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, carbamazepine and sodium valporate carry with them several serious side effects notably neurotoxicity [4]. As majority of anti-epileptic drugs are consumed lifelong, 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...
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 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 ± 2°C 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 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 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 (Sigma, USA), Zonisamide (Sun Pharma, Mumbai, India), BRL 50481 (Tocris Bioscience, UK), and Except gabapentin, methylene blue 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:** Swiss Albino mice were divided into seven groups with six animals in each. Treatment protocol and group description is mentioned as follows.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Mice served as solvent control, received vehicle i.e 10% w/v of dimethylsulfoxide (DMSO) (5 ml/kg, i.p.)</td>
</tr>
<tr>
<td>II</td>
<td>Mice received gabapentin (2.5 mg/kg, i.p), treated as positive control.</td>
</tr>
<tr>
<td>III</td>
<td>Mice received A-350619 (100 µM/kg, i.p), an guanylate cyclase activator.</td>
</tr>
<tr>
<td>IV</td>
<td>Mice received methylene blue (50 mg/kg, i.p), an guanylate cyclase inhibitor.</td>
</tr>
<tr>
<td>V</td>
<td>Mice received BRL 50481 (2 mg/kg, i.p), a PDE-7 inhibitor.</td>
</tr>
</tbody>
</table>

**B. Maximal electroshock (MES) method for rats**

Wistar strain rats were divided into seven groups with six animals in each. Treatment protocol and group description is mentioned as follows.

<table>
<thead>
<tr>
<th>Group</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rats served as solvent control, received 10% w/v of dimethylsulfoxide (DMSO) (3.5 ml/kg, i.p.)</td>
</tr>
<tr>
<td>II</td>
<td>Rats received zonisamide (35 mg/kg, i.p), treated as positive control.</td>
</tr>
<tr>
<td>III</td>
<td>Rats received A-350619 (70 µM/kg, i.p), an guanylate cyclase activator.</td>
</tr>
<tr>
<td>IV</td>
<td>Rats received methylene blue (35 mg/kg, i.p), an guanylate cyclase inhibitor.</td>
</tr>
<tr>
<td>V</td>
<td>Rats received BRL 50481 (1.4 mg/kg, i.p), a PDE-7 inhibitor.</td>
</tr>
<tr>
<td>VI</td>
<td>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.</td>
</tr>
<tr>
<td>VII</td>
<td>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.</td>
</tr>
</tbody>
</table>

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-
7 inhibitor beside with guanylate cyclase activator and inhibitor on chemonock 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 (<0.01) in methylene blue alone treated (69.2%) group, compared to DMSO received group (100%). The data shows that 83.3% of protection (<0.01) in methylene blue alone treated (69.2%) group, compared 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 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 (<0.01) in methylene blue alone treated (69.2%) group, compared 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].
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 significantly (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 significant (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 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 thiazine 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 endogenous 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].

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

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

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

<table>
<thead>
<tr>
<th>Treatment groups</th>
<th>Drug name</th>
<th>Total duration of convulsion (Sec)</th>
<th>% change from control (Convulsive time)</th>
<th>Mortality (%)</th>
<th>Protection (%)</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10% DMSO</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>-</td>
<td>NS</td>
</tr>
<tr>
<td>II</td>
<td>Zonisamide</td>
<td>15.1</td>
<td>33.7</td>
<td>66.7</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>A-350619</td>
<td>11.2</td>
<td>83.3</td>
<td>16.7</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Methylene blue</td>
<td>40.9</td>
<td>P&lt;0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>BRL50481</td>
<td>14.1</td>
<td>83.3</td>
<td>16.7</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>A-350619 + BRL50481</td>
<td>3.40</td>
<td>50.0</td>
<td>50.0</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>Methylene blue + BRL50481</td>
<td>39.7</td>
<td>16.7</td>
<td>83.3</td>
<td>P&lt;0.01</td>
<td></td>
</tr>
</tbody>
</table>
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 myelogenesis [47]. Ishikawa et al., showed that, two major pathways have been reported by which a cGMP increases intracellular Ca2+ concentration [Ca2+]i. cGMP can activate Ca2+ influx, by a process involving PKG regulation of ion channels. It can also activate Ca2+ release from ryanodine-sensitive intracellular stores by a pathway involving PKG and cyclic ADF-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 (IP3) production resulting in Ca2+ release from endoplasmic reticulum and to NOS activation. This data supported the view that NO/cGMP signal transduction has a crucial role in Ca2+ homeostasis [48]. This increase the level of cGMP and the regulation of Ca2+ homeostasis provokes 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.

References


