Design, synthesis, and pharmacological evaluation of aryl oxadiazole linked 1,2,4-triazine derivatives as anticonvulsant agents

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Abstract

A series of new clubbed aryl oxadiazole-1,2,4-triazine derivatives **(6a-l)** were designed and synthesized using appropriate chemical routes. The structures were designed having the required structural elements for any compounds to be potential anticonvulsant. Preliminary (Phase I) screening of anticonvulsant activity was performed by means of maximal electroshock seizure (MES), subcutaneous pentylenetetrazole-induced seizure (scPTZ) and behavioral activity was assessed by motor impairment test and actophotometer test. The derivatives 6-((5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione **(6f)** and 6-((5-(4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione **(6g)** revealed significant activity against both MES and scPTZ indicating that the compounds are effective against both generalized tonic-clonic and absence seizure. The lead compound (**6g)** was further evaluated for quantitative (Phase II) evaluation and emerged as most effective anticonvulsant with median effective dose of 28.5 mg/kg (MES ED_{50}), 76.6 mg/kg (scPTZ) and toxic dose (TD_{50}) was found to be > 500 mg/kg. In the GABA estimation study results showed significantly increased GABA concentration.

1. Introduction

Epilepsy is a complex disorder of CNS characterized by spontaneous and recurrent seizures. An epileptic seizure is a brief episode of altered brain function due to abnormal and excessive electrical discharge from the brain cells [1]. It may involve all parts (generalized seizure) or one part of the brain (focal or partial seizure) [2]. International League against Epilepsy (ILAE) has given the following definition of the patient diagnosed with epilepsy if having a) two unprovoked or reflex seizure occurring more than 24 hours apart explain that they are at higher risk of further seizure in the future b) who has a single unprovoked or reflex seizure and the probability of further seizure similar to general recurrence risk (at least 60%) after two unprovoked seizures over the next 10 years means that these also recognizes as seizure even without having actual second seizures (person at risk of having further seizure *i.e.* CNS infection, stroke, epileptiform activity on EEG and potential epileptogenic abnormality on brain imaging) c) Diagnosis of an epilepsy syndrome, describe that an epileptic syndrome has been identified by consultation and investigation through clinicians [3, 4]. According to WHO around 50 million peoples worldwide have epilepsy making it the most common neurological disorder and 80% of them are living in low to middle-income countries [5–7]. A number of newer antiepileptic drugs (AEDs) were made available in the market in the 1990s with favorable tolerability, pharmacokinetics, and potential for drug interactions [8]. Regardless of the newly introduced anti-epileptic drugs (AEDs) such as lamotrigine, felbamate, vigabatrin, tiagabine, zonisamide, and topiramate, still these drugs lack effectiveness in about 20 to 30% of patients. Drug resistance and undesirable side effects such as neurotoxicity have been associated with nearly all current AEDs. This creates a need for drug companies and scientists around the globe to develop newer AEDs with more efficacies and lesser side-effects [9, 10].

The 1,3,4-oxadiazole heterocyclic moiety serves as hydrogen bonding domains, having potential sites for the interaction inside the receptors increasing the pharmacological activities and considered as

bioisoesteres of ester and amide groups [11]. Phenyl oxadiazole derivatives were reported to provide a hydrophobic unit in promising anticonvulsant compounds in tested animal models. On the other hand, the 1,2,4-triazine ring represents a core moiety providing different pharmacological activities including anticonvulsant (lamotrigine), anticancer (tirapazamine), antiviral (azaribine), and antibacterial (dihydromethyl furalazine) [12]. Lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine) is an anticonvulsant drug approved for the treatment of partial seizures, secondarily generalized tonic-clonic seizures adjunct therapy for the treatment of generalized seizures associated with Lennox-Gastaut syndrome [13]. The drug acts by the prolongation of voltage-sensitive Na^+ channel's inactivation [14]. It is associated with hypersensitivity reaction due to epoxide generation from the *O*-dichlorophenyl potential site present in the compound [12].

Dimmock and Pandeya *et al.* have suggested the pharmacophoric model and indicated that these essential pharmacophoric elements are necessary for good anticonvulsant activity. These are a) hydrophobic unit *i.e.* lipophilic aryl ring **A** b) hydrogen bonding domain depicted as **HBD** c) electron donor **D** [15–17]. Therefore we tried to clubbed aryloxadiazole with 1,2,4-triazine having NH as linker proposed skeleton fulfills the pharmacophoric requirements for any drug to act as an anticonvulsant [18, 19]. The rationale behind the design and the pharamacophoric elements in the clubbed aryloxadiazole-1,2,4-triazine derivatives have been represented **(**Figure 1**)**. The compounds synthesized were assessed for *in vivo* anticonvulsant activity using MES, scPTZ test and motor impairment by using rotarod test.

2. Result And Discussion

2.1 Chemistry

The synthesis of clubbed aryl oxadiazole-1,2,4-triazine derivatives *i.e.* 6-((5-aryl-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione derivatives **(6a-l)** follows the reaction sequence as shown in **Scheme I**. The very first step was the synthesis of 2-(substituted benzylidene)hydrazine-1-carboxamide **(3a-l)** carried out utilizing easily available different substituted arylaldehyde and semicarbazides in the basic medium of base sodium acetate. The drop-wise addition of bromine in sodium acetate mixture with the synthesized carboxamide derivatives **(3a-l)** afford the cyclized 5-substitutedaryl-1,3,4-oxadiazol-2-amines **(4a-l)**. The syntheses were also performed by using different oxidation methods such as trituration of semicarbazone with $KBr/KBrO_3$, $FeCl_3$ and ceric ammonium nitrate methods mentioned in literature but not able to get the product in high yields. These free amino group-containing derivatives of substituted aryloxadizole amine **(4a-l)** were acetylated to *N*-(5-substitutedaryl-1,3,4-oxadiazol-2-yl)acetamide **(5a-l)** followed by the addition of chloroacetyl chloride in small proportion using base potassium carbonate in solvent DCM. The confirmation of the synthesized acetylated compounds were difficult by (toluene: ethyl acetate and formic acid; T:E:F) solvent system in TLC but was resolved using a different composition of the solvent system of chloroform and methanol. The compound **(5a-l)** get cyclized by the use of thiosemicarbazide to 6-((5-(substituted-aryl)-1,3,4-oxadiazol-2-yl)amino)-4,5-dihydro-1,2,4-triazin-3(2H)-thione in 10% ethanolic NaOH. The sodium hydroxide is dissolved in a minimum quantity of water and

then sufficient aldehyde free ethanol is added to make 10% concentration and was left for 24 h in a tightly closed container. The solution was filtered and used in the reaction. The derivatives were then washed with bromine water solution to get stable final compound; 6-((5-substitutedaryl-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione **(6a-l)**. The obtained final compounds were filtered, washed with water (2-3 times), dried, and recrystallized with ethanol. These reactions to the final compounds were carried out through facile synthetic strategies and are economical. The final synthesized structures were characterized with the help of modern analytical techniques using mass spectrometry, ^{13}C NMR, ^{1}H NMR and IR spectral data.

2.2 Pharmacology

The results of the *in vivo* preliminary phase I screening are displayed in Table 1. The activities for the compounds to be anticonvulsant were checked by maximal electroshock seizure *i.e.* MES and chemshock seizure induced by subcutaneously pentylenetetrazole drug *i.e.* scPTZ test. The results of maximal electroshock seizure (MES) and subcutaneous pentylenetetrazole induced seizure (scPTZ) model confirms for the compounds to be active against generalized tonic-clonic and absence seizures. The synthesized phenyloxadiazole clubbed-1,2,4-triazines derivatives were injected into mice intraperitoneally with a dose of 30, 100, and 300 mg/kg of body weight. After 0.5h and 4.0h of the test drug administration, the anticonvulsant effects were recorded. The neurological impairment test is performed by the rotarod test. The compound found to be most potent was tested for quantitative determination in the phase II study. In this protective index (PI) were calculated dividing the median toxic dose (TD_{50}) with median effective dose (ED_{50}) (Table 2).

Table 1
Preliminary (Phase I) screening result for compound **(6a-l)**.

Compd.	Dose injected in mice[a]					
	MES screen		scPTZ screen		Behavioural toxicity screen	
	0.5h	4h	0.5h	4h	0.5h	4h
6a	100	300	300	-	-	-
6b	100	300	100	300	-	-
6c	100	100	300	300	-	-
6d	30	100	300	300	-	-
6e	100	-	300	-	300	X
6f	30	100	100	100	-	-
6g	30	30	100	100	-	-
6h	300	-	X	X	X	X
6i	100	300	100	300	-	-
6j	100	100	300	300	-	-
6k	300	300	X	X	-	-
6l	300	300	X	X	-	-
Phenytoin[b]	30	30	-	-	100	100
Carbamazepine[b]	30	100	100	300	100	300

[a]Doses of 30, 100, and 300 mg/kg injected (*i.p*) in mice. The results in the table showed the minimum dose found to be effective in 50% or more mice in any group. The activity was checked after 0.5 and 4 h of the synthesized test drug administration. At a maximum dose of 300 mg/kg dash (-) shows that the compounds lack any anticonvulsant effect or absence of any behavioral toxicity. The compounds not undertaken for study are marked by X. [b]marketed drugs.

Table 2
Phase II study showing quantitative anticonvulsant evaluation of the selected compound.

Compd.	ED_{50}[a] (mg/kg)		TD_{50}[b] (mg/kg)	PI[c]	
	MES	scPTZ		MES	scPTZ
6g	28.5 (26.5-32.7)	76.6 (61.9-95.4)	>500	>17.54	>6.5
Phenytoin	9.5 (8.1-10.4)	>300	65.5 (52.5-72.9)	6.9	<0.22
Carbamazepine	8.8 (5.5-14.1)	>100	71.6 (45.9-135)	8.1	<0.72
Each group contains animals used = 10; Drug dissolved in polyethylene glycol (0.1 ml, *i.p*).					
[a] ED_{50} median effective dose found to be effective as anticonvulsant in 50% or more animals.					
[b] TD_{50} median toxic dose found to show absence of behavioral toxicity in 50% or more animals.					
[c] PI = Protective index (TD_{50}/ED_{50}).					

2.2.1 Phase I studies

In the preliminary study, all the synthesized compounds showed some degree of protection against MES activity indicates the potential of these compounds against generalized tonic-clonic seizure (Table 1). The compound **6g** showed protection against seizure-induced by the MES model at a dose of 30 mg/kg body weight of mice after both 0.5 and 4.0h of the drug administration. This indicates that compound **6g** has a rapid onset and prolonged duration of action at a lower dose. Compound **6d** and **6f** showed rapid onset at a lower dose of 30 mg/kg (after 0.5h) but a long duration of action at a relatively higher dose of 100 mg/kg (after 4.0h) of the drug administration. Compound **6c** and **6j** were more active at 100 mg/kg after both the reported time intervals after both the time interval of 0.5h and 4.0h. The compounds **6a**, **6b**, and **6i** were found to be active at higher dose value 100 mg/kg (after 0.5h) and 300 mg/kg (after 4.0h). The anticonvulsant activity of compound **6e** lasted after 0.5h at a dose of 100 mg/kg indicative of short-acting as the compounds do not show any activity after 4.0h even at the maximum dose of 300 mg/kg. The compounds **6k** and **6l** were weak anticonvulsant agents than all other compounds as these compounds were effective at the maximal dose of 300 mg/kg after both time intervals. The compound **6h** was found to possess no significant anticonvulsant activity at the maximum dose.

In another chemshock induced seizures test *i.e.*, scPTZ, compounds **6b**, **6f**, **6g**, and **6i** showed significant protection. Among these compounds, **6f** and **6g** raised the seizure threshold against the absence seizure at both the time interval of 0.5 and 4.0h time intervals at a dose of 100 mg/kg. Compounds **6b** and **6i** showed protection after 0.5h at 100 mg/kg dose and a maximal dose of 300 mg/kg after 4.0h. The compounds showed rapid onset at 100 mg/kg dose but were longer acting at higher dose of 300 mg/kg.

Compounds **6c**, **6d** and **6j** raised the seizure threshold at a higher dose of 300 mg/kg after both the time intervals. The compounds **6a** and **6e** lasted after 0.5h time interval indicative of short acting at the maximum dose (300 mg/kg).

The varying substituents on the aryl ring attached at the C2 position of oxadiazole ring in clubbed aryl oxadiazole-1,2,4-triazine derivatives were studied for its anticonvulsant potential. The 1,2,4-triazine-2-thione ring was selected rather than 1,2,4-triazine-2-one as they better fit into the receptors confirmed through docking studies and literature available. The introduction of different electron-withdrawing or electron-donating groups on the aryl ring showed varying degrees of anticonvulsant effect. The most active compounds with $-OCH_3$ **(6f)** and -OH **(6g)** substitution evidenced that electron-donating substituents contribute to anticonvulsant action. Electron withdrawing group such as $-NO_2$ **(6i)** also lead to an increase in the activity but the results were more pronounced when the nitro group is at *para* position of aryl ring rather than *meta* or *ortho*. The other EWG such as chloro derivatives **(6e)** led to a decrease in anticonvulsant activity and was also associated with neurotoxicity. This can be in agreement with metabolism to electrophilic arene oxide which may covalently bind with antioxidant glutathione (GSH). Compounds with naphthyl/furan substitution showed promising activity. This may be reasoned because of an increase in hydrophobicity or electronic parameters and better interaction within the receptors. The SAR of synthesized clubbed aryl-oxadiazole-1,2,4-triazine derivatives is represented **(**Figure 2**)**.

2.2.2 Phase II quantitative anticonvulsant evaluation in mice

Compound **6g** ought to be most potent in preliminary (phase I) screening with the absence of any neurotoxic (minimal motor impairment) or hepatotoxic effect. This led us to further investigate and quantify its pharmacological properties in quantitative anticonvulsant estimation (phase II) screening (Table 2). The results showed that compound **6g** exhibited moderate efficacy with an ED_{50} of 28.5 mg/kg against MES screens, which is higher than marketed drugs phenytoin and carbamazepine with ED_{50} value of 9.5 mg/kg and 8.8 mg/kg. Conversely, in the scPTZ study the compound **6g** showed protection with an ED_{50} of 76.6 mg/kg, a lower value than the standard drugs carbamazepine and phenytoin. The median toxic dose (TD_{50}) of the drug **6g** in the rotarod test was above 500 mg/kg which was significantly higher than the standard drugs. Calculation of protective indices (PI) resulted in higher PI values of 17.54 in MES and 6.5 in scPTZ screen, showing that compound **6g** is indeed a safer and effective anticonvulsant agent.

2.2.3 Actophotometer test

The titled compounds were also evaluated for CNS study (locomotor) using actophotometer (Table 3). Phenytoin did not show significant behavioral anguish effect after 0.5 (241 ± 10.87) and 1 h (251 ± 13.29) when compared to control (255 ± 11.61). Compound **6d** unveiled decreased locomotor activity after post treatment of 0.5 h interval but did not exhibit remarkable behavioral despair after 1 hour of post

treatment. All other compounds were found to displayed safe and did not show any behavioral anguish effect after both interval (post treatment 0.5 and 1h) when they were compared to 24 hours prior reading.

Table 3
Behavioral study data of titled compounds (**6k-l**) in actophotometer test.

Compounds	Control (24 h prior)	Post treatment (0.5h)	Post treatment (1h)
6a	263±25.44	247±24.19	257±20.97
6b	278±22.06	256±26.32	255±15.84
6c	267±20.59	245±16.70	263±19.53
6d	260±20.5	154±18.73	256±18.73
6e	245±25.57	235±13.30	241±11.16
6f	264±28.01	248±18.56	248±20.40
6g	281±19.4	265±20.49	269±17.7
6h	249±10.39	240±8.01	244±11.72
6i	258±31.00	243±28.6	239±11.6
6j	273±14.40	244±13.78	254±16.19
6k	246±11.61	240±10.17	244±13.19
6l	258±11.91	239±10.27	250±13.20
Standard (Phenytoin)	255±11.61	241±10.87	251±13.89
The results are represented as mean ± SEM with six animals in each group.			

2.2.4 Hepatotoxicity study

Hepatotoxicity is an adverse drug reaction associated with liver damage or injury and is caused by an increase in levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) than the upper limit of normal. The elevated levels of liver enzymes of AEDs drugs are rare but serious. Abnormalities in the level of the enzymes are signs for early prediction of liver toxicity. Therefore, the most potent compound **6g** was selected to study their hepatotoxicity profile. In the study most potent compound **6g** was selected for further analysis of their hepatotoxic profiles. As shown in Table 4 results the levels of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT) remained almost like that with control values indicate a non-toxic effect on liver ($p < 0.01$).

Table 4
The effect compound **6g** on levels of alanine aminotransferase (ALT; SGPT) and aspartate aminotransferase (AST; SGOT) enzymes.

Compd.	SGOT (units/mL)	SGPT (units/mL)
6g	37.97±1.15^{ns}	36.41±1.14*
Control[a]	37.18±1.71	34.23±1.27
* P < 0.01, ns - not significant. The mean level of SGOT/SGPT ± SEM was calculated using test ANOVA and followed by Dunnett's multiple comparison test (n = 4)		
[a]Control group was treated with 0.5% methyl cellulose for 15 days.		

2.2.5 Neurochemical study: Estimation of the GABA level

GABA is an important neurotransmitter and show CNS-inhibitory action. It is strictly related to the manifestations and occurrence of convulsions. To further estimate the possible mechanism of most potent compound (**6g**), whole brain GABA estimation was carried out. The standard drugs clobazam (111.23 ± 4.64) and diazepam (94.72 ± 5.85) significantly increased the GABA concentration as compared to control (47.22 ± 2.52). In the similar manner compound **6g** (80.64 ± 7.74) also increased the GABA level concentrations (Table 5).

Table 5
GABA estimation of **6g** in mice whole brain

Compounds	GABA concentration in mice whole brain (mg/100 mg tissue)[a]
	7-days Post treatment
Control	47.22 ± 2.52
Clobazam	111.23±4.64
Diazepam	94.72±5.85
6g	80.64±7.74
[a] Each value represents the mean ± SEM of six mice	

2.3 Antioxidant activity

2.3.1 FRAP assay

In FRAP assay, augmented absorbance of the samples with concentration indicates amplified in reducing power. The compounds were assayed for four concentrations (25, 50, 75, 100 µg/ml). The results were presented in IC_{50} for each sample (Table 6). Ascorbic acid was used as standard and showed 19.54 ± 34 IC_{50}. Among all the derivatives **6a**, **6d**, **6e**, **6f** and **6g** found as most compelling compounds with IC_{50} value 16.50 ± 34, 14.09 ± 78, 17.64 ± 67, and 15.55 ± 56 respectively.

2.3.2 DPPH assay

According to DPPH assay antioxidant effect is proportional to the fading of DPPH in the test samples. DPPH test was also performed using four different concentrations (25, 50, 75, 100 µg/ml. The IC_{50} for standard was found to be 13.53±76 (Table 6). Among the synthesized compounds four compounds (**6a**, **6e**, **6f** and **6g**) were found to displayed most active antioxidant with IC_{50} value 12.10 ± 95, 11.79 ± 78, 14.54 ± 89 and 13.00 ± 54.

Table 6
Results of *in-vitro* antioxidant assay in terms of IC_{50} for FRAP and DPPH assay for titled compounds **6a-6l.**

Compounds	Antioxidant activity	
	FRAP IC_{50}	DPPH IC_{50}
6a	16.50±34	12.10±95
6b	48.97±39	28.11±90
6c	30.23±94	30.97±75
6d	14.09±78	12.10±15
6e	17.64±67	11.79±78
6f	38.97±04	14.54±89
6g	15.55±56	42.15±35
6h	18.95±45	20.17±56
6i	17.64±78	14.54±45
6j	15.55±70	13.00±54
6k	18.09	29.05
6l	17.88±23	15.01±00
Ascorbic acid	19.54±34	13.53±76

2.4. Computational study

2.4.1. Pharmacophore distance mapping

Pharmacophore distance mapping results analysis showed the average distance between (R-D), (R-HBD) and (D-HBD) calculated after optimizing the 3D structures using with the help of software ChemDraw Professional PerkinElmer featuring SciFinder/3D viewer version 15.0. According to Unverferth *et al* [[20]] distance between an electron donor (D) and aryl ring (R) and donor (D) and HAD unit are smaller in

comparison to the distance between R and HAD which further depends upon different calculation methods. The distance calculations between the essential structural elements by *ab initio* MO data obtained by the CHARMm force field are in agreement. The distance between essential structural elements for standard compounds by molecular dynamic distance calculations are represented **(**Figure 3**)**. The distance between essential pharmacophoric elements were also checked for the synthesized compounds **(6a-l)** to confirm their pharmacophoric models in relation to established AEDs. Our compounds **(6a-l)** fulfilled the structural distance required for the pharmacophoric features essential for anticonvulsant action. The distance mapping data of compound **6g** and standard drugs are represented.

3. Experimental

3.1 Chemistry

The chemicals used in carrying out the steps in the synthesis were supplied by S.D. Fine and lobachemie. Hicon melting point apparatus was used to check the melting points of the synthesized clubbed aryl oxadiazole-1,2,4-triazine derivatives. The Hicon melting point apparatus uses an open capillary tube method to check the melting point and are uncorrected. The purity and reaction completion were checked with thin layer chromatography on silica gel G (Merck) coated plates by using solvents system toluene: ethyl acetate: formic acid (5:4:1) and chloroform: methanol (9:1). The completion of the reaction is confirmed by visualization of TLC spots either by UV lamp or in the iodine chamber. Thermo scientific Nicolet iS5 FT-IR spectrometer (KBr pellets) was used for generating IR spectra. The ^{1}H NMR spectra were measured on a Bruker Avance NEO-500 instrument (500 MHz) and ^{13}C NMR spectra were measured on Bruker Avance NEO-500 instrument (125 MHz) with complete proton decoupling in DMSO-∂_6/$CDCl_3$ solutions. Chemical shift results were shown in ppm downfield from tetramethylsilane (TMS) as the internal standard. Splitting in NMR is classified as: m, multiplet; d, doublet; s, singlet. Chemical shifts (δ) values are given in parts per million (ppm). The coupling constant is measured in Hz and designated as *J* values. Waters synapt LC/MS instrument using electron impact ionization and the data are represented as m/z and the mass spectra recorded as M^+ and M^+ +1 peak.

3.1.1. General procedure for 2-(substituted-arylidene)hydrazine-1-carboxoamide (3a-l)

In a flat-bottomed flask, a solution of semicarbazide hydrochloride (1.11 g, 0.01 M) and sodium acetate (1.64 g, 0.02 M) were dissolved in 15–20 ml of distilled water. Different aryldehyde (0.01M) **(1a-l)** was taken in aldehyde-free alcohol and added slowly to the stirred mixture/solution of semicarbazide hydrochloride. The precipitate formed immediately was further stirred for another half an hour with constant stirring. More of the solvent was added if stirring was stopped due to the formation of an immediate precipitate. The precipitate obtained was filtered and recrystallized from ethanol (95%). A distinct single spot in thin layer chromatography (TLC) was used to initially confirm the reaction completion and purity of the compounds [11].

3.1.2. General procedure for 5-substituted-aryl-1,3,4-oxadiazol-2-amines (4a-l)

A mixture of synthesized compound 2-(substituted-arylidene)hydrazine-1-carboxamide (0.01 M) **(3a-l)** and sodium acetate (0.02 M) were stirred continuously in 30–40 ml of glacial acetic acid. In the reaction mixture bromine (1.4 ml in 10 ml of GAA) was added dropwise and stirred for another 1-4 h. After completion, it was poured on ice and the solid gets separated. The solid was filtered and leftover for drying. Finally, it was recrystallized from ethanol to get 5-substituted-aryl-1,3,4-oxadiazol-2-amines **(4a-l)** [11].

3.1.3. General procedure for 2-chloro-N-(5-substituted-aryl-1,3,4-oxadiazol-2-yl)acetamide (5a-l)

The obtained compounds **(4a-l)** (0.016 M) were dissolved in DCM (30 ml) and to this solution powdered anhydrous K_2CO_3 (1g) was added. Chloroacetyl chloride (0.03 M) was added dropwise with constant stirring to the reaction mixture maintained at 10 ºC in an ice-water bath. The reaction mixture was stirred continuously for another 2-4 h. After stirring it was left open overnight so that excess DCM left over the reaction mixture gets evaporated. After that water was added to the residue and stirred for another 20 min. The crude precipitate obtained was filtered, washed twice or thrice with water, and then dried. The obtained product was recrystallized from ethanol. The purity and progress of the reaction were checked throughout by TLC using TEF (5:4:1) as mobile phase [21].

3.1.4. General procedure for 6-((5-substituted-aryl-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6a-l)

A mixture of the chloroacetylated derivatives **(5a-l)** (0.01 M) and thiosemicarbazide (0.01 M) in 10% ethanolic sodium hydroxide (20 ml) was refluxed for 8-12 h. After the completion, the reaction mixture was transferred onto the ice and acidified with few drops of hydrochloric acid. The formed precipitate was shaken with bromine water solution (1.5 g of bromine in 40 ml of water). The solid product obtained **(6a-g)** was filtered off and washed with an excess water. The compound was treated with 5% sodium thiosulphate (20 mL) and extracted with $CHCl_3$:CH_3OH (9:1) (3 x 30 mL). The organic layer was combined and dried over anhydrous sodium sulphate, concentrated, and recrystallized from ethanol.

3.1.4.1. 6-((5-phenyl-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6a)

White color; solid; R_f value 0.41 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 272.29; yield 58%; mp 203-204ºC; IR (KBr) υ_{max} cm^{-1}: 3365 (N-H str), 3319 (NH str cyclic CSNH), 3016 (C-H str), 1661 (C=C str), 1581 (C=N str), 1266 (C=S str); ^{1}H NMR (DMSO-d_6) δ(ppm): 11.12 (s, 1H, -NH-C=S), 7.82-7.38 (m, 5H, Ar-

H), 7.23 (s, 1H, =CH 1,2,4-triazine), 7.08 (s, 1H, -NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 162.53, 161.15, 159.87, 158.33, 156.63, 136.42, 131.76, 125.05, 122.24. M^+, 272 (100); M^+ + 1, 273 (12)..

3.1.4.2. 6-((5-(p-tolyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6b)

White color; solid; R_f value 0.56 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 286.32; yield 49%; mp 192-193^{o}C; IR (KBr) υ_{max} cm^{-1}: 3373 (N-H str), 3322 (NH str cyclic CSNH), 3041 (C-H str), 1659 (C=C str), 1597 (C=N str), 1260 (C=S str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.09 (s, 1H, -NH-C=S), 7.93-7.90 (d, 2H, Ar-H, J = 9.8), 7.85-7.87 (d, 2H, Ar-H, J = 7), 7.51 (s, 1H, =CH 1,2,4-triazine), 7.21 (s, 1H, -NH), 2.27 (s, 3H, -CH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 165.15, 164.03, 161.91, 159.61, 158.03, 136.32, 129.23, 128.22, 119.34, 22.13. M^+, 286 (100); M^+ + 1, 287 (13).

3.1.4.3. 6-((5-(furan-2-yl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6c)

Cream color; solid; R_f value 0.45 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 262.25; yield 54%; mp 217-218^{o}C; IR (KBr) υ_{max} cm^{-1}: 3364 (N-H str), 3335 (NH str cyclic CSNH), 3047 (C-H str), 1665 (C=C str), 1548 (C=N str), 1245 (C=S str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.10 (s, 1H, -NH-C=S), 7.72 (d, 1H, furan, J = 3.5), 7.68-7.64 (m, 2H, furan), 7.47 (s, 1H, =CH 1,2,4-triazine), 6.98 (s, 1H, -NH), ^{13}C NMR (DMSO-d_6) δ (ppm): 165.11, 161.97, 159.78, 156.54, 154.26, 145.17, 141.12, 127.42, 123.22. M^+, 262 (100); M^+ + 1, 263 (10).

3.1.4.4. 6-((5-(naphthalen-2-yl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6d)

White color; solid; R_f value 0.61 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 322.35; yield 47%; mp 224-225^{o}C; IR (KBr) υ_{max} cm^{-1}: 3378 (N-H str), 3318 (NH str cyclic CSNH), 3047 (C-H str), 1659 (C=C str), 1554 (C=N str), 1261 (C=S str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.18 (s, 1H, -NH-C=S), 7.69-7.77 (m, 4H, Ar-H), 7.44 (s, 1H, =CH 1,2,4-triazine), 7.16-7.21 (m, 3H, Ar-H), 7.10 (s, 1H, -NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 166.54, 165.30, 162.43, 160.16, 158.32, 139.62, 133.55, 133.01, 131.55, 129.38, 128.92, 128.02, 126.74, 125.41, 123.53. M^+, 322 (100); M^+ + 1, 323 (16).

3.1.4.5. 6-((5-(4-chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6e)

White color; solid; R_f value 0.55 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 306.74; yield 47%; mp 186-187^{o}C; IR (KBr) υ_{max} cm^{-1}: 3363 (N-H str), 3334 (NH str cyclic CSNH), 3022 (C-H str), 1672 (C=C str), 1544 (C=N str), 1248 (C=S str), 689 (C-Cl str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.01 (s, 1H, -NH-C=S), 8.03-7.99 (d, 2H, Ar-H, J = 15), 7.53 (s, 1H, =CH 1,2,4-triazine), 7.18-7.16 (d, 2H, Ar-H, J = 8.9), 7.01 (s, 1H, -NH),

^{13}C NMR (DMSO-d_6) δ (ppm): 165.01, 162.87, 161.45, 159.11, 156.73, 152.07, 134.03, 128.22, 121.23. M^+, 306 (100); M^+ + 1, 308 (32).

3.1.4.6. 6-((5-(4-methoxyphenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6f)

White color; solid; R_f value 0.58 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 302.32; yield 64%; mp 204-205^{o}C; IR (KBr) υ_{max} cm^{-1}: 3392 (N-H str), 3327 (NH str cyclic CSNH), 3032 (C-H str), 1667 (C=C str), 1590 (C=N str), 1268 (C=S str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.04 (s, 1H, -NH-C=S), 7.72-7.74 (d, 2H, Ar-H, J = 9), 7.14 (s, 1H, =CH 1,2,4-triazine), 7.07-7.09 (d, 2H,Ar-H, J = 2), 6.92 (s, 1H, -NH), 3.82 (s, 3H, -OCH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 164.29, 163.40, 160.71, 159.27, 157.19, 150.62, 136.26, 126.68, 116.85, 55.26. M^+, 302 (100); M^+ + 1, 303 (13).

3.1.4.7. 6-((5-(4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6g)

White color; solid; R_f value 0.47 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 288.29; yield 42%; mp 211-212^{o}C; IR (KBr) υ_{max} cm^{-1}: 3342 (N-H str), 3315 (NH str cyclic CSNH), 3042 (C-H str), 1660 (C=C str), 1585 (C=N str), 1270 (C=S str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.20 (s, 1H, -NH-C=S), 7.80-7.82 (d, 2H, Ar-H, J = 8.7), 7.65 (d, 2H, Ar-H, J = 2.3), 7.40 (s, 1H, =CH 1,2,4-triazine), 7.17 (s, 1H, -NH), 6.29 (s, 1H, -OH); ^{13}C NMR (DMSO-d_6) δ (ppm): 167.83, 160.30, 157.26, 154.90, 140.83, 133.53, 129.13, 127.44, 126.64. MS m/z (%): M^+, 288 (100); M^+ + 1, 289 (12).

3.1.4.8. 6-((5-(2-chlorophenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6h)

White color; solid; R_f value 0.53 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 306.74; yield 51%; mp 188-189^{o}C; IR (KBr) υ_{max} cm^{-1}: 3341 (N-H str), 3328 (NH str cyclic CSNH), 3019 (C-H str), 1670 (C=C str), 1565 (C=N str), 1272 (C=S str), 696 (C-Cl str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.11(s, 1H, -NH-C=S), 7.71-7.65 (m, 4H, Ar-H), 7.50 (s, 1H, =CH 1,2,4-triazine), 6.92 (s, 1H, -NH), ^{13}C NMR (DMSO-d_6) δ (ppm): 165.11, 162.07, 160.05, 157.23, 154.08, 143.17, 139.06, 133.34, 130.23, 128.43, 126.13. M^+, 306 (100); M^+ + 1, 308 (32).

3.1.4.9. 6-((5-(4-nitrophenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6i)

Yellow color; solid; R_f value 0.51 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 317.29; yield 53%; mp 198-199^{o}C; IR (KBr) υ_{max} cm^{-1}: 3378 (N-H str), 3316 (NH str cyclic CSNH), 3021 (C-H str), 1665 (C=C str), 1576 (C=N str), 1261 (C=S str); ^{1}H NMR (DMSO-d_6) δ (ppm): 11.15 (s, 1H, -NH-C=S), 7.78-7.77 (d, 2H, Ar-H, J = 5.5), 7.56 (s, 1H, =CH 1,2,4-triazine), 7.61-7.60 (d, 2H, Ar-H, J = 7.1), 6.87 (s, 1H, -NH); ^{13}C NMR (DMSO-

d_6) δ (ppm): 164.78, 161.76, 158.15, 151.27, 147.08, 142.92, 132.04, 129.91, 122.18. M^+, 317 (100); M^+ + 1, 318 (12).

3.1.4.10. 6-((5-(2,5-dimethoxyphenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6j)

White color; solid; R_f value 0.60 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 332.35; yield 59%; mp 214-215^oC; IR (KBr) υ_{max} cm^{-1}: 3377 (N-H str), 3340 (NH str cyclic CSNH), 3029 (C-H str), 1671 (C=C str), 1567 (C=N str), 1250 (C=S str); ^1H NMR (DMSO-d_6) δ (ppm): 11.17 (s, 1H, -NH-C=S), 7.44 (d, 2H, Ar-H, J = 4), 7.27 (s, 1H, Ar-H), 7.19 (s, 1H, =CH 1,2,4-triazine), 7.15 (d, 2H, Ar-H, J = 4.5), 6.98 (s, 1H, -NH), 3.81 (s, 6H, -OCH_3); ^{13}C NMR (DMSO-d_6) δ (ppm): 165.13, 162.07, 160.31, 158.17, 156.08, 150.62, 147.26, 129.06, 126.12, 121.17, 119.78, 56.08, 54.19. M^+, 332 (100); M^+ + 1, 333 (14).

3.1.4.11. 6-((5-(3-nitrophenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6k)

Yellow color; solid; R_f value 0.52 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 317.29; yield 50%; mp 199-200^oC; IR (KBr) υ_{max} cm^{-1}: 3378 (N-H str), 3316 (NH str cyclic CSNH), 3021 (C-H str), 1665 (C=C str), 1576 (C=N str), 1254 (C=S str); ^1H NMR (DMSO-d_6) δ (ppm): 11.21 (s, 1H, -NH-C=S), 7.86 (s, 1H, Ar-H), 7.76-7.84 (m, 3H, Ar-H), 7.59 (s, 1H, =CH 1,2,4-triazine), 7.08 (s, 1H, -NH); ^{13}C NMR (DMSO-d_6) δ (ppm): 167.22, 163.06, 161.32, 157.08, 153.33, 145.67, 132.04, 129.91, 126.18, 123.12, 120.54. M^+, 317 (100); M^+ + 1, 318 (12).

3.1.4.12. 6-((5-(2,4-dichlorophenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione (6l)

White color; solid; R_f value 0.57 (toluene: ethyl acetate: formic acid 5:4:1); Mol wt. 341.18; yield 48%; mp 193-194^oC; IR (KBr) υ_{max} cm^{-1}: 3364 (N-H str), 3324 (NH str cyclic CSNH), 3039 (C-H str), 1669 (C=C str), 1571 (C=N str), 1265 (C=S str), 680 (C-Cl str); ^1H NMR (DMSO-d_6) δ (ppm): 11.23(s, 1H, -NH-C=S), 7.76 (s, 1H, Ar-H), 7.50 (s, 1H, =CH 1,2,4-triazine), 7.37 (d, 1H, Ar-H, J = 5.5), 7.29 (d, 1H, Ar-H, J= 6), 7.12 (s, 1H, -NH), ^{13}C NMR (DMSO-d_6) δ (ppm): 166.28, 163.14, 161.34, 158.06, 155.31, 141.65, 138.43, 132.34, 130.56, 127.23, 125.08. M^+, 339 (100); M^+ + 1, 341 (12).

3.2 Biological activity

All the pharmacological activities were done in albino mice (either sex) (26-34 g) *in vivo.* The albino mice were kept with sufficient food and water access under standard conditions at a normal temperature of 25 ± 2°C, except at the time they were brought out of the cage. The experiments were carried out after

permission from the Institutional Animal Ethics Committee (IAEC). The animals are provided from the Animal House Facility, ISF College of Pharmacy after their approval. Registration with form no is ISFCP/IAEC/CPCSEA/Meeting No.26/2020/Protocol No. 446. Phenytoin and Carbamazepine were administered in 0.5% w/v methylcellulose in water. These drugs were administered either orally (*p.o*) or intraperitoneally (*i.p*) in a volume of 0.01 ml/g body weight in mice. The chemical convulsants were administered subcutaneously. For statistical analysis, ANOVA followed by Dunnett's method with the aid of Graph pad Prism 5.0 software, (inc., San Diego, USA) was used. The animal test includes preliminary investigation (phase I screening) of MES and scPTZ and minimal motor inhibitory activity of the synthesized final compounds as approved protocols provided by the Antiepileptic Drug Development (ADD) program by NINDS, USA. The dose of the test compounds was selected 30, 100 and 300mg/kg and were given to mice through intraperitoneal route and the response of the animals were recorded after an interval of 0.5 and 4 h [22–24]. The compounds found most potent in preliminary screening were further tested for phase II quantitative screening. The results were calculated as ED_{50}, TD_{50} and further stated in terms of the protective index (PI) [25]. To evaluate the behavioral effects (locomotor activity) the titled compounds **6a-l** were also estimated by actophotometer test. The locomotor activity was recorded by actophotometer (IMCORP, Ambala, India) photocell as a digital score. Animal were placed individually in the activity chamber for 3 minutes as habitation periods before making the actual reading [26]. The liver toxicity results of the potent compound in the series as SGOT and SGPT were evaluated by serum enzyme activity assay [27, 28]. For the development of the possible mechanisms of the derivatives. The most potent compounds were screened for estimation of GABA level in extracted tissue of mice brain. The enzymatic spectrophotometric method was very used for the analysis as it is selective and specific. In this method, 2 h after the administration of drug (30 mg/kg, *i.p.*) the animals were decapitated. The brains were removed, washed with ice-cold isotonic saline and then homogenized with 0.1 mmol/L phosphate buffer (pH 7.4). The homogenate (10% w/v) was then centrifuged and supernatant formed was screened for GABA estimation as previously described [29].

3.3 Anti-oxidant activity

The synthesized compounds were evaluated for in vitro antioxidant activity by FRAP (Ferric-Reducing antioxidant power) and DPPH (1,1-diphenyl-2-picrylhydrazyl). In FRAP method four concentrations (25, 50, 75, 100 µg/ml) of each sample and standard in methanol were prepared and mixed (2.5 ml) with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1.0% potassium ferricyanide (2.5 ml). The mixture was incubated at 50°C for 20 min. Aliquots of 10% trichloro acetic acid (2.5 ml) were added to the mixture, centrifuged at 5000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with distilled water (2.5 ml) and a freshly prepared ferric chloride solution (0.5 ml, 0.1%) and allowed to stand for 30 min in dark to complete the reaction. The control solution was prepared as above, taking water in place of samples. The absorbance was measured at λ_{max} 700 nm. The reducing power of each compound were expressed as percentage of most active reference compound in current assay, based on the formula given below and the result obtained was averaged and expressed as mean ± standard deviation. DPPH method is principally work on the theory of hydrogen donor is an antioxidant. It measures compound that are radical scavengers. The antioxidant effect is comparative to the disappearance of DPPH in the test

samples. DPPH shows a strong absorption maximum at 517 nm (purple). The color turns from purple to yellow followed by formation of DPPH upon absorption of hydrogen from an antioxidant. This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm. Freshly Prepared the DPPH solution 0.1 mM in methanol and was kept in dark for 2 h. Prepared the four different concentrations (5, 10, 15, 20 µg/mL) of each sample. 2 mL of different concentrations of test samples were taken in a set of test tubes and to this, 2 mL of freshly prepared DPPH solution was added and mixed thoroughly. This final solution is then incubated for 30 min at room temperature and the absorbance was recorded at 517 nm in UV spectrophotometrically All tests and analysis were run in triplicates [30].

4. Conclusion

Various novel 6-((5-substituted-aryl-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione derivatives were designed and synthesized. The designed compounds possess structural elements of standard drugs such as to increase the anticonvulsant potential and decrease the toxicity. The synthesized clubbed aryl oxadiazole-1,2,4-triazine derivatives were characterized by mass spectrometry, ^{13}C NMR, ^{1}H NMR, IR. After characterization compounds were assessed for preliminary anticonvulsant activity using MES, scPTZ and motor impairment test by rotarod test. Compounds were effective and displayed substantial protection against both models *i.e.*, MES and scPTZ. Among them, compounds, 6-((5-(4-hydroxyphenyl)-1,3,4-oxadiazol-2-yl)amino)-1,2,4-triazine-3(2H)-thione **(6g)** possess to be most potent in preventing seizure spread and elevates seizure threshold. The compound **6g** was further subjected to quantitative study in phase II screening and the results showed a higher protective index as compared to the standard drug. The series of compounds were also reviled no sign of locomotor effect in actophotometer test. The compounds **6g** showed no sign of hepatotoxicity estimated through the liver enzyme estimation. The compounds **6g** revealed to increase GABA level in the whole brain GABA estimation test. The synthesized 1,2,4-triazine derivatives possess potent anticonvulsant activity with no neurotoxicity as well as hepatotoxicity and may be regarded as strong candidates for future investigation.

Declarations

Declaration of Conflicts of interest/Competing interests (include appropriate disclosures): The authors declare no conflicts of interest, financial or otherwise.

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Scheme

Scheme 1 is available in the Supplementary Files section.

Figures

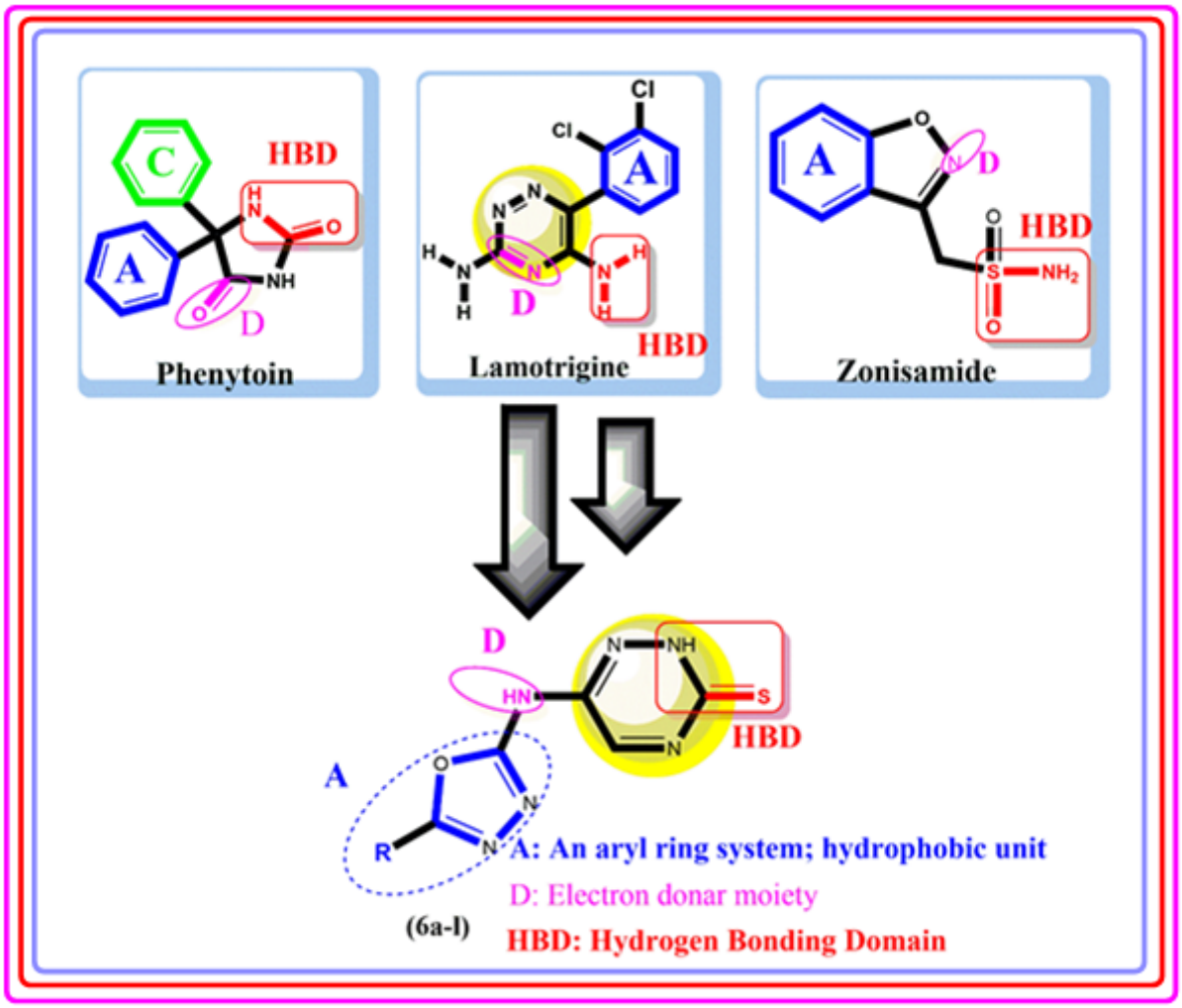

Figure 1

Design of the newly clubbed aryl oxadiazole-1,2,4-triazine derivatives and the structures of marketed antiepileptic drugs.

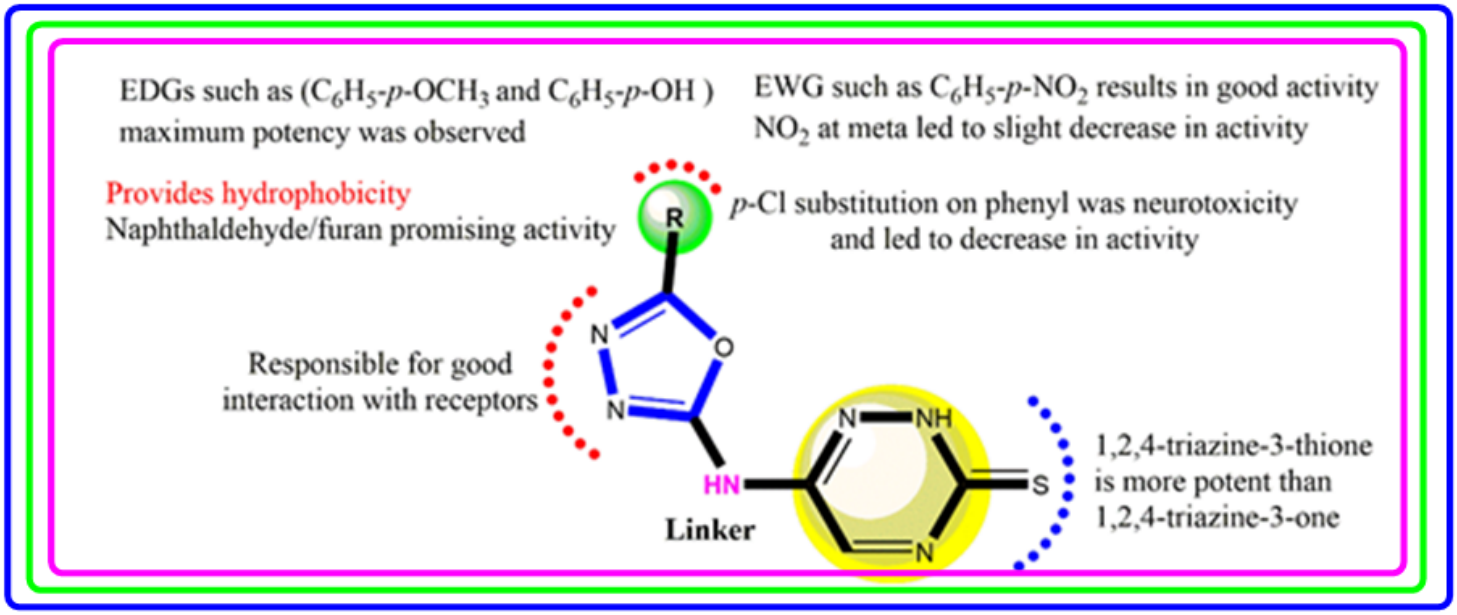

Figure 2

SAR of clubbed aryl-oxadiazole-1,2,4-triazine

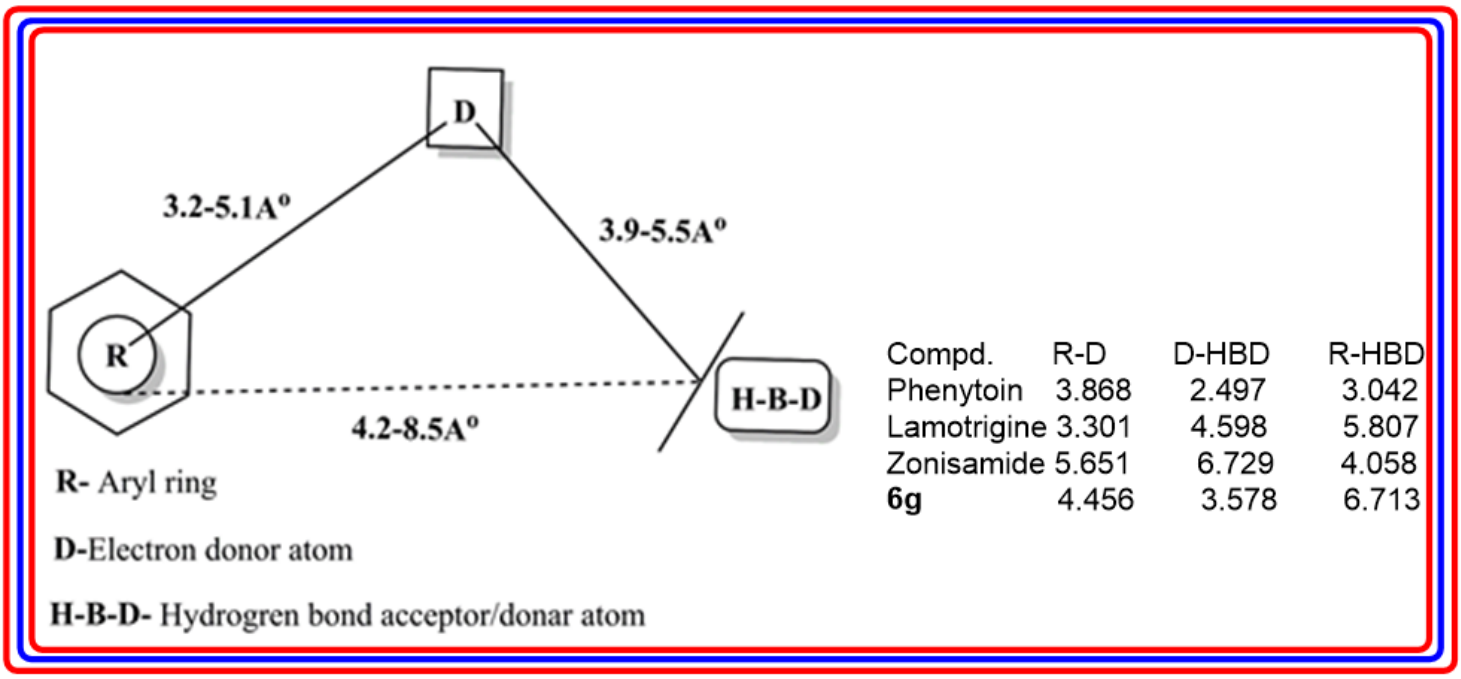

Figure 3

Three-point pharmacophore model of standard compounds.

Supplementary Files

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