

***In vivo* anticonvulsant activity of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene in pilocarpine and strychnine induced-seizure models**

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Abstract: An imbalance between inhibitory (GABA) and excitatory (Glutamate) neurotransmission contribute to the development of epilepsy. Earlier studies reported that dysregulation of GABA and glutamatergic activities resulted in status epilepticus (SE) and ultimately support the development of temporal lobe epilepsy (TLE), a type of resistant epilepsy. In the earlier work, 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene demonstrated anticonvulsant activity against pentylenetetrazole (PTZ)-induced seizures. Apart from the PTZ-induced TLE, the dysregulation muscarinic receptors and glycine receptors are also widely reported phenomena in the development of temporal lobe epilepsy. Keeping the role of these two receptors in epilepsy, the present work investigated the effect of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene in pilocarpine-induced and strychnine-induced seizure models. Our results demonstrated that 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene significantly delayed the onset of seizure with maximum protection from SE in pilocarpine-induced seizure model. However, the test compound did not revealed any effect on strychnine-induced seizures in mice. Based on these observations, we suggest that 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene could be a potential candidate in reduction of SE and treatment of temporal lobe epilepsy (TLE) in future.

Keywords: Pilocarpine, Strychnine, epilepsy, status epilepticus, temporal lobe epilepsy.

INTRODUCTION

Epilepsy, from the time of its origin to the recent advancement, still a debatable and major concern of the world. It is categorized as most prevalent chronic neurological disorder that affects nearly 80 million of the worldwide population including both developing and underdeveloped countries (De Boer *et al.*, 2008; Sahu *et al.*, 2012; Sirven 2015). In comparison to other neurological disorders such as multiple sclerosis, stroke and cerebral palsy, its prevalence is high and it ranked as 3rd most prevalent after Alzheimer's disease. In Pakistan, about 1.38 million people was recorded to have epilepsy in 2005 (Khatri *et al.*, 2003), out of which there is about 10 epileptic patients in every 1000 sample (Sheerani, 2005), and is prevailed more in rural than in urban areas.

Temporal lobe epilepsy (TLE), also known as resistant epilepsy, is a kind of complex partial seizures initiated by status epilepticus and approximately 60% of all epileptic patients are suffering from TLE (French *et al.*, 1993; Helmstaedter *et al.*, 2016; Langa *et al.*, 2018). Status epilepticus is an emergency medical condition characterized by continuous seizures, lasts for approximate five to six minutes without intervals (Nair *et*

al., 2011) or with loss of consciousness accompanied by intermittent seizures. Earlier studies reported that people living with epilepsy usually die (Sperling *et al.*, 2005) and these kinds of unexplained deaths are more common in the young individuals residing with refractory epilepsy (Ryvlin *et al.*, 2006).

Typical features of TLE can be mimicked in chronic animal models such as kindling or SE, which closely resembles with the characteristics discussed above (Morimoto *et al.*, 2004; Redy *et al.*, 2018; Crans *et al.*, 2019). Strychnine develops seizures by antagonizing receptors that facilitates the inhibition of the neurotransmitter glycine (Loscher and Schmidt, 1993, 1994; McCracken *et al.*, 2017). Pilocarpine (PLP) induced SE seems to be highly homologous with the human diseases (Turski *et al.*, 1983 a,b). Activated M1 muscarinic receptors subtypes are responsible for PLP-induced SE. Subsequently, studies proved that the M1 receptor knockout mice were not developing seizures in response to PLP (Hamilton *et al.*, 1997). Furthermore, in vitro studies done on hippocampal neurons determined that PLP though acting through muscarinic receptors, creates disproportion among the excitatory and inhibitory neuronal transmission, thus results in the development of SE (Priel and Albuquerque, 2002). While *in vivo* studies

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support the PLP-induced rise in glutamate levels which is followed by seizure generation (Smolders *et al.*, 1997).

There are many treatment options for SE, such as gamma knife radiosurgery, ketogenic diet, vagus nerve stimulation (VNS) and other complementary therapies along with conventional antiepileptic drugs (AEDs) (Connor, 2004; Tolman and Faulkner, 2009; Coppola *et al.*, 2010; Bao *et al.*, 2011; Glauser *et al.*, 2013). The currently available AEDs are associated with multiple side effects and only provide symptomatic relief in minimum subset of population. It has been reported that more than 30% of epileptic patients living with inadequate seizure control, while others having adequate control struggling with bothersome adverse side effects (McCabe, 2000). On the other hand, AEDs only reduces the spreading of seizure, they are unable to reduce the process of epileptogenesis that goes against their use (Schachter, 1999; Schachter, 2002; Klitgaard and Pitkänen, 2003). Keeping these in mind, it is necessary to search for new potential drugs, as 80% of the patients with epilepsy completely trust on the AEDs for relief on medication and they are unable to bear the high cost (Uijl *et al.*, 2005). Another important reason that highlights the new invention of AEDs is totally based on the rationale that for any disease the development of a new entity should have better and improved efficacy and also it should be devoid of any potential adverse effect (Chadwick, 1997; Gilliam, 2005). In our earlier studies, we have already established the anticonvulsant activity of isomeric compounds [E/Z]-2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene fig. 1) isolated from aqueous fraction of *Delphinium denudatum* roots both in acute and kindling model of scPTZ-induced seizures in mice (Simjee *et al.*, 2010; Simjee, *et al.*, 2012a, 2012b; Ashraf *et al.*, 2013). In the present study, we carried out anticonvulsant activity of the test compound against other chemical convulsants such as pilocarpine (PLP) and strychnine (STN) to explore its action on glycine and muscarinic receptors participating in epilepsy.

MATERIALS AND METHODS

The NMRI (Naval Medical Research Institute, Sweden) male mice of (20-25g) were used in this study. The animals were housed in the animal housing facility of International Center for Chemical and Biological Sciences, University of Karachi and provided with appropriate conditions i.e., 12hr. light and dark cycle with optimum temperature at $21 \pm 1^\circ\text{C}$. All the experiments were performed according to International guidelines and Institutional Animal Care and Use Committee (IACUC) under the protocol number 2015-0007, assigned by Advisory Committee on Animal Standards, of ICCBS.

Pilocarpine (PLP) and Strychnine (STN) were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Diazepam (DZP) was a gift from Roche Pharmaceuticals (Roche Pakistan Ltd. Pakistan). The test compound 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene) was provided by our chemist collaborators working at ICCBS, University of Karachi, Pakistan.

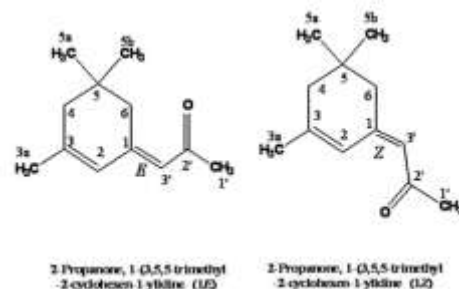


Fig.1: Structure of the isomeric mixture of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene.

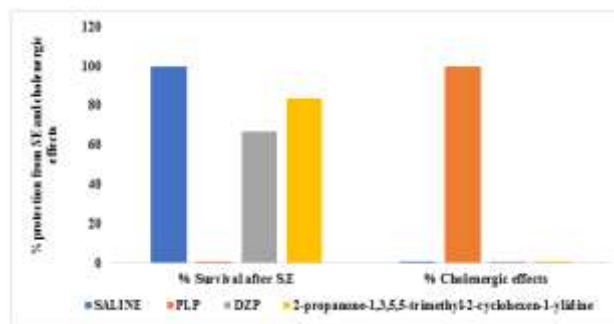


Fig 2: % protection from SE and % cholinergic effects of the mice in Pilocarpine-induced seizure test (PLP 10 mg/kg, i.p.). Each bar represents Mean \pm SD of 6 animals per group.

Anticonvulsant screening

In order to perform the acute seizure test, 30mg/kg dose of the test compound 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene was administered intraperitoneally at least 35 minutes prior to the administration of chemoconvulsant (PLP and strychnine).

Intraperitoneal PLP- and STN-induced seizure tests

The animals for both PLP- induced and STN-induced acute seizure tests were divided into four groups (table 1) with each group having six mice. In both test, PLP (400 mg/kg) and STN (2mg/kg) was injected in their respective groups, 35 minutes after the administration of test compound and animals were observed for a period of sixty minutes. In pilocarpine induced seizure test, animals were treated with 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene (30mg/kg, i.p.) before administration of PLP (400mg/kg, i.p.). Animals were placed in separate cages for an observational period of 60 minutes. Since pilocarpine is a cholinomimetic drug, it also manifest the peripheral cholinergic effects that include miosis, piloerection and urination etc), stereotyped movements (rearing, repetitive sniffing and paw licking), seizures, initiation of status epilepticus, and

lethality for 24 hours. We observed latency to first episode of seizure, Latency to status epilepticus and mortality protection in the animals and also behavioural changes in mice after pilocarpine injection, as earlier studies reported the occurrence of seizures and deaths up to 24 hours after pilocarpine administration (Santos *et al.*, 2008; Lopim *et al.*, 2016). In STN-induced test, seizures were induced by using 2mg/kg of strychnine, administered by i.p. route. The extension in the hind limb was observed in the animals of each group for about 45-60 minutes following administration of convulsants.

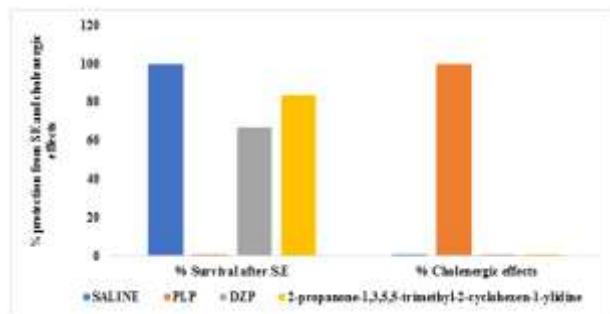


Fig 3: % protection from SE and % cholinergic effects of the mice in Pilocarpine-induced seizure test (PLP 10 mg/kg, i.p.). Each bar represents Mean \pm SD of 6 animals per group.

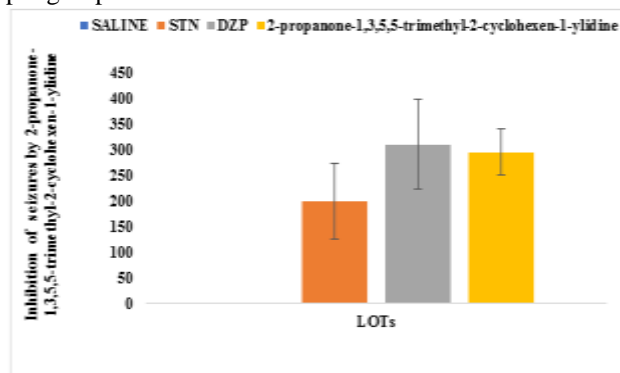


Fig. 4: Effects of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene on onset of Acute seizure in Strychnine-induced seizure test (PLP 10mg/kg, i.p.). Each bar represents Mean \pm SD of 6 animals per group.

Toxicity profile

Acute neurotoxicity

The neurotoxic manifestation of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene was determined by inverted screen acute neurotoxicity test developed by Coughenour *et al.* 1977. The apparatus used for the testing consisted of six 13cm square platforms of wire mesh supported by metal bars. The rod was supported at both ends and was inverted through an arc of 180°. Before testing, the mice were pretested on the apparatus and those failing the task were not included in drug testing. Test was carried out at 5, 30, 60 and 120 minutes following i.p. administration of 20mg/kg, 30mg/kg, 50 mg/kg and 100mg/kg of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene (6 mice/group). Animals unable to

climb to an upright position for 1min duration were rated as failures.

Behavioural assessment

For behavioural analysis, modified procedure as described by Turner, 1972 was adopted. The effects were recorded using a scoring system (scores were allocated according to the intensity of the symptoms from 0-4) as described by Turner i.e. for stereotype behavior 0: No effect; 1: intermittent; 2: continues 3: intense; 4: severe and for spontaneous activity 0: reduced activity and 4: increased activity. Animals were transferred into individual cages to allow them to acclimatize to the new environment prior to the experiment. Animals were observed in these cages for 1-2 hr after 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene treatment for the signs of behaviours including ataxia, biting/ licking or grooming, hyper-locomotion, head weaving, hyper-excitability, and sedation. In order to avoid any biased interpretations, we made sure to employ the blind-testing i.e., the experimenter conducting this study was blinded to the treatment given.

Muscle relaxant activity

This was examined by traction test. Forepaws of the mouse were placed on a small twisted wire rigidly supported above a bench top. Normal mice grasped the wire with forepaws and when allowed to hang free, placed at least one hind foot on wire within 5 seconds. The inability to put at least one hind foot on the wire was considered failure to the test. The test was conducted at 30 minutes and 1 hour after administration of diazepam and 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene.

Gross effects

Following the administration of the test compound, the animals were observed continuously for 2h in order to check the effect (if any) on the gross behaviour. The final observations were made after 24h. The CNS stimulation was judged by the signs and symptoms including ataxia, catalepsy, crouching clonic and tonic convulsions, lacrimation, locomotor activity, salivation or any other signs which deviate from the normal behavior of the animal under observation, and sedation.

STATISTICAL ANALYSIS

The data was analyzed statistically using Statistical Package for the Social Sciences (SPSS) (version 20) with significant level set at $P < 0.05$. Values are represented as mean \pm SEM. Data is analyzed statistically using one-way ANOVA with Tukey's post hoc test.

RESULTS

Anti-convulsant activity of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene against PLP and STN

Animals in the pilocarpine treated group exhibited seizures between 6-8 minutes from the time of

Table 1: Experimental groups in pilocarpine and strychnine-induced seizures test

Groups	Treatment	Dose	Route of Administration
Group I	Normal saline	0.5 mg/kg	i.p.
Group II	Pilocarpine	400 mg/kg	i.p.
Group III	2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene /PLP	30 mg/kg	i.p.
Group IV	Diazepam/PLP	7.5 mg/kg	i.p.
Group V	Strychnine	2 mg/kg	i.p.
Group VI	Diazepam/STN	7.5 mg/kg	i.p.
Group VII	2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene /STN	30 mg/kg	i.p.

Where i.p. represents intraperitoneal route of administration

Table 2: Anticonvulsant effect of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene in pilocarpine and strychnine-induced seizures

Group (s)	Treatment	Dose	Latency to First Seizure (sec)	% Stereotype Behaviour	% Seizure	Latency to SE (sec)	% Survival Rate
Group I	SALINE	0.5ml/kg	0	0	0	0	100
Group II	PLP	400mg/kg	438.33±15***	100	0	826.66±26***	0
Group III	DIAZEPAM/PLP	7.5mg/kg	948.33±34***	50	16.67	1610±18***	83.33
Group IV	2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene /PLP	30mg/kg	880 ±17***	50	33.33	1813.33±38***	83.33
Group V	STN	2mg /kg	262.5±35	-	100	-	0
Group VI	DIAZEPAM/STN	7.5mg/kg	333.75±30	-	100	-	33.33
Group VII	2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene /STN	30mg/kg	295±17	-	100	-	16.66

Where PLP and STN refers pilocarpine and strychnine respectively. Effect of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene on the onset of seizures and development of status epilepticus (induced by pilocarpine) expressed as mean ± S.E.M. of the number of experiments shown in the table. The strychnine group was compared with normal control and the drug and test compound treated groups were compared with strychnine group. The *** represents the significance level of $P < 0.001$.

administration of PLP (400mg/kg i.p.). After half an hour from seizure onset all the animals developed extensive limbic seizures (status epilepticus) and ultimately died. Pre-treatment with 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene (30mg/kg) in pilocarpine-induced seizures, significantly increased ($***P < 0.001$) the duration of onset of seizure (880 sec/15 minutes) as compared to PLP-treated animals and also able to prolong the duration of HLTE (fig. 2, table 2). Only 33% of the animals in the 2-propanone-1, 3, 5, 5-trimethyl-2-cyclohexen-1-ylidene treated group exhibited seizures as compared to PLP-controlled animals and are comparable to diazepam. After administration of PLP in the 2-propanone-1, 3, 5, 5-trimethyl-2-cyclohexen-1-ylidene treated animals, some animals briefly exhibited limbic seizures and 83.33% of the animals in the treated group survived up to 24 hours notably than pilocarpine injected animal and this survival rate is also comparable to standard treatment (table 2). None of the diazepam or 2-propanone-1, 3, 5, 5-trimethyl-2-cyclohexen-1-ylidene treated animals demonstrated the peripheral cholinergic signs (fig. 3) produced by the pilocarpine treated mice.

Further, minimization of stereotype movements in 50% of the 2-propanone-1, 3, 5, 5-trimethyl-2-cyclohexen-1-ylidene and DZP- treated groups was observed (table 2).

Unlike pilocarpine, 2-propanone-1, 3, 5, 5-trimethyl-2-cyclohexen-1-ylidene was unable to exhibit any anticonvulsant activity against strychnine induced seizures. Furthermore, the test dose was also failed to provide protection against strychnine-induced mortality (fig. 4, table 2).

DISCUSSION

The inhibition of GABAergic and hyper-excitation of glutamatergic transmissions constitute the underlying factors in the epilepsy (Westmoreland *et al.*, 1994). In addition to these major neurotransmitters involved in epilepsy, studies have reported that increased cholinergic neurotransmission because of cholinergic hyper activation can also initiate seizures. It is also evident that SE results from an increase in glutamatergic activity (excitatory pathway) and reduction in inhibitory transmission

(GABA), rendering as one of the proposed underlying pathophysiology of SE (Westmoreland *et al.*, 1994). In our earlier studies, we have investigated the role of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene in inhibiting PTZ-induced seizures (Simjee *et al.*, 2010; Simjee, *et al.*, 2012a, 2012b; Ashraf *et al.*, 2013). Keeping in mind the role of muscarinic and glycine receptors in epileptic seizures, the present study has explored the activity of the 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene in pilocarpine and strychnine-induced seizures in mice.

Pilocarpine is a nonspecific muscarinic receptor agonist and it was reported in previous studies that some cerebral structures have high density of muscarinic type receptors that are responsible for pilocarpine induced seizures (Clifford *et al.*, 1987). The glutamate levels were also found to be upregulated in hippocampus after seizure induction by pilocarpine (Smolders *et al.*, 1997). When pilocarpine-induced SE is not disturbed by administration of drugs, it produces pronounce anatomical damage and reorganization that is similarly seen in human TLE cases (Curia *et al.*, 2008). Furthermore, intraperitoneal administration of high dose (400mg/kg) of pilocarpine hydrochloride in rodents exhibited continuous limbic seizures and status epilepticus (Quintans *et al.*, 2008). We have observed that 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene not only reduced the first latency to seizures but also delayed the SE onset and decreased its duration. These results point our attention towards the potential of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene acting on muscarinic receptors. Status epilepticus (SE) characterized as a state of prolonged and continuous convulsion or continuous seizure activity last for about 30-60 minutes (DeLorenzo *et al.*, 1983) and can lead to brain death (Lothman 1990; DeLorenzo *et al.*, 1992; Knake *et al.*, 2009; Hocker *et al.*, 2016; Danzer 2019). 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene delayed the initiation and reduced the frequency of SE which proves that it also have neuroprotective effects. In the CNS, there is another inhibitory pathway i.e. glycine neurotransmission pathway. Keeping the role of glycine as inhibitory neurotransmitter, we decided to evaluate the effect of 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene on strychnine-induced seizure which blocks the glycine transmission (Larson, 1969). It was clearly observed that 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene was unable to protect animals against STN-induced seizures, this suggest that, 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene does not act on glycine receptor.

CONCLUSION

Based on current observations, it can be speculated that, 2-propanone-1,3,5,5-trimethyl-2-cyclohexen-1-ylidene has a potential to halt the seizures induced by PLP. Moreover, it has ability to reduce the SE that justify its use in TLE

and this action might be attributed partially by blockade of central cholinergic receptors or excitatory pathway, further elucidation is still required.

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