolysis	Abundant in skeletal muscle; present in heart and kidney

Table 3: PDE isoenzyme family with regulatory characteristics, with known function effects.

In general, the cyclic adenosine 3',5'- monophosphate (cAMP) plays a major role in the generation of seizure activity. The adenylate cyclase, an important transmembrane enzyme possesses certain activity in the brain, which promotes the intracellular level of cAMP, from adenosine triphosphate (ATP). An elevation in cAMP content has been reported in the cerebral cortex accompanying chemically induced epileptic activity (Ferrendelli, 1980; Krivanek, 1977). Cyclic AMP plays a key function by controlling a wide variety of cellular processes (Houslay, 2001; Houslay*,* 1998), also which acts as a ubiquitous second messenger and modulator of signal transduction processes (Houslay, 1998). This cAMP is generated by the action of adenylyl cyclase (Houslay, 1997) and degraded by hydrolysis process, which is regulated by a family of cyclic nucleotide phosphodiesterases (PDEs) (Conti, 1999; Soldering, 2000). The neuronal cell signaling structure of cytoskeleton plays a vital role in the development of seizures. Cyclic

nucleotides have been extensively studied as second messengers of intracellular events initiated by activation of many types of hormone and neurotransmitter receptors. Cyclic guanosine monophosphate (cGMP) also serves as a second messenger in a manner similar to that observed with cAMP. Peptide hormones, such as natriuretic factors, activate receptors that are associated with membrane bound guanylate cyclase (GC). Receptor activation of GC leads to the conversion of GTP to cGMP. Nitric oxide (NO) also stimulates cGMP production by activating soluble GC, perhaps by binding to the heme moiety of the enzyme. Similar to cAMP, cGMP mediates most of its intracellular effects through the activation of specific cGMP dependent protein kinases (PKG). Several families of PDEs act as regulatory switches by catalyzing the degradation of cGMP to guanosine -5′-monophosphate (5′-GMP). (Francis, 1999; Juilfs, 1999; Simonds, 1999).

Phosphodiesterase enzymes regulate the degradation of cyclic AMP a product of the adenylate cyclase activation and could contribute to the pathophysiology of the seizure mechanisms (Conti, 1999). Seizures, especially chemically induced are associated with increased expression in discrete brain regions like the hippocampus and subi-culum and cerebral cortical structures. Twelve members of the family have been identified and these can be further divided into a number of subtypes and splice variants. The PDE types differ in their amino acid sequence, substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities and in their organ, tissue and sub cellular distribution (Beavo, 1994). On the other hand the cellular levels of cAMP hydrolyze into 5′-nucleotide monophosphates by PDEs. By blocking phosphodiesterase hydrolysis, PDE inhibition results in higher levels of cyclic AMP. Therefore, PDE inhibitors may have considerable therapeutic utility as anti-inflammatory agents, anti-asthmatics, vasodilators, smooth muscle relaxants, cardiotonic agents, antidepressants, antithrombotics and agents for improving memory and other cognitive functions (Donell, 1999; McGeer, 1995; Hulley *et al*, 1995; Watchtel, 1983).

Calcium ions are responsible for many important physiological functions, such as in muscle contraction. It is normally bound to intracellular components, even though a secondary messenger is a plasma membrane receptor. Calcium regulates the protein calmodulin, and when bound to calmodulin, it produces an alpha helical structure. This is

also important in muscle contraction. The enzyme phospholipase C produces diacylglycerol and inositol triphosphate, which increases calcium ion permeability into the membrane. Active G-protein open up calcium channels to let calcium ions enter the plasma membrane. Active G-protein open up calcium channels to let calcium ions enter the plasma membrane. The other product of phospholipase C, diacylglycerol, activates protein kinase C, which assists in the activation of cAMP (another second messenger). So, both neuronal calcium currents and neurotransmitter release play pivotal roles in the generation and propagation of seizures (Meyer, 1986 (a); Meyer *et al.,* 1987; Meyer, *et al.,* 1986 (b))

Pharmacologic Therapy:

Bromide salts were the first effective anti-epileptic medications. Discovered in 1850, the bromides were employed on the mistaken notion that by reducing sexual drive they would reduce seizures. Although bromides inhibit seizures, they are toxic and were abandoned when phenobarbital was introduced 60 years latter. Phenobarbital was first used as a sedative / hypnotic; it was serendipity that led to discovery of its anti-epileptic potential. Many seizure medications were developed that are chemical derivatives of Phenobarbital, an example of which is phenytoin, developed in 1938 as the first nonsedating anti-epileptic. By contrast, carbamazepine was developed in the 1950s as a medication for treating depression and pain and valproic acid was a solute found by chance to be anti-epileptic when it was used for dissolving compounds to be tested as anti-epileptic agents (Enna, 1998).

Anti-epileptic drug development has passed through several stages. Bromides represented the era of wrong theory, Phenobarbital the era of serendipity, primidone and mephobarbital the phenobarbital imitation era, and phenytoin the MES screening era. Most newer medications were designed for their ability to selectively modify neurochemical systems in brain. Thus, vigabatrin and tiagabine increase the synaptic availability of GABA, the former by blocking GABA metabolism and the latter by blocking the reuptake of GABA into neurons and glia. Lamotrigine and remacemide work, in part, by blocking the release of, or the receptor for, glutamic acid. Pheytoin, carbamazepine, valproic acid, felbamate, lamotrigine, and several other medications appear to prolong the time during which the neuronal sodium channel is closed after

inactivation. This prolongation prevents the axon from generating another action potential too quickly, diminishing the rapidity of firing (Enna, 1998). The mechanism of action of most AEDs can be categorized as affecting ion channels, augmenting inhibitory neurotransmission, or modulating excitatory neurotransmission. The ion channels affected include the sodium and calcium channels. Augmentation inhibitory neurotransmission includes increasing CNS concentration of GABA, whereas efforts to decrease excitatory neurotransmission are primarily focused on decreasing (or antagonizing) glutamate and aspartate neurotransmission. The ultimate goal of treatment for epilepsy is no seizures and no side effects with an optimal quality of life. The best quality of life is associated with a seizure free state (Vickery *et al.,* 1994).

Current trends in anti-epileptic drug therapy:

Over the last two decades, drug therapy for epilepsy has improved substantially. This can be ascribed to a large extent to three factors,

1. Including the demonstration of the advantages of monotherapy; the realization of the need for dosage tailoring,
2. coupled [for some antiepileptic drugs (AEDs)] with control of pharmacokinetic variability through therapeutic drug monitoring; and
3. The introduction of newer agents with improved tolerability profiles. What further advances should we expect for the future?

Current trends that are expected to increasingly affect our prescribing patterns include greater reliance on evidence-based medicine and treatment guidelines, a trend that will be facilitated by completion of therapeutically meaningful randomized trials (including cost-effectiveness studies) and high-quality observational studies (including multinational pregnancy registries), as well as initiatives from scientific societies and government organizations aimed at condensing the most relevant information into therapeutic guidelines. The explosion in communication technology will accelerate dissemination of this information and its application to clinical practice. Other factors include a more rational patient-tailored AED selection and dose individualization, aided by characterization of predictors of outcome as defined by clinical parameters (sex, age,

epilepsy syndrome, and etiology), pathophysiological mechanisms, and newly discovered genetic markers of outcome; improved definition of the role of new AEDs, resulting in their increased use in newly diagnosed epilepsy; and reappraisal of the value of combination therapy in refractory epilepsies, based on evidence produced by experimental and clinical studies designed to identify favorable pharmacodynamic interactions. Additional important developments may come from the discovery of novel, more efficacious AEDs and from exploration of potential new targets, such as prevention of epileptogenesis.

Limitations of Current Therapeutic Approaches:

Despite the major advances previously summarized, the current approach to AED therapy still has many limitations. For example, inadequate understanding of the pathophysiological mechanisms underlying seizure disorders, coupled with the still incomplete knowledge of the mechanisms of action of individual AEDs, prevents a mechanistic approach to AED therapy. Indeed, drug selection in epilepsy remains based on empirical assessment of the probability of achieving seizure freedom and associated side-effect profiles, rather than on the rational application of a treatment that corrects a specific functional or biochemical abnormality. For the same reasons, we have no reliable tools to predict which AED, among the many known to be active against a given seizure type, will control the seizures in an individual patient. Therefore, at the current state of knowledge, AED therapy is based mainly on a trial-and-error approach. Clearly, however, the most important limitation of current pharmacological treatments is that, in at least one third of patients, seizures cannot be controlled completely by available AEDs. The phenomenon of drug resistance has hardly been affected by the advent of second generation AEDs, as no more than 10–15% of patients with severe refractory epilepsy become seizure free by treatment with these agents. Therefore, the search for newer and more effective AEDs should continue. The purpose of this research is to provide a concise overview of a number of strategies that are currently being implemented in an attempt to overcome the limitations discussed previously and further improve the effectiveness of AED therapy.

The Future: Novel Tools and Novel Approaches:

The fact that over one third of patients with epilepsy cannot achieve seizure control with available AEDs provides a powerful stimulus to research aimed at identifying new therapies. There is also a need for novel AEDs and AED formulations with improved tolerability profiles, which could replace older medications even in patients with currently responsive forms of the disease. Although the consolidation taking place in the pharmaceutical industry may not encourage investment in therapeutic areas not perceived as strategic, there is no shortage of incentives to develop new medications for epilepsy. Because of the limitations of current treatments, a novel AED with an improved efficacy and tolerability profile could easily capture a large segment of the market. Many AEDs are also efficacious in other indications, so that the potential use of a new AED may extend far beyond its prescriptions for epilepsy. Finally, the cost of bringing an AED to the market is considerably lower than in other therapeutic areas, and special programs such as the National Institutes of Health Anti-convulsant Drug Development Program are in place to foster drug development in epilepsy. Among the latter, important breakthroughs could emerge from progress made in the on-line computerized analysis of EEG data used to predict the occurrence of seizures with an anticipation of several minutes before their clinical onset. Eventually, this could lead to "ondemand" intermittent administration of AEDs through a computer-driven device, an approach for which proof-of concept has already been provided in animal models. Other exciting advances could come from the development of compounds that can interfere with the process of epileptogenesis, thereby preventing the occurrence of epilepsy in high-risk patients or antagonizing the progression of the seizure disorder that may occur in certain situations. Treatments aimed at providing a cure, such as gene therapy, may also become available in the future.

Against this background, this study was designed to examine and investigate the possible roles of specific inhibitors of PDE along with adenylate cyclase inhibitors, soluble guanylate cyclase activator and inhibitor, calcium channel blocker and activator in the generation of convulsive seizures.

2. AIM

This study was undertaken to explore the possible roles of the inhibitors of PDE isozymes affecting the actions of adenylate cyclase as well as guanylate cyclase enzymes. The objective was to elucidate whether the PDE inhibition in conjunction with other signaling elements could affect elicitation or inhibition of seizures.

Moreover, a detailed investigation was carried out with regard to brain function improvement as well as therapy of epilepsy in future by using cGMP mediated phosphodiesterase.

The role of ion channel modulators both sodium and calcium channel modulators where taken into consideration in the animal models of epilepsy. It was carried out to investigate the role of sodium and calcium channel modulators in delaying the onset of seizures.

3. OBJECTIVES

A family of cyclic nucleotide PDEs used to regulate the process of cAMP generation and degradation by hydrolysis with the action of adenylyl cyclase activators and inhibitors. Cyclic AMP plays a key role in controlling a wide variety of cellular processs. A study was carried out to explore the inducement of epileptic activity of cAMP at elevated level. Apart from cAMP, cGMP also possess certain intracellular effects through the activation of specific cGMP dependent protein kinases (PKG) by way of soluble guanylate cyclase (sGC). In view of these findings the present study was undertaken to examine and investigate the possible roles of specific inhibitors of PDEs along with adenylate cyclase inhibitors, soluble guanylate cyclase activator and inhibitors to evaluate the effect on chemical convulsant and maximal electroshock induced seizures in mice and rats.

Ion channels mediate and regulate crucial electrical functions throughout the body. There are therapeutic drug targets for a variety of disorders and the direct cause of unwanted side effects. In the view of these principles the present study also designed to examine and investigate the role of different ion channel modulators such as sodium channel modulator, calcium channel modulator, blocker and activator in the generation of convulsive seizures.

4. REVIEW OF LITERATURES

A literature review is a summary of previous research on a topic. The review of literature aims to describe the 'state of play' in the area selected for study. Our literature review focused on the pharmacological potential of phosphodisesterase inhibitors, adenylate cyclase inhibitor, guanylate cyclase activator and inhibitor, ion channel modulators, calcium channel blocker and activator. Such literature reviews describe what has been written about the area, the major research findings across studies, and the major debates in terms of substantive and methodological issues.

4.1 PHOSPHODIESTERASE INHIBITORS

- The bipyridine phosphodiesterase III inhibitors amrinone and milrinone form a new class of positive inotropic vasodilator agents, which inhibit the intracellular hydrolysis of cAMP, there by promoting cAMP catalyzed phosphorylation of sarcolemmal calcium channels and activating the calcium pump (Honerjaer, 1991). A study was carried out to analyze the possible roles of selective inhibition of cyclic nucleotide PDE isozymes, adenylate cyclase activation and tissue cAMP elevation in the positive inotropic action of five new cardiotonic drugs such as amrinone, milrinone, AR-L 115BS, MDL 17, 043 and RMI 82,249. The study results showed that the specific inhibition of cAMP PDE3 isozyme and the consequent elevation of tissue cAMP levels in cardiac tissue are an important mechanism of action of amrinone, milrinone and others (Ahn *et al.,* 1986).

- A research study results suggest that milrinone exerts inotropic effects by inhibiting cAMP-PDE selectively in the human heart tissues and that this compound can also be used to evaluate different forms of cAMP-PDEs present in human tissues (Ito *et al.,* 1988). Milrinone, which is a PDE3 inhibitor, enhances the calcium dependency of the positive inotropic effects on the amphibian and mammalian myocardium at the dose range of 10 to 300 µg (Juvekar & Vadlamudi., 1998). Milrinone, which inhibits cAMP-specific phosphodiesterase, so as which increases the level of cAMP in vascular smooth muscles. Adenylate cyclase activity also significantly increased by milrinone at the dose of 10 µM (Rabbani *et al.,* 2007).

- Cilostazol, is a PDE3 inhibitor that is a US FDA approved therapy for claudication, owing to its activity on both platelets and endothelium (Kambayashi *et al.,* 2003). Cilostazol is a selective PDE3 phosphodiesterase inhibitor with therapeutic focus on increasing cAMP. An increase in cAMP results in an increase in PKA, which is directly related with an inhibition in platelet aggregation (Taniguchi *et al.,* 2007). Cilostazol, a selective inhibitor of phosphodiesterase 3, exerts neuroprotective effects on acute brain injury after cerebral ischemia in rats. The study conclude that cilostazol protects against not only the acute injury, but also the late injury in mice with focal cerebral ischemia at the dose level of 3-10 mg/kg, i.p. especially it can modify brain remodeling, astrogliosis and angiogenesis (Luye *et al.,* 2007).

- Cone et al., compared the cilostazol and milrinone, both caused a concentration-dependent increase in the cAMP level in rabbit and human platelets with similar potency. Furthermore, cilostazol and milrinone were equally effective in inhibiting human platelet aggregation with a median inhibitory concentration (IC50) of 0.9 and 2 microM, respectively. In rabbit ventricular myocytes, however, cilostazol elevated cAMP levels to a significantly lesser extent $p < 0.05$ vs. milrinone (Cone *et al.,* 1999). Matousovic et al., study evidenced that intracellular cAMP levels are indeed increased in response to cilostazol, PDE-III inhibitors without detecting change of total cAMP content. Also the findings pointed out to the possibility that the PDE-III regulated cAMP pool is functionally related to PKA activation, phosphorylation and decrease in the activity of Raf-l proteinkinase (Matousovic *et al.,* 1995).

- Rolipram, is a PDE4 inhibitor, increases the intracellular level of cAMP by inhibiting its metabolism (Beavo, 1998). Rolipram increased cAMP accumulation more effectively than forskolin, isoproterenol or adenosine derivatives alone, extensive synergism effect was seen with combined agents (Thompson *et al.,* 2002). Araki T et al., result suggest that cAMP and rolipram binding sites are predominantly distributed on the pyramidal cell layer of the hippocampal CA1 sector of rats and that transient cerebral ischemia can cause marked reduction in these binding sites in the hippocampus (Araki *et al.,* 1992).

- Sildenafil, a cGMP-specific PDE5 was first identified in rat platelets in 1978 and was originally known as cGMP-PDE. PDE could be specifically inhibited by zaprinast (PDE5 inhibitor) and this was widely used to explore the functional role of what we now know as the PDE5 isoenzyme (Murray, 1993). Sildenafil, is a selective oral PDE5 inhibitor effective in erectile dysfunction, which also shown a proconvulsant effect on seizure threshold, interacting with exogenously and endogenously released nitric oxide (Riazi *et al.,* 2006). Proconvulsant activity of sildenafil was previously reported in humans, and also the experimental evidence pointed out the involvement of the NO/cGMP pathway (Gilad *et al.,* 2002). The early report suggest that high levels of cGMP, as caused by inhibiting PDE5 with sildenafil, may affect neuronal excitability and eventually predispose to convulsion (Folbergrova, 1980). An increase in the concentration of cyclic nucleotides, PDE5 inhibitor such as sildenafil, including cGMP, was noted in different brain structures and in the cerebrospinal fluid in laboratory animals made to convulse (Nomikos *et al.,* 2000).

- Smith *et al.,* demonstrated that BRL 50481 is a PDE7 inhibitor with nanomolar potency and is sufficiently selective against other PDE isoenzyme families for *in-vitro* pharmacological studies. BRL 50481 was poorly active in suppressing human T cell proliferation and TNF release from monocytes and macrophages but, nevertheless, acted in at least an additive manner with the PDE4 inhibitor rolipram. These findings suggest that hybrid PDE4/PDE7 inhibitors may be more efficacious and display a superior therapeutic index than a PDE4 inhibitor alone

(Smith *et al.,* 2004). Lee *et al.,* analyzed by using enzyme kinetic analysis, BRL 50481, a competitive inhibitor (with respect to substrate) of hrPDE7A1 with a K_i value in the high nanomolar range. At a substrate concentration of 50 nM, where the IC_{50} value of cAMP PDE inhibitors closely approximates to the K_i value (Lee *et al.,* 2002). A research study observations suggest that selective PDE7 inhibitor such as BRL 50481 could be useful in the treatment of T cell mediated autoimmune diseases, which is also responsible for depletion of cAMP which broadly suppresses cell functions and cellular responses to many activation stimuli (Yang *et al.,* 2003).

- Dipyridamole, a selective PDE6 inhibitor, the interaction between dipyridamole and nitric oxide (NO) was studied using isolated rabbit platelets and segments of the rabbit aorta. Dipyridamole clearly enhanced the dilation caused by exogenous NO from four different sources, including endothelial cells (Bult *et al.,* 1991). Harker, found that dipyridamole appears to act *in-vivo* by synergistically modifying several biochemical pathways, including inhibition of platelet cAMP phosphodiesterase, and also potentiation of adenosine inhibition of platelet function by blocking reuptake by vascular and blood cells (Harker *et al.,* 1983). Dipyridamole, a platelet inhibitor, which binds to the adenosine with adenosine receptor, stimulates adenylate cyclase activity and production of cAMP. Elevated cAMP impairs platelets aggregation and also causes arteriolar smooth muscle relaxation (Geiger, 2001).

- Dipyridamole having ability to increase the intracellular cAMP and cGMP in a time and dose dependent manner through PDE inhibition. In the presence of dipyridamole the effect of cGMP and protein kinase G (PKG) is more than cAMP and protein kinase A (PKA) (Zhuplatov *et al.,* 2006). Dipyridamole has been reported to enhance NO mediated effects. It appears to regulate both NO and superoxide release from vascular cells, which possess anti-oxidant, and anti-inflammatory properties as well as their subsequent effect on cell signaling (Chakrabarti *et al.,* 2008). The effects of selective and non selective cyclic nucleotide PDE inhibitors on cGMP and cAMP accumulation were studied in rat hippocampal slices incubated *in-vitro.* The result showed that the cGMP levels were increased in the presence of different concentrations of IBMX, EHNA, dipyridamole, vinpocetine and rolipram (Staveren *et al.,* 2001).

- Wang *et al.,* discovered that the etazolate is a phosphodiesterase inhibitor selective for the PDE4 subtype (Wang *et al.,* 1997). Etazolate, inhibitor of cyclic nucleotide phosphodiesterase and subtype-selective $GABA_A$ receptor modulator. Stimulates the neurotrophic α-secretase (non-amyloidogenic) pathway and inhibits β-amyloid-induced neuronal death. May ultimately reduce amyloid plaques (Marcade, *et al.,* 2008). Etazolate is a pyrazolopyridine compound that belongs to a family of molecules with anxiolytic-like properties, which selectively modulates the $GABA_A$ receptor (Barnes *et al.,* 1983; Patel *et al.,* 1985; Whiting *et al.,* 1997; Thompson *et al.,* 2002).

- Etazolate binding to the $GABA_A$ receptor can be blocked by bicuculine (Bic), an allosteric inhibitor of $GABA_A$ receptor opening. Therefore, the potential neuroprotective properties of etazolate against Aβ42 toxicity were investigated in the presence or absence of Bic (10 lM) (Barnes *et al.,* 1983). Etazolate modulates the $GABA_A$ receptor pharmacology in a subunit-selective manner with IC50 around 1 μM. However, it should be noted that etazolate is also known to have a phosphodiesterase 4 (PDE4) inhibitor activities. By stimulating cAMP levels, the PDE4 inhibitor rolipram and the PDE4- CREB pathway show beneficial effect at counteracting Aβ induced memory, synaptic and dendritic alterations (Gong *et al.,* 2004; Wang *et al.,* 1997; Shrestha *et al.,* 2006).

4.2 ADENYLATE CYCLASE INHIBITOR

- SQ22536, an adenylate cyclase inhibitor had no significant effect on forskolin induced relaxation but markedly inhibited the elevation of cAMP content in new born ovine pulmonary veins (Gao *et al.,* 2001). SQ22536 has been recently shown that, in guinea pig aortas, which eliminated the elevation of cAMP induced by an iloprost (a prostaglandin I_2 analog) but had no effect on relaxation induced by this agent (Turcato *et al.,* 1999). Sunahara et al., discovered that the increase in cAMP content and in adenylyl cyclase activity caused by forskolin was largely inhibited by SQ22536 (Sunahara *et al.,* 1996). The adenylate cyclase activity and the cAMP content in the ipsilateral amygloid complex were significantly increased during preconvulsive and convulsive states (Kakita *et al.,* 1979).

4.3 GUANYLATE CYCLASE ACTIVATOR AND INHIBITOR

- A-350619: a noval activator of soluble guanylyl cyclase, consistant with its biochemical activity, at the dose level of 1 µmol/kg alone induced penile erection in a conscious rat model via a key mediator of nitric oxide (NO). Also observed that activation of sGC by A-350619 was partially inhibited by ODQ, a specific inhibitor of sGC by oxidation of the enzyme heme (Miller *et al.,* 2003). Recent study demonstrated the potential use of heme dependent sGC activator such as A-350619 in the treatment of cardiovascular diseases. The study also explains that Nitric oxide (NO) sensitive soluble guanylyl cyclase (sGC) is the receptor that catalyzes the formation of the intracellular messenger cGMP. Binding of the physiological activator, NO, to the reduced heme moiety sGC increases the conversion of GTP to cGMP and engages crucial effector systems such as protein kinases, phosphodiesterases and ion channels (Gur *et al.,* 2010).
- The study reviewed the discovery, biochemistry, pharmacology and clinical potential of haem-dependent sGC stimulators such as A-350619, YC-1, BAY 41-8543, BAY 41-8543 and CFM-1571. sGC is a key signal transduction enzyme activated by NO. Impaired bioavailability and responsiveness to endogenous NO has been implicated in the pathogenesis of cardiovascular and other diseases (Evgenov *et al.,* 2006). A-350619, sGC activator synergistically enhance the effects of NO by stimulating cGMP synthesis without altering the breakdown of cGMP (Brioni *et al.,* 2002).

- Methylene blue, a soluble GC inhibitor, ICV (Intracerebroventricular) administration of 1, 5 and 25 µg/rat was given in a dose dependent manner, which specifically decreases the brain cGMP contents in rats (Masaki *et al.,* 1999). The cellular level of cGMP concentration in samples of platelet rich plasma increased significantly after incubation with dipyridamole alone or with aspirin. Methylene blue significantly decreased this effect, where as L-Name and hemoglobin had no effect (Cruz *et al.,* 2000). Lo *et al.,* studied the effect of methylene blue as a soluble GC inhibitor and reported that the level of cGMP concentration was decreased (Lo *et al.,* 2005). Riazi *et al.,* observed that the pretreatment with either methylene blue, a guanylate cyclase inhibitor, inhibited the proconvulsant effect of sildenafil, indicating the mediation of this effect of NO-cGMP pathway (Riazi *et al.,* 2006).

- Investigators showed that sodium nitroprusside significantly prevents the penicillin induced seizures and that this effect is blocked by methylene blue and by a NO scavenger, hemoglobin. The authors have concluded that NO may be an endogenous anti-convulsant agent (Marangoz *et al.,* 1994). Bahremand et al., demonstrated that the anti-convulsant effect of lithium were prevented by pretreatment with sildenafil (10 and 20 mg/kg) in mice and also methylene blue (0.5 and 1 mg/kg) enhances the anti-convulsant effect of sub convulsive dose of lithium (10 mg/kg, i.p). It is also explained that NO-cGMP pathway could be involved in the anti-convulsant properties of the lithium chloride (Bahremand *et al.,* 2010).

4.4 ION CHANNEL MODULATORS

- Walaas and Greengard discovered that cAMP and Ca^{+2} calmodulin dependent protein kinases can phosphorylate key intracellular proteins: ion channels, receptors, enzymes, transcription factor, regulate, thus, which causes neuronal excitability (Walaas *et al.,* 1991). Ion channels mediate and regulate crucial electrical functions throughout the body. They are therapeutic drug targets for a variety of disorders and in some cases, the direct cause of unwanted side effects (Bennett *et al.,* 2003). News in physiological sciences explains that the inherited alterations of ion channels (ion channelopathies) in peripheral nerves that lead to bizarre neuromuscular disorders in experimental animal models. Now modifications of the similar channels in the brain are being recognized as causes of hyperexcitability in the CNS and hence of an ancient phenotype, epilepsy. The bursting of neuron is the cardinal feature of all seizure disorders (Valtin, 1998).

- A study explained the influx of calcium and sodium from glutamate receptor stimulation results in membrane depolarization, which can also activate voltage-dependent calcium channels. These other calcium channels then allow further calcium influx, aggravating the intracellular calcium overload initiated by over stimulation of the glutamate receptors and opening of the associated ion channels (Mark *et al.,* 2001). Amiloride, a sodium-hydrogen exchanger (NHE) inhibitor, can protect against seizure development of pentylenetetrazole induced kindling mice. Further, it protected against the subconvulsant dose of PTZ in kindled mice, suggesting that it not only inhibits the development of epilepsy but also seizure severity even when the disease state fully developed (Ali, 2005). Amiloride, with

regards to various *in-vitro* reports where reduced epileptiform activity in hippocampal slices elicited by different pharmacological strategies such as bicuculline, caffeine or zero magnesium (Bonnet *et al.,* 2000).

- Sokolova *et al.,* reported an inhibitory effect of amiloride on epileptic activity in the rat temporal cortex slices (Sokolva *et al.,* 1992). Recent study suggests that a protective action of amiloride in *in-vitro* seizure models in rodents including increasing current electroshock seizure threshold and PTZ tests (Ali *et al.,* 2004). Class I anti-arrhythmic drugs such as lidocaine, quinidine, and flecainide as well as some anti-convulsants including phenytoin and carbamazepine act by inhibiting ionic currents through voltage-gated Na^+ channels (Williams, 1984; Harrison, 1985).

- Flecainide, having ability to selectively inhibit Na^+ channels during abnormal membrane depolarizations and rapid bursts of action potentials that characterize cardiac and neuronal pathologies (Catterall, 1987). Ragsdale *et al*., explained the voltage-gated sodium channels mediate regenerative inward currents that are responsible for the initial depolarization of action potentials in brain neurons. Many of the most widely used anti-epileptic drugs, as well as a number of promising new compounds suppress the abnormal neuronal excitability associated with seizures by means of complex voltage- and frequency-dependent inhibition of ionic currents through sodium channels (Ragsdale *et al.,* 1998).

4.5 CALCIUM CHANNEL BLOCKER AND ACTIVATOR

- Nifedipine, is calcium channel blocker, at the dose level of 2.5-10 mg/kg offered some protection against PTZ induced seizure activity in mice (Wajtowicz, *et al.,* 1991). Khanna *et al.,* observed that the nifedipine is a calcium channel blocker, possess anti-convulsant activity, which was explained via electrophysiological evidence has indicated that calcium ion currents in hippocampal pyramidal cells are sensitive to voltage dependent calcium channel agonist and antagonist (Khanna, 2000). The intraperitoneal administration of nifedipine (5mg/kg), a specific L-type calcium channel blocker, inhibits picrotoxin induced seizure activity in adult female Sprague- Dawley rats (Otoom *et al.,* 2006).

- BAY K 8644, S(-)- is an L-type Ca^{2+} channel agonist. Bay K 8644 has been shown to cause an increase in myosin light chain phosphorylation and induce vasoconstriction in rabbit aorta *in- vitro* (Franckowiak *et al.,* 1985). BAY K 8644, S(-)- is a dihydropyridine (DHP) calcium channel activator were investigated on clonic convulsion by the administration of PTZ in mice. BAY K 8644, S(-)- shown a proconvulsant effect by increasing the number of animals dying from tonic extension convulsions to PTZ, a result which might be expected since it enhances neuronal calcium currents. In summary, DHP calcium activator BAY K 8644 increased the convulsant activity of PTZ (O' Neill *et al.,* 1990).

- Both DHP calcium channel activator (BAY K8644) and antagonist (Nifedipine) possess the devoid of behavioral effects in mice (Bolger et al., 1985). Low dose (2-12 mg/kg, i.p. in mice) of BAY K 8644 elicit marked behavioral changes in

rodents and dogs which include limb clonus and tonus and self limiting grand mal like seizures (Hoffmiester, 1986). Shelton et al., demonstrated the low dose of intracerebroventricular administration of BAY K 8644, a calcium channel agonist induced the proconvulsant action in mice (Shelton *et al.,* 1987).

- Amiodarone, is a multiple ion-channel blocker drug, inhibiting sodium and calcium inward currents and potassium outward current, and having noncompetitive adrenergic blocking effect (Kodama *et al.,* 1999). Dengiz, *et al.,* evaluated anti-convulsant effects of amiodarone against phentylenetetrazole (100 mg/kg) or caffeine (300 mg/kg) induced models in mice. In both models, amiodarone prolonged both latency period and time to death, and acted as an anti-convulsant drug. It was found to be more effective in the PTZ model than in the caffeine model (Dengiz *et al.,* 2009). Turovaya *et al.,* 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 (Turovaya *et al.,* 2005). Amiodarone, is a potassium channel blocker, increasing the intracellular calcium and extracellular potassium ion concentrations. The anti-convulsant activity was exerted by inhibiting the outward K^+ currents at the neuron-like cardiac cells (Heinemann *et al.,* 1977; Onozuko *et al.,* 1989; Piredda *et al.,* 1985).

5. SCOPE AND PLAN OF WORK

Epilepsy, a group of disorders of the central nervous system possesses a threat to the human community. It is prevalent all across the world. The therapeutic measures where so far unfolded to overcome this disorder have limitations.

A detailed study was undertaken in a systematic manner to explore the assuring roles of PDE isozymes to control epilepsy by affecting the actions of adenylate cyclase as well as guanylate cyclase ezymes. The PDE isozymes in conjunction with cell signalling molecules exhibited the significance role in the inhibition of convulsions. Amongst the other PDE isozymes, the cGMP mediated was specifically used to achieve delay the onset of seizure activity.

Ion channel mediate and regulate crucial electrical functions throughout the body. These are therapeutic drug targets for a variety of disorders and the direct cause of unwanted side effects. In view of this, the present study was also extended to examine and investigate the role of sodium and calcium ion channel modulator and blocker.

The pharmacological study on animal models of epilepsy where carried out on mice and rats. For this study the mice and rats were divided into groups (n=6) in each. The drugs were administrated through intraperitoneally 30 mins prior to the convulsion test. The study was carried out by the following two methods.

1. **Chemoconvulsant method:**

   To carryout the study the following chemoconvulsants were used.

   1. Pentylenetetrazole (PTZ)
   2. Picrotoxin (PTx)
   3. Isoniazid (INH)
   4. Kainic acid
   5. Pilocarpine

2. **Maximal electroshock method:**

Here also the drugs were administrated intraperitoneally 30 mins prior to the electroshock by subjecting the animal to a current of 55 mA to mice and 150 mA to rats for 0.2 sec duration through electro-convulsiometer using corneal electrodes. The present study design was carried out with the following models, carrying protocols significantly for each model as furnished below,

1. Phosphodisesterase (PDEs) inhibitors alone:

1.1. PDE-3 inhibitors alone:

Protocol – I : (Only Mice)

I. Chemoconvulsant method:

PTZ & - Amrinone (3 doses)
INH - Milrinone (3 doses)

II. Maximal electroshock method:

MES - Amrinone (3 doses)
- Milrinone (3 doses)

1.2. PDE-3, 4 & 5 inhibitors alone:

Protocol – II : (Both Mice and Rats)

I. Chemoconvulsant method- (Only Mice)

INH - Cilostazol (PDE-3 inhibitor),
- Rolipram (PDE- 4 inhibitor),
- Sildenafil (PDE-5 inhibitor).

II. Maximal electroshock method- (Both Mice and Rats)

MES - Cilostazol (PDE-3 inhibitor),
- Rolipram (PDE- 4 inhibitor),
- Sildenafil (PDE-5 inhibitor).

2. PDEs inhibitors along with adenylate cyclase (AC) inhibitor:

Protocol – III : (Both Mice and Rats)

I. Chemoconvulsant method- (Only Mice)

PTZ - SQ 22536 (AC inhibitor)
- Dipyridamole (PDE- 5/6/8/10/11 inhibitor)
- BRL 50481 (PDE- 7 inhibitor)
- SQ 22536 + Dipyridamole
- SQ 22536 + BRL 50481

II. Maximal electroshock method- (Only Rats)

MES - SQ 22536 (AC inhibitor)
- Dipyridamole (PDE- 5/6/8/10/11 inhibitor)
- BRL 50481 (PDE- 7 inhibitor)
- SQ 22536 + Dipyridamole
- SQ 22536 + BRL 50481

3. PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor:

Protocol – IV : (Both Mice and Rats)

I. Chemoconvulsant method- (Only Mice)

PTZ
- A 350619 (sGC activator)
- Methylene blue (sGC inhibitor)
- BRL 50481 (PDE-7 inhibitor)
- A 350619 + BRL 50481
- Methylene blue + BRL 50481

II. Maximal electroshock method- (Only Rats)

MES
- A 350619 (sGC activator)
- Methylene blue (sGC inhibitor)
- BRL 50481 (PDE-7 inhibitor)
- A 350619 + BRL 50481
- Methylene blue + BRL 50481

4. Ion channel modulators alone (Both sodium and calcium ions):

Protocol – V : (Only Mice)

I. Chemoconvulsant method – (Only Mice)

Kainic acid & PTZ
- Lacosamide (Std)- Sodium Channel Modulator
- Amiloride (SCM)
- Amiodarone (Calcium Channel Modulator)
- Flecainide (SCM)
- Flecainide + Amiodarone

II. Maximal electroshock method – (Only mice)

MES - Lacosamide (Std)- Sodium Channel Modulator
- Amiloride (SCM)
- Amiodarone (Calciu Channel Modulator)
- Flecainide (SCM)
- Flecainide + Amiodarone

Protocol – VI : (Only Mice)

I. Chemoconvulsant method – (Only Mice)

PTZ - Lacosmide
PTx - Flecainide - Test Dose- I
Kainic acid- Flecainide - Test Dose- II

II. Maximal electroshock method – (Only mice)

MES - Lacosmide
- Flecainide - Test Dose- I
- Flecainide - Test Dose- II

5. Calcium channel blocker and activator alone:

Protocol – VII (Only Mice)

I. Chemoconvulsant method – (Only Mice)

PTx & - Nifedipine (Std.,)
Kainic acid - Amiodarone (L-type Calcium Channel blocker)
- Bay K 8644, S(-) (L-type Calcium Channel activator)

II. Maximal electroshock method – (Only mice)

MES - Nifedipine (Std.,)
- Amiodarone (L-type Calcium Channel blocker)
- Bay K 8644, S(-) (L-type Calcium Channel activator)

6. PDEs inhibitors along with sGC inhibitor, calcium channel modulator and calcium channel blocker:

6.1. PDE-7 inhibitor with the presence of sGC activator and inhibitor:

Protocol – VIII (Only Mice)

I. Chemoconvulsant method- (Only Mice)

Pilocarpine,	-	A 350619 (sGC activator)
Kainic acid &	-	Methylene blue (sGC inhibitor)
PTx	-	BRL 50481 (PDE-7 inhibitor)
	-	A 350619 + BRL 50481
	-	Methylene blue + BRL 50481

6.2. PDE-4 inhibitor with the presence of PDE-7 inhibitor, sGC inhibitor, calcium channel modulator and blocker:

Protocol – IX (Only Mice)

I. Chemoconvulsant method – (Only Mice)

PTZ,	-	Etazolate Alone (PDE-4 inhibitor)		
Kainic acid &	-	Etazolate	+	BRL 50481
Pilocarpine	-	Etazolate	+	Methylene blue
	-	Etazolate	+	Amiodarone
	-	Etazolate	+	Nifedipine

II. Maximal electroshock method – (Only mice)

MES	-	Etozolate Alone (PDE-4 inhibitor)		
	-	Etozolate	+	BRL 50481
	-	Etazolate	+	Methylene blue
	-	Etazolate	+	Amiodarone
	-	Etazolate	+	Nifedipine

6. MATERIALS AND METHODS

6.1. DRUGS & CHEMICALS USED:

Rolipram, Cilostazol, SQ 22536, A-350619, Methylene blue, Pentylenetetrazole or metrazol (PTZ), Picrotoxin (PTx), Kainic acid and 10 % w/v Dimethyl sulfoxide (DMSO) were obtained from Sigma chemical Co.(St.Louis, MO, USA), Dipyridamole, BRL-50481 and Etazolate were bought from Tocris Bioscience (UK), Zonisamide and Sildenafil were obtained from Sun Pharma (Mumbai, India), Amrinone was bought from Samarth Pharma (India), Milrinone was obtained from Sanofi Synthelab Ltd (Mumbai, India), Gabapentin was purchased from Micro labs Ltd. (Bangalore, India), Lacosamide was bought from Cayman Chemicals (USA), Amiloride was obtained from Micro Nova Pharmaceuticals (Bangalore, India), Amiodarone was purchased form Zydus Alidac (Ahemedabad, India), Flecainide was obtained from Shreeji Pharma International, (Gujarat, India), Nifedipine was purchased from Bayer India Ltd (Mumbai, India), Bay K8644 S(-) was bought from Santa Cruz Biotechnology Inc. (USA), Isoniazid (INH) was obtained from Fourts India Ltd (Chennai, India), Pilocarpine was bought from FDC Limited, (Mumbai, India), Normal saline (0.9 %) received from CMC (Vellore, India), Sterile water for injection was obtained from Nirmal Prime Health Care (Mumbai, India), Electro Convulsiometer (Techno, India).

6.2. EXPERIMENTAL ANIMALS & ETHICAL CONSIDERATION:

Swiss Albino mice (22 – 26gm) and Wistar strain rats (180-220gm) of either sex were used for this study. The animals were obtained from animal house, Christian Medical College, Vellore. After the arrival, the animals were placed randomly and allocated to treatment groups in polypropylene cages with paddy husk as bedding.

Animals were housed at a temperature of 24 ± 2°C and relative humidity of 30 – 70 % at 12:12 light: dark cycle was followed. All animals were allowed to free access to water and fed with standard commercial pelleted rat/mice chaw (M/s. Hindustan Lever Ltd, Mumbai). All the experimental procedures and protocols used in this study were reviewed by the Institutional Animal Ethics Committee and were in accordance with the guidelines of the IAEC.

6.3. PHARMACOLOGICAL STUDY:

ANIMAL MODELS OF EPILEPSY: (BOTH RATS AND MICE)

6.3.1. CHEMOCONVULSANT METHOD:

The animals both mice and rats were divided into various groups with six animals (n=6) in each. Treatment schedule and group description were prepared as per protocols. All the drugs were administered intraperitoneally 30 min prior to the administration of chemoconvulsants. 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 Yemitan and Salahdeen, 2005; Salahdeen and Yemiten, 2006 method. The following chemoconvulsants were used for our study,

1. Pentylenetetrazole (PTZ)
2. Picrotoxin (PTx)
3. Isoniazid (INH)
4. Kainic acid
5. Pilocarpine

6.3.1. (1). Pentylenetetrazole (PTZ) or Metrazol induced convulsion:

Pentylenetetrazole induced clonic convulsion in rats or mice are similar to the convulsions observed in petit mal epilepsy. PTZ antagonizes the action of gamma-amino butyric acid (GABA) and induces convulsions. The convulsions are inhibited by drugs like diazepam, sodium valproate, gabapentin, etc., (Swinyard, 1982).

6.3.1. (2). Picrotoxin (PTx) induced convulsion:

Picrotoxin induced convulsions are used to further evaluate CNS-active compounds. Picrotoxin is regarded as a $GABA_A$-antagonist modifying the function of the chloride ion channel of the $GABA_A$ receptor complex (Usunoff *et al.,* 1969)

6.3.1. (3). Isoniazid (INH) induced convulsion:

Isoniazid can precipitate convulsions in patients with seizure disorders. The compound is regarded as a GABA-synthesis inhibitor (Costa *et al*., 1975). Clonic tonic seizures are elicited in mice which are antagonized by anxiolytic drugs.

6.3.1. (4). Kainic acid induced convulsion:

Glutamate is the predominant excitatory neurotransmitter in the central nervous system. The involvement of glutamate in key neurological processes such as synaptogenesis (McDonald, 1990), as well as neurodegenerative disorders and neurotoxicity (Meldrum, 1990) has stimulated research focused on identifying glutamate receptors and understanding their functions.

Glutamate receptors are classified into two groups, metabotropic and ionotropic. Ionotropic glutamate include NMDA and non-NMDA subclasses. Receptors preferring kainic acid (KA receptors) are categorized within the non-NMDA subclass along with

AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) receptors (Wisden, 1993)

Kainic acid is a conformationally restricted analog of L-glutamic acid and is the prototype agonist at the kainate class of ionotropic glutamate receptors. Despite having lower affinity at kainate receptors than the kainic acid analog, domoic acid, kainic acid is the preferred agonist due to its greater selectivity (Hampson, 1998). Kainic acid has been used to delineate differences between AMPA and KA recognition sites. It is an excitotoxin that has been used to model experimental epilepsy and neurodegenerative diseases *in- vivo* (Buckmaster, 1997; Gluck *et al.,* 2000; Routbort, 1999).

6.3.1. (5). Pilocarpine induced convulsion:

Pilocarpine administration has been used as an animal model for temporal lobe epilepsy since it produces several morphological and synaptic features in common with human complex partial seizures. Pilocarpine is a cholinergic drug, non-selective muscarinic agonist, which induces seizures are initiated via muscarinic receptors and further mediated via NMDA receptors. Sustained increases in extracellular glutamate levels after pilocarpine administration are related to the limbic seizures. Pilocarpine initially decreased extracellular hippocampal glutamate and GABA levels. During the subsequent pilocarpine-induced limbic convulsions extracellular glutamate, GABA and dopamine concentrations in hippocampus were significantly increased. (Ilse *et al*., 1997; Turski *et al*., 1989; Lauren *et al*., 2006)

6.3.2. MAXIMAL ELECTROSHOCK METHOD (MES):

The animals both mice and rats were divided into various groups with six animals (n=6) in each. Treatment schedule and group description were prepared as per protocols. All the drugs were administered intraperitoneally 30 min prior to the electroshock. The electroshock will be induced in animal by passing a current of 55 mA to mice and 150 mA to rats for 0.2 sec duration through electroconvulsiometer using corneal electrodes. The incidence of seizures, tonic limb flexion, tonic extensor, clonus, stupor and recovery / mortality of the animals will be observed and noted as per Achliya *et al.,* 2005.

Apart from the above findings the study also summarizes the total duration of convulsion, percentage change from control, mortality and protection in incredible levels of percentage in both animal models of epilepsy.

6.4. EXPERIMENTAL DESIGN:

The present experimental study design was carried out with the following models.

1. Phosphodisesterase (PDEs) inhibitors alone,
2. PDEs inhibitors along with adenylate cyclase (AC) inhibitor,
3. PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor,
4. Ion channel modulators alone (both sodium and calcium ions),
5. Calcium channel blocker and activator alone,
6. PDEs inhibitors along with sGC inhibitor, calcium channel modulator and calcium channel blocker.

The above said all the experimental models were preceded with various protocols in both animal models of epilepsy.

6.4.1. DOSE SELECTION & DRUG TREATMENT:

The selection of dose for all drugs and pharmacological tools for the animal models of epilepsy were based on the previous research work. The route of administration of almost all the drugs and chemicals were intra peritoneal (i.p.).

6.5. STATISTICAL ANALYSIS:

All the datas were expressed as mean ± SEM. The statistical analysis were carried out by one way analysis of variance (ANOVA) followed by Dunnett's test as well as Tukey-Kramer Multiple Comparisons Test using Graphpad Instat version 3. Statistically significant difference was ascertained by 'P' value which is considered significant at the level of $P<0.05$ and highly significant at $P<0.001$.

7. RESULTS AND ANALYSIS

The present study results were explained as per the experimental study design.

7.1 Effect of Phosphodiesterase Inhibitors :

7.1.1. PDE-3 inhibitors alone:

Evaluation of onset of seizures:

(i) INH induced seizures:

Table 4 and 5 shows the data obtained from experiments conducted with INH induced seizures. In animals treated with normal saline onset of action were noticed 2830 ± 52.33 sec and convulsions appeared 3065 ± 45.43 sec after INH. Amrinone in a dose of 0.5 mg/kg significantly potentiated the onset of action, jerky movements and convulsions ($p<0.05$) where as the rate of onset of action, jerky movements and convulsions time was reduced significantly in the doses like 0.6 mg/kg and 0.7 mg/kg of amrinone ($p<0.001$).

Simultaneously the rate of onset of action, jerky movements and convulsion time was reduced at the great extent even in the low doses like (200 μg/mg and 300 μg/mg) of milrinone ($p<0.001$) considerable mortality (67%) was observed while using amrinone (0.6 mg/kg and 0.7 mg/kg) and milrinone (100 μg/kg, 200 μg/kg and 300 μg/kg).

Table 4: Action of various dose levels of amrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	CHEMO-CONVULSANT ISONIAZID (INH) Onset Time Of Various Phases Of Convulsions (in sec.)		
	Action	**Jerky Movements**	**Convulsions**
Normal Saline (5 ml/kg, i.p)	2830 ± 52.33	3000 ± 46.47	3065 ± 45.43
Amrinone (0.5 mg/kg, i.p)	2190 ± 45.82 *	2293.33 ± 44.24 *	2320 ± 43.42 *
Amrinone (0.6 mg/kg, i.p)	1938 ± 37.62 **	2025 ± 39.30 **	2063.33 ± 34.79 **
Amrinone (0.7 mg/kg, i.p)	1601.67 ± 17.78 **	1681.67 ± 20.56 **	1730 ± 15.27 **

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to chemical convulsant injection of INH (500 mg/kg, s.c.). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05 and ** p<0.001, compared to the normal saline treated group.*

Table 5: Action of various dose levels of milrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	**CHEMO-CONVULSANT ISONIAZID (INH)** **Onset Time Of Various Phases Of Convulsions (in sec.)**		
	Action	**Jerky Movements**	**Convulsions**
Normal Saline (5 ml/kg, i.p)	2830 ± 52.33	3000 ± 46.47	3065 ± 45.43
Milrinone (50 µg/kg, i.p)	2520 ± 34.65	2860 ± 25.31	3060 ± 21.92
Milrinone (100 µg/kg, i.p)	2220 ± 34.65 *	2483.3 ± 26.04 *	2610 ± 25.71 *
Milrinone (200 µg/kg, i.p)	1870 ± 36.04 **	2100 ± 30.98 **	2270 ± 36.04 **
Milrinone (300 µg/kg, i.p)	1670 ± 21.91 **	1890 ± 25.71 **	2010 ± 33.76 **

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to chemical convulsant injection of INH (500 mg/kg, s.c.). The data were analysed by one-way ANOVA followed by Dunnett's test. * $p<0.05$ and ** $p<0.001$, compared to the normal saline treated group.*

(ii). PTZ induced seizures:

Table 6 and 7 summarizes the data obtained from experiments conducted with PTZ induced seizures. In animals treated with normal saline onset of action were observed 86.5 ± 1.29 sec after PTZ and convulsions appeared 171.5 ± 1.61 sec after PTZ. Amrinone in a dose of 0.6 mg/kg significantly enhanced onset of action (p<0.05) and stimulate the convulsions (p<0.001). The results show that there was a significant increase in onset of action of seizure activity when increased the dose (0.7 mg/kg) of amrinone (p<0.001). At the same time milrinone in a dose of 300 µg/kg significantly potentiated onset of action (p<0.001) and produce the convulsion phenomenon much faster (p<0.001) than amrinone. Even at a very low dose 33% of morality was observed while using amrinone (0.6 mg/kg and 0.7 mg/kg) and milrinone (200 µg/kg and 300 µg/kg).

Table 6: Action of various dose levels of amrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	**CHEMO-CONVULSANT Pentylenetetrazole (PTZ) Onset Time Of Various Phases Of Convulsions (in sec.)**		
	Action	**Jerky Movements**	**Convulsions**
Normal Saline (5 ml/kg, i.p)	86.5 ± 1.29	140.33 ± 1.36	171.5 ± 1.61
Amrinone (0.5 mg/kg, i.p)	65 ± 1.24	96 ± 1.07*	125 ± 1.91*
Amrinone (0.6 mg/kg, i.p)	58 ± 0.93*	76.83 ± 1.33**	93.83 ± 0.87**
Amrinone (0.7 mg/kg, i.p)	47.5 ± 0.92**	55.83 ± 2.24**	75.33 ± 1.82**

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p.). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05 and ** p<0.001, compared to the normal saline treated group.*

Table 7: Action of various dose levels of milrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	**CHEMO-CONVULSANT Pentylenetetrazole (PTZ) Onset Time Of Various Phases Of Convulsions (in sec.)**		
	Action	**Jerky Movements**	**Convulsions**
Normal Saline (5 ml/kg, i.p)	86.5 ± 1.29	140.33 ± 1.36	171.5 ± 1.61
Milrinone (50 µg/kg, i.p)	103.8 ± 4.37	138 ± 4.41	128.3 ± 5.88*
Milrinone (100 µg/kg, i.p)	62.7 ± 1.92	76.3 ± 2.78*	79.8 ± 2.65**
Milrinone (200 µg/kg, i.p)	54.5 ± 1.35*	60.8 ± 1.26**	63.8 ± 1.38**
Milrinone (300 µg/kg, i.p)	47.3 ± 1.18**	49.8 ± 1.43**	52.2 ± 1.51**

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p.). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05 and ** p<0.001, compared to the normal saline treated group.*

(iii) Maximal Electroshock Test:

Table 8 and 9 illustrates the action of various dose levels of amrinone and milrinone against MES induced seizures. In which 0.6 mg/kg and 0.7 mg/kg of amrinone produced a gradual reduction in tonic limb flexion significantly ($p<0.05$) when compared with normal saline. Significant ($p<0.001$) was observed in stupor phase of convulsion at the dose of 0.6 mg/kg and 0.7 mg/kg of amrinone. Likewise milrinone treated animals showed a significant ($p<0.001$) reduction in tonic limb tonic extensor and stupor flexion, phases of convulsion in the 200 μg/kg and 300 μg/kg dose levels.

Milrinone in the doses like 200 μg/kg and 300μg/kg treated animals produced the significantly reduced the clonus phases of convulsion at the level of $p<0.005$ and $p<0.001$ respectively. Mortality (67%) was observed in both doses like 200 μg/kg and 300 μg/kg of milrinone.

Table 8: Action of various dose levels of amrinone on maximal electroshock induced convulsions in mice (n=6)

Treatment (mg/kg, i.p)	**Onset Time Of Various Phases Of Convulsions (in sec.)**				
	Tonic Limb Flexion	**Tonic Extensor**	**Clonus**	**Stupor**	**Recovery / Death**
Normal Saline (5 ml/kg, i.p)	5.67 ± 0.33	23.33 ± 0.67	36.83 ± 1.38	66.67 ± 1.23	196.25 ± 5.55
Amrinone (0.5 mg/kg, i.p)	4.33 ± 0.21	19.83 ± 0.31	37 ± 0.73	59.83 ± 0.6 *	226 ± 0.82
Amrinone (0.6 mg/kg, i.p)	3.5 ± 0.22 *	15.5 ± 0.43 *	33.33 ± 0.42	53.83 ± 0.6 **	207 ± 3.72
Amrinone (0.7 mg/kg, i.p)	3.17 ± 0.17 *	9.83 ± 0.31 **	28 ± 0.51 *	43 ± 0.86 **	207.33 ± 0.42

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to maximal electroshock (60 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * $p<0.05$ and ** $p<0.001$, compared to the normal saline treated group.*

Table 9: Action of various dose levels of milrinone on maximal electroshock induced convulsions in mice (n=6)

Treatment (mg/kg, i.p)	**Onset Time Of Various Phases Of Convulsions (in sec.)**				
	Tonic Limb Flexion	**Tonic Extensor**	**Clonus**	**Stupor**	**Recovery/ Death**
Normal Saline (5 ml/kg, i.p)	5.67 ± 0.33	23.33 ± 0.67	36.83 ± 1.38	66.67 ± 1.23	196.25 ± 5.55
Milrinone (50 µg/kg, i.p)	4.8 ± 0.33	18.3 ± 0.41	36.8 ± 0.41	57.5 ± 0.78 *	218.3 ± 0.86
Milrinone (100 µg/kg, i.p)	3.3 ± 0.20 *	15.2 ± 0.49 *	32.7 ± 0.82	53.5 ± 0.65 **	280 ± 3.14
Milrinone (200 µg/kg, i.p)	2.7 ± 0.20 **	8.7 ± 0.3 3 **	25.8 ± 0.33 *	47.7 ± 0.65 **	235.0 ± 1.71
Milrinone (300 µg/kg, i.p)	2.2 ± 0.17 **	7.3 ± 0.33 **	20 ± 0.57 **	39.8 ± 0.69 **	237.5 ± 1.43

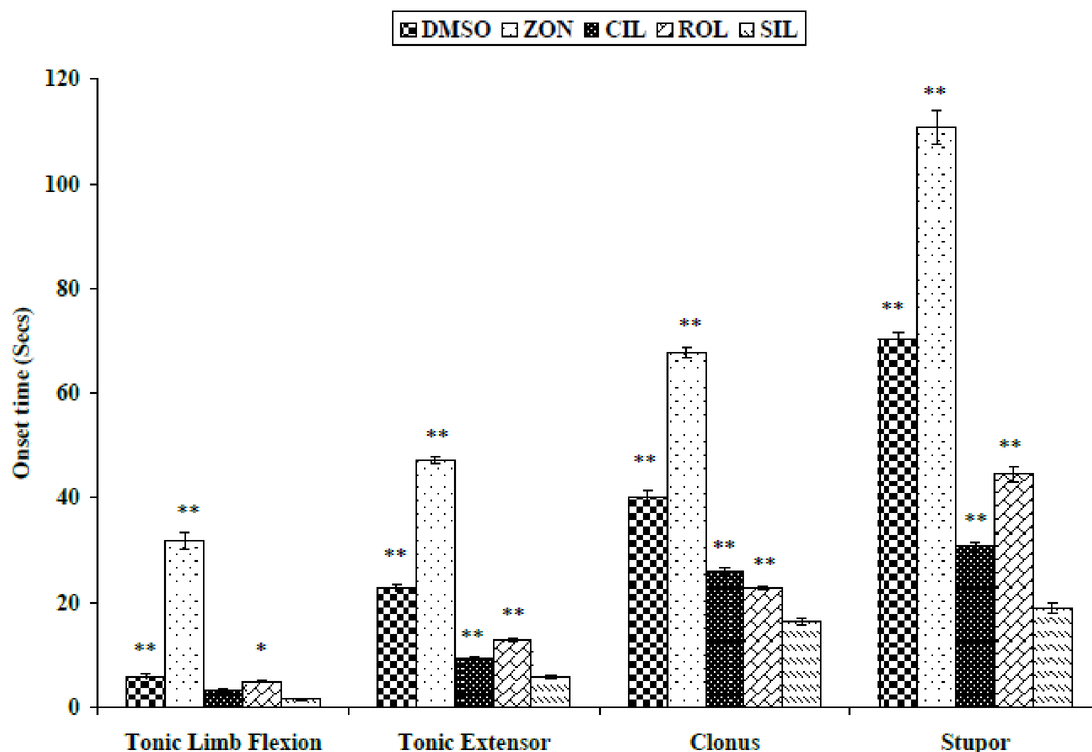
*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to maximal electroshock (60 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * p <0.05 and ** p<0.001, compared to the normal saline treated group.*

7.1.2. Effect of PDE-3, 4 & 5 inhibitors :

A. Maximal Electroshock Test:

Fig. 17 illustrates the action of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil against MES induced seizures in mice. In which sildenafil (5 mg/kg, i.p.) produced a reduction in the tonic limb flexion significantly (p<0.01) when compared to other groups. Like wise, rolipram (2.4 mg/kg, i.p.) treated animals showed significant (p<0.05) reduction in tonic limb flexion. In the similar manner, sildenafil produced a reduction in the tonic extensor, clonus and stupor phases of convulsion significantly (p<0.01) when compared to other groups.

Fig. 17: Effect of PDE-3, PDE-4 & PDE-5 inhibitors on maximal electroshock induced convulsions in mice (n=6)



*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 25 mins prior to maximal electroshock (45 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05, ** p<0.01 and *** p<0.001 Vs Sildenafil treated group.*

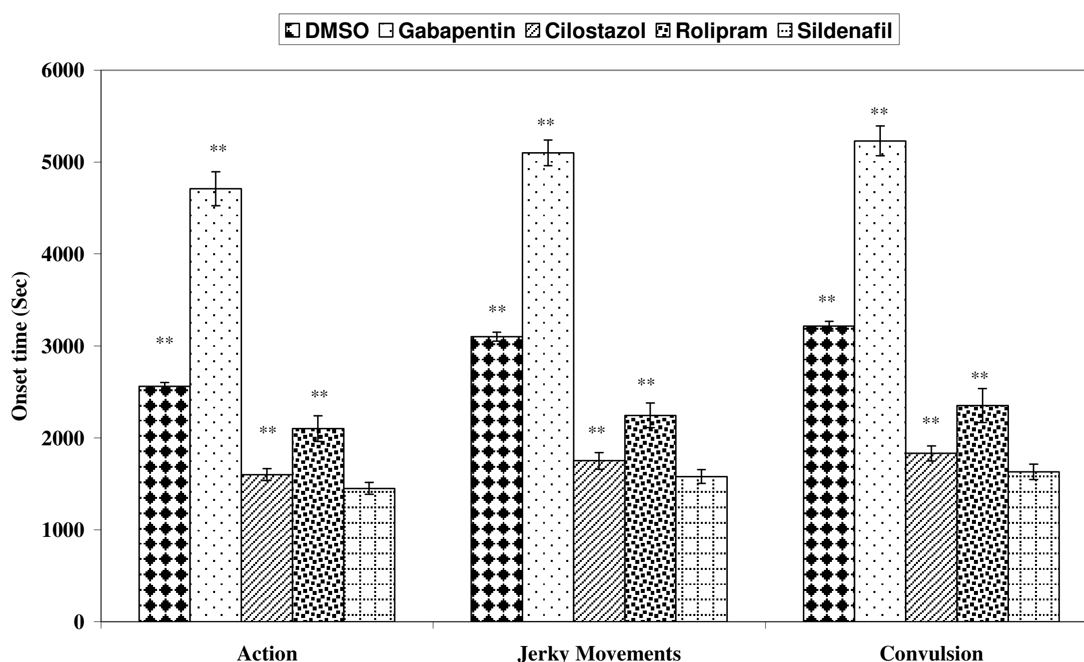
B. Chemical convulsant method:

Isoniazid (INH) induced seizures:

Fig. 18 exhibits the action of PDE-3, 4 and 5 inhibitors on INH induced seizures in mice. Sildenafil (5 mg/kg, i.p.) showed a gradual reduction in the onset of action, jerky movements and convulsion significantly (p<0.01) when compared to other PDE

inhibitors such as cilostazol (10 mg/kg, i.p.) and rolipram (2.4 mg/kg, i.p.), DMSO (5 ml/kg, i.p.) and gabapentin (2.5 mg/kg, i.p.).

Fig. 18: Effect of PDE-3, PDE-4 & PDE-5 inhibitors on chemoshock seizures in mice (n=6)

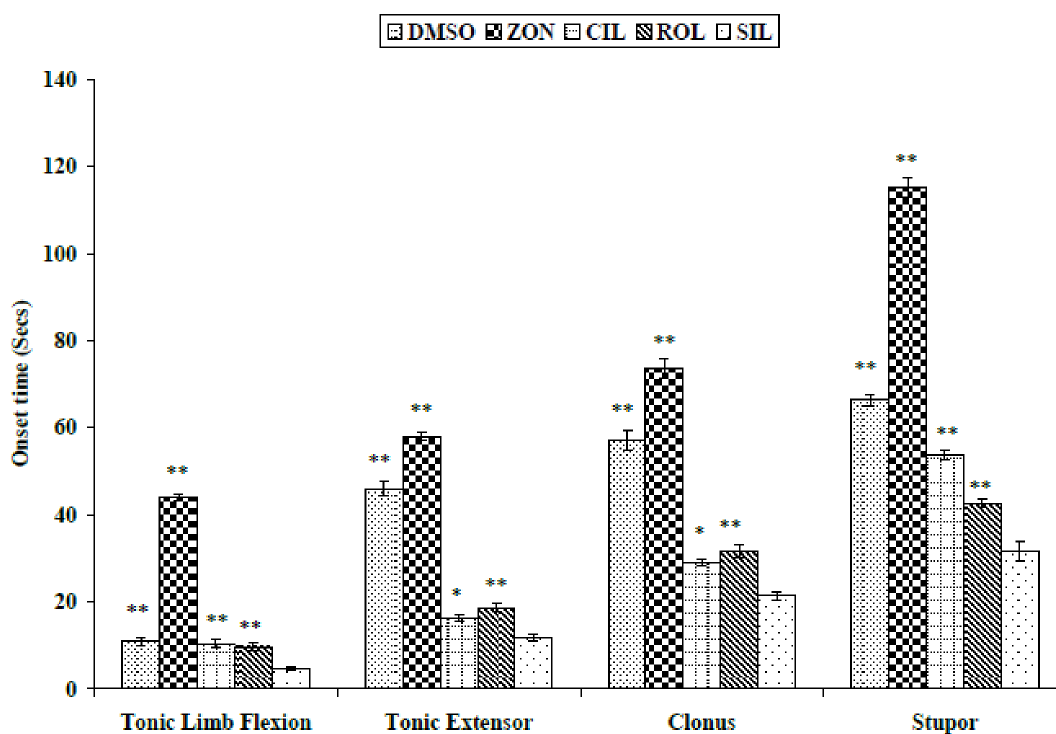


*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 25 mins prior to chemical convulsant injection of INH (500 mg/kg, s.c). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05, ** p<0.01 and *** p<0.001 Vs Sildenafil treated group.*

C. Maximal electroshock (MES) method for rats:

Fig. 19 exhibits the action of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil against MES induced seizures in rats. In which sildenafil (3.5 mg/kg, i.p.) produced a gradual reduction in the tonic limb flexion significantly ($p<0.01$) when compared to other groups. Similarly, sildenafil produced a gradual reduction in the tonic extensor, clonus and stupor phases of convulsion significantly ($p<0.01$) when compared to other groups except cilostazol (7 mg/kg, i.p.) treated rats. Table 10 shows that the treatment protocol of MES model for mice and rats, and INH model for mice by using PDE-3, 4 and 5 inhibitor.

Fig. 19: Action of PDE-3, PDE-4 & PDE-5 inhibitors on maximal electroshock induced convulsions in rats (n=6)



*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 25 mins prior to maximal electroshock (150 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05, ** p<0.01 and *** p<0.001 Vs Sildenafil treated group.*

***Table 10: Treatment protocol of maximal electroshock (MES) model for mice & rats and chemoconvulsant (INH) model for mice*.**

Treatment Groups	Drugs used	Category	MES for Mice (45 mA for 0.2 sec)	Chemoconvulsant (INH) for Mice (500 mg/kg, s.c)	MES for Rats (150 mA, 0.2 sec)
Group- I	10% DMSO	Solvent Control	5 ml/kg, i.p.	5 ml/kg, i.p.	3.5 ml/kg, i.p.
Group- II	Zonisamide/ Gabapentin	Positive Control	(Zonisamide) 50 mg/kg, i.p.	(Gabapentin) 2.5 mg/kg, i.p.	(Zonisamide) 35 mg/kg, i.p.
Group- III	Cilostazol	PDE-3 inhibitor (cGMP-inhibited)	10 mg/kg, i.p.	10 mg/kg, i.p.	7 mg/kg, i.p.
Group- IV	Rolipram	PDE-4 inhibitor (cAMP-specific)	2.4 mg/kg, i.p.	2.4 mg/kg, i.p.	1.7 mg/kg, i.p.
Group- V	Sildenafil	PDE-5 inhibitor (cGMP-specific)	5 mg/kg, i.p.	5 mg/kg, i.p.	3.5 mg/kg, i.p.

Data explains the different groups receiving various drugs and its appropriate dose levels with respect to animal models.

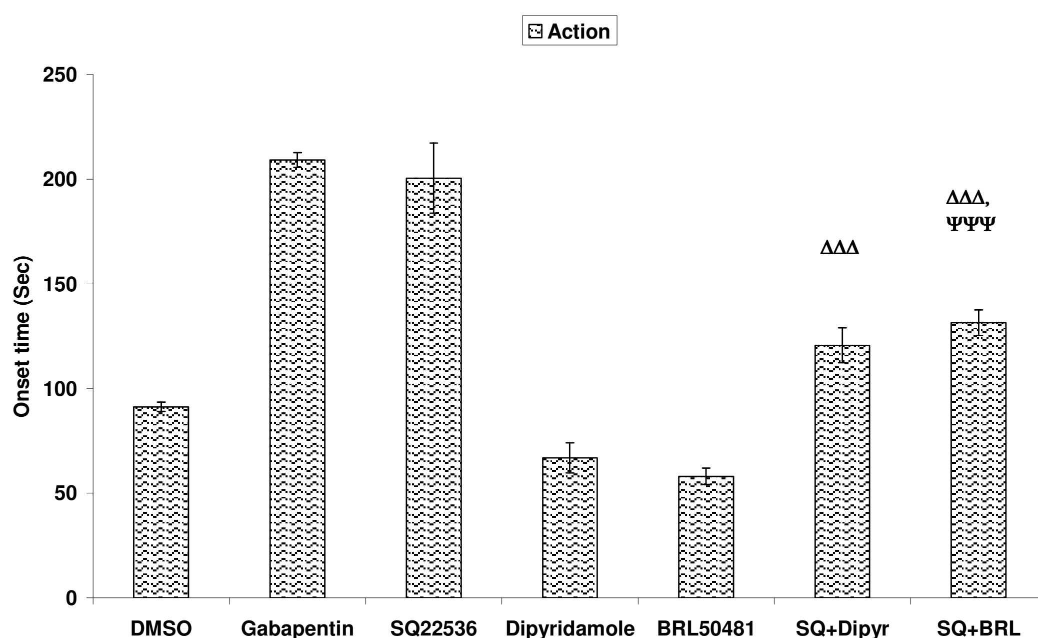
7.2. Effect of PDEs inhibitors in combination with adenylate cyclase (AC) inhibitor:

A. Chemoshock method:

Pentylenetetrazole (PTZ) or Metrazol (MTZ) induced seizure model in mice:

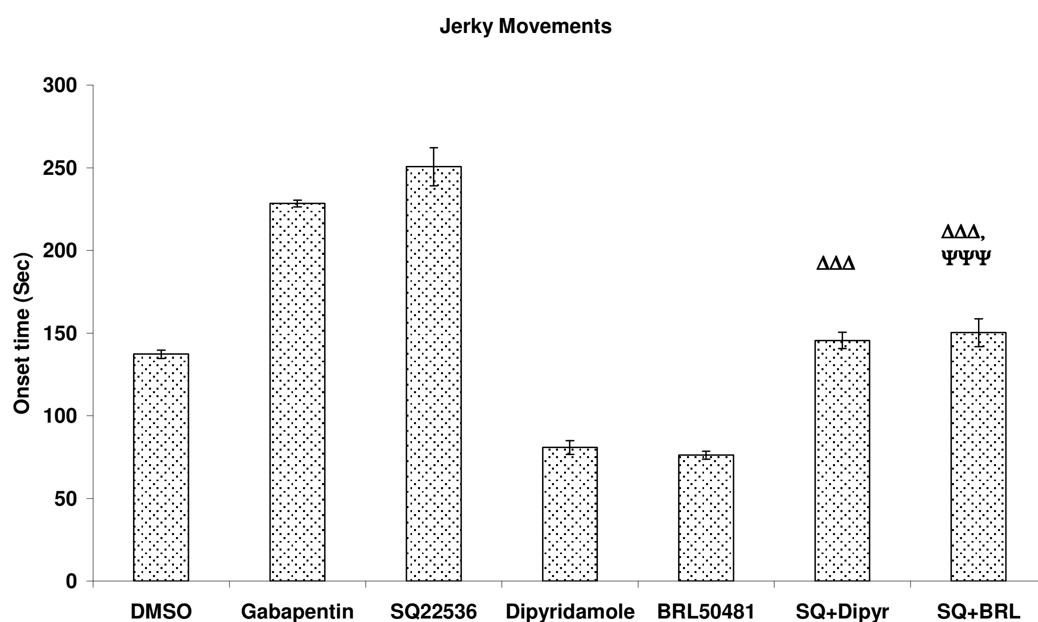
Fig. 20, 21 and 22 summarizes the data obtained from experiments conducted with PDE- 5/6/7/8/10/11 inhibitors along with adenylate cyclase activator and inhibitor on chemoshock such as PTZ (60 mg/kg, i.p.) induced seizures in mice**.** The highlights of the findings are the data obtained with combination of AC inhibitor, SQ22536 and dipyridamole which showed a good reduction ($P<0.001$) in onset of action, jerky movements and convulsion against PTZ induced seizures in mice when compared to SQ22536 alone received group of animals (Fig. 20, 21 & 22). The combination of SQ22536 and PDE-7 inhibitor, BRL50481 received mice showed a significant ($P<0.001$) decrease in seizure activity when compared to SQ22536 and BRL50481 alone treated mice (Fig. 20, 21 & 22). The overall highlights of Fig. 20, 21 and 22 explicit the individual effect of AC inhibitor, SQ22536 which delays the onset of action of seizures as well as prolongs the total duration of convulsive time (Table 11).

Fig. 20: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on chemoshock seizures in mice.



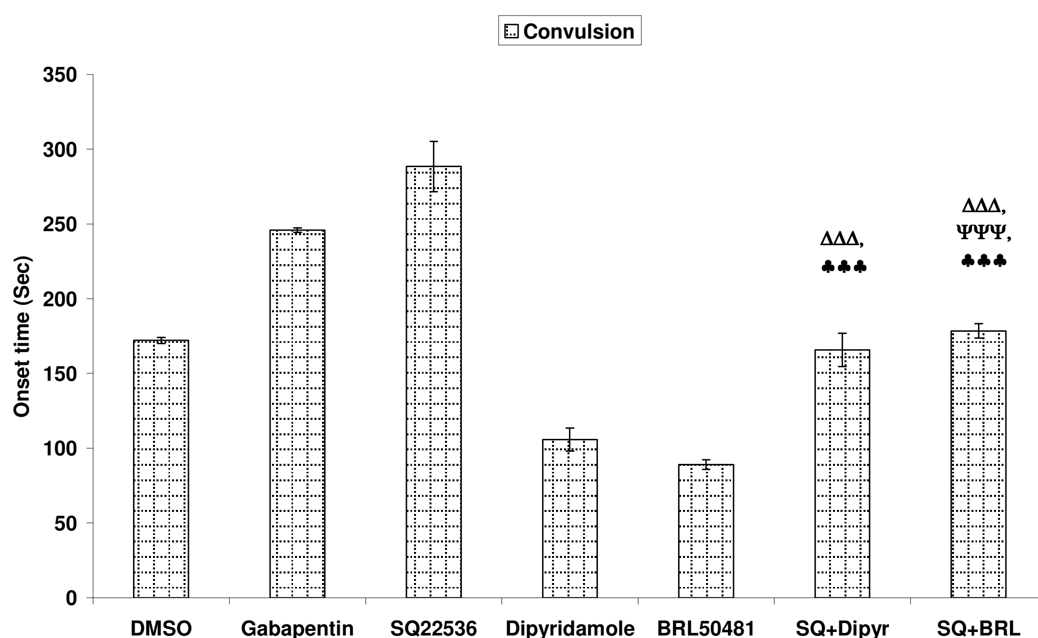
Data represented as mean ± SEM (n=6), which represents onset time of action phase of convulsion in seconds. Treatments were given 30 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p.). **ΔΔΔ** denotes $p<0.001$ compared with SQ22536 received group, **ΨΨΨ** denotes $p<0.001$ compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 21: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on chemoshock seizures in mice.



Data represented as mean ± SEM (n=6), which represents onset time of jerky movement phase of convulsion in seconds. Treatments were given 30 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p.). **ΔΔΔ** denotes $p<0.001$ compared with SQ22536 received group, **ΨΨΨ** denotes $p<0.001$ compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 22: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on chemoshock seizures in mice.



Data represented as mean ± SEM (n=6), which represents onset time of convulsion phase in seconds. Treatments were given 30 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p.). **ΔΔΔ** denotes p<0.001 compared with SQ22536 received group, **ΨΨΨ** denotes p<0.001 compared with BRL50481 received group, ♣ and ♣♣♣ denotes p<0.05 and p<0.001, respectively, compared with dipyridamole received group group and **ns** denotes non significant (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 11 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 SQ22536 alone treated (60.2%) and combination of SQ22536 with BRL50481 treated (27.4%) groups compared to DMSO received group (100%). The data shows that 83.3% and 66.7% of protection of animals were noticed in SQ22536 and i.p injection of SQ22536 followed by dipyridamole treated groups against PTZ induced seizures in mice. The results show that there was an increase in seizure activity (0.9%) in BRL50481 treated alone animals. Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown), Fig 20, 21 and 22 expresses the action of animals against PTZ induced seizures as follows, gabapentin treated group showed significant (P<0.001) reduction in onset of action and jerky movements of seizures, when compare to all groups except SQ22536 (NS). The data shown in Table 11 also demonstrates that i.p administration of SQ22536 (1 nmol/kg, i.p) greatly increased the anti-convulsant activity (P<0.01) along with higher protection (83.3%) range. Simultaneously, the combined effect of SQ22536 with exogenously administered BRL50481 (2 mg/kg, i.p.) and SQ22536 with dipyridamole (2 mg/kg, i.p.) showed a significant (P<0.01 & P<0.05) anti-convulsant activity with moderate protection (50% & 66.7%) range respectively (Table 11). A similar trend was noted in the results obtained from SQ22536 received groups explicit mild reduction (P<0.05) in convulsion compared to gabapentin. SQ22536, dipyridamole and BRL50481 treated groups showed a significant reduction (P<0.001) in jerky movements against DMSO received mice (data not shown).

Table 11: **Effect of drugs on pentylenetetrazole (60 mg/kg, i.p.) induced seizures in mice.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	212.50	100	83.3	16.7	--
II	Gabapentin	275.00	29.4	33.3	66.7	*P*<0.01
III	SQ22536	340.05	60.2	16.7	83.3	*P*<0.01
IV	Dipyridamole	240.31	13.2	50.0	50.0	NS
V	BRL50481	210.40	0.9	66.7	33.3	NS
VI	SQ22536 + Dipyridamole	260.18	22.5	33.3	66.7	*P*<0.05
VII	SQ22536 + BRL50481	270.42	27.4	50.0	50.0	*P*<0.01

The group of mice (n=6) were injected with 60 mg/kg, i.p. of PTZ 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.), SQ22536 (1 nmol/kg, i.p.), Dipyridamole (2 mg/kg, i.p.) and BRL50481 (2 mg/kg, i.p.). (One way ANOVA followed by Dunnett's test compared with DMSO treated mice)

Table 12: **Effect of drugs on maximal electroshock induced seizures in rats.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	236.50	100	100	--	--
II	Zonisamide	285.00	20.6	33.3	66.7	$P<0.05$
III	SQ22536	335.00	41.7	16.7	83.3	$P<0.01$
IV	Dipyridamole	260.81	10.3	33.3	66.7	NS
V	BRL50481	283.45	19.9	83.3	16.7	$P<0.05$
VI	SQ22536 + Dipyridamole	290.40	22.8	50.0	50.0	$P<0.01$
VII	SQ22536 + BRL50481	330.47	39.7	33.3	66.7	$P<0.01$

The group of rats (n=6) were subjected to 150 mA (0.2 sec) electroshock and 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 injected intraperitoneally. The drugs used were administered in the following doses. DMSO (3.5 ml/kg, i.p.), zonisamide (35 mg/kg, i.p.), SQ22536 (0.7 nmol/kg, i.p.), Dipyridamole (1.4 mg/kg, i.p.) and BRL50481 (1.4 mg/kg, i.p.). (One way ANOVA followed by Dunnett’s test compared with DMSO treated rats).

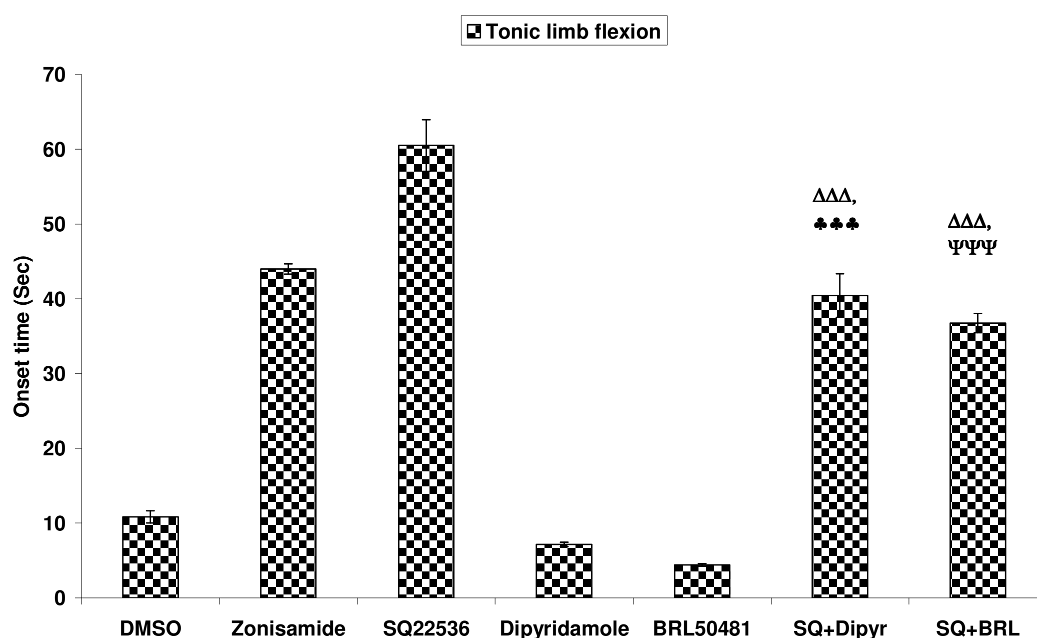
B. Maximal electroshocks (MES) method for rats:

Fig. 23, 24, 25 and 26 illustrate the data obtained from experiments conducted with maximal electroshock induced seizures in rats**.** It is evident from the data displayed in fig. 23, 24 and 25 that combination of AC inhibitor, SQ22536 and dipyridamole effectively (P<0.001) decreased the tonic limb flexion, tonic extensor and clonus stage of convulsion, compared to SQ22536 alone treated rats. The same significant level (P<0.001) was obtained in SQ22536 combined with BRL50481, instead of dipyridamole (Fig. 23, 24 & 25). The overall highlights of fig. 23, 24, 25 and 26 explicit the BRL50481 alone received group, potentiates the seizure activity against MES induced convulsion. Emphasis was also seen on the independent effect of AC inhibitor, SQ22536 in delaying the onset of seizure activity (Fig. 23, 24, 25 & 26) as well as prolonging the total duration of convulsive time (Table 12).

Table 12 demonstrated the total duration of convulsion, percentage change from control, mortality and protection in marked levels of percentage. The total convulsive time was long lasting significantly (P<0.01) in SQ22536 alone treated (41.7%) and combination of SQ22536 with BRL50481 treated group increase significantly (P<0.01) the duration of convulsion (39.7%), compared to DMSO received group (100%). The data showed that 83.3% and 66.7% of protection of animals were noticed in SQ22536 and i.p injection of SQ22536 followed by BRL50481 treated groups against MES induced seizures in rats. From Table 12 it was evident that there was a significant increase in seizure activity (10.3%) when dipyridamole treated alone. Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown) the action of animals against MES induced seizures. Gabapentin, SQ22536, SQ22536 with dipyridamole, SQ22536 with BRL50481 treated groups showed significant (P<0.001) reduction in onset of tonic limb flexion phase of convulsion, when compare to DMSO. Simultaneously, the individual effect of SQ22536 and dipyridamole received groups showed a significant (P<0.001) reduction in tonic extensor phase of convulsion, against gabapentin treated group. Table 12 reveals that i.p administration of SQ22536 (0.7 nmol/kg, i.p.) greatly enhances the anti-convulsant activity (P<0.01) along with higher protection (83.3%) range. At the same time, the combined effect of SQ22536

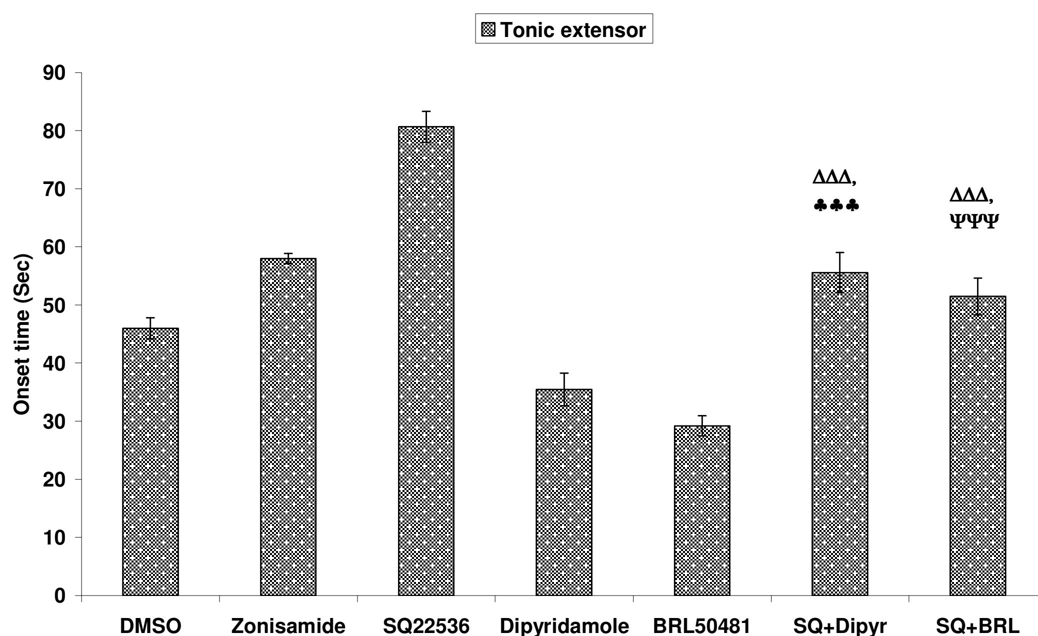
with exogenously administered BRL50481 (1.4 mg/kg, i.p.) and SQ22536 with dipyridamole (1.4 mg/kg, i.p.) showed a significant (P<0.01 and P<0.01) anti-convulsant activity with judicious protection (66.7% and 50%) range respectively (Table 12).

Fig. 23: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



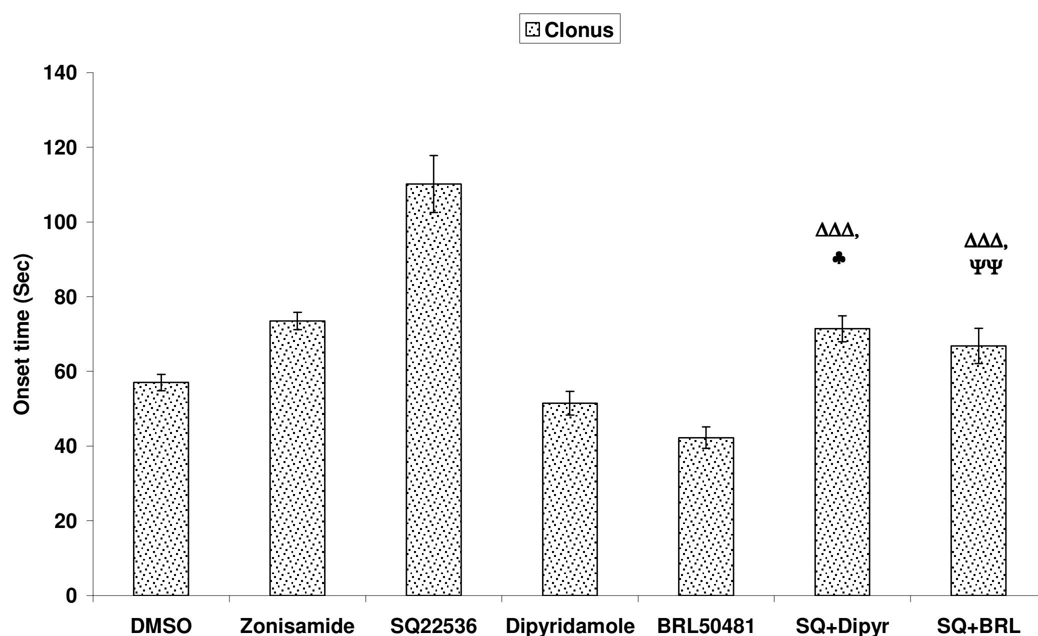
Data represented as mean ± SEM (n=6), which represents onset time of tonic limb flexion phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). **ΔΔΔ** denotes $p<0.001$ compared with SQ22536 received group, **ΨΨΨ** denotes $p<0.001$ compared with BRL50481 received group, ♣♣♣ denotes $p<0.001$ compared with dipyridamole received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 24: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



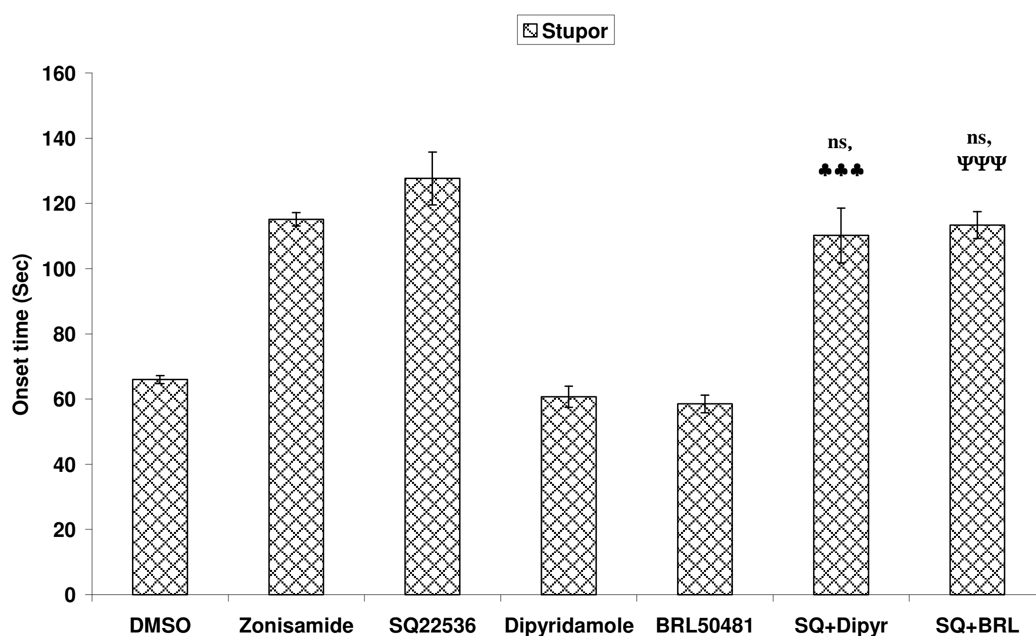
Data represented as mean ± SEM (n=6), which represents onset time of tonic extensor phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). **ΔΔΔ** denotes $p<0.001$ compared with SQ22536 received group, **ΨΨΨ** denotes $p<0.001$ compared with BRL50481 received group, ♣♣♣ denotes $p<0.001$ compared with dipyridamole received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 25: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



Data represented as mean ± SEM (n=6), which represents onset time of clonus phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). **ΔΔΔ** denotes $p<0.001$ compared with SQ22536 received group, **ΨΨ** denotes $p<0.01$ compared with BRL50481 received group, ♣ denotes $p<0.05$ compared with dipyridamole received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 26: Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



Data represented as mean ± SEM (n=6), which represents onset time of stupor phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). **ΨΨΨ** denotes $p<0.001$ compared with BRL50481 received group, ♣♣♣ denotes $p<0.001$ compared with dipyridamole received group and **ns** denotes non significant (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.3. PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor:

A. Chemoshock Method:

Pentylenetetrazole (PTZ) or Metrazol (MTZ) induced seizure model in mice:

Table 13 summarizes the data attained from experiments conducted with PDE-5/6/7/8/10/11 inhibitors beside with guanylate cyclase activator and inhibitor on chemoshock such as PTZ (60 mg/kg, i.p.) induced seizures in mice**.** The combination of methylene blue and PDE-7 inhibitor, BRL50481 received mice showed a significant (*P*<0.001) delay in onset of action, jerky movements and convulsion when compare to A-350619 and BRL 50481 alone treated mice. The overall highlights of Table 13 exhibits the individual effect of methylene blue delays the onset of action of seizures as well as prolongs the total duration of convulsive time.

Table 15 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 methylene blue alone treated (69.2%) group, compared to DMSO received group (100%). The data shows that 83.3% of protections of animals were noticed in both methylene blue treated groups against PTZ induced seizures in mice. The result shows that there was a increase in seizure activity (8.0% and 3.2%) when A-350619 and BRL 50481 treated alone while compare to DMSO received group (100%). Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown), Table 13 expresses the action of animals against PTZ induced seizures as follows, gabapentin treated group showed significant (*P*<0.001) reduction in onset of action and jerky movements of seizures, when compare to all groups. The data shown in Table 15 also demonstrates that i.p. administration of 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 methylene blue with exogenously administered BRL50481 (2 mg/kg, i.p.) showed a significant (*P*<0.05) anti-convulsant activity with moderate protection (66.7% and 83.3%) range respectively (Table 15).

Table 13: Effect of PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor on PTZ (60 mg/kg, i.p) induced seizures in mice (n=6)

Groups	Treatment	Onset time in seconds		
		Pentylenetetrazole (PTZ)		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p) Solvent Control	101.37 ± 3.29	142.38 ± 4.67	170.27 ± 5.76
II	Gabapentin (2.5 mg/kg, i.p) Positive Control	212.90 ± 3.46	225.37 ± 3.57	255.67 ± 7.81
III	A-350619 (100 µM/ kg, i.p) sGC activator	96.02 ± 2.19	117.56 ± 1.78	140.32 ± 5.29
IV	Methylene blue (50 mg/kg, i.p) sGC inhibitor	171.29 ± 5.30	218.52 ± 6.78	260.39 ± 7.30
V	BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	62.12 ± 4.27	80.38 ± 4.67	96.27 ± 5.06
VI	A-350619 (100 µM/ kg,i.p) + BRL 50481 (2 mg/kg, i.p)	89.02 ± 4.09**ΔΔΔ,ΨΨΨ**	103.36 ± 3.98 **ΔΔΔ,Ψ**	118.22 ± 7.25 **ΔΔΔ**
VII	Methylene blue (50 mg/kg, i.p) + BRL 50481 (2 mg/kg, i.p)	158.29 ± 5.30*****,ΨΨΨ**	187.35 ± 5.13*****, ΔΔΔ,ΨΨΨ**	230.49 ± 9.74*****,ΨΨΨ**

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 30 mins prior to chemical -convulsant injection of PTZ (60 mg/kg, i.p). *** denotes $p<0.001$ compared with A-350619 received group, **ΔΔΔ** denotes $p<0.001$ compared with methylene blue received group, **Ψ** and **ΨΨΨ** denotes $p<0.01$ and $p<0.001$, respectively, compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 14: Effect of PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor on MES induced seizures in rats (n=6)

Groups	**Treatment**	**Onset time (sec) in various phases of convulsion**			
		Tonic limb flexion	**Tonic extensor**	**Clonus**	**Stupor**
I	10% w/v DMSO (3.5 ml/kg, i.p) Solvent Control	11.26 ± 0.79	43.54 ± 1.27	54.28 ± 2.89	70.37 ± 3.29
II	Zonisamide (35 mg/kg, i.p) Positive Control	48.27 ± 0.85	62.89 ± 2.71	76.14 ± 4.15	120.13 ± 6.28
III	A 350619 (70 µM/ kg,i.p) sGC activator	11.18 ± 0.39	36.78 ± 1.93	45.29 ± 2.07	54.39 ± 2.39
IV	Methylene blue (35 mg/kg, i.p) sGC inhibitor	65.21 ± 2.14	85.13 ± 3.28	117.45 ± 5.37	134.26 ± 5.06
V	BRL 50481 (1.4 mg/kg, i.p) PDE-7 inhibitor	6.26 ± 0.67	28.78 ± 1.13	31.29 ± 2.15	42.09 ± 3.28
VI	A 350619 (70 µM/ kg,i.p) + BRL 50481 (1.4 mg/kg, i.p)	10.27 ± 0.79**ΔΔΔ**	21.78 ± 2.61 **, ΔΔΔ**	28.69 ±3.37 **ΔΔΔ**	39.39 ± 3.81 **ΔΔΔ**
VII	Methylene blue (35 mg/kg, i.p) + BRL 50481 (1.4 mg/kg, i.p)	51.21 ± 2.12*****, ΔΔΔ, ΨΨΨ**	75.11 ± 3.28*****, ΔΔΔ,ΨΨΨ**	103.25 ± 5.89*****,ΨΨΨ**	121.26 ± 7.08*****,ΨΨΨ**

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA for 0.2 secs). ******* denotes $p<0.001$, compared with A-350619 received group, **Δ** and **ΔΔΔ** denotes $p<0.05$ and $p<0.001$ compared with methylene blue received group, **ΨΨΨ** denotes $p<0.001$, compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 15: **Effect of drugs on pentylenetetrazole induced seizures in mice.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	213.50	100	83.3	16.7	Nil
II	Gabapentin	279.00	30.7	33.3	66.7	$P<0.05$
III	A-350619	196.40	8.0	83.3	16.7	NS
IV	Methylene blue	360.06	69.2	16.7	83.3	$P<0.01$
V	BRL50481	220.43	3.2	66.7	33.3	NS
VI	A-350619 + BRL50481	230.31	8.2	83.3	16.7	NS
VII	Methylene blue + BRL50481	276.12	29.8	33.3	66.7	$P<0.05$

The group of mice (n=6) were injected with 60 mg/kg, i.p. of PTZ 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.), A-350619 (100 μM/kg, i.p.), methylene blue (50 mg/kg, i.p.) and BRL50481 (2 mg/kg, i.p.). (One way ANOVA followed by Dunnett's test compared with DMSO treated mice)

Table 16: **Effect of drugs on maximal electroshock induced seizures in rats.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	242.60	100	100	-	-
II	Zonisamide	280.72	15.1	33.7	66.7	NS
III	A-350619	216.54	11.2	83.3	16.7	NS
IV	Methylene blue	343.67	40.9	16.7	83.3	$P<0.01$
V	BRL50481	278.18	14.1	83.3	16.7	NS
VI	A-350619 + BRL50481	235.67	3.40	50.0	50.0	NS
VII	Methylene blue + BRL50481	340.72	39.7	16.7	83.3	$P<0.01$

The group of rats (n=6) were subjected to 150 mA (0.2 sec) electroshock and 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 injected intraperitoneally. The drugs used were administered in the following doses. DMSO (3.5 ml/kg, i.p.), zonisamide (35 mg/kg, i.p.), A-350619 (70 µM/kg, i.p.), methylene blue (35 mg/kg, i.p.) and BRL50481 (1.4 mg/kg, i.p.). (One way ANOVA followed by Dunnett's test compared with DMSO treated rats).

B. Maximal electroshock (MES) method for rats:

Table 14 depicts the data obtained from experiments conducted with maximal electroshock induced seizures in rats**.** It is evident from the data shown in table 14 that combination of methylene blue and BRL50481 effectively (P<0.001) decreased the tonic limb flexion, tonic extensor, clonus and stupor stage of convulsion, compared to methylene blue alone treated rats. The overall highlights of table 14 exhibits the combined effects of A-350619 with BRL50481 received groups and BRL50481 alone received group, potentiates the seizure activity against in MES induced convulsion. Also this emphasizes that methylene blue delays the onset of seizure activity as well as prolongs the total duration of convulsive time (Table 14).

Table 16 demonstrates the total duration of convulsion, percentage change from control, mortality and protection in marked levels of percentage. The total convulsive time was long lasting significantly (P<0.01) in methylene blue alone treated (40.9%) and combination of methylene blue with BRL50481 treated groups increases significantly (P<0.01) the total duration of convulsion (39.7%), compared to DMSO received group (100%). The data shows that 83.3% of protections of animals were noticed in both methylene blue and i.p. injection of methylene blue followed by BRL50481 treated groups against MES induced seizures in rats. From Table 16, it was evident that there was a significant increase in seizure activity (14.1%) when BRL50481 treated alone. Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown), the action of animals against MES induced seizures. Methylene blue, methylene blue with BRL50481 treated groups showed significant (P<0.01) reduction in onset of various phases of convulsion, when compare to DMSO. Simultaneously, A-350619, methylene blue, A-350619 with BRL50481 received groups showed a significant (P<0.001) reduction in tonic extensor phase of convulsion, against zonisamide treated group. The data shown in table 16, exposed that i.p. administration of methylene blue (35 mg/kg, i.p.), greatly enhances the anti-convulsant activity (P<0.01) along with higher protection (83.3%) range. At the same time, the combined effect of methylene blue with exogenously administered BRL50481 (1.4 mg/kg, i.p.) showed a

significant (*P*<0.01) anti-convulsant activity with judicious protection (83.3%) range for both groups (Table 16).

7.4.1 Ion channel modulators alone (both sodium and calcium ions):

7.4.1. A. Chemoshock method:

7.4.1. A.1. Pentylenetetrazole (PTZ) or Metrazol (MTZ) induced seizure model in mice:

Table 17 summarizes the data obtained from experiments conducted with ion channel modulators such as sodium and calcium channel modulators like amiloride (75 mg/kg, i.p), amiodarone (40 mg/kg, i.p.) and flecainide (50 mg/kg, i.p.) on chemoshock such as PTZ (60 mg/kg, i.p) induced seizures in mice**.** The highlights of the findings are the data obtained with individual effect of amiloride and flecainide showed a good remarkable delay (P<0.001) in onset of action, jerky movements and convulsion against PTZ induced seizures in mice when compared to DMSO received group, amiodarone alone received group. The individual effect of amiodarone showed a significant (P<0.001) delay in onset of action, jerky movements, convulsion when compared DMSO, lacosamide received group, but it doesn't meet with up to the level of sodium channel modulator alone. The combination of flecainide and amiodarone received mice showed a significant (P<0.01) decrease in seizure activity when compared to CCM such as amiodarone. The overall highlights of Table 17 indicate the individual effect of SCM, flecainide, which delays the onset of action of seizures as well as prolongs the total duration of convulsive time.

Table 17: Effect of ion channel modulators on PTZ (60 mg/kg, i.p) induced seizures in mice (n=6).

Groups	**Treatment**	**Onset time in seconds**		
		Pentylenetetrazole (PTZ)		
		Action	**Jerky movements**	**Convulsion**
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	120.5 ± 6.41	145.1 ± 8.73	156.72 ± 9.26
II	Lacosamide (4.5 mg/kg, i.p) Standard	330.4 ± 6.76***	420.4 ± 11.27***	460.47 ± 9.8***
III	Amiloride (75 mg/ kg, i.p.) Sodium channel modulator	280.67 ± 8.4***, ΔΔ	330.6 ± 8.01***,ΔΔΔ	360.13 ± 10.32***,ΔΔΔ
IV	Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	210.49 ± 8.31***, ΔΔΔ,ΨΨΨ	240.7 ± 8.21***,ΔΔΔ,ΨΨΨ	270.14 ± 11.21***,ΔΔΔ,ΨΨΨ
V	Flecainide (50 mg/kg, i.p.) Sodium channel modulator	310.43 ± 10.24***,♣♣♣	380.4 ± 14.32***,Ψ,♣♣♣,	410.40 ± 13.7 ***,ΔΔΔ,ΨΨΨ,♣♣♣
VI	Flecainide (50 mg/ kg, i.p.) + Amiodarone (40 mg/kg, i.p.)	260.74 ± 13.49***, ΔΔΔ,♣♣, ΣΣ	310.4 ± 13.71***,ΔΔΔ,♣♣,ΣΣ	340.70 ± 16.3***,ΔΔΔ,♣♣♣, ΣΣΣ

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 PTZ (60 mg/kg, i.p). *** denotes $p<0.001$ compared with DMSO received group, **ΔΔ** and **ΔΔΔ** denotes $p<0.01$ and $p<0.001$, respectively, compared with lacosamide received group, **Ψ** and **ΨΨΨ** denotes $p<0.05$ and $p<0.001$, respectively, compared with amiloride received group, ♣♣ and ♣♣♣ denotes $p<0.01$ and $p<0.001$, respectively, compared with amiodarone received group, **ΣΣ** and **ΣΣΣ** denotes $p<0.01$ and $p<0.001$, respectively, compared with flecainide received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.4.1. A.2. Kainic acid induced seizure model in mice:

Table 18 demonstrated the data obtained from experiments conducted with sodium and calcium channel modulators like amiloride (75 mg/kg, i.p), amiodarone (40 mg/kg, i.p.) and flecainide (50 mg/kg, i.p.) on chemical convulsant such as kainic acid (20 mg/kg, i.p.) induced seizures in mice**.** The main highlights of the findings are the data obtained with individual effect of amiloride and flecainide showed a significant delay ($P<0.001$) in onset of action, jerky movements and convulsion against kainic acid induced seizures in mice when compared to DMSO received group. The mice treated with flecainide alone showed a gradual ($P<0.01$) delay in jerky movements and convulsion against kainic acid induced seizures while compared to amiodarone alone received group. The individual effect of CCM such as amiodarone showed a significant ($P<0.001$) delay in onset of action, jerky movements, convulsion when compared DMSO, lacosamide received group. The combination of flecainide and amiodarone received mice showed a significant ($P<0.01$) decrease in seizure activity when compared to CCM such as amiodarone. The overall highlights of Table 18 indicate that the individual effect of SCM, flecainide, which delays the onset of action of seizures as well as prolongs the total duration of convulsive time, which closely match with the positive control of lacosamide.

Table 18: Effect of ion channel modulators on kainic acid (20 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Kainic acid		
		Action	**Jerky movements**	**Convulsion**
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	204.37 ± 12.13	230.47 ± 11.14	260.51 ± 8.17
II	Lacosamide (4.5 mg/kg, i.p) Standard	410.67 ± 13.15***	440.70 ± 16.7***	490.63 ± 18.91***
III	Amiloride (75 mg/ kg, i.p.) Sodium channel modulator	350.71 ± 14.67***	380.17 ± 16.78 ***	420.70 ± 19.2***,**Δ**
IV	Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	290.67 ± 13.67**,**ΔΔΔ**	340.08 ± 18.61 ***,**ΔΔΔ**	380.71 ± 15.65***,**ΔΔΔ**
V	Flecainide (50 mg/kg, i.p.) Sodium channel modulator	394.23 ± 17.67***,♣♣♣	430.60 ± 16.78***,♣♣	470.37 ± 13.71***,♣♣
VI	Flecainide (50 mg/ kg, i.p.) + Amiodarone (40 mg/kg, i.p.)	326.08 ± 18.62***,**ΔΔ, Σ**	360.04 ± 9.84***,**ΔΔ,Σ**	397.43 ± 10.93***,**ΔΔ,Σ**

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 kainic acid (20 mg/kg, i.p). ** and *** denotes $p<0.01$ and $p<0.001$, respectively, compared with DMSO received group, **Δ, ΔΔ** and **ΔΔΔ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with lacosamide received group, ♣♣ and ♣♣♣ denotes $p<0.01$ and $p<0.001$, respectively, compared with amiodarone received group, **Σ** denotes $p<0.05$ compared with flecainide received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.4.1. B. Maximal electroshock (MES) method for mice:

Table 19 demonstrated the data obtained from experiments conducted with sodium and calcium channel modulators like amiloride (75 mg/kg, i.p.), amiodarone (40 mg/kg, i.p.) and flecainide (50 mg/kg, i.p.) on maximal electroshock induced seizures in mice**.** It is evident from the data shown in Table 19 that individual effect of flecainide effectively ($P<0.001$) delays the tonic limb flexion, tonic extensor, clonus and stupor stage of convulsion, compared to amiodarone alone received group as well as combination of flecainide and amiodarone received group of animals. The overall highlights of Table 19 exhibits the individual effect of amiodarone (CCM) received group, potentiates the seizure activity against in MES induced convulsion. Also this emphasizes that flecainide delays the onset of seizure activity as well as prolongs the total duration of convulsive time in individual as well as combined with amiodarone.

Table 19: Effect of ion channel modulators on maximal electroshock induced seizures in mice (n=6)

Groups	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	8.21 ± 0.63	18.32 ± 1.4	36.32 ± 2.47	57.0 ± 4.1
II	Lacosamide (4.5 mg/kg, i.p) Standard	31.38 ± 2.47***	40.72 ± 3.71***	54.31 ± 4.31**	70.31 ± 4.7
III	Amiloride (75 mg/ kg, i.p.) Sodium channel modulator	25.67 ± 3.14***	31.42 ± 3.92*	40.71 ± 3.17**Δ,**	58.47 ± 2.48
IV	Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	20.43 ± 1.24**,**ΔΔ**	26.73 ± 1.76**Δ,**	32.31 ± 2.67**ΔΔΔ**	39.67 ± 2.67*,**ΔΔΔ,♣♣**
V	Flecainide (50 mg/kg, i.p.) Sodium channel modulator	28.41 ± 1.97***	32.43 ± 2.40**	39.67 ± 2.77**Δ**	50.23 ± 4.31**ΔΔ**
VI	Flecainide (50 mg/ kg, i.p.) + Amiodarone (40 mg/kg, i.p.)	24.17 ± 1.8***	25.47 ± 1.86**ΔΔ,**	30.13 ± 2.63**ΔΔΔ,**	41.47 ± 2.30*,**ΔΔΔ,♣**

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, **Δ, ΔΔ** and **ΔΔΔ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with lacosamide received group, ♣ and ♣♣ denotes $p<0.05$ and $p<0.01$, respectively, compared with amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.4.2 Sodium channel modulator alone:

7.4.2.A. Chemoshock method:

7.4.2.A.1. Pentylenetetrazole (PTZ) induced seizure model in mice:

Table 20 summarizes the data obtained from experiments conducted with PTZ induced seizures. In animals treated with DMSO onset of action were observed 130.5 ± 7.4 sec after PTZ (60 mg/kg, i.p.) and convulsions appeared 165.72 ± 8.43 sec after PTZ. Flecainide in a dose of 50 mg/kg significantly enhanced the delay in onset of action ($p<0.001$) and stimulate the convulsions ($p<0.001$) compared to DMSO received group. The results show that there was a significant increase in onset of action of seizure activity when increased the dose (100 mg/kg) of flecainide ($p<0.001$) when compared to DMSO received group. At the same time there was no much more difference was observed in between flecainide test dose 1 and 2. Low profile of mortality was observed while using flecainide (50 mg/kg, i.p.) and flecainide (100 mg/kg, i.p.).

Table 20: Effect of sodium channel modulators on PTZ (60 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	**Treatment**	**Onset time in seconds**		
		Pentylenetetrazole (PTZ)		
		Action	**Jerky movements**	**Convulsion**
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	130.5 ± 7.4	150.1 ± 9.75	165.72 ± 8.43
II	Lacosamide (4.5 mg/kg, i.p) Standard	345.64 ± 12.4***	430.7 ± 10.97***	450.61 ± 15.4***
III	Flecainide (50 mg/kg, i.p.)- T.D1 Sodium channel modulator	302.9 ± 13.47***	335.34 ± 13.74***,ΔΔΔ	350.51 ± 15.45***,ΔΔΔ
IV	Flecainide (100 mg/kg, i.p.)- T.D2	340.7 ± 11.54***	360.81 ± 14.7***,ΔΔ	396.21 ± 12.14***,Δ

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 PTZ (60 mg/kg, i.p). *** denotes $p<0.001$ compared with DMSO received group, **Δ, ΔΔ** and **ΔΔΔ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with lacosamide received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.4.2. A.2. Picrotoxin induced seizure in mice:

Table 21 demonstrated the data obtained from experiments conducted with various dose level of sodium channel modulator like flecainide (50 & 100 mg/kg, i.p.) on chemical convulsant of picrotoxin (5.7 mg/kg, i.p.) induced seizures in mice**.** In animals treated with DMSO onset of action were observed 190.92 ± 12.4 sec after picrotoxin (5.7 mg/kg, i.p.) and convulsions appeared 276.1 ± 14.71 sec after PTx. Flecainide in a dose of 50 mg/kg significantly enhanced the delay in onset of action ($p<0.001$) and stimulate the convulsions ($p<0.01$) compared to DMSO received group. The results show that there was a significant increase in onset of action of seizure activity i.e 370.43 ± 15.57, when increased the dose (100 mg/kg) of flecainide ($p<0.001$) when compared to DMSO received group. At the same time there was no much more difference was observed in between flecainide test dose 1 and 2. Flecainide test dose 1 (50 mg/kg, i.p.) significantly ($p<0.05$) potentiates the anti-convulsant activity when compared to standard drug of lacosamide (4.5 mg/kg, i.p.). Low profile of mortality was observed while using flecainide (50 mg/kg, i.p.) and flecainide (100 mg/kg, i.p.).

Table 21: Effect of sodium channel modulators on picrotoxin (PTx) (5.7 mg/kg, i.p) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Picrotoxin (PTx)		
		Action	**Jerky movements**	**Convulsion**
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	190.92 ± 12.4	250.78 ± 13.72	276.1 ± 14.71
II	Lacosamide (4.5 mg/kg, i.p) Standard	394.14 ± 19.32***	430.71 ± 18.47***	445.6 ± 17.36***
III	Flecainide (50 mg/kg, i.p.)- T.D1 Sodium channel modulator	336.73 ± 18.72***	350.65 ± 19.31**,Δ	372.65 ± 13.71**,Δ
IV	Flecainide (100 mg/kg, i.p.)- T.D2	370.43 ± 15.57***	390.43 ± 16.74***	412.35 ± 14.52***

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 picrotoxin (5.7 mg/kg, i.p.). ** and *** denotes $p<0.01$ and $p<0.001$, respectively, compared with DMSO received group, **Δ** denotes $p<0.05$ compared with lacosamide received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.4.2. A.3. Kainic acid induced seizure model in mice:

Table 22 demonstrated the data obtained from experiments conducted with various dose level of sodium channel modulator like flecainide (50 & 100 mg/kg, i.p.) on chemical convulsant of kainic acid (20 mg/kg, i.p.) induced seizures in mice**.** In animals treated with DMSO onset of action were observed 204.37 ± 12.13 sec after kainc acid injection and convulsions appeared after 260.51 ± 8.17 sec. Flecainide at the dose of 50 mg/kg significantly potentiates the delay in onset of action (p<0.001) and stimulate the convulsions (p<0.001) compared to DMSO received group. The main highlights of the findings are the animals received lacosamide, flecainide test dose 1 and 2 showed that the onset of various phases of seizure threshold is significantly (p<0.001) enhanced when compared to DMSO received group. The result indicates that there was a significant increase in onset of action of seizure activity i.e 430.72 ± 16.73, when increased the dose (100 mg/kg) of flecainide (p<0.001) when compared to DMSO received group. At the same time there was no much more difference was observed in between flecainide test dose 1 and 2.

Table 22: Effect of sodium channel modulators on kainic acid (20 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Kainic acid		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	204.37 ± 12.13	230.47 ± 11.14	260.51 ± 8.17
II	Lacosamide (4.5 mg/kg, i.p) Standard	410.67 ± 13.15***	440.70 ± 16.7***	490.63 ± 18.91***
III	Flecainide (50 mg/kg, i.p.)- T.D1 Sodium channel modulator	394.23 ± 17.67***	430.60 ± 16.78***	470.37 ± 13.71***
IV	Flecainide (100 mg/kg, i.p.)- T.D2	430.72 ± 16.73***	452.7 ± 16.7***	467.12 ± 18.17***

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 kainic acid (20 mg/kg, i.p). *** denotes $p<0.001$ compared with DMSO received group, (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.4.2.B. Maximal electroshock (MES) method for mice:

Table 23 illustrates the effect of various dose level of sodium channel modulator such as flecainide (50 mg/kg, i.p. & 100 mg/kg, i.p.) on MES induced seizures in mice. In which 100 mg/kg of flecainide produced a gradual increase in tonic limb flexion and tonic extensor significantly ($p<0.001$) when compared with DMSO received group. Flecainide (50 mg/kg, i.p) produced a significant $p<0.001$ and $p<0.01$ delay onset in tonic limb flexion and tonic extensor respectively, when compared to DMSO received group. Significant ($p<0.05$) was observed in clonus and stupor phase of convulsion at the dose of 50 mg/kg of flecainide, when compared to standard lacosamide.

Table 23: Effect of sodium channel modulators on maximal electroshock induced seizures in mice (n=6)

Groups	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	8.21 ± 0.63	18.32 ± 1.4	36.32 ± 2.47	57.0 ± 4.1
II	Lacosamide (4.5 mg/kg, i.p) Standard	31.38 ± 2.4***	40.72 ± 3.71***	54.31 ± 4.31**	70.31 ± 4.7
III	Flecainide (50 mg/kg, i.p.)- T.D1 Sodium channel modulator	28.41 ± 1.97***	32.43 ± 2.40**	39.67 ± 2.77**Δ**	50.23 ± 4.31**Δ**
IV	Flecainide (100 mg/kg, i.p.)- T.D2	34.36 ± 2.48***	39.68 ± 2.71***	48.73 ± 3.48	56.73 ± 4.8

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 $p<0.01$ and $p<0.001$, respectively, compared with DMSO received group, **Δ** denotes $p<0.05$ compared with lacosamide received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.5. Calcium channel blocker and activator alone:

7.5. A. Chemoshock method:

7.5. A.1. Picrotoxin (PTx) induced seizure model in mice:

Table 24 demonstrated the data obtained from experiments conducted with calcium channel modulator like amiodarone (40 mg/kg, i.p.), L-type Calcium Channel blocker and Bay K 8644, S(-) (2 mg/kg, i.p.), L-type Calcium Channel activator on chemical convulsant of picrotoxin (5.7 mg/kg, i.p.) induced seizures in mice**.** In animals treated with DMSO onset of action were observed 190.92 ± 12.4 sec after picrotoxin (5.7 mg/kg, i.p.) and convulsions appeared 276.1 ± 14.71 sec after PTx. Amiodarone (40 mg/kg, i.p.) as well as standard drug nifedipine (20 mg/kg, i.p.) significantly (p<0.001) potentiates the delay in all phases of seizure activity, compared to DMSO received group. It is evident from the Table 24, the animals received Bay K 8644, S(-) (2 mg/kg, i.p.), showed that there was a significant quick on set of seizure threshold p<0.001 and p<0.001 respectively, when compared to nifedipine and amiodarone received group. This indicates that Bay K 8644, S(-) (2 mg/kg, i.p.), L-type Calcium Channel activator having proconvulsant action. On the other hand amiodarone (40 mg/kg, i.p.) does not produce any significant level of difference with standard drug of nifedipine received group.

Table 24: Effect of calcium channel modulators on picrotoxin (PTx) (5.7 mg/kg, i.p) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Picrotoxin (PTx)		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	190.92 ± 12.4	250.78 ± 13.72	276.1 ± 14.71
II	Nifedipine (20 mg/kg, i.p) L-type calcium channel blocker	502.47 ± 16.71***	521.79 ± 18.31***	552.61 ± 18.32***
III	Amiodarone (40 mg/kg, i.p.) L-type calcium channel blocker	475.71 ± 18.63***	503.71 ± 18.41***	534.45 ± 21.73***
IV	Bay K 8644, S(-) (2 mg/kg, i.p.) L-type calcium channel activator	120.79 ± 8.45*,**ΔΔΔ,ΨΨΨ**	139.42 ± 10.21***,**ΔΔΔ,ΨΨΨ**	152.96 ± 9.72***,**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 picrotoxin (5.7 mg/kg, i.p.). * and *** denotes $p<0.05$ and $p<0.001$, respectively, compared with DMSO received group, **ΔΔΔ** denotes $p<0.001$ compared with nifedipine received group, **ΨΨΨ** denotes $p<0.001$ compared with amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.5. A.2. Kainic acid (KA) induced seizures in mice:

Table 25 demonstrated the data obtained from experiments conducted with calcium channel modulator like amiodarone (40 mg/kg, i.p.), L-type Calcium Channel blocker and Bay K 8644, S(-) (2 mg/kg, i.p.), L-type Calcium Channel activator on chemical convulsant of kainic acid (20 mg/kg, i.p.) induced seizures in mice**.** In animals treated with DMSO onset of action were observed 204.37 ± 12.13 sec after kainic acid (20 mg/kg, i.p.) and convulsions appeared 260.51 ± 8.17 sec after KA. Amiodarone (40 mg/kg, i.p.) as well as standard drug nifedipine (20 mg/kg, i.p.) significantly ($p<0.001$) potentiates the delay in all phases of seizure activity, compared to DMSO received group. The main high light was found from the Table 25, the animals received Bay K 8644, S(-) (2 mg/kg, i.p.), showed that there was a significant quick on set of seizure threshold $p<0.001$ and $p<0.001$ respectively, when compared to nifedipine and amiodarone received group. This indicates that Bay K 8644, S(-) (2 mg/kg, i.p.), L-type Calcium Channel activator having the role of proconvulsant activity. On the other hand amiodarone (40 mg/kg, i.p.) does not produce any significant level of difference with standard drug of nifedipine received group.

Table 25: Effect of calcium channel modulators on kainic acid (20 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Kainic acid		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	204.37 ± 12.13	230.47 ± 11.14	260.51 ± 8.17
II	Nifedipine (20 mg/kg, i.p) L-type calcium channel blocker	526.73 ± 16.76***	552.61 ± 13.98***	570.12 ± 19.75***
III	Amiodarone (40 mg/kg, i.p.) L-type calcium channel blocker	480.41 ± 19.81***	507.21 ± 13.15***	524.01 ± 21.75***
IV	Bay K 8644, S(-) (2 mg/kg, i.p.) L-type calcium channel activator	139.47 ± 8.49***,ΔΔΔ,ΨΨΨ**	172.41 ± 10.43***,ΔΔΔ,ΨΨΨ**	189.47 ± 12.15***,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 kainic acid (20 mg/kg, i.p). * and *** denotes $p<0.05$ and $p<0.001$ compared with DMSO received group, **ΔΔΔ** denotes $p<0.001$ compared with nifedipine received group, **ΨΨΨ** denotes $p<0.001$ compared with amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.5. B. Maximal electroshock (MES) method for mice:

Table 26 illustrates the data obtained from experiments conducted with calcium channel modulator like amiodarone (40 mg/kg, i.p.), L-type Calcium Channel blocker and Bay K 8644, S(-) (2 mg/kg, i.p.), L-type Calcium Channel activator on maximal electroshock method in mice. In which standard drug nifedipine (20 mg/kg, i.p.) as well as test drug amiodarone (40 mg/kg, i.p.) produced a gradual increase in tonic limb flexion, tonic extensor, clonus significantly ($p<0.001$) when compared with DMSO received group. It is evident form the Table 26, the animals received Bay K 8644, S(-) (2 mg/kg, i.p.), showed that there was a significant quick on set of seizure threshold $p<0.001$ and $p<0.001$ respectively, when compared to nifedipine and amiodarone received group. Which indicates that Bay K 8644, S(-) (2 mg/kg, i.p.), L-type Calcium Channel activator possess the proconvulsant effect. On the other hand amiodarone (40 mg/kg, i.p.) produced, a significant ($p<0.01$) level of tonic limb flexion and clonus was observed, compared with standard drug nifedipine received group.

Table 26: Effect of calcium channel modulators on maximal electroshock induced seizures in mice (n=6).

Groups	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	8.21 ± 0.63	18.32 ± 1.4	36.32 ± 2.47	57.00 ± 4.1
II	Nifedipine (20 mg/kg, i.p) L-type calcium channel blocker	48.41 ± 2.48***	56.43 ± 2.81***	76.91 ± 3.81***	84.91 ± 3.45***
III	Amiodarone (40 mg/kg, i.p.) L-type calcium channel blocker	39.81 ± 2.23***,**ΔΔ**	46.74 ± 2.54***,**Δ**	58.56 ± 2.71***,**ΔΔ**	72.27 ± 4.79*,
IV	Bay K 8644, S(-) (2 mg/kg, i.p.) L-type calcium channel activator	4.13 ± 0.08**ΔΔΔ,ΨΨΨ**	11.47 ± 1.14**ΔΔΔ,ΨΨΨ**	20.69 ± 1.82**,**ΔΔΔ,ΨΨΨ**	38.40 ± 2.41*,**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 $p<0.05$ and $p<0.001$, respectively, compared with DMSO received group, **Δ, ΔΔ, ΔΔΔ** denotes $p<0.05$, $p<0.01$ and $p<0.001$ compared with nifedipine received group, **ΨΨΨ** denotes $p<0.001$ compared with amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.6. PDEs inhibitors along with sGC activator and inhibitor, calcium channel modulator and calcium channel blocker:

7.6.1. PDE-7 inhibitor with the presence of sGC activator and inhibitor:

7.6.1. A. Chemoconvulsant method:

7.6.1. A. 1. Pilocarpine induced seizure model in mice:

Table 27 summarizes the data attained from experiments conducted with PDE- 7 inhibitors beside with guanylate cyclase activator and inhibitor on chemoshock such as pilocarpine (500 mg/kg, i.p.) induced seizures in mice**.** The combination of methylene blue (50 mg/kg, i.p) and PDE-7 inhibitor, BRL50481 (2 mg/kg, i.p.) received mice showed a significant (P<0.001) delay in onset of action, jerky movements and convulsion when compare to A-350619 (100 µM/ kg, i.p.), BRL 50481(2 mg/kg, i.p.) alone received mice as well as combination of A-350619 and BRL 50481 received group. The overall highlights of Table 27 exhibits the individual effect of methylene blue delays significantly (P<0.001) the onset of action of seizures as well as prolongs the total duration of convulsive time against DMSO as well as A-350619 received group. On the other hand, BRL 50481 alone received group showed significant level (P<0.001) of quick on set of seizure threshold in all phases of convulsion when compared to DMSO, gabapentin and methylene blue received groups.

Table 27: Effect of PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor 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	138.76 ± 3.4	161.42 ± 6.53	186.53 ± 7.91
II	Gabapentin (2.5 mg/kg, i.p.) Positive Control	238.27 ± 10.23∗∗∗	258.83 ± 9.87∗∗∗	285.92 ± 8.64∗∗∗
III	A-350619 (100 µM/ kg, i.p.) sGC activator	116.05 ± 5.87**ΔΔΔ**	127.64 ± 5.07**ΔΔΔ**	164.03 ± 8.38**ΔΔΔ**
IV	Methylene blue (50 mg/kg, i.p.) sGC inhibitor	194.54 ± 8.98∗∗∗,**ΔΔ,ΨΨΨ**	239.14 ± 11.64∗∗∗,**ΨΨΨ**	281.37 ± 11.26∗∗∗,**ΨΨΨ**
V	BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	82.39 ± 4.65∗∗∗,**ΔΔΔ,Ψ,♣♣♣**	103.43 ± 6.31∗∗∗,**ΔΔΔ,♣♣♣**	116.38 ± 4.98∗∗∗,**ΔΔΔ,ΨΨ,♣♣♣**
VI	A-350619 (100 µM/ kg, i.p.) + BRL 50481 (2 mg/kg, i.p.)	108.23 ± 5.69 **ΔΔΔ,♣♣♣**	124.67 ± 6.73**ΔΔΔ,♣♣♣**	145.35 ± 5.93∗,**ΔΔΔ,♣♣♣**
VII	Methylene blue (50 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p.)	176.83 ± 8.59∗,**ΔΔΔ,ΨΨΨ, ΣΣΣ,**□□□	207.85 ± 12.86∗,**ΔΔ,ΨΨΨ, ΣΣΣ,**□□□	256.98 ± 12.53∗∗∗,**ΨΨΨ, ΣΣΣ,**□□□

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 $p<0.05$ and $p<0.001$, respectively, compared with DMSO received group, **ΔΔ** and **ΔΔΔ** denotes $p<0.01$ and $p<0.001$ compared with gabapentin received group, **Ψ, ΨΨ** and **ΨΨΨ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with A-350619 received group, ♣♣♣ denotes $p<0.001$ compared with methylene blue received group, **ΣΣΣ** denotes $p<0.001$ compared with BRL 50481 received group, □□□ denotes $p<0.001$ compared with A-350619 and BRL 50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.6.1. A. 2. Kainic acid induced seizure model in mice:

Table 28 explains the data attained from experiments conducted with PDE- 7 inhibitors beside with guanylate cyclase activator and inhibitor on chemoshock such as kainic acid (20 mg/kg, i.p.) induced seizures in mice**.** The combination of methylene blue (50 mg/kg, i.p) and PDE-7 inhibitor, BRL50481 (2 mg/kg, i.p.) received mice showed a significant (P<0.001) delay in onset of action, jerky movements and convulsion when compare to A-350619 (100 μM/ kg, i.p.), BRL 50481(2 mg/kg, i.p.) alone received mice as well as combination of A-350619 and BRL 50481 received group. The overall highlights of Table 28 exhibits the individual effect of methylene blue delays significantly (P<0.001) the onset of action of seizures as well as prolongs the total duration of convulsive time against DMSO as well as A-350619 received group. On the other hand, BRL 50481 alone received group showed significant level (P<0.001) of quick on set of seizure threshold in all phases of convulsion when compared to DMSO, gabapentin, A-350619 and methylene blue received groups.

Table 28: Effect of PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor on kainicacid (20 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Kainicacid (20 mg/kg, i.p.)		
		Action	**Jerky movements**	**Convulsion**
I	10% w/v DMSO (5 ml/kg, i.p) Solvent Control	204.28 ± 8.96	224.08 ± 9.56	251.65 ± 10.28
II	Gabapentin (2.5 mg/kg, i.p.) Positive Control	391.24 ± 13.28***	424.56 ± 15.38***	480.35 ± 17.29***
III	A-350619 (100 µM/ kg, i.p.) sGC activator	192.09 ± 10.28**ΔΔΔ,**	226.67 ± 11.27**ΔΔΔ**	260.67 ± 11.64**ΔΔΔ**
IV	Methylene blue (50 mg/kg, i.p.) sGC inhibitor	331.78 ± 14.85***,**Δ,ΨΨΨ**	376.06 ± 16.53***,**ΨΨΨ**	408.38 ± 17.3***,**ΔΔ,ΨΨΨ**
V	BRL 50481 (2 mg/kg, i.p.) PDE-7 inhibitor	121.45 ± 8.56***,**ΔΔΔ,ΨΨ,♣♣♣**	145.76 ± 7.2**,**ΔΔ,ΨΨΨ,♣♣♣**	172.96 ± 9.37**,**ΔΔΔ,ΨΨΨ,♣♣♣**
VI	A-350619 (100 µM/ kg, i.p.) + BRL 50481 (2 mg/kg, i.p.)	182.35 ± 9.27**ΔΔΔ,♣♣♣, Σ**	208.03 ± 9.78 **ΔΔΔ,♣♣♣, Σ**	228.53 ± 11.62**ΔΔΔ,♣♣♣**
VII	Methylene blue (50 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p.)	316.74 ± 14.65***,**ΔΔ,ΨΨΨ, ΣΣΣ,** □□□	347.56 ± 14.32***,**ΔΔ,ΨΨΨ, ΣΣΣ,** □□□	386.54 ± 13.49***,**ΔΔΔ,ΨΨΨ, ΣΣΣ,** □□□

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<0.01 and p<0.001, respectively, compared with DMSO received group, **Δ, ΔΔ** and **ΔΔΔ** denotes p<0.05, p<0.01 and p<0.001, respectively, compared with gabapentin received group, **ΨΨ** and **ΨΨΨ** denotes p<0.01 and p<0.001, respectively, compared with A-350619 received group, **♣♣♣** denotes p<0.001 compared with methylene blue received group**, Σ** and **ΣΣΣ** denotes p<0.05 and p<0.001, respectively, compared with BRL 50481 received group, □□□ denotes p<0.001 compared with A-350619 and BRL 50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.6.1. A.3. Picrotoxin induced seizure model in mice:

Table 29 summarizes the data obtained from experiments conducted with PDE- 7 inhibitors beside with guanylate cyclase activator and inhibitor on chemoshock such as picrotoxin (5.7 mg/kg, i.p.) induced seizures in mice**.** The combination of methylene blue (50 mg/kg, i.p) and PDE-7 inhibitor, BRL50481 (2 mg/kg, i.p.) received mice showed a significant ($P<0.001$) delay in onset of action, jerky movements and convulsion when compare to BRL 50481(2 mg/kg, i.p.) alone received mice as well as combination of A-350619 and BRL 50481 received group ($P<0.01$). The overall highlights of Table 29 exhibits the individual effect of methylene blue delays significantly ($P<0.001$) the onset of action of seizures as well as prolongs the total duration of convulsive time against DMSO as well as A-350619 received group. At the same time, BRL 50481 alone received group showed significant level ($P<0.001$) of quick on set of seizure threshold in all phases of convulsion when compared to DMSO, gabapentin and methylene blue alone received groups.

Table 29: Effect of PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor on picrotoxin (5.7 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Picrotoxin (5.7 mg/kg, i.p.)		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p.) Solvent Control	191.24 ± 11.28	248.26 ± 13.28	275.17 ± 12.67
II	Gabapentin (2.5 mg/kg, i.p.) Positive Control	289.23 ± 13.74 ***	332.64 ± 11.23***	365.29 ± 13.29 ***
III	A-350619 (100 µM/ kg, i.p.) sGC activator	178.14 ± 7.94ΔΔΔ	202.34 ± 11.76ΔΔΔ	248.56 ± 11.36ΔΔΔ
IV	Methylene blue (50 mg/kg, i.p.) sGC inhibitor	271.45 ± 9.73 ***,ΨΨΨ	318.27 ± 19.34 **,ΨΨΨ	361.28 ± 14.86 ***,ΨΨΨ
V	BRL 50481 (2 mg/kg, i.p.) PDE-7 inhibitor	120.93 ± 4.67 ***,ΔΔΔ,ΨΨ,♣♣♣	143.25 ± 3.72 ***,ΔΔΔ,Ψ,♣♣♣	169.24 ± 9.34 ***,ΔΔΔ,ΨΨΨ,♣♣♣
VI	A-350619 (100 µM/ kg,i.p.) + BRL 50481 (2 mg/kg, i.p.)	180.24 ± 9.53ΔΔΔ,♣♣♣, ΣΣ	206.23 ± 9.51ΔΔΔ,♣♣♣, Σ	219.31 ± 10.85 *,ΔΔΔ,♣♣♣
VII	Methylene blue (50 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p.)	235.73 ± 10.54 *,ΔΔ,ΨΨ, ΣΣΣ, □□	249.05 ± 10.32 ΔΔΔ,♣♣, ΣΣΣ	290.63 ± 11.37 ΔΔ,♣♣, ΣΣΣ, □□

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 picrotoxin (5.7 mg/kg, i.p.). *, ** 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 gabapentin received group, **Ψ, ΨΨ** and **ΨΨΨ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with A-350619 received group, ♣♣ and ♣♣♣ denotes $p<0.01$ and $p<0.001$ compared with methylene blue received group**, Σ, ΣΣ** and **ΣΣΣ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with BRL 50481 received group, □□ denotes $p<0.01$ compared with A-350619 and BRL 50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.6.2. PDE-4 inhibitor with the combination of PDE-7 inhibitor, sGC inhibitor, calcium channel modulator and blocker:

7.6.2. A. Chemoconvulsant method:

7.6.2. A.1. Pentylenetetrazole induced seizure model in mice:

Table 30 summarizes the data attained 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 PTZ (60 mg/kg, i.p.) induced seizures in mice**.** The etazolate (7 mg/kg, i.p.) alone received mice showed a significant (*P*<0.001) quick onset of action 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.) received mice showed a significant (*P*<0.001) delay in onset of action, jerky movements and convulsion when compare to gabapentin as well as combination of etazolate with BRL 50481. 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 etazolate with BRL 50481 received mice. There was no much more difference was observed in between etazolate with calcium channel blocker and modulator.

Table 30: Effect of PDE-4 inhibitor along with PDE-7 inhibitor, soluble guanylate cyclase (sGC) inhibitor, calcium channel modulator and blocker on PTZ (60 mg/kg, i.p.) induced seizures in mice (n=6).

Groups	Treatment	Onset time in seconds		
		Pentylenetetrazole (60 mg/kg, i.p.)		
		Action	Jerky movements	Convulsion
I	10% w/v DMSO (5 ml/kg, i.p) Solvent Control	130.37 ± 3.29	145.38 ± 2.67	189.27 ± 4.76
II	Gabapentin (2.5 mg/kg, i.p.) Positive Control	236.90 ± 3.46***	255.37 ± 3.57***	285.67 ± 7.81***
III	Etazolate (7 mg/kg, i.p.) PDE-4 inhibitor	150.90 ± 3.46**ΔΔΔ**	207.50 ± 7.44***,**ΔΔ**	230.56 ± 6.46*,**ΔΔ**
IV	Etazolate (7 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	130.34 ± 7.67**ΔΔΔ**	168.27 ± 10.23**ΔΔΔ,Ψ**	192.45 ± 6.56**ΔΔΔ**
V	Etazolate (7 mg/kg, i.p.) + Methylene blue (50 mg/kg, i.p.) sGC inhibitor	180.46 ± 10.43**,**ΔΔΔ,♣♣**	212.63 ± 8.49***,**ΔΔ,♣♣**	255.71 ± 11.71***,**♣♣♣**
VI	Etazolate (7 mg/kg, i.p.) + Amiodarone (40 mg/kg, i.p.) Calcium channel modulator	130.34 ± 7.67*,**ΔΔΔ,♣**	215.13 ± 9.13***,**Δ,♣♣**	240.63 ± 12.67**,**Δ,♣♣**
VII	Etazolate (7 mg/kg, i.p.) + Nifedipine (20 mg/kg, i.p.) Calcium channel blocker	130.34 ± 7.67**,**ΔΔΔ,♣♣**	223.69 ± 10.13***,**♣♣♣**	255.14 ± 9.72***,**♣♣♣**

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 pentylenetetrazole (60 mg/kg, i.p.). *, ** and *** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with DMSO received group, **Δ, ΔΔ** and **ΔΔΔ** denotes $p<0.05$, $p<0.01$ and $p<0.001$, respectively, compared with gabapentin received group, **Ψ** denotes $p<0.05$ 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 (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.6.2. A.2. Kainic acid induced seizure model in mice:

Table 31 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 etazolate (7 mg/kg, i.p.) alone received 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.) received mice 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 more difference was observed in between etazolate with calcium channel blocker and modulator

Table 31: 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).

7.6.2. A.3. Pilocarpine induced seizure model in mice:

Table 32 summarizes the data attained 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 received mice 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 showed a significant (P<0.001) quick onset of action, jerky movements and convulsion when compare to gabapentin received group. The combination of etazolate (7 mg/kg, i.p.) and methylene blue (50 mg/kg, i.p.) received mice 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) received mice.

Table 32: 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 ± 6.71**ΔΔΔ**	147.24 ± 8.67**ΔΔΔ**	169.73 ± 9.73**ΔΔΔ**
IV	Etazolate (7 mg/kg, i.p.) + BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	107.81 ± 4.67**ΔΔΔ**	135.67 ± 7.45**ΔΔΔ**	156.31 ± 7.39**ΔΔΔ**
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 ± 8.52**ΔΔΔ,ΨΨ,♣♣♣**	187.32 ± 7.37**ΔΔΔ,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,♣♣♣,**□	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, □ denotes $p<0.05$ compared with etazolate and amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

7.6.2. B. Maximal electroshock (MES) method for mice:

Table 33 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 33 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 33 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 33). 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 33: 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).

Groups	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, □□	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, □□ denotes $p<0.01$ compared with etazolate and amiodarone received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

8. DISCUSSION

8.1. Phosphodiesterase inhibitors alone:

8.1.1. PDE-3 inhibitors alone:

The therapeutic use of theophylline/ aminophylline is associated with the incidence of intractable seizures and mortality (Barnes, 1995; Barnes, 1998). The mechanisms involved in these seizures are not well understood and the treatment of the life threatening condition is unsatisfactory. The results of this study suggest that PDE-3 inhibitors potentiate the electroshock and chemical induced seizures. The bipyridine derivative of selective PDE-3 inhibitors such as amrinone and milrinone is a new class of positive inotropic drugs chemically and pharmacologically distinct from digitalis and catecholamines (Peter, 1991; Masaaki *et al.,* 1988). The mechanism of the positive inotropic effect of PDE inhibitors is similar to that of β-adrenergic agents (Cruickshank, 1993). Milrinone has been the most studied and used extensively as PDE-3 inhibitor and it is currently used in the acute treatment of heart failure to diminish long term risk (Wetzel, 1998). This study demonstrates the importance of the PDE-3 inhibitors such as amrinone and milrinone in the generation of seizure activity with the accumulation of cellular levels of cAMP and cGMP by inhibiting its metabolism. cAMP accumulation is considered to be anticonvulsant and cGMP is considered to be proconvulsant.

The data obtained from this study show that pre-treatment with PDE-3 inhibitors potentiates the onset of action and various phases of convulsions against INH, PTZ and maximal electroshock induced convulsions as depicted in Table 4 till Table 9. Our study

results also clearly suggest that rate of onset of convulsive time was significantly ($p<0.05$ and $p<0.001$) reduced with increasing the dose levels of both amrinone and milirinone against INH, PTZ and MES induced seizures.

Earlier studies suggest that the elevated levels of cGMP was found in cortical structure in some experimental models of epilepsy (Vullimoz, 1983; Riazi *et al.,* 2006), and the neuronal excitability was regulated by cGMP and Ca^{2+}/calmodulin dependent protein kinase and its phosphorylation process (Walaas, 1991). Apart from these findings, PDE-3 inhibitors possess transmembrane influx of Ca^{2+}. This influx of Ca^{2+} is responsible for the phosphorylation process of intracellular proteins, such as ion channels, receptors, enzymes and transcription factors which exhibit significant neuronal excitability and epileptic seizures (Butler *et al.,* 1995).

On the other hand, phosphorylation of variety of substrates regulates the myriad of physiological process, such as immune responses, cardiac and smooth muscle contraction, visual response, glycogenolysis, platelet aggregation, ion channel conductance, apoptosis and growth control (Francis, 2001). The present study results also early correspond with the generation of seizure activity due to the breakdown of hydrolysis of cGMP which promotes protein kinase phosphorylation process.

8.1.2. PDE-3, 4 & 5 inhibitors alone:

This study was conducted to evaluate the effect of phosphodiesterase isozymes 3, 4 and 5 inhibitors on the maximal electroshock and isoniazid induced convulsions. The results of this study are depicted in figures 17-19. The results of this study suggest that

zonisamide and gabapentin are anticonvulsant drugs were able to attenuate both the MES and isoniazid induced chemical convulsion. On the other hand, PDE 4 inhibitor rolipram and PDE 5 inhibitor, sildenafil was actually potentiating the convulsive phenomenon i.e the onset of epileptic threshold was reduced as tested by MES and isoniazid induced convulsions. The mechanism of its action is through the inhibition of T-type Ca^{2+} currents. In addition zonisamide inhibits sustained repetitive firing of spinal cord neurons, presumably by prolonging the inactivated state of voltage gated sodium channels in a manner similar to actions of phenytoin and carbamazepine. Recent evidence suggests that the cyclic nucleotide PDE exist in several molecular forms and that these isozymes are unequally distributed in various tissues. Phosphodiesterase (PDE) activity is found in every cell in the body, although there is distinct cellular and subcellular distribution of the 12 isoenzymes, which has provided many possibilities for increasingly selective therapeutic targets (Degerman, 1996). In identifying isoenzyme selective targets for specific diseases, a substantial amount of work was undertaken by pharmacologists working in the U.K., particularly in characterizing tissue expression, subcellular distribution and modulation of tissue function by isoenzyme selective inhibitors. The PDE-3, PDE-4 and PDE-5 belong to cGMP inhibitory, c-AMP specific and cGMP specific types of affinity to cyclic nucleotides respectively. According to Riazi *et al* sildenafil is a proconvulsant drug in the rodents and the role of nitric oxide cGMP pathway is implicated in these actions. Other reports also suggest the proconvulsant action of sildenafil in humans and animals. Studies conducted by Demchenko *et al.*, showed that PDE5 blockers oppose the protective vasoconstriction that is the initial response to hyperbaric hyperoxia, decreasing the safety of hyperbaric oxygen and

hastening onset of CNS oxygen toxicity. The present study mainly focuses the onset of seizures against the prior administration of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil. PDE-3 has high affinity for cAMP but can also hydrolyse cGMP. However, since the *K*m for cGMP has generally been reported to be lower than that for cAMP, and the Vmax is ten times greater for cAMP than for cGMP, cGMP readily inhibits the hydrolysis of cAMP by PDE3 by acting as a potent competitive inhibitor at the catalytic site (Shakur *et al.,* 2001; Movsesian, 2002). Thus expression of PDE3 allows stimuli that elevate cGMP levels to augment cAMP-mediated signaling (Movsesian, 2002). There are two PDE3 genes, PDE3A and PDE3B. Cilostazol is a PDE3 inhibitor that is a U.S. FDA - approved therapy for intermittant claudication, owing to its activity on both platelets and endothelium (Johnston, 2004). PDE-4 enzymes are cAMP-specific and play an important role in the biology of haematopoietic cells. These PDEs all hydrolyse cAMP with *K*m values in the range 1–4 μM (Baillie, 2005). Earlier reported study suggest that G-proteins and PKAs (cAMP-dependent protein kinase), is essential for controlling localized concentrations of cAMP (Brunton, 2003; Houslay, 2001; Houslay, 1998; Wallis, 1999). PDE-5, a cGMP-specific PDE family of considerable importance in regulating smooth muscle and endothelial cell function, is also present in platelets. Although having no effect on platelet function when used alone, PDE5 inhibitors augment nitroprusside's anti-platelet aggregation activity *in vitro* (Lamb, 1986).

The data obtained from this study show that pre-treatment with PDE-3, 4 and 5 inhibitors potentiates the onset of action and various phases of convulsions against INH and maximal electroshock induced convulsions as depicted in Fig 17 to 19. At the same

time the effect of onset of action, after administration of PDE-3 and 4 were less when compared to PDE- 5 inhibitor. Our study results also clearly suggest that rate of onset of convulsive time was significantly ($p<0.05$ and $p<0.01$) reduced with sildenafil against INH and MES induced seizures in both mice and rats. It has recently been reported that the elevation of cGMP levels provides a depolarized state at the rod outer segment of retina (Hescheler, 1987) and a GTP-binding protein (a G-protein, Go) regulates the neuronal Ca^{2+} channel (Butler, *et al.,* 1995). This Ca^{2+} is responsible for the phosphorylation process of intracellular proteins, such as ion channels, receptors, enzymes and transcription factors which exhibit significant neuronal excitability and epileptic seizures (Kohno, *et al.,* 1987). These events were associated with a significant increase in intracerebellar cyclic GMP (Ronit, 2002).

8.2. PDEs inhibitors in combination with adenylate cyclase (AC) inhibitor:

The data obtained from this study showed that pre-treatment with adenylate cyclase inhibitor, SQ22536 alone and along with the PDE-5/6/7/8/10/11 inhibitors such as dipyridamole and BRL 50481, potentiated the anticonvulsant activity against the PTZ and MES induced convulsions as depicted in Fig. 20-26. PDE-5/6/8/10/11 inhibitor, dipyridamole is an adenosine transport inhibitor, which acts mainly in two ways: (i) by increasing cyclic nucleotides as a result of the inhibition of phosphodiesterase (especially type 5, which is cGMP dependent) (Lugnier, *et al.,* 1986) and (ii) by increasing extracellular levels of adenosine (Roos, 1972) which leads to the activation of adenylate cyclase (Gresele, 1986) to convert adenosine into cAMP. Secondly, it also inhibits

cGMP- phosphodiesterase, increasing the amount of intracellular cGMP which may augment the downstream signalling effects of nitric oxide (NO), a vasodilator and inhibitor of platelet aggregation (Gamboa, *et al.,* 2005; Liao, 2007). Dipyridamole also increases cAMP by inhibiting the cellular uptake of adenosine (Roos, 1972). Our results support these findings in such a way that this combination showed a good reduction ($P<0.001$) in induction of seizure activity against PTZ and MES induced seizures in animals when compared to SQ22536 alone received group of animals (Fig. 20-26).

SQ22536 is a specific adenylate cyclase (AC) inhibitor (Reyes, 2005) which was employed to inhibit the activity of AC. Recent study explains that SQ22536 abolished the elevation of cAMP (Gao, 2001). Our study also explains that the BRL50481 showed a quick onset of seizure responses with increase in the mortality range in both animal models of epilepsy and this shows the potential role of this agent for therapeutic purpose. Murray (1990) discovered that adenylate cyclase assay reveals the direct effect of AC activator providing the net effect of measurement of cAMP production by AC and cAMP degradation by PDEs (Salahdeen, 2006). In mammalian cells, AC consists of at least 10 isoforms (Sunahara, *et al.,* 1996) some isoforms are stimulated by Ca^{2+}-calmodulin and inhibited by calmodulin antagonists (Mons, *et al.,* 1998). Since the decrease in cAMP was largely based on usage of SQ22536, acting predominantly by Ca^{2+}-calmodulin dependent (Sunahara *et al.,* 1996). Recent study shows that SQ22536 abolished the elevation of cAMP content by Iloprost (a prostaglandin I_2 analog) in guinea-pig which supports our findings (Turcato, 1999).

BRL50481 is a selective inhibitor of PDE-7, a novel subtype of PDE that is expressed in a number of cell types, including T lymphocytes. There are at least two genes coding for PDE7, each with several splice variants (Adkinson, 2008). Two PDE7 genes (PDE7A & PDE7B) have been identified in humans (Gardner, *et al.,* 2000; Hetman *et al.,* 2000). Li *et al.* (1999) suggested that PDE 7 may modulate human T-cell function. PDE7 is highly expressed in brain regions, including the hippocampus and olfactory bulb (Miro, *et al.,* 2001; Reyes, 2005). The distribution of PDE 7A3 is largely unknown, but it has been found in human T-lymphocytes (Glavas, *et al.,* 2001) and may also be present in many PDE7A1-expressing cells as both transcripts are probably regulated by the same promoter (Torras, 2003). In contrast, PDE7B is abundant in the brain, liver, heart, thyroid glands, and skeletal muscles, but it is not found in leukocytes (Gardner, 2000). Our study reports concurrence with combined effects of SQ22536 with exogenously administered BRL50481 (1.4 mg/kg, i.p) and SQ22536 with dipyridamole (1.4 mg/kg, i.p) showing a significant ($P<0.01$ and $P<0.01$) anticonvulsant activity with judicious protection (66.7% & 50%) range respectively against MES model as depicted in Table 12. Fig. 23, 24, 25 and 26 illustrates the PDE-7 inhibitor, BRL50481 showed a marked ($P<0.01$) decrease in onset of tonic extensor phase of convulsion in MES model of epilepsy. The total convulsive time was prolonged significantly ($P<0.01$) in SQ22536 alone treated (60.2%) and combination of SQ22536 with BRL50481 treated (27.4%) groups, compared to DMSO received group (100%) as in Table 12.

8.3. PDE-7 inhibitor in combination with soluble guanylate cyclase (sGC) activator and inhibitor:

The data obtained from this study shown that pre-treatment with soluble guanylate cyclase inhibitor, methylene blue alone and with the existence of PDE-7 inhibitor such as BRL 50481, potentiates the anti-convulsant activity against the PTZ and MES induced convulsions as described in Table 13 & 14. And also our study explains that the combination of A-350619 with BRL50481 as well as the individual effect of A-350619 and BRL 50481 alone showed a quick onset of seizures responses with increased the mortality range in both animal models of epilepsy. Methylene blue, is a guanylate cyclase inhibitor and is a thaizine dye (Furian, *et al.,* 2007). Pretreatment with either MB or L-NAME inhibited the proconvulsant effect of sildenafil, indicated the mediation of this effect by NO–cGMP pathway (Kiarash, *et al.,* 2006). Nitric oxide (NO) is a highly reactive and unstable free radical, which diffuses easily through the cell membrane (Garthwaite, 1991; Dawson, 1994). It contributes to intercellular signal transduction in many tissues. In the central nervous system, it acts as a neuronal retrograde messenger (Garthwaite, 1991; Moncada, 1991). NO is an endogeneous activator of guanylate cyclase, which synthetizes cGMP (Moncada, 1991; Garthwaite, 1988). It activates guanylate cyclase by binding to the iron of the heme, which is located at the active site of the enzyme and by changing its conformation (Moncada, 1989). In the CNS, NO is formed from L-arginine, by calcium/calmodulin- dependent constitutive NO synthase, which is mainly activated by the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Moncada, 1991; Moncada, 1989). Paul *et al*., reported that, ion channels mediate and regulate crucial electrical functions throughout the body. They are

therapeutic drug targets for a variety of disorders (Bennett, 2003). In living tissues, extracellular calcium is essential for the secretion of NO from NMDA-stimulated neurons (Moncada, 1991). NMDA receptor activation causes an influx of a large amount of calcium into the cell through receptor associated ion channels; calcium binds to calmodulin and activates NO synthase (Mayer, 1990; Alkaike, 1994). This mechanism might be the reason for the protective role and anti-convulsant activity of methylene blue. Our results support this findings in such a way that this combination showed a good reduction (P<0.001) in induction of seizure activity against PTZ and MES induced seizures in animals when compare to methylene blue alone received group of animals (Table 13 & 14).

BRL50481 is a selective inhibitor of PDE-7, a novel subtype of PDE that is expressed in a number of cell types, including T lymphocytes. There are at least two genes coding for PDE7, each with several splice variants (Adkinson, 2008). Two PDE7 genes (PDE7A and PDE7B) have been identified in humans (Gardner, 2000; Hetman, 2000). Li et al., suggest that PDE 7 may modulate human T-cell function (Li, 2000). PDE7 is highly expressed in brain regions, including the hippocampus and olfactory bulb (Miro, *et al.,* 2001; Reyes, 2005).

The distribution of PDE7A3 is largely unknown, but it has been found in human T-lymphocytes (Glavas, *et al.,* 2001) and may also be present in many PDE7A1-expressing cells as both transcripts are probably regulated by the same promoter (Torras, 2003). In contrast, PDE7B is abundant in the brain, liver, heart, thyroid glands, and

skeletal muscles, but it is not found in leukocytes (Gardner, 2000). Our study reports concurrence with combined effect of methylene blue with exogenously administered BRL50481 (1.4 mg/kg, i.p.) showed a significant ($P<0.01$) anti-convulsant activity with judicious protection (83.3%) range respectively against MES model as depicted in Table 16. The total convulsive time was prolonged significantly ($P<0.01$) in methylene blue alone treated (69.2%) and combination of methylene blue with BRL50481 treated (29.8%) groups, compared to DMSO received group (100%) as illustrates in Table 15.

A-350619, a heme-dependent soluble guanylate cyclase activator, sGC is a key signal transduction enzyme activated by nitric oxide (NO). Impaired bioavailability and/or responsiveness to endogenous NO have been implicated in the pathogenesis of cardiovascular and other diseases (Evgenov, *et al.,* 2006). In mature brain cGMP acts mainly in cortex, caudate-putamen, cerebellum and hippocampus. In immature brain cGMP is involved in guiding neurons to achieve their destination and it plays important role in myelinogenesis (Domek, 2005). Ishikawa et al., expressed that, two major pathways have been reported by which a cGMP increases intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. cGMP can activate Ca^{2+} influx, by a process involving PKG regulation of ion channels. It can also activate Ca^{2+} release from ryanodine-sensitive intracellular stores by a pathway involving PKG and cyclic ADP-ribose. The cholinergic and β-adrenergic receptors stimulated production of NO that activates a rise in cGMP concentration. Activation of phospholipase C (PLC) leads to inosital (1,4,5) triphosphate (IP_3) production resulting in Ca^{2+} release from endoplasmic reticulum and to NOS activation. This data supported the view that NO/cGMP signal transduction has a crucial

role in Ca^{2+} homeostasis (Ishikawa, 2002). This increase the level of cGMP and the regulation of Ca^{2+} homeostasis provocates seizures in our animal models of epilepsy. From Table 15 it was illustrated, there was an increase in seizure activity (8% and 3.2%) when A-350619 and BRL50481 treated alone against PTZ model.

8.4. Ion channel modulators alone:

The results of this study suggest that ion channel modulators, very particular sodium channel modulator like amiloride and flecainide potentiate the delay onset of seizure ($P<0.001$) threshold against PTZ, kainic acid and MES induced seizures (Table 17-19). Inherited alterations of ion channels in peripheral nerves that lead to bizarre neuromuscular disorders in experimental animal models. Modifications of similar channels in the brain are being recognized as causes of hyperexcitability in the central nervous system and hence of an ancient phenotype, epilepsy (Heinz, 1998). The involvement of sodium hydrogen exchangers in modulation of seizure activity in neuronal cells is well established (Bonnet, 2000; Whittingam, 1989). Among the Na^+/H^+ exchangers especially subtype 1 and 4 are highly aboundant within pyramidal cells of the hippocampus (Lin *et al.,* 1996; Ma, 1997), a region important for epileptic activity. Amiloride is known to block these subtypes in the brain (Lin *et al.,* 1996; Wakabayashi, 1997). Recently, a study has reported a protective action of amiloride in *in-vivo* seizure models in rodents including increasing current electroshock seizures and PTZ tests. Further, another study suggested that inhibition of NHE results in sustained intracellular acification (Bonnet et al., 2000). The later is known to terminate epileptiform discharges (Xiong *et al.,* 2000) and to attenuate glutamate neurotoxicity as well as Ca^{2+} mediated neuronal injury observed in epileptics (Giffard, 1990; Puka,1994) Though intracellular

acidification appears to be a primary mechanism for the anti-convulsant action of amiloride, others effects may not be ruled out, including inhibition of transmembrane low threshold Ca^{2+} channels (Higashima *et al*., 1998) and inhibition of voltage gated Na^+ channels (Velly *et al.,* 1988), the latter being an important mechanism for many of the established anti-epileptic drugs. So, we can come to a conclusion this might be the reason for anti-convulsant activity of amiloride.

Similarly, Table 20-23 explains how flecainide also works as an anti-convulsant (P< 0.001) as like amiloride even with different dose (50 mg/kg, i.p. and 100 50 mg/kg, i.p.) levels against different chemoconvulsants like PTZ, picrotoxin, kainic acid and MES induced seizures in mice. David and co-workers studied, Voltage-gated sodium channels mediate regenerative inward currents that are responsible for the initial depolarization of action potentials in brain neurons. Many of the most widely used anti-epileptic drugs, as well as a number of promising new compounds suppress the abnormal neuronal excitability associated with seizures by means of complex voltage and frequency dependent inhibition of ionic currents through sodium channels (David, 1998). This might be one of the reasons for the existence of anti-convulsant activity of flecainide with the given test dose 1 as well as 2. In general voltage gated Na^+ channels are the molecular targets of local anaesthetics, class I anti-arrhythmic drugs, and some anti-convulsants. These voltage gated Na^+ channels are responsible for the initiation and propagation of action potentials in both nerve and muscle cells (Catterall, 1992; Hille, 1992). These chemically diverse drugs inhibit Na^+ channels with complex voltage and frequency dependent properties that reflect preferential drug binding to open and inactivated channel states. In other hand, which also emphasis Na^+ channel function is regulated by

voltage-dependent transitions among three sets of functionally distinct conformational states. At hyperpolarized membrane potentials, most Na^+ channels are in closed resting states. In response to membrane depolarization, channels rapidly convert to an open state that conducts Na^+ ions and then to a nonconducting, inactivated state. The opening and subsequent inactivation of Na^+ channels results in a transient inward current that inactivates within a few milliseconds. This propagates anti-convulsant activity of flecainide.

8.5. Calcium channel blocker and activator:

Based on Table 24, 25 & 26 it can be suggested that calcium channel blocker, amiodarone potentiate the delay onset of seizure ($P<0.001$) threshold against chemo convulsant injection of picrotoxin, kainic acid and MES induced seizures in mice. Amiodarone, is a multiple ion-channel blocker drug, inhibiting sodium and calcium inward currents and potassium outward current, and having noncompetitive adrenergic blocking effect (Kodama, 1999; Herbette, 1988) in particular which inhibit binding of 1,4-dihydropyridine to L-type Ca^{2+} channels, a/b adrenergic receptor antagonist and coronary vasodilator. The anti-convulsant effect of amiodarone was clearly proven, how ever, animals were treated with a calcium channel activator, BAY K 8644, S(-), a potent, direct acting, voltage-sensitive Ca^{2+} channel activator. It exerts positive inotropic, vasoconstrictive and positive chronotropic activity in a concentration range from 10^{-9}-10^{-7} M, BAY K 8644, S(-), clearly showed that proconvulsant activity as like PDE inhibitor. The probable explanation could be that since dihydropyridine channels modulate the release of neurotransmitter (Middlemiss, 1985) and electrophysiological evidence has indicated that calcium ion currents in hippocampal pyramidal cells are

sensitive to voltage dependent calcium channel agonists and antagonist (Gahwiler, 1987). Moreover, the fact that the central actions of voltage dependent CCBs show anti-convulsant activity, depending on the kind of experimental convulsions investigated, the protection offered by nifedipine in kindled seizures is in accordance with the literature. In general glutamate receptor overstimulation increases intracellular calcium by directly opening ion channels and secondarily affecting calcium homeostatic mechanisms. As mentioned, the initial glutamate receptor opening of the sodium/calcium but also causes membrane depolarization. The depolarization would in turn activate the voltage dependent calcium channels, which would further increase the intracellular calcium levels (Mark *et al.,* 2001). Glutamate excitotoxicity is the final common pathway resulting in neuronal injury for many seemingly unrelated disorders, including ischemia, trauma, seizures, hypoglycemia, hypoxia, and even some neural degenerative disorders (Mark *et al.,* 2001). So, as of now the development of seizures were based on the above mechanism.

8.6. PDEs inhibitors along with sGC inhibitor, calcium channel modulator and calcium channel blocker:

The data obtained from this study shown that pre-treatment with soluble guanylate cyclase inhibitor, methylene blue alone and with the existence of PDE-7 inhibitor such as BRL 50481, potentiates ($P<0.001$) the anti-convulsant activity against the pilocarpine, kainic acid, picrotoxin and MES induced convulsions as described in Table 27, 28 & 29. And also our study explains that the combination of A-350619 with BRL50481 as well as the individual effect of A-350619 and BRL 50481 alone showed a

quick onset of seizures responses with increased the mortality range in both animal models of epilepsy. The Table 27,28 & 29 depict the effect and mechanisms which resembles same as explained already in Table 13, only difference is chemoconvulsant action instead of PTZ and we used pilocarpine, kainic acid and picrotoxin.

Based on Table 30, 31, 32 & 33 it was 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 PTZ, KA, pilocarpine and MES induced seizures in mice**.** The combination of sGC inhibitor, methylene blue with etazolate showed significant ($P<0.001$) a delay 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 (Moncada, 1993). 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 (Bagetta *et al.,* 2002). Inducible NOS (iNOS), a Ca-independent enzyme, is involved in various inflammation and pathophysiological processes including ischemia, stroke, trauma, infection, and autoimmunity (Garthwaite, 1995). NO is also a major stimulator of cGMP generation via guanylate cyclase, which is assumed to play a major role in seizure (Snyder, 1991). 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 proconvulsant activity. Etazolate is a pyrazolopyridine compound (Patel, 1985), which selectively modulates the $GABA_A$ receptor (Barnes, 1983; Whiting *et al.,* 1997; Thompson *et al.,*

2002). The study demonstrates that etazolate 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.

9. SUMMARY

Epilepsy is a generally a chronic neurological disorder. Neither an effective prophylaxsis nor a permanent cure of any of these disorders is available except neurosurgical resection of epileptic tissue in selected instances. There is hope that understanding the cellular and molecular mechanisms of the epilepsies will lead to improved therapies as well as new insights into brain structure and function. Because of the inherent diversity of the epilepsies and their models and the wide range of techniques used for investigating their cellular and molecular basis, I had focused on selected models and issues, attempted to bring some coherency to the findings, and sought to draw conclusions that may be generally relevant to epilepsy. I made a special effort to show how investigations of the epilepsies with methods from diverse disciplines can be complementary and mutually reinforcing. By use of the tools of electrophysiology it was initially demonstrated that the epilepsies are disorders of neuronal excitability, which were subsequently characterized in populations of neurons, in individual neurons, and single ion channels. Next, I addressed the cellular mechanisms of seizures. Finally, I present ways of utilizing PDE inhibitors for the study of epilepsy in animals.

PDE-3 inhibitors related study shows a definite relationship between the specific PDE-3 inhibitors and increase the cellular level of cGMP and Ca^{2+} ions with the generation of seizures. The releases of free radicals have been implicated in many drug and chemical induced toxicities. It is possible that increased production of reactive oxygen species could result in oxidant/ anti-oxidant imbalance and thus, precipitate neurotoxicity. Therefore it appears that non nucleotide mechanism although not well

defined could also be contributing significantly to the seizure activity of phosphodiesterase 3 inhibitors.

The present study finds that PDE-5 inhibitor, sildenafil having strong pro-convulsant activitsy in MES and INH induced animal models of epilepsy and also PDE-3 & 4 inhibitors, such as cilostazol and rolipram possess less pro-convulsant action. The probable mode of action may be the cGMP levels elevated by certain excitatory amino acids and may allow one to imply that an excitable state exists in the neuronal cells through the action of the G-protein as well as the ion channel. Although the findings here seem to indicate divergent features of the excitable neuronal cells, they may provide clues to observing a close association of receptors, enzymes and channels of the membranes in exploring the provocation of seizures.

The present study reflects the individual effect of adenylate cyclase (AC) inhibitor, SQ22536 delay the onset of action of seizures as well as prolonging the total duration of convulsive time in both PTZ and MES models of epilepsy. The SQ22536 greatly increased the anti-convulsant activity along with higher percentage protection range of animals in both models of epilepsy. Further studies can be conducted using specific neuronal cell lines and elucidating the exact signal transduction mechanisms responsible for anti-convulsant effects.

The study reflects, (i) the individual effect of guanylate cyclase (GC) inhibitor, methylene blue delays the onset of action of seizures as well as prolong the total duration of convulsive time in both PTZ and MES models of epilepsy. The methylene blue greatly increased the anti-convulsant activity along with higher percentage protection range of animals in both models of epilepsy; (ii) onset of action and the incidence of seizures are

quick in sGC activator, A-350619 alone as well as combination with PDE-7 inhibitor, BRL-50481 treated groups; (iii) the occurrence of high percentage of mortality is associated with quick onset of seizures resembles pro-convulsant action in A-350619 alone as well as combination with BRL-50481 treated groups in both animal models of epilepsy; (iv) This study also reflects the identification and investigation of new cGMP mediated phosphodiesterases family members will offers a new strategy for improvement of brain function and for the new therapy for epilepsy in future.

Sodium channel modulators such as amiloride and flecainide having more distinct role in the generation of delay onset of seizures in all the phases of convulsion while compared to calcium channel modulator alone treated group. This result reflects the SCM having more potency of anti-convulsant activity while compared to CCM. The combination of SCM along with CCM also enhances the delay onset of seizures when compared to CCM alone treated group. This indicates SCM having more potency of anti-convulsant activity. The animals which exposed increase the anti-convulsant activity while increase the dose levels of SCM even with different chemo convulsants like PTZ and picrotoxin (PTX).

Calcium channel blocker and activator related study explained that delay in onset of various phases of convulsion in both models was enhanced by calcium channel blocker (amiodarone) alone treated animals. This shows that CCB having anti-convulsant activity. The difference in onset of time of various phases of convulsion was clearly explained with help of calcium channel activator [Bay K8644, S (-)], which enhances the quick onset of convulsive threshold. So, Bay K8644, S (-)] possess proconvulsant activity.

The data explains that methylene blue alone, methylene blue with BRL50481 greatly increased the anti-convulsant activity along with higher protection range of animals were seen in both models. The study also explains that the combination of A-350619 with BRL50481 as well as the individual effect of A-350619 and BRL 50481 alone showed a quick onset of seizures response with increased the mortality range in both animal models of epilepsy.

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. 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.

10. CONCLUSION

Advances in our understanding of the molecular pharmacology of cyclic nucleotide PDE isozymes have led to the development of first and second generation selective inhibitors for a variety of clinical indications. While it is difficult to predict the extent to which each of these strategies will eventually affect the management of epilepsy, it is clear that exciting times are ahead of us. Thus in conclusion, phosphodiesterase(PDEs) ioszymes regulate the degradation of cyclic GMP and cAMP a product of the guanylate cyclase and adenylate cyclase activation and could contribute to the pathophysiology of the seizure mechanisms. PDE isozymes 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. Recent evidence that the cyclic nucleotide phosphodiesterases exist in several molecular forms and that these isozymes are unequally distributed in various tissue. 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. As per our study results, PDE inhibitors works as a proconvulsant, so phosphodiesterase (PDEs) isozymes might used as anti-convulsant molecule for better therapeutic response in future.

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12. LIST OF PUBLICATIONS

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Evaluation of cyclic nucleotide phosphodiesterase III inhibitors in animal models of epilepsy

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Key words: PDE-3 inhibitors, amrinone, milrinone, mice, seizures.

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Abstract

This study was conducted to investigate the role of specific phosphodiesterase-3 (PDE-3) inhibitors like amrinone and milrinone in the generation of seizures in albino Swiss mice. Generation of seizures were carried out in the animals by subjecting them to injection of a chemical convulsant, isoniazid (INH) at the dose of 500mg/kg, s.c and by subjecting them to maximal electroshock (MES) at 60 mA for 0.2 sec. The animals were pre-treated with various dose levels of amrinone (0.5 mg/kg, 0.6 mg/kg and 0.7 mg/kg, i.p) and milrinone (50µg/kg, 100 µg/kg, 200µg/kg, 300 µg/kg, i.p) 20 mins prior to the INH or MES. The control group of animals received normal saline (5 ml/kg i.p) 20 mins prior to the injection of INH , or before subjecting the animals to MES. PDE-3 inhibitors significantly enhanced the onset of seizures induced by INH and MES. In particular, milrinone potentiated the convulsive phenomenon more significantly ($p<0.05$ and $p< 0.001$) when compared with amrinone.

Introduction

Epilepsy is one of the most common affliction of human beings with a prevalence rate of approximately 1 % of the total population [1]. Seizure is a characteristic feature in epilepsy and is associated with disordered and rhythmic high frequency discharge of impulses by a group of neurons in the brain. The pathophysiological basis for epileptic disorders is both complex and intricate. The search for newer antiepileptic drugs have focussed the research on cell signaling elements like the cytoskeletal structures, transmembrane enzymes and ion channel modulators.

There is recent evidence that the cyclic nucleotide phosphodiesterases exist in several molecular forms and that these isozymes are unequally distributed in various tissue makes the phosphodiesterases particularly suitable targets for pharmacologic manipulation, for it suggests that by finding selective inhibitors of the different phosphodiesterase isozymes, one may be able to raise the concentration of cyclic nucleotides in discrete cell types [2]. Through the selective inhibition of the major phosphodiesterase isozyme of a diseased tissue, it may then be possible to alter the course of diseases characterized by an abnormal metabolism of cyclic nucleotides.

Twelve members of the family have been identified and these can be further divided into a number of subtypes and splice variants. The PDE types differ in their aminoacid sequence, substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities and in their organ, tissue and sub cellular distribution [3,4]. PDE-3 is characterized by its high affinity for cAMP and cGMP. cAMP is postulated to be anticonvulsant while cGMP is considered to be proconvulsant [5]. PDE-3 enzyme is highly expressed in the hippocampus, striatum and other discrete sites of the brain and may affect the influx of Ca^{2+} ions [6,7].

In mammals, PDE are encoded by at least 19 different genes and PDE isoforms are expressed differently in different tissues [8]. Electroshock shock has been reported to increase the expression of PDE 4 isoform in rat brains suggesting ECS regulates the activity of cAMP system by modifying PDE isoform expression [9,10]. However these studies were limited to PDE 4 family in the cerebral cortex and the hippocampus.

This study was hitherto designed carefully to examine and investigate the role of cyclic phosphodiesterase III in the generation of convulsive seizures. We used pharmacological tools like amrinone and milrinone to block the PDE-3 and evaluate the effect on maximal electroshock and chemical convulsant induced seizures in mice.

Materials and Methods

Animals used

Swiss Albino mice of either sex weighing between 22-25 g were utilized for this study. The animals were kept under standard laboratory conditions. A 12:12 dark: light cycle was followed during the experiments. Animals had free access to food and water *ad libitum*. The Institutional Animal Ethical Committee approved the protocol of this study.

Drugs and Chemicals

The following drugs and chemicals were used for conducting this study. Normal saline (0.9%), Nandha College of Pharmacy, Erode. Pentylenetetrazole (Sigma, USA), Isoniazid (Fourts India Ltd, Chennai, India), Amrinone (Samarth Pharma, India), Milrinone (Sanofi Synthelabo Ltd, Mumbai, India). Both amrinone and milrinone were diluted with sterile water for injection. Normal saline was administered in a volume of 5ml/kg, i.p.

(i) Isoniazid (INH) induced seizures

Albino mice were divided into different groups each containing six animals (n=6). Seizures were induced in the animals by using chemical convulsant, Isoniazid (INH). INH is a GABA synthesis inhibitor, which was injected to induce seizures at the dose of 500 mg/kg, s.c as described earlier [11]. 15 mins prior to the injection of INH the animals were pre-treated with varying doses of amrinone (0.5 mg/kg, 0.6 mg/kg and 0.7 mg/kg, i.p) and milrinone (50μg/kg, 100 μg/kg, 200 μg/kg and 300 μg/kg, i.p). Onset of action, myoclonic jerks, clonus, tonic flexion and mortality were noted and tabulated.

(ii) Maximal Electroshock (MES) Method

MES were induced in the animals using a technique described earlier [12]. The animals were pre-treated with aminone and milrinone in the same dose as mentioned in the corneal electrodes. The animals were subjected to electroshock (60mA/0.2 secs) via the corneal electrodes. After induction of seizures, tonic limb flexion, tonic extensor, clonus, stupor and recovery/mortality of the animals were observed and tabulated.

Biometric Analysis

The data is represented as mean ± SEM. Statistically significant difference was ascertained by ‘P’ value which is considered significant of $P<0.05$ and highly significant of $P<0.01$ as comparisons of different groups were done using one way ANOVA followed by Dunnett’s test.

Result

Evaluation of onset of seizures

(i) INH induced seizures

Table 1 and 3 shows the data obtained from experiments conducted with INH induced seizures. In animals treated with normal saline onset of action were noticed 2830 ± 52.33 sec and convulsions appeared 3065 ± 45.43 sec after INH. Amrinone in a dose of 0.5 mg/kg significantly potentiated the onset of action, jerky movements and convulsions ($p<0.05$) where as the rate of onset of action, jerky movements and convulsions time was reduced significantly in the doses like 0.6 mg/kg and 0.7 mg/kg of amrinone ($p<0.001$).

Simultaneously the rate of onset of action, jerky movements and convulsion time was reduced at the great extent even in the low doses like (200 μg/mg and 300 μg/mg) of milrinone ($p<0.001$) considerable mortality (67%) was observed while using amrinone (0.6 mg/kg and 0.7 mg/kg) and milrinone (100 μg/kg, 200 μg/kg and 300 μg/kg).

(ii) Maximal Electroshock Test

Table 2 and 4 illustrates the action of various dose levels of amrinone and milrinone against MES induced seizures. In which 0.6 mg/kg and 0.7mg/kg of amrinone produced a gradual reduction in tonic limb flexion significantly ($p<0.05$) when compared with normal saline. Significant ($p<0.001$) was observed in stupor phase of convulsion at the dose of 0.6 mg/kg and 0.7 mg/kg of amrinone. Likewise milrinone treated animals showed a significant ($p<0.001$) reduction in tonic limb tonic extensor and stupor flexion, phases of convulsion in the 200μg/kg and 300 μg/kg dose levels.

Milrinone in the doses like 200 μg/kg and 300μg/kg treated animals produced the significantly reduced the clonus phases of convulsion at the level of $p<0.005$ and $p<0.001$ respectively. Mortality (67%) was observed in both doses like 200μg/kg and 300μg/kg of milrinone.

Table 1: Action of various dose levels of amrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	**CHEMO-CONVULSANT ISONIAZID (INH) Onset Time Of Various Phases Of Convulsions (in sec.)**		
	Action	**Jerky Movements**	**Convulsions**
Normal Saline (5 ml/kg, i.p)	2830 ± 52.33	3000 ± 46.47	3065 ± 45.43
Amrinone (0.5 mg/kg, i.p)	2190 ± 45.82 *	2293.33 ± 44.24 *	2320 ± 43.42 *
Amrinone (0.6 mg/kg, i.p)	1938 ± 37.62 **	2025 ± 39.30 **	2063.33 ± 34.79 **
Amrinone (0.7 mg/kg, i.p)	1601.67 ± 17.78 **	1681.67 ± 20.56 **	1730 ± 15.27 **

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to chemical convulsant injection of INH (500 mg/kg, s.c.). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05 and ** p<0.001, compared to the normal saline treated group.*

Table 2: Action of various dose levels of amrinone on maximal electroshock induced convulsions in mice (n=6)

Treatment (mg/kg, i.p)	**Onset Time Of Various Phases Of Convulsions (in sec.)**				
	Tonic Limb Flexion	**Tonic Extensor**	**Clonus**	**Stupor**	**Recovery / Death**
Normal Saline (5 ml/kg, i.p)	5.67 ± 0.33	23.33 ± 0.67	36.83 ± 1.38	66.67 ± 1.23	196.25 ± 5.55
Amrinone (0.5 mg/kg, i.p)	4.33 ± 0.21	19.83 ± 0.31	37 ± 0.73	59.83 ± 0.6 *	226 ± 0.82
Amrinone (0.6 mg/kg, i.p)	3.5 ± 0.22 *	15.5 ± 0.43 *	33.33 ± 0.42	53.83 ± 0.6 **	207 ± 3.72
Amrinone (0.7 mg/kg, i.p)	3.17 ± 0.17 *	9.83 ± 0.31 **	28 ± 0.51 *	43 ± 0.86 **	207.33 ± 0.42

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to maximal electroshock (60 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05 and ** p<0.001, compared to the normal saline treated group.*

Table 3: Action of various dose levels of milrinone on chemoshock seizures in mice (n=6)

Treatment (mg/kg, i.p)	**CHEMO-CONVULSANT ISONIAZID (INH) Onset Time Of Various Phases Of Convulsions (in sec.)**		
	Action	**Jerky Movements**	**Convulsions**
Normal Saline (5 ml/kg, i.p)	2830 ± 52.33	3000 ± 46.47	3065 ± 45.43
Milrinone (50 µg/kg, i.p)	2520 ± 34.65	2860 ± 25.31	3060 ± 21.92
Milrinone (100 µg/kg, i.p)	2220 ± 34.65 *	2483.3 ± 26.04 *	2610 ± 25.71 *
Milrinone (200 µg/kg, i.p)	1870 ± 36.04 **	2100 ± 30.98 **	2270 ± 36.04 **
Milrinone (300 µg/kg, i.p)	1670 ± 21.91 **	1890 ± 25.71 **	2010 ± 33.76 **

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to chemical convulsant injection of INH (500 mg/kg, s.c.). The data were analysed by one-way ANOVA followed by Dunnett's test. * p<0.05 and ** p<0.001, compared to the normal saline treated group.*

Table 4: Action of various dose levels of milrinone on maximal electroshock induced convulsions in mice (n=6)

Treatment (mg/kg, i.p)	Onset Time Of Various Phases Of Convulsions (in sec.)				
	Tonic Limb Flexion	Tonic Extensor	Clonus	Stupor	Recovery/ Death
Normal Saline (5 ml/kg, i.p)	5.67 ± 0.33	23.33 ± 0.67	36.83 ± 1.38	66.67 ± 1.23	196.25 ± 5.55
Milrinone (50 µg/kg, i.p)	4.8 ± 0.33	18.3 ± 0.41	36.8 ± 0.41	57.5 ± 0.78 *	218.3 ± 0.86
Milrinone (100 µg/kg, i.p)	3.3 ± 0.20 *	15.2 ± 0.49 *	32.7 ± 0.82	53.5 ± 0.65 **	280 ± 3.14
Milrinone (200 µg/kg, i.p)	2.7 ± 0.20 **	8.7 ± 0.3 3 **	25.8 ± 0.33 *	47.7 ± 0.65 **	235.0 ± 1.71
Milrinone (300 µg/kg, i.p)	2.2 ± 0.17 **	7.3 ± 0.33 **	20 ± 0.57 **	39.8 ± 0.69 **	237.5 ± 1.43

*Values are mean ± SEM, represents onset time of various phases of convulsion in seconds. Treatments were given 20 mins prior to maximal electroshock (60 mA, 0.2 sec). The data were analysed by one-way ANOVA followed by Dunnett's test. * p <0.05 and ** p<0.001, compared to the normal saline treated group.*

Discussion

The therapeutic use of theophylline/ aminophylline is associated with the incidence of intractable seizures and mortality [13,14]. The mechanisms involved in these seizures are not well understood and the treatment of the life threatening condition is unsatisfactory. The results of this study suggest that PDE-3 inhibitors potentiate the electroshock and chemical induced seizures. The bipyridine derivative of selective PDE-3 inhibitors such as amrinone and milrinone is a new class of positive inotropic drugs chemically and pharmacologically distinct from digitalis and catecholamines [15,16]. The mechanism of the positive inotropic effect of PDE inhibitors is similar to that of β-adrenergic agents [17]. Milrinone has been the most studied and used extensively as PDE-3 inhibitor and it is currently used in the acute treatment of heart failure to diminish long term risk [18]. This study demonstrates the importance of the PDE-3 inhibitors such as amrinone and milrinone in the generation of seizure activity with the accumulation of cellular levels of cAMP and cGMP by inhibiting its metabolism. cAMP accumulation is considered to be anticonvulsant and cGMP is considered to be proconvulsant.

The data obtained from this study show that pre-treatment with PDE-3 inhibitors potentiates the onset of action and various phases of convulsions against INH and maximal electroshock induced convulsions as depicted in Table 1 to 4. Our study results also clearly suggest that rate of onset of convulsive time was significantly ($p<0.05$ and $p<0.001$) reduced with increasing the dose levels of both amrinone and milirinone against INH and MES induced seizures.

Earlier studies suggest that the elevated levels of cGMP was found in cortical structure in some experimental models of epilepsy [19,20], and the neuronal excitability was regulated by cGMP and Ca^{2+}/calmodulin dependent protein kinase and its phosphorylation process [21]. Apart from these findings, PDE-3 inhibitors possess transmembrane influx of Ca^{2+}. This influx of Ca^{2+} is responsible for the phosphorylation process of intracellular proteins, such as ion channels, receptors, enzymes and transcription factors which exhibit significant neuronal excitability and epileptic seizures [22].

On the other hand, phosphorylation of variety of substrates regulates the myriad of physiological process, such as immune responses, cardiac and smooth muscle contraction, visual response, glycogenolysis, platelet aggregation, ion channel conductance, apoptosis and growth control [23]. The present study results also early correspond with the generation of seizure activity due to the breakdown of hydrolysis of cGMP which promotes protein kinase phosphorylation process.

Thus, in conclusion the study shows a definite relationship between the specific PDE-3 inhibitors and increase the cellular level of cGMP and Ca^{2+} ions with the generation of seizures. The release of free radicals have been implicated in many drug and chemical induced toxicities [24]. It is possible that increased production of reactive oxygen species could result in oxidant/ anti-oxidant imbalance and thus, precipitate neurotoxicity. Therefore it appears that non nucleotide mechanism although not well defined could also be contributing significantly to the seizure activity of phosphodiesterase 3 inhibitors.

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Characterization of the Effects of Phosphodiesterases (PDEs) Isozyme Inhibitors in Animal Models of Epilepsy

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Abstract

Epilepsy is a neurological disorder. Phosphodiesterase (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 signaling in the various body systems This study was conducted to evaluate the effect of phosphodiesterase isozymes 3, 4 and 5 inhibitors on the maximal electroshock and isoniazid induced convulsions. The results of this study suggested that zonisamide and gabapentin are anticonvulsant drugs were able to attenuate both the MES and isoniazid induced chemical convulsion respectively. On the other hand, PDE 4 inhibitor rolipram and PDE 5 inhibitor, sildenafil was actually potentiating the convulsive phenomenon that the onset of epileptic threshold was reduced as tested by MES and isoniazid induced convulsions. These studies ascertain the action of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil against MES induced seizures in mice. In which sildenafil (5 mg/kg, i.p.) produced a reduction in the tonic limb flexion significantly ($p<0.01$) when compared to other groups. Like wise, rolipram (2.4 mg/kg, i.p.) treated animals showed significant ($p<0.05$) reduction in tonic limb flexion. In the similar manner, sildenafil produced a reduction in the tonic extensor, clonus and stupor phases of convulsion significantly ($p<0.01$) when compared to other groups and the action of PDE-3, 4 and 5 inhibitors on Isoniazid (INH) induced seizures in mice. Sildenafil (5 mg/kg, i.p.) showed a gradual reduction in the onset of action, jerky movements and convulsion significantly ($p<0.01$) when compared to other PDE inhibitors. The PDE-3, 4 and 5 inhibitors against MES induced seizures in rats. In which sildenafil (3.5 mg/kg, i.p.) produced a gradual reduction in the tonic limb flexion significantly ($p<0.01$) when compared to other groups. Similarly, sildenafil produced a gradual reduction in the tonic extensor, clonus and stupor phases of convulsion significantly ($p<0.01$) when compared to other groups except cilostazol (7 mg/kg, i.p.) treated rats. This study concludes that PDE-5 inhibitor, sildenafil having strong proconvulsant activity in MES and INH induced animal models of epilepsy.

Keywords: Epilepsy; phosphodiesterase isozymes; maximal electroshock; isoniazid induced convulsions; anticonvulsant drug; inhibitors; tonic limb flexion; tonic extensor; clonus.

Introduction

Epilepsy is a 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 and molecular biology aspects of this neurological disorder. 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 epilepsy. Many receptors, when stimulated by various neurotransmitters and hormones, may stimulate second messengers to elicit biological reactions. In cell physiology, a second messenger system is a method of cellular signaling whereby a diffusible signaling molecule is rapidly produced, which can then go on to activate effector proteins within the cell to exert a cellular response. Secondary messengers are a component of signal transduction cascades. Secondary messenger

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systems can be activated by diverse means, either by activation of enzymes that synthesize them, as is the case with the activation of cyclases that synthesize cyclic nucleotides, or by opening of ion channels to allow influx of metal ions, such as in Ca^{2+} signaling. These small molecules may then go on to exert their effect by binding to and activating effector molecules such as protein kinases, ion channels, and a variety of other proteins, thus continuing the signaling cascade [3]. The messengers first reported are cyclic nucleotides, e.g. cAMP and cGMP, and those recently discovered are the reaction products of inositide (PI) response [4], i.e. inositol triphosphate and diacylglycerol. The products of the former release stored intracellular Ca2+ and the latter activate protein kinase C. Phosphodiesterase (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 signalling in the various body systems [5]. Recent evidence that the cyclic nucleotide phosphodiesterases exist in several molecular forms and that these isozymes are unequally distributed in various tissues [6]. Twelve members of the PDE family have been identified and these can be further divided into a number of subtypes and splice variants. The PDE types differ in their amino acid sequence, substrate specificities, kinetic properties, allosteric regulators, inhibitor sensitivities and in their organ, tissue and sub cellular distribution [7, 8]. Through the selective inhibition of the major phosphodiesterase isozyme of a diseased tissue, it may then be possible to alter the course of diseases characterized by an abnormal metabolism of cyclic nucleotides. Out of the twelve PDE gene families PDE-3, PDE-4 and PDE-5 are belongs to cGMP-inhibited [9-11], cAMP-specific [12-16] and cGMP-specific [17-22] types of affinity to cyclic nucleotides respectively. PDE-3 and PDE-4 enzyme are expressed in the hippocampus, striatum and other discrete sites of the brain and may affect of calcium ions and electroshock may modify their activity. Therefore, the present study examined the influence of cyclic nucleotide PDE inhibitors, especially PDE-3, 4 and 5, in order to prove the effective role in the induction of convulsive seizures. We used pharmacological tools like cilostazol, rolipram and sildenafil to block the PDE 3, 4 and 5 isozymes respectively to evaluate the effect on maximal electroshock and chemical convulsant induced seizures in mice and rats.

Methods

Animals Used: Male Swiss Albino mice weighing between 23-26 g and male Wistar strain rats weighing between 160-220 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 Degrees Celcius 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 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, Cilostazol (Sigma, USA), Rolipram (Sigma, USA), Sildenafil (Sun Pharma, Mumbai, India), Zonisamide (Sun Pharma, Mumbai, India) and Gabapentin (Micro labs Ltd., Bangalore, India). Except rolipram and cilostazol, other drugs are soluble in sterile water for injection, rolipram and cilostazol is soluble in DMSO.

A. Maximal electroshock (MES) method: The mice were divided into five groups with six animals (n=6) in each. Group I served as solvent control, received 10 % w/v of dimethylsulfoxide [DMSO] (5 ml/kg, i.p.), group II received zonisamide (50 mg/kg, i.p.), treated as positive control and Group III, IV and V received PDE- 3 inhibitor such as cilostazol (10 mg/kg, i.p.), PDE-4 inhibitor such as rolipram (2.4 mg/kg, i.p.) and PDE-5 inhibitor such as sildenafil (5 mg/kg, i.p.) respectively. All the drugs were administered intraperitoneally 25 min prior to the electroshock. The electroshock was induced in animal by passing a current of 45 mA for 0.2 sec duration through electroconvulsiometer (Techno India) using corneal electrodes [23]. The incidence of seizures, tonic limb flexion, tonic extensor, clonus, stupor of the animals were observed and noted as per earlier described method [24].

B. Chemical convulsant method: Isoniazid (INH) induced seizures: Albino mice were divided into five groups each containing six animals (n=6). Seizures were induced in the animals by using chemical convulsant, isoniazid (INH). INH is a GABA synthesis inhibitor, which was injected to induce seizures at the dose of 500 mg/kg, s.c as described earlier [25]. All the drugs were administered intraperitoneally 25 minutes prior to the chemoshock in the same dose as mentioned in MES, except positive control (Gabapentin 2.5mg/kg, i.p.). Onset of action, myoclonic jerks, clonus, and tonic flexion were observed and noted.

C. Maximal electroshock (MES) method for rats: Rats were divided into five groups with six animals (n=6) in each. Group I served as solvent control, received 10 % w/v of dimethylsulfoxide [DMSO] (3.5 ml/kg, i.p.), group II received zonisamide (35 mg/kg, i.p.), treated as positive control and Group III, IV and V received PDE-3 inhibitor such as cilostazol (7 mg/kg, i.p.), PDE-4 inhibitor such as rolipram (1.7 mg/kg, i.p.) and PDE-5 inhibitor such as sildenafil (3.5 mg/kg, i.p.) respectively. All the drugs were administered intraperitoneally 25 min prior to the electroshock. The electroshock was induced in animals by passing a current of 150 mA for 0.2 sec duration through electroconvulsiometer (Techno India) using corneal electrodes according to a previously described method [23]. The incidence of seizures, tonic limb flexion, tonic extensor, clonus, stupor of the animals was observed and noted as per earlier described method [24].

Statistical analysis: The values were expressed as mean ± SEM. The statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's test. P values <0.05 were considered significant.

Results

Evaluation of onset of seizures

A. Maximal Electroshock Test: Fig. 1 illustrates the action of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil against MES induced seizures in mice. In which sildenafil (5 mg/kg, i.p.) produced a reduction in the tonic limb flexion significantly ($p<0.01$) when compared to other groups. Like wise, rolipram (2.4 mg/kg, i.p.) treated animals showed significant ($p<0.05$) reduction in tonic limb flexion. In the similar manner, sildenafil produced a reduction in the tonic extensor, clonus and stupor phases of convulsion significantly ($p<0.01$) when compared to other groups.

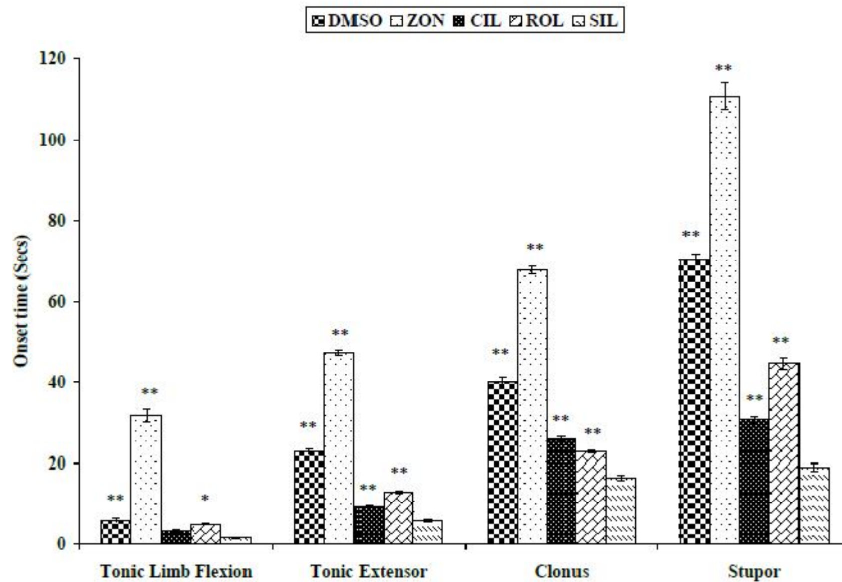
B. Chemical convulsant method:
Isoniazid (INH) induced seizures Fig. 2 exhibits the action of PDE-3, 4 and 5 inhibitors on INH induced seizures in mice. Sildenafil (5 mg/kg, i.p.) showed a gradual reduction in the onset of action, jerky movements and convulsion significantly ($p<0.01$) when compared to other PDE inhibitors such as cilostazol (10 mg/kg, i.p.) and rolipram (2.4 mg/kg, i.p.), DMSO (5 ml/kg, i.p.) and gabapentin (2.5 mg/kg, i.p.).

C. Maximal electroshock (MES) method for rats: Fig. 3 exhibits the action of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil against MES induced seizures in rats. In which sildenafil (3.5 mg/kg, i.p.) produced a gradual reduction in the tonic limb flexion significantly ($p<0.01$) when compared to other groups. Similarly, sildenafil produced a gradual reduction in the tonic extensor, clonus and stupor phases of convulsion significantly ($p<0.01$) when compared to other groups except cilostazol (7 mg/kg, i.p.) treated rats.

Discussion

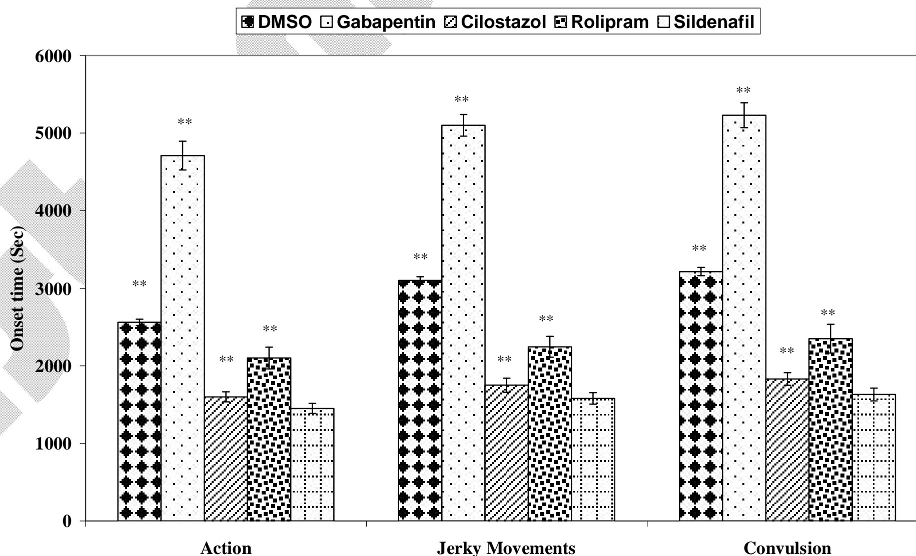
This study was conducted to evaluate the effect of phosphodiesterase isozyme 3, 4 and 5 inhibitors on the maximal electroshock and isoniazid induced convulsions. The results of this study are depicted in figures 1-3. The results of this

Fig. 1: Effect of PDE-3, PDE-4 and PDE-5 inhibitors on maximal electroshock induced convulsions in mice (n=6).



Values are mean ± SEM, represent onset time of various phases of convulsion in seconds. Treatments were given 25 mins prior to maximal electroshock (45 mA, 0.2 sec). The data were analyzed by one-way ANOVA followed by Dunnett's test. * *p*<0.05, ** *p*<0.01 and *** *p*<0.001 vs Sildenafil treated group.

Fig. 2: Effect of PDE-3, PDE-4 & PDE-5 inhibitors on chemoshock seizures in mice (n=6).

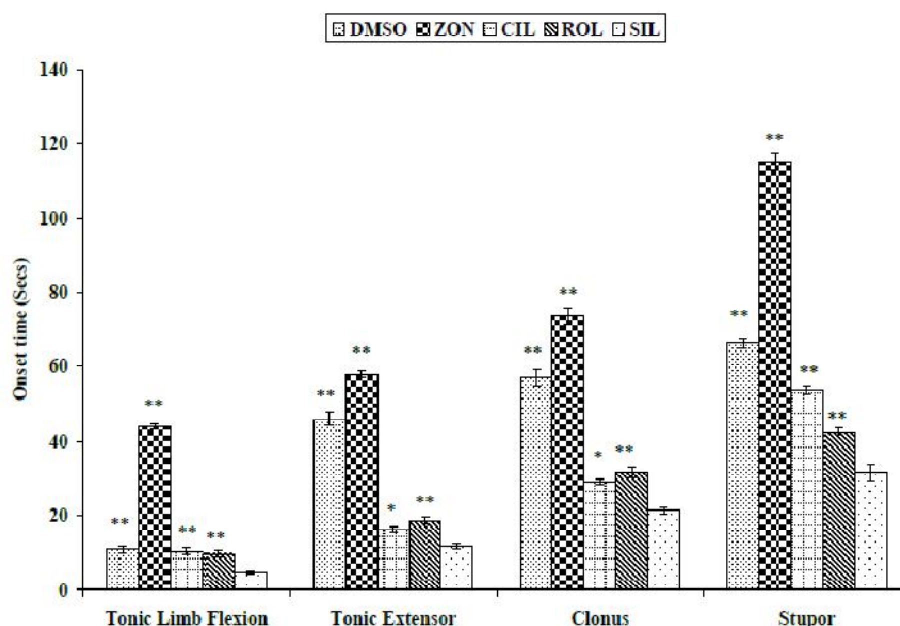


Values are mean ± SEM, represent onset time of various phases of convulsion in seconds. Treatments were given 25 mins prior to chemical convulsant injection of INH (500 mg/kg, s.c). The data were analyzed by one-way ANOVA followed by Dunnett's test. * *p*<0.05, ** *p*<0.01 and *** *p*<0.001 vs Sildenafil treated group.

study suggest that zonisamide and gabapentin are anticonvulsant drugs were able to attenuate both the MES and isoniazid induced chemical convulsion. On the other hand, PDE 4 inhibitor rolipram and PDE 5 inhibitor, sildenafil was actually potentiating the convulsive phenomenon i.e. the onset of epileptic threshold was reduced as tested by MES and isoniazid induced convulsions. The mechanism of its action is through the inhibition of T-type Ca 2+ currents. In addition, zonisamide inhibits sustained repetitive firing of spinal cord neurons, presumably by prolonging the inactivated state of voltage gated sodium channels in a manner similar to actions of phenytoin and carbamazepine. Recent evidence suggests that the cyclic nucleotide PDE exist in several molecular forms and that these isozymes are unequally distributed in various tissues. Phosphodiesterase (PDE) activity is found in every cell in the body, although there is distinct cellular and subcellular distribution of the 12 isoenzymes, which has provided many possibilities for increasingly selective therapeutic targets [27]. In identifying isoenzyme selective targets for specific diseases, a substantial amount of work was undertaken by pharmacologists working in the U.K., particularly in characterizing tissue expression, subcellular distribution and modulation of tissue function by isoenzyme selective inhibitors. The PDE-3, PDE-4 and PDE-5 belong to cGMP inhibitory, c-AMP specific and cGMP specific types of affinity to cyclic nucleotides respectively. According to Riazi et al sildenafil is a proconvulsant drug in the rodents and the role of nitric oxide cGMP pathway is implicated in these actions. Other reports also suggest the proconvulsant action of sildenafil in humans and animals. Studies conducted by Demchenko et al showed that PDE5 blockers oppose the protective vasoconstriction that is the initial response to hyperbaric hyperoxia, decreasing the safety of hyperbaric oxygen and hastening onset of CNS oxygen toxicity. The present study mainly focuses the onset of seizures against the prior administration of PDE-3, 4 and 5 inhibitors such as cilostazol, rolipram and sildenafil. PDE-3 has high affinity for cAMP but can also hydrolyse cGMP. However, since the *K*m for cGMP has generally been reported to be lower than that for cAMP, and the *V*max is ten times greater for cAMP than for cGMP, cGMP readily inhibits the hydrolysis of cAMP by PDE3 by acting as a potent competitive inhibitor at the catalytic site [28–29]. Thus, expression of PDE3 allows stimuli that elevate cGMP levels to augment cAMP-mediated signalling [29]. There are two PDE3 genes, PDE3A and PDE3B. Cilostazol is a PDE3 inhibitor that is a U.S. FDA - approved therapy for intermittent claudication, owing to its activity on both platelets and endothelium [30]. PDE-4 enzymes are cAMP-specific and play an important role in the biology of haematopoietic cells. These PDEs all hydrolyse cAMP with *K*m values in the range 1–4 μM [31]. Earlier reported study suggest that G-proteins and PKAs (cAMP-dependent protein kinase), is essential for controlling localized concentrations of cAMP [32-35]. PDE-5, a cGMP-specific PDE family of considerable importance in regulating smooth muscle and endothelial cell function, is also present in platelets. Although having no effect on platelet function when used alone, PDE5 inhibitors augment nitroprusside's anti-platelet aggregation activity *in vitro* [36].

The data obtained from this study show that pre-treatment with PDE-3, 4 and 5 inhibitors potentiates the onset of action and various phases of convulsions against INH and maximal electroshock induced convulsions as depicted in Fig 1 to 3. At same time the effect of onset of action, after administration of PDE-3 and 4 were less when compared to PDE- 5 inhibitor. Our study results also clearly suggest that rate of onset of convulsive time was significantly ($p<0.05$ and $p<0.01$) reduced with sildenafil against INH and MES induced seizures in both mice and rats. It has recently been reported that the elevation of cGMP levels provides a depolarized state at the rod outer segment of retina [37] and a GTP-binding protein (a G-protein, Go) regulates the neuronal Ca2+ channel [38]. This Ca2+ is responsible for the phosphorylation process of intracellular proteins, such as ion channels, receptors, enzymes and transcription factors that exhibit significant neuronal excitability and epileptic seizures [39]. These events were associated with a significant increase in intracerebellar cyclic GMP [40]. Thus, in conclusion the present study finds that PDE-5 inhibitor, sildenafil having strong proconvulsant activity in MES and INH induced animal models of epilepsy and also PDE-3 & 4 inhibitors, such as cilostazol and rolipram possess less proconvulsant action. The probable mode of action may be the cGMP levels elevated by certain excitatory amino acids and may allow one to imply that an excitable state exists in the neuronal cells through the action of the G-protein as well as the ion channel. Although the findings here seem to indicate divergent features of the excitable neuronal cells, they may provide clues to observing a close association of receptors, enzymes and channels of the membranes in exploring the provocation of seizures.

Fig. 3: Action of PDE-3, PDE-4 & PDE-5 inhibitors on maximal electroshock induced convulsions in rats (n=6).



Values are mean ± SEM, represent onset time of various phases of convulsion in seconds. Treatments were given 25 mins prior to maximal electroshock (150 mA, 0.2 sec). The data were analyzed by one-way ANOVA followed by Dunnett's test. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ vs Sildenafil treated group.

Table 1: Treatment protocol of maximal electroshock (MES) model for mice and rats, and chemoconvulsant (INH) model for mice.

Treatment Groups	Drugs used	Category	MES for Mice (45 mA for 0.2 sec)	Chemoconvulsant (INH) for Mice (500 mg/kg, s.c)	MES for Rats (150 mA, 0.2 sec)
Group- I	10% DMSO	Solvent Control	5 ml/kg, i.p.	5 ml/kg, i.p.	3.5 ml/kg, i.p.
Group- II	Zonisamide/ Gabapentin	Positive Control	(Zonisamide) 50 mg/kg, i.p.	(Gabapentin) 2.5 mg/kg, i.p.	(Zonisamide) 35 mg/kg, i.p.
Group- III	Cilostazol	PDE-3 inhibitor (cGMP-inhibited)	10 mg/kg, i.p.	10 mg/kg, i.p.	7 mg/kg, i.p.
Group- IV	Rolipram	PDE-4 inhibitor (cAMP-specific)	2.4 mg/kg, i.p.	2.4 mg/kg, i.p.	1.7 mg/kg, i.p.
Group- V	Sildenafil	PDE-5 inhibitor (cGMP-specific)	5 mg/kg, i.p.	5 mg/kg, i.p.	3.5 mg/kg, i.p.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

JN: experimental work, manuscript preparation; AK: experimental work; MGT: conception of research work, manuscript preparation.

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Evaluation of seizure activity after phospho-diesterase and adenylate cyclase inhibition (SQ22536) in animal models of epilepsy

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Abstract

The role of adenylate cyclase (AC) inhibitor (SQ22536) was evaluated in the presence of PDE-5/6/8/10/11 and PDE-7 inhibitors such as dipyridamole and BRL-50481 in animal models of epilepsy. Seizures were induced in the animals by subjecting them to injection of chemical convulsant, pentylenetetrazole (PTZ) and maximal electroshock (MES). The study mainly comprises of the onset of seizures, mortality/recovery, percentage of prevention of seizures (anti-convulsant) and total duration of convulsive time. Present study mainly highlights the combined effects of AC inhibitor SQ22536 with dipyridamole as well as BRL50481 showed a good reduction ($P<0.001$) in incidence of seizures, compared to SQ22536 and BRL50481 alone treated mice against PTZ (60 mg/kg, i.p.) model. The total convulsive time was prolonged significantly ($P<0.01$) in SQ22536 alone treated (60.2%) and in with combination of SQ22536 with BRL50481 treated (27.4%) groups, compared to DMSO received group (100%). The study also demonstrates that SQ22536 alone, SQ22536 followed by dipyridamole and SQ22536 with BRL50481 greatly increased the anticonvulsant activity ($P<0.01$, $P<0.05$ and $P<0.01$) along with higher protection 83.3%, 66.7% and 50% range respectively. SQ22536 with dipyridamole effectively ($P<0.001$) decreased the MES (150 mA, 0.2 sec) induced convulsion, compared to SQ22536. The data shows that SQ22536 alone, SQ22536 followed by dipyridamole and SQ22536 with BRL50481 greatly increased the anti-convulsant activity ($P<0.01$, $P<0.01$ and $P<0.01$) along with higher protection 83.3%, 50% and 66.7% range respectively in animals pre-treated with MES. The results suggest the possible involvement of SQ22536 alone and with presence of dipyridamole and BRL50481, delays the onset of seizure activity as well as prolongs the total duration of convulsive time in both models.

Keywords: Adenylate cyclase, PDE, SQ22536, dipyridamole, BRL50481, seizures

Introduction

Epilepsy is a common health problem and affects more than 50 million people worldwide, 5 million of them have seizures more than once per month (Porter, 1988). Approximately 5-10% of the population usually develops seizure at least once during their lifetime, with the highest incidence occurring in early childhood and late adulthood (Lowenstein, 2001). A seizure is a sudden change in behaviour characterized by changes in sensory perception (sense of feeling) or motor activity (movement) due to an abnormal firing of nerve cells in the brain. Epilepsy is a condition characterized by recurrent seizures that may include repetitive muscle jerking called convulsions. Epilepsy is a complex disease with diverse clinical characteristics that preclude a singular mechanism. One way to gain insight into potential mechanisms is to reduce the features of epilepsy to its basic components: seizures, epileptogenesis and the state of recurrent unprovoked seizures that defines epilepsy itself. 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 exist 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 (Scharfman, 2007).

The cyclic adenosine 3', 5'-monophosphate (cAMP) plays a major role in the generation of seizure activity. An elevation in cAMP content has been reported in the cerebral cortex accompanying chemically induced epileptic activity (Walker *et al*., 1973; Krivanek & Mares, 1977; Ferrendelli *et al.,* 1980). The adenylate cyclase (AC), an important transmembrane enzyme possesses certain activity in the brain which promotes the intracellular level of cAMP, from adenosine triphosphate (ATP) (Seamon *et al.,* 1981; Higashima *et al.,* 2002). In epileptic conditions the cAMP concentration in the cerebrospinal fluid is also elevated after an attack (Myllyla *et al.,* 1975). cAMP plays a key function by controlling a wide variety of cellular processes (Houslay *et al.,* 1998; Houslay, 2001) also which acts as a ubiquitous second messenger and modulator of signal transduction processes (Houslay, 1998). This cAMP is generated by the action of adenylate cyclase (Houslay & Milligan, 1997) and degraded by hydrolysis process, which is

regulated by a family of cyclic nucleotide phosphodiesterases (PDEs) (Conti & Jin 1999; Soldering & Beavo, 2000).

PDE enzymes regulate the degradation of cAMP a product of the adenylate 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 (Maurice *et al.,* 2003). Recent evidence shows that the cyclic nucleotide phosphodiesterases exist in several molecular forms and that these isozymes are unequally distributed in various tissues (Jeon *et al.,* 2005). Twelve members of the PDE family have been identified and these can be further divided into 50 isoforms of subtypes and splice variants (Wallace *et al.,* 2005). Out of the twelve PDE gene families, PDE-5 & 6 belong to cGMP-specific (Francis *et al.,* 1990; Loughney *et al.,* 1998; Wang *et al.,* 2001) PDE-7 & 8 are cAMP-specific (Michaeli *et al.,* 1993; Soderling *et al.,* 1998), PDE-10&11 related with cGMP-sensitive and dual specificity (Loughney *et al.,* 1999; Yuasa *et al.,* 2000). 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 (Fisher & Coyle, 1991).

The present study examines the role of adenylate cyclase in the presence of cyclic nucleotide phosphodiesterase-5/6/7/8/10/11 inhibitors in the generation of seizure threshold. We used pharmacological tools like SQ-22536 (adenylate cyclase inhibitor), Dipyridamole (PDE-5/6/8/10/11 inhibitor) and BRL-50481 (PDE-7 inhibitor) to block and attenuate the effects of PDE and evaluate the effect on chemical convulsant and maximal electroshock induced seizures in mice and rats.

Materials and methods

Either sex of Swiss Albino mice weighing between 24-26 g and Wistar strain rats weighing between 160-220 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 chow (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), SQ22536 (Sigma, USA), zonisamide (Sun Pharma, Mumbai, India), dipyridamole (Tocris Bioscience, UK), BRL50481 (Tocris Bioscience, UK) and except gabapentin and zonisamide, other drugs are soluble in DMSO, gabapentin and zonisamide are soluble in sterile water for injection.

A. Chemoshock method

Pentylenetetrazole (PTZ) or metrazol (MTZ) induced seizure model in mice

Swice Albino mice were divided into 7 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 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 SQ22536 (1nmol/kg, i.p) an adenylate cyclase inhibitor.

Group-IV : Mice received dipyridamole (2 mg/kg, i.p) a PDE-5/6/8/10/11 Inhibitor.

Group-V : Mice received BRL50481 (2mg/kg, i.p) a PDE-7 inhibitor.

Group-VI : Mice received SQ22536 (1nmol/kg, i.p) along with dipyridamole (2 mg/kg, i.p) combination of adenylate cyclase inhibitor and PDE-5/6/8/10/11 inhibitor.

Group-VII: Mice received SQ22536 (1 nmol/kg, i.p) along with BRL50481 (2 mg/kg, i.p) combination of adenylate cyclase inhibitor and PDE-7 inhibitor.

All the drugs were administered intraperitoneally 30 min prior to the administration of pentylenetetrazole (60 mg/kg, i.p). The animals were observed for 1 h 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 (Yemitan & Salahdeen, 2005; Salahdeen & Yemiten, 2006) method.

B. Maximal electroshocks (MES) method for rats

Wistar strain rats were divided into 7 groups with six animals (n=6) in each. Treatment protocol and group description is mentioned as follows:

Group- I: Rats served as solvent control, received 10 % w/v of DMSO (3.5 ml/kg, i.p).

Group- II: Rats received zonisamide (35 mg/kg, i.p.), treated as positive control.

Group- III : Rats received SQ22536 (0.7 nmol/kg, i.p) an adenylate cyclase inhibitor,

Group- IV: Rats received dipyridamole (1.4 mg/kg, i.p) a PDE-5/6/8/10/11 inhibitor.

Group-V: Rats received BRL50481 (1.4 mg/kg, i.p) a PDE-7 inhibitor.

Group-VI: Rats received SQ22536 (0.7 nmol/kg, i.p) along with dipyridamole (1.4 mg/kg, i.p) combination of adenylate cyclase inhibitor and PDE-5/6/8/10/11 inhibitor.

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Group-VII: Rats received SQ22536 (0.7 nmol/kg, i.p) along with BRL50481 (1.4 mg/kg, i.p) combination of adenylate cyclase inhibitor and PDE-7 inhibitor.

All the drugs were administered intraperitoneally 30 min prior to the electroshock. The electroshock was induced in animals by passing a current of 150 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).

Statistical analysis

All the results were expressed as mean ± SEM. One way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test was applied. 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 used. To compare with DMSO treated group the estimation of total duration of convulsion time in seconds and percentage of change from control were analysed in Table 1 and 2. P values <0.05 were considered as statistically significant.

Results

Evaluation of onset of seizures

A. Chemoshock method

Pentylenetetrazole (PTZ) or Metrazol (MTZ) induced seizure model in mice: Fig. 1, 2 and 3 summarizes the data obtained from experiments conducted with PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase activator and inhibitor on chemoshock such as PTZ (60 mg/kg, i.p) induced seizures in mice. The highlights of the findings are the data obtained with combination of AC inhibitor, SQ22536 and dipyridamole which showed a good reduction (P<0.001) in onset of action, jerky movements and convulsion against PTZ induced seizures in mice when compared to SQ22536 alone received group of animals (Fig. 1, 2 & 3). The combination of SQ22536 and PDE-7 inhibitor, BRL50481 received mice showed a significant (P<0.001) decrease in seizure activity when compared to SQ22536 and BRL50481 alone treated mice (Fig. 1, 2 & 3). The overall highlights of Fig. 1, 2 and 3 explicit the individual effect of AC inhibitor, SQ22536 which delays the onset of action of seizures as well as prolongs the total duration of convulsive time (Table 1).

Table 1 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 SQ22536 alone treated (60.2%) and combination of SQ22536 with BRL50481 treated (27.4%) groups compared to DMSO received group (100%). The data shows that 83.3% and 66.7% of protection of animals were noticed in SQ22536 and i.p injection of SQ22536 followed by dipyridamole

Table 1. Effect of drugs on pentylenetetrazole induced seizures in mice.

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	212.50	100	83.3	16.7	--
II	Gabapentin	275.00	29.4	33.3	66.7	*P*<0.01
III	SQ22536	340.05	60.2	16.7	83.3	*P*<0.01
IV	Dipyridamole	240.31	13.2	50.0	50.0	NS
V	BRL50481	210.40	0.9	66.7	33.3	NS
VI	SQ22536+Dipyridamole	260.18	22.5	33.3	66.7	*P*<0.05
VII	SQ22536 + BRL50481	270.42	27.4	50.0	50.0	*P*<0.01

The group of mice (n=6) were injected with 60 mg/kg, i.p. of PTZ 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.), SQ22536 (1 nmol/kg, i.p.), Dipyridamole (2 mg/kg, i.p.) and BRL50481 (2 mg/kg, i.p.). (One way ANOVA followed by Dunnett's test compared with DMSO treated mice)

Table 2. Effect of drugs on maximal electroshock induced seizures in rats.

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	236.50	100	100	--	--
II	Zonisamide	285.00	20.6	33.3	66.7	P<0.05
III	SQ22536	335.00	41.7	16.7	83.3	P<0.01
IV	Dipyridamole	260.81	10.3	33.3	66.7	NS
V	BRL50481	283.45	19.9	83.3	16.7	P<0.05
VI	SQ22536 + Dipyridamole	290.40	22.8	50.0	50.0	P<0.01
VII	SQ22536 + BRL50481	330.47	39.7	33.3	66.7	P<0.01

The group of rats (n=6) were subjected to 150 mA (0.2 sec) electroshock and 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 injected intraperitoneally. The drugs used were administered in the following doses. DMSO (3.5 ml/kg, i.p), zonisamide (35 mg/kg, i.p), SQ22536 (0.7 nmol/kg, i.p), Dipyridamole (1.4 mg/kg, i.p) and BRL50481 (1.4 mg/kg, i.p). (One way ANOVA followed by Dunnett's test compared with DMSO treated rats).

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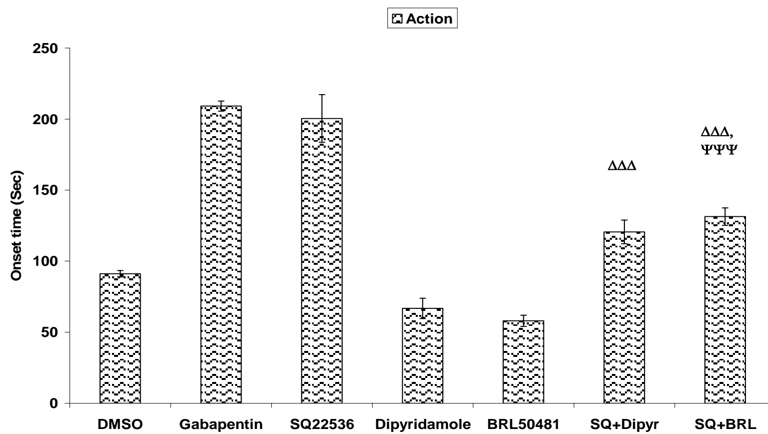
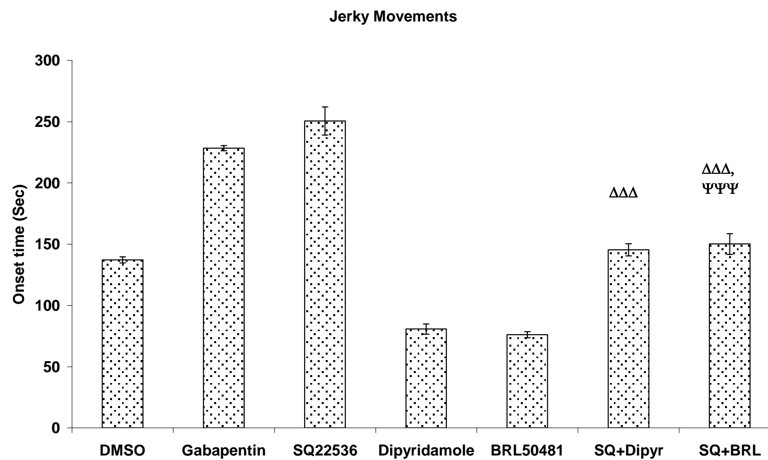
Fig. 1. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on chemoshock seizures in mice.



Data represented as mean ± SEM (n=6), which represents onset time of action phase of convulsion in seconds. Treatments were given 30 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p). ΔΔΔ Denotes p<0.001 compared with SQ22536 received group, ΨΨΨ denotes p<0.001 compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 2. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase Inhibitor on chemoshock seizures in mice.



Data represented as mean ± SEM (n=6), which represents onset time of jerky movement phase of convulsion in seconds. Treatments were given 30 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p). ΔΔΔ Denotes p<0.001 compared with SQ22536 received group, ΨΨΨ denotes p<0.001 compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

treated groups against PTZ induced seizures in mice. The results show that there was an increase in seizure activity (0.9%) in BRL50481 treated alone animals. Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown), Fig 1, 2 and 3 expresses the action of animals against PTZ induced seizures as follows, gabapentin treated group showed significant (P<0.001) reduction in onset of action and jerky movements of seizures, when compare to all groups except SQ22536 (NS). The data shown in Table 1 also demonstrates that i.p administration of SQ22536 (1 nmol/kg, i.p) greatly increased the anticonvulsant activity (P<0.01) along with higher protection (83.3%) range. Simultaneously, the combined effect of SQ22536 with exogenously administered BRL50481 (2 mg/kg, i.p) and SQ22536 with dipyridamole (2 mg/kg, i.p) showed a significant (P<0.01 & P<0.05) anti-convulsant activity with moderate protection (50% & 66.7%) range respectively (Table 1). A similar trend was noted in the results obtained from SQ22536 received groups explicit mild reduction (P<0.05) in convulsion compared to gabapentin. SQ22536, dipyridamole and BRL50481 treated groups showed a significant reduction (P<0.001) in jerky movements against DMSO received mice (data not shown).

Maximal electroshocks (MES) method for rats

Fig. 4, 5, 6 and 7 illustrate the data obtained from experiments conducted with maximal electroshock induced seizures in rats. It is evident from the data displayed in fig. 4, 5 and 6 that combination of AC inhibitor, SQ22536 and dipyridamole effectively (P<0.001) decreased the tonic limb flexion, tonic extensor and clonus stage of convulsion, compared to SQ22536 alone treated rats. The same significant level (P<0.001) was obtained in SQ22536 combined with BRL50481, instead of dipyridamole (Fig. 4, 5 & 6). The overall highlights of fig. 4, 5, 6 and 7 explicit the BRL50481 alone received group, potentiates the seizure activity against MES induced convulsion. Emphasis was also seen on the independent effect of AC inhibitor, SQ22536 in delaying the onset of seizure activity (Fig. 4, 5, 6 & 7) as well as prolonging the total duration of convulsive time (Table 2).

Table 2 demonstrated the total duration of convulsion, percentage change from control, mortality and protection in marked levels of percentage. The total convulsive time was long lasting significantly (P<0.01) in SQ22536 alone treated (41.7%) and combination of SQ22536 with BRL50481 treated group increase significantly (P<0.01) the duration of convulsion (39.7%), compared to DMSO received group (100%). The data showed that 83.3% and 66.7% of protection of animals were noticed in SQ22536 and i.p injection of SQ22536 followed by BRL50481 treated groups against MES induced seizures in rats. From Table 2 it was evident that there was a significant increase in seizure activity (10.3%) when dipyridamole treated alone. Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown) the action of animals against MES induced seizures. Gabapentin, SQ22536, SQ22536 with

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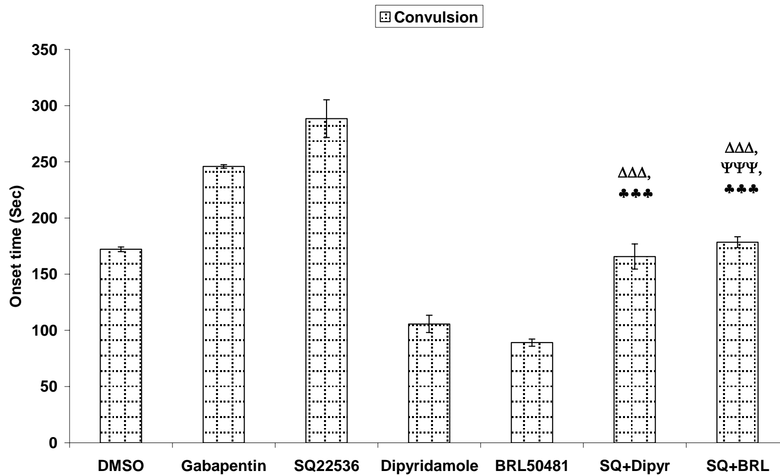
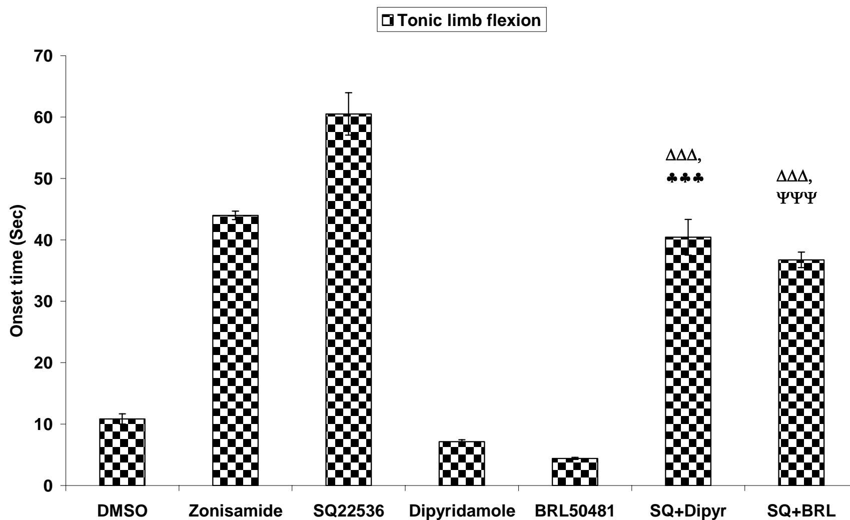
Fig. 3. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on chemoshock seizures in mice.



Data represented as mean ± SEM (n=6), which represents onset time of convulsion phase in seconds. Treatments were given 30 mins prior to chemical convulsant injection of PTZ (60 mg/kg, i.p.). ΔΔΔ Denotes p<0.001 compared with SQ22536 received group, ΨΨΨ denotes p<0.001 compared with BRL50481 received group, ♣ and ♣♣♣ denotes p<0.05 and p<0.001, respectively, compared with dipyridamole received group group and ns denotes non significant (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 4. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



Data represented as mean ± SEM (n=6), which represents onset time of tonic limb flexion phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). ΔΔΔ denotes p<0.001 compared with SQ22536 received group, ΨΨΨ denotes p<0.001 compared with BRL50481 received group, ♣♣♣ denotes p<0.001 compared with dipyridamole received group group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

dipyridamole, SQ22536 with BRL50481 treated groups showed significant (P<0.001) reduction in onset of tonic limb flexion phase of convulsion, when compare to DMSO. Simultaneously, the individual effect of SQ22536 and dipyridamole received groups showed a significant (P<0.001) reduction in tonic extensor phase of convulsion, against gabapentin treated group. Table 2 reveals that i.p administration of SQ22536 (0.7 nmol/kg, i.p) greatly enhances the anti-convulsant activity (P<0.01) along with higher protection (83.3%) range. At the same time, the combined effect of SQ22536 with exogenously administered BRL50481 (1.4 mg/kg, i.p) and SQ22536 with dipyridamole (1.4 mg/kg, i.p) showed a significant (P<0.01 and P<0.01) anti-convulsing activity with judicious protection (66.7% and 50%) range respectively (Table 2.).

Discussion

The data obtained from this study showed that pre-treatment with adenylate cyclase inhibitor, SQ22536 alone and along with the PDE-5/6/7/8/10/11 inhibitors such as dipyridamole and BRL 50481, potentiated the anticonvulsant activity against the PTZ and MES induced convulsions as depicted in Fig. 1-7. PDE-5/6/8/10/11 inhibitor, dipyridamole is an adenosine transport inhibitor, which acts mainly in two ways: (i) by increasing cyclic nucleotides as a result of the inhibition of phosphodiesterase (especially type 5, which is cGMP dependent) (Lugnier *et al.,* 1986) and (ii) by increasing extracellular levels of adenosine (Roos & Pleger, 1972) which leads to the activation of adenylate cyclase (Gresele *et al.,* 1986) to convert adenosine into cAMP. Secondly, it also inhibits cGMP-phosphodiesterase, increasing the amount of intracellular cGMP which may augment the downstream signalling effects of nitric oxide (NO), a vasodilator and inhibitor of platelet aggregation (Gamboa *et al.,* 2005; Liao, 2007). Dipyridamole also increases cAMP by inhibiting the cellular uptake of adenosine (Roos & Pleger, 1972). Our results support these findings in such a way that this combination showed a good reduction (P<0.001) in induction of seizure activity against PTZ and MES induced seizures in animals when compared to SQ22536 alone received group of animals (Fig. 1-7).

SQ22536 is a specific adenylate cyclase (AC) inhibitor (35) which was employed to inhibit the activity of AC. Recent study explains that SQ22536 abolished the elevation of cAMP (Gao & Usha Raj, 2001).

Our study also explains that the BRL50481 showed a quick onset of seizure responses with increase in the mortality range in both animal models of epilepsy and this shows the potential role of this agent for therapeutic purpose. Murray (1990) discovered that

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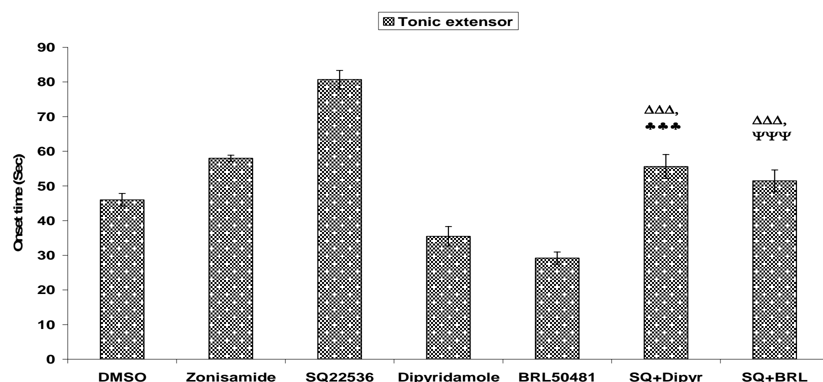
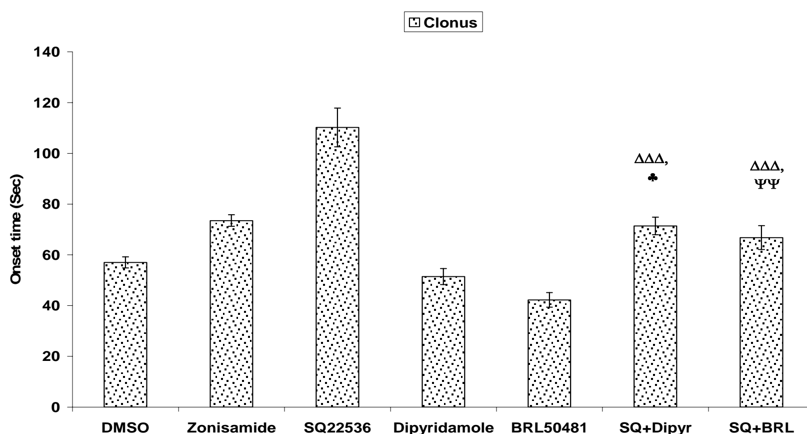
Fig. 5. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



Data represented as mean ± SEM (n=6), which represents onset time of tonic extensor phase of convulsion in seconds. Treatments were given 30 min prior to maximal electroshock (150 mA, 0.2 sec). ΔΔΔ Denotes p<0.001 compared with SQ22536 received group, ΨΨΨ denotes p<0.001 compared with BRL50481 received group, ♣♣♣ denotes p<0.001 compared with dipyridamole received group group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Fig. 6. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



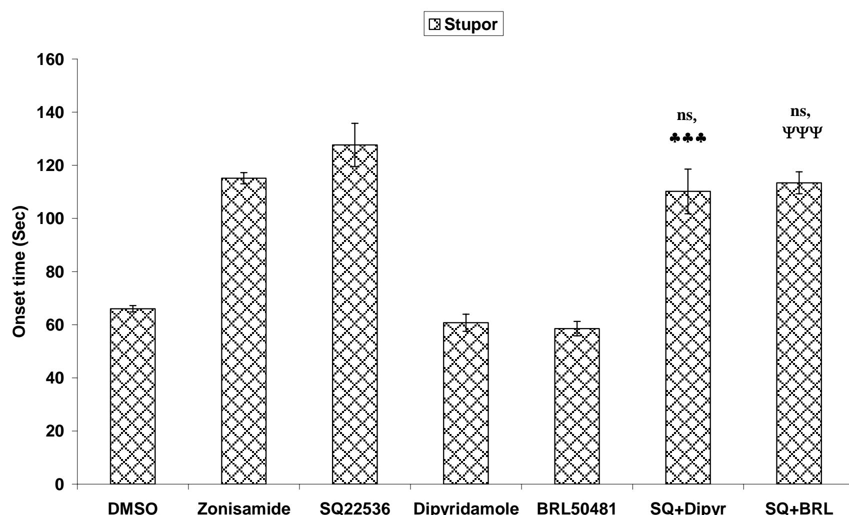
Data represented as mean ± SEM (n=6), which represents onset time of clonus phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). ΔΔΔ Denotes p<0.001 compared with SQ22536 received group, ΨΨ denotes p<0.01 compared with BRL50481 received group, ♣ denotes p<0.05 compared with dipyridamole received group group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

adenylate cyclase assay reveals the direct effect of AC activator providing the net effect of measurement of cAMP production by AC and cAMP degradation by PDEs (37). In mammalian cells, AC consists of at least 10 isoforms (Sunahara *et al.,* 1996) some isoforms are stimulated by Ca^{2+}-calmodulin and inhibited by calmodulin antagonists (Mons *et al.,* 1998). Since the decrease in cAMP was largely based on usage of SQ22536, acting predominantly by Ca^{2+}-calmodulin dependent (Sunahara *et al.,* 1996). Recent study shows that SQ22536 abolished the elevation of cAMP content by iloprost (a prostaglandin I_2 analog) in guinea-pig which supports our findings (Turcato & Clap, 1999).

BRL50481 is a selective inhibitor of PDE-7, a novel subtype of PDE that is expressed in a number of cell types, including T lymphocytes. There are at least two genes coding for PDE7, each with several splice variants (Adkinson, 2008). Two PDE7 genes (PDE7A & PDE7B) have been identified in humans (Gardner *et al.,* 2000; Hetman *et al.,* 2000). Li *et al.* (1999) suggested that PDE 7 may modulate human T-cell function. PDE7 is highly expressed in brain regions, including the hippocampus and olfactory bulb (Miro *et al.,* 2001; Irisarri *et al.,* 2005). The distribution of PDE 7A3 is largely unknown, but it has been found in human T-lymphocytes (Glavas *et al.,* 2001) and may also be present in many PDE7A1-expressing cells as both transcripts are probably regulated by the same promoter (Torras-Llort & Azorin, 2003). In contrast, PDE7B is abundant in the brain, liver, heart, thyroid glands, and skeletal muscles, but it is not found in leukocytes (Gardner *et al.,* 2000). Our study reports concurrence with combined effects of SQ22536 with exogenously administered BRL50481 (1.4 mg/kg, i.p) and SQ22536 with dipyridamole (1.4 mg/kg, i.p) showing a significant (P<0.01 and P<0.01) anticonvulsant activity with judicious protection (66.7% & 50%) range respectively against MES model as depicted in Table 2. Fig. 4, 5, 6 and 7 illustrates the PDE-7 inhibitor, BRL50481 showed a marked (P<0.01) decrease in onset of tonic extensor phase of convulsion in MES model of epilepsy. The total convulsive time was prolonged significantly (*P*<0.01) in SQ22536 alone treated (60.2%) and combination of SQ22536 with BRL50481 treated (27.4%) groups, compared to DMSO received group (100%) as in Table 1.

Thus, in conclusion the study reflects the individual effect of adenylate cyclase (AC) inhibitor, SQ22536 delay the onset of action of seizures as well as prolonging the total duration of convulsive time in both PTZ and MES models of epilepsy. The SQ22536 greatly increased the anti-convulsant activity along with higher percentage protection range of animals in both models of epilepsy. Further studies can be conducted using specific neuronal cell lines and elucidating the exact signal transduction mechanisms responsible for anti-convulsant effects.

Fig. 7. Effect of PDE-5/6/7/8/10/11 inhibitors along with adenylate cyclase inhibitor on maximal electroshock induced convulsions in rats.



Data represented as mean ± SEM (n=6), which represents onset time of stupor phase of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA, 0.2 sec). ΨΨΨ denotes $p<0.001$ compared with BRL50481 received group, ♣♣♣ denotes $p<0.001$ compared with dipyridamole received group group and ns denotes non significant (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

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Research Article

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Evaluation of Seizure Activity After Phospho-diesterase Inhibition (BRL 50481) with Guanylate Cyclase Activation (A-350619) and Inhibition (Methylene blue) in Animal Models of Epilepsy

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Abstract

The role of soluble guanylate cyclase (GC) activator (A-350619) and inhibitor (methylene blue) was evaluated in the presence of phosphodiesterase-7 (PDE-7) inhibitor such as BRL-50481, in animal models of epilepsy. Seizures were induced in the animals by subjecting them to an injection of chemical convulsant, pentylenetetrazole (PTZ) and maximal electroshock (MES). The study mainly comprises the onset of seizures, mortality/recovery, percentage of prevention of seizures (anti-convulsant) and total duration of convulsive time. The combination of GC inhibitor, methylene blue with BRL 50481 showed a delay onset ($P<0.001$) in incidence of seizures, compared to A-350619 and BRL 50481 alone treated group. The total convulsive time was prolonged significantly ($P<0.01$) in methylene blue alone treated (69.2%) groups, compared to DMSO received group (100%). The study also demonstrates that methylene blue alone and methylene blue with BRL 50481 greatly increased the anti-convulsant activity ($P<0.01$ and $P<0.05$) along with higher protection 83.3% and 66.7% range respectively in PTZ model. Methylene blue with BRL 50481 effectively ($P<0.01$) decreased the MES (150 mA, 0.2 sec) induced convulsion, compared to DMSO. The data shows that methylene blue alone, methylene blue with BRL 50481 greatly increased the anti-convulsant activity ($P<0.01$ and $P<0.01$) along with higher protection 83.3% range in animals treated with MES. The present result suggested of the possible involvement of methylene blue alone and with presence of BRL 50481, delays the onset of seizure activity as well as prolongs the total duration of convulsive time in both models.

Keywords: Guanylate cyclise; PDE; A-350619; Methylene blue; BRL 50481; Seizures

Introduction

Epilepsy is a serious neurological disorder that affects a wide range of people throughout the world. It is a disorder of brain characterized by unpredictable and periodic occurrence of a transient alteration of behaviour due to the disordered, synchronous and rhythmic firing of populations of brain 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]. It has been observed that the presently available anti-epileptic drugs are unable to control seizures effectively in as many as 25% of the patients [3]. The conventional anti-epileptic agents like phenytoin, carbamazeipine and sodium valporate carry with them several serious side effects notably neurotoxicity [4]. As majority of anti-epileptic 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 exist 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 [5-7]. The 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 cGMP 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 cyclic adenosine monophosphate (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

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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-5 & 6 belong to cGMP-specific [16-18], PDE-7 & 8 are cAMP-specific [19,20], PDE-10&11 related with cGMP-sensitive and dual specificity [21,22]. 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 [23].

Thus, it is necessary to investigate for an anti-epileptic agent that is highly efficacious as well as safe in terms of drug related toxicity. The aim of treating anti- 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 guanylate cyclase in the presence of cyclic nucleotide phosphodiesterase-7 inhibitor in the generation of seizure threshold. We used pharmacological tools like A-350619 (guanylate cyclase activator), methylene blue (guanylate cyclase inhibitor), and BRL 50481 (PDE-7 inhibitor) to block and attenuate the effect of PDE and evaluate the effect on chemical convulsant and maximal electroshock induced seizures in mice and rats.

Materials and Methods

Animals used

Swiss Albino mice of either sex weighing between 24-26 g and Wistar strain rats weighing between 160-220 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 ± 2C and relative humidity of 30-70%. A 12:12 dark: light cycle was followed during the experiments. All the animals were allowed to 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 *committee for the purpose of control and supervision of experiments in animals* (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), A-350619 (Sigma, USA), methylene blue (Sigma, USA), Zonisamide (Sun Pharma, Mumbai, India), BRL 50481 (Tocris Bioscience, UK), and Except gabapentin, methylene blue and zonisamide, other drugs are soluble in DMSO, rest of others are soluble in sterile water for injection.

A. Chemoshock method

Pentylenetetrazole (PTZ) or Metrazol (MTZ) 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 vehicle i.e 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 A-350619 (100 µM/kg, i.p), an guanylate cyclase activator.

Group - IV: Mice received methylene blue (50 mg/kg, i.p), an guanylate cyclase inhibitor.

Group -V: Mice received BRL 50481 (2 mg/kg, i.p), a PDE-7 nhibitor.

Group -VI: Mice received A-350619 (100 µM/kg, i.p) along with BRL 50481 (2 mg/kg, i.p), combination of guanylate cyclase activator and PDE-7 inhibitor.

Group -VII: Mice received methylene blue (50 mg/kg, i.p) along with BRL 50481 (2 mg/kg, i.p), combination of guanylate cyclase inhibitor and PDE-7 inhibitor.

All the drugs were administered intraperitoneally 30 min prior to the administration of pentylenetetrazole (60 mg/kg, i.p). The animals were observed for 1 hour by placing them 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 shown by Yemitan and Salahdeen, 2005; Salahdeen and Yemiten, 2006 method [24,25]. All the drugs used are known to cross the blood brain barrier.

B. Maximal electroshock (MES) method for rats

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

Group - I: Rats served as solvent control, received 10% w/v of dimethylsulfoxide [DMSO] (3.5 ml/kg, i.p).

Group - II: Rats received zonisamide (35 mg/kg, i.p), treated as positive control.

Group - III: Rats received A-350619 (70 µM/kg, i.p), an guanylate cyclase activator.

Group - IV: Rats received methylene blue (35 mg/kg, i.p), an guanylate cyclase inhibitor.

Group -V: Rats received BRL 50481 (1.4 mg/kg, i.p), a PDE-7 inhibitor.

Group -VI: Rats received A-350619 (70 µM/kg, i.p) along with BRL 50481 (1.4 mg/kg, i.p), combination of guanylate cyclase activator and PDE-7 inhibitor.

Group -VII: Rats received methylene blue (35 mg/kg, i.p), along with BRL 50481 (1.4 mg/kg, i.p), combination of guanylate cyclase inhibitor and PDE-7 inhibitor.

All the drugs will be administered intraperitoneally 30 min prior to the electroshock. The electroshock will be induced in animal by passing a current of 150 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 will be observed and tabulated as per Achliya et al., 2005 [26].

Statistical analysis

All the results 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 in Table 3 and 4. P values <0.05 were considered as statistically significant.

Results

Evaluation of onset of seizures

Chemoshock method

Pentylenetetrazole (PTZ) induced seizure model in mice: Table 1 summarizes the data attained from experiments conducted with PDE-

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7 inhibitor beside with guanylate cyclase activator and inhibitor on chemoshock such as PTZ (60 mg/kg, i.p.) induced seizures in mice**.** The combination of methylene blue and PDE-7 inhibitor, BRL 50481 received mice showed a significant (*P*<0.001) delay in onset of action, jerky movements and convulsion when compare to A-350619 and BRL 50481 alone treated mice.The overall highlights of Table 1 exhibits the individual effect of methylene blue delays the onset of action of seizures as well as prolongs the total duration of convulsive time.

Table 3 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 methylene blue alone treated (69.2%) group, compared to DMSO received group (100%). The data shows that 83.3% of protection in animals were noticed in methylene blue treated groups against PTZ induced seizures in mice. The result shows that there was an increase in seizure activity (8.0% and 3.2%) when A-350619 and BRL 50481 treated alone while compare to DMSO received group (100%). Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown), Table 1 expresses the action of animals against PTZ induced seizures as follows, gabapentin treated group showed significant (*P*<0.001) reduction in onset of action and jerky movements of seizures, when compare to all groups. The data shown in Table 3 also demonstrates that i.p administration of 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 methylene blue with exogenously administered BRL 50481 (2 mg/kg, i.p.) showed a significant (*P*<0.05) anti-convulsant activity with moderate protection (66.7%) range respectively [Table 3].

Maximal electroshock (MES) method for rats

Table 2 depicts the data obtained from experiments conducted with maximal electroshock induced seizures in rats**.** It is evident from the data shown in Table 2 that combination of methylene blue and BRL 50481 effectively (*P*<0.001) decreased the tonic limb flexion, tonic extensor, clonus and stupor stage of convulsion, compared to alone methylene blue treated rats. The overall highlights of Table 2 exhibits the combined effects of A-350619 with BRL 50481 received groups and BRL 50481 alone received group, potentiates the seizure activity against in MES induced convulsion. Also this emphasizes that methylene blue delays the onset of seizure activity as well as prolongs the total duration of convulsive time (Table 2).

Table 4 demonstrates the total duration of convulsion, percentage

Groups	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	11.26 ± 0.79	43.54 ± 1.27	54.28 ± 2.89	70.37 ± 3.29
II	Zonisamide (35 mg/kg, i.p) Positive Control	48.27 ± 0.85	62.89 ± 2.71	76.14 ± 4.15	120.13 ± 6.28
III	A 350619 (100 µM/ kg,i.p) sGC activator	11.18 ± 0.39	36.78 ± 1.93	45.29 ± 2.07	54.39 ± 2.39
IV	Methylene blue (50 mg/kg, i.p) sGC inhibitor	65.21 ± 2.14	85.13 ± 3.28	117.45 ± 5.37	134.26 ± 5.06
V	BRL 50481 (2 mg/kg, i.p) PDE-7 inhibitor	6.26 ± 0.67	28.78 ± 1.13	31.29 ± 2.15	42.09 ± 3.28
VI	A 350619 (100 µM/ kg,i.p) + BRL 50481 (2 mg/kg, i.p)	10.27 ± 0.79ΔΔ	21.78 ± 2.61 **, ΔΔΔ	28.69 ±3.37 ΔΔΔ	39.39 ± 3.81 ΔΔΔ
VII	Methylene blue (50 mg/kg, i.p) + BRL 50481 (2 mg/kg, i.p)	51.21 ± 2.12***, ΔΔ, ΨΨΨ	75.11 ± 3.28***, ΔΔΔ,ΨΨΨ	103.25 ± 5.89***,ΨΨΨ	121.26 ± 7.08***,ΨΨΨ

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA for 0.2 secs). *** denotes *p*<0.001, compared with A-350619 received group, Δ and ΔΔΔ denotes *p*<0.05 and *p*<0.001 compared with methylene blue received group, ΨΨΨ denotes *p*<0.001, compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 1: Effect of PDE 7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor on PTZ (60 mg/kg, i.p) induced seizures in mice (n=6).

Groups	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	11.26 ± 0.79	43.54 ± 1.27	54.28 ± 2.89	70.37 ± 3.29
II	Zonisamide (35 mg/kg, i.p) Positive Control	48.27 ± 0.85	62.89 ± 2.71	76.14 ± 4.15	120.13 ± 6.28
III	A 350619 (100 µM/ kg,i.p) sGC activator	11.18 ± 0.39	36.78 ± 1.93	45.29 ± 2.07	54.39 ± 2.39
IV	Methylene blue (50 mg/kg, i.p) sGC inhibitor	65.21 ± 2.14	85.13 ± 3.28	117.45 ± 5.37	134.26 ± 5.06
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VI	A 350619 (100 µM/ kg,i.p) + BRL 50481 (2 mg/kg, i.p)	10.27 ± 0.79ΔΔ	21.78 ± 2.61 **, ΔΔΔ	28.69 ±3.37 ΔΔΔ	39.39 ± 3.81 ΔΔΔ
VII	Methylene blue (50 mg/kg, i.p) + BRL 50481 (2 mg/kg, i.p)	51.21 ± 2.12***, ΔΔ, ΨΨΨ	75.11 ± 3.28***, ΔΔΔ,ΨΨΨ	103.25 ± 5.89***,ΨΨΨ	121.26 ± 7.08***,ΨΨΨ

Data represented as mean ± SEM (n=6), which represents onset time of various phases of convulsion in seconds. Treatments were given 30 mins prior to maximal electroshock (150 mA for 0.2 secs). *** denotes *p*<0.001, compared with A-350619 received group, Δ and ΔΔΔ denotes *p*<0.05 and *p*<0.001 compared with methylene blue received group, ΨΨΨ denotes *p*<0.001, compared with BRL50481 received group (One-way ANOVA followed by Tukey-Kramer multiple comparisons test).

Table 2: Effect of PDE-7 inhibitor along with soluble guanylate cyclase (sGC) activator and inhibitor on MES induced induced seizures in rats (n=6).

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	213.50	100	83.3	16.7	Nil
II	Gabapentin	279.00	30.7	33.3	66.7	*P*<0.05
III	A-350619	196.40	8.0	83.3	16.7	NS
IV	Methylene blue	360.06	69.2	16.7	83.3	*P*<0.01
V	BRL50481	220.43	3.2	66.7	33.3	NS
VI	A-350619 + BRL50481	230.31	8.2	83.3	16.7	NS
VII	Methylene blue + BRL50481	276.12	29.8	33.3	66.7	*P*<0.05

The group of mice (n=6) were injected with 60 mg/kg, i.p of PTZ 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), A-350619 (100 μM/kg, i.p), methylene blue (50 mg/kg, i.p) and BRL50481 (2 mg/kg, i.p). (One way ANOVA followed by Dunnett's test compared with DMSO treated mice)

Table 3: **Effect of drugs on pentylenetetrazole induced seizures in mice.**

Treatment groups	Drug name	Total duration of convulsion (Sec)	% change from control (Convulsive time)	Mortality (%)	Protection (%)	Significance
I	10% DMSO	242.60	100	100	-	-
II	Zonisamide	280.72	15.1	33.7	66.7	NS
III	A-350619	216.54	11.2	83.3	16.7	NS
IV	Methylene blue	343.67	40.9	16.7	83.3	*P*<0.01
V	BRL50481	278.18	14.1	83.3	16.7	NS
VI	A-350619 + BRL50481	235.67	3.40	50.0	50.0	NS
VII	Methylene blue + BRL50481	340.72	39.7	16.7	83.3	*P*<0.01

The group of rats (n=6) were subjected to 150 mA (0.2 sec) electroshock and 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 injected intraperitoneally. The drugs used were administered in the following doses. DMSO (3.5 ml/kg, i.p), zonisamide (35 mg/kg, i.p), A-350619 (100 μM/kg, i.p), methylene blue (50 mg/kg, i.p) and BRL50481 (1.4 mg/kg, i.p). (One way ANOVA followed by Dunnett's test compared with DMSO treated rats)

Table 4: Effect of drugs on maximal electroshock induced seizures in rats.

change from control, mortality and protection in marked levels of percentage. The total convulsive time was long lasting significantly (*P*<0.01) in methylene blue alone treated (40.9%) and combination of methylene blue with BRL 50481 treated groups increases significantly (*P*<0.01) the total duration of convulsion (39.7%), compared to DMSO received group (100%). The data shows that 83.3% of protections of animals were noticed in both methylene blue and i.p injection of methylene blue followed by BRL 50481 treated groups against MES induced seizures in rats. From Table 4, it was evident that there was a significant increase in seizure activity (14.1%) when BRL 50481 treated alone. Apart from these highlighted points, the author would like to discuss few things from the data obtained (data not shown), the action of animals against MES induced seizures. Methylene blue, methylene blue with BRL 50481 treated groups showed significant (*P*<0.01) reduction in onset of various phases of convulsion, when compare to DMSO. Simultaneously, A-350619, methylene blue, A-350619 with BRL 50481 received groups showed a siginificant (*P*<0.001) reduction in tonic extensor phase of convulsion, against zonisamide treated group. The data shown in Table 4, exposed that i.p administration of methylene blue (35 mg/kg, i.p), greatly enhances the anti-convulsant activity (*P*<0.01) along with higher protection (83.3%) range. At the same time, the combined effect of methylene blue with exogenously administered BRL 50481 (1.4 mg/kg, i.p.) showed a significant (*P*<0.01) anti-convulsant activity with judicious protection (83.3%) range in both groups (Table 4).

Discussion

The data obtained from this study shown that pre-treatment with soluble guanylate cyclase inhibitor, methylene blue alone and with the PDE-7 inhibitor such as BRL 50481, potentiates the anti-convulsant activity against the PTZ and MES induced convulsions as described in Table 1 & 2. And also our study shows that the combination of A-350619 with BRL50481 as well as the individual effect of A-350619 and BRL 50481 alone showed a quick onset of seizures responses with increased the mortality range in both animal models of epilepsy. Methylene blue, is a guanylate cyclase inhibitor and belongs to thaizine dye [27]. Pretreatment with either methylene blue or L-Nitro-Arginine Methyl Ester (L-NAME) inhibited the proconvulsant effect of sildenafil, indicated the mediation of this effect by NO–cGMP pathway [28]. Nitric oxide (NO) is a highly reactive and unstable free radical, which diffuses easily through the cell membrane [29,30]. It contributes to intercellular signal transduction in many tissues. In the central nervous system, it acts as a neuronal retrograde messenger [29,31]. NO is an endogeneous activator of guanylate cyclase, which synthesizes cGMP [31,32]. It activates guanylate cyclase by binding to the iron of the heme, which is located at the active site of the enzyme and by changing its conformation [33]. In the CNS, NO is formed from L-arginine, by calcium/calmodulin- dependent constitutive NO synthase, which is mainly activated by the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors [31,33]. Paul et al., reported that, ion channels mediate and regulate crucial electrical functions throughout the body. They are therapeutic drug targets for a variety of disorders [34]. In living tissues, extracellular calcium is essential for the secretion of NO from NMDA-stimulated neurons [31]. NMDA receptor activation causes an influx of a large amount of calcium into the cell through receptor associated ion channels; calcium binds to calmodulin and activates NO synthase [35,36]. This mechanism might be the reason for the protective role and anti-convulsant activity of methylene blue. Our results support this findings in such a way that this combination showed a good reduction (*P*<0.001) in induction of seizure activity against PTZ and MES induced seizures in animals when compare to methylene blue alone received group of animals (Table 1&2).

BRL50481 is a selective inhibitor of PDE-7, a novel subtype of PDE that is expressed in a number of cell types, including T lymphocytes. There are at least two genes coding for PDE7, each with several splice variants [37]. Two PDE7 genes (PDE7A and PDE7B) have been identified in humans [38,39]. Li et al. 1999 suggest that PDE 7 may modulate human T-cell function [40]. PDE7 is highly expressed in brain regions, including the hippocampus and olfactory bulb [41,42].

The distribution of PDE7A3 is largely unknown, but it has been found in human T-lymphocytes [43] and may also be present in many PDE7A1-expressing cells as both transcripts are probably regulated by the same promoter [44]. In contrast, PDE7B is abundant in the brain, liver, heart, thyroid glands, and skeletal muscles, but it is not found in leukocytes [45]. Our study reports concurrence with combined effect of methylene blue with exogenously administered BRL50481 (1.4 mg/kg, i.p) showed a significant (P<0.01) anti-convulsant activity with judicious protection (83.3%) range respectively against MES model as depicted in Table 4. The total convulsive time was prolonged significantly (P<0.01) in methylene blue alone treated (69.2%) and combination of methylene blue with BRL50481 treated (29.8%) groups, compared to DMSO received group (100%) as illustrated in Table 3.

A-350619, a heme-dependent soluble guanylate cyclase activator, sGC is a key signal transduction enzyme activated by nitric oxide (NO). Impaired bioavailability and/or responsiveness to endogenous NO have been implicated in the pathogenesis of cardiovascular and other diseases [46]. In mature brain cGMP acts mainly in cortex, caudate-putamen, cerebellum and hippocampus. In immature brain cGMP is involved in guiding neurons to achieve their destination and it plays important role in myelinogenesis [47]. Ishikawa et al., showed that, two major pathways have been reported by which a cGMP increases intracellular Ca^{2+} concentration $[Ca^{2+}]_i$. cGMP can activate Ca^{2+} influx, by a process involving PKG regulation of ion channels. It can also activate Ca^{2+} release from ryanodine-sensitive intracellular stores by a pathway involving PKG and cyclic ADP-ribose. The cholinergic and β-adrenergic receptors stimulated production of NO that activates a rise in cGMP concentration. Activation of phospholipase C (PLC) leads to inositol (1,4,5) triphosphate (IP_3) production resulting in Ca^{2+} release from endoplasmic reticulum and to NOS activation. This data supported the view that NO/cGMP signal transduction has a crucial role in Ca^{2+} homeostasis [48]. This increase the level of cGMP and the regulation of Ca^{2+} homeostasis provocates seizures in our animal models of epilepsy. From Table 3. it was illustrated, there was a increase in seizure activity (8% and 3.2%) when A-350619 and BRL50481 treated alone against PTZ model.

Thus, in conclusion the study reflects, (i) the individual effect of guanylate cyclase (GC) inhibitor, methylene blue delays the onset of action of seizures as well as prolongs the total duration of convulsive time in both PTZ and MES models of epilepsy. The methylene blue greatly increased the anti-convulsant activity along with higher percentage protection of animals in both models of epilepsy; (ii) onset of action and the incidence of seizures are quick in sGC activator, A-350619 alone as well as combination with PDE-7 inhibitor, BRL-50481 treated groups; (iii) the occurrence of high percentage of mortality is associated with quick onset of seizures resembles pro-convulsant action in A-350619 alone as well as combination with BRL-50481 treated groups in both animal models of epilepsy; (iv) This study also reflects that the identification and investigation of new cGMP mediated phosphodiesterases family members offers a new strategy for the novel therapy of epilepsy in future.

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