Anticonvulant Potentail of the Oxazetidine Derivatives

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ABSTRACT:

Anticonvulsant potential of the 2-[3-chloro-2-(4-chlorophenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(2)), 2-[3-chloro-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(3)), 2-[3-chloro-2-(3-nitrophenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(4)) has been reported. The anticonvulsant activity have been carried out via maximal electroshock (MES) induced convulsion and Pentylenetetrazole (PTZ) induced convulsion model. Biochemical analysis for both models have been performed via analysis of Malondialdehyde (MDA), reduced Glutathione (GSH) reagent estimation and measurement of *Catalase* activity and SOD activity. Derivative 2-[3-chloro-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(3)) exhibited potent and having comparable bioactivity to the standard in both animal models.

KEY WORDS:

Anticonvulsant, Oxazetidine, maximal electroshock (MES) induced convulsion, Pentylenetetrazole (PTZ) induced convulsion

INTRODUCTION: [1-2]

Epilepsy is one most abundant neurological disorder which is affecting mankind. The hyperactivity or excessive neuronal discharge which can lead to the abnormality in the brain or electrical activity in brain is called as epilepsy. The change in the neurotransmitter discharge or imbalance in the electrical activity of the brain is one of the major reasons for the epileptic attack. The epileptic attack is often classified in the major and minor seizures according to the part of the brain involved. Although these epileptic seizures are short lived but sometimes they can cause considerable damage to the patient. Due to the complexity in their generation and involvement of the central nervous system development of the antiepileptic or anticonvulsant drugs is an uphill task. The treatment regime for Epilepsy includes number of the heterocyclic derivatives like phenytoin, carbamazepine which signifies the importance of the heterocyclic systems. In the heterocyclic system the nitrogen and sulphur containing molecules gaining importance over others due to their wide range of the biological applications. In a generalized observation the nitrogen is most abundant heterocycles in the number of medicinal compounds. Due to the significant contribution of the nitrogen containing heterocycles theses are contagiously researched and explored for number of significant biological applications. Saturated heterocycles of nitrogen like azetidine are key component of the marketed drugs like azelnidipine. Azeditines makes an important candidature for the neurological drug discovery due to their strong molecular rigidity and stability. Here we report the anticonvulsant potential of the in house synthesized -[3-chloro-2-(4-chlorophenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(2)), 2-[3-chloro-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(3)), 2-[3-chloro-2-(3-nitrophenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(4)) molecules using maximal electroshock (MES) induced convulsion bioassay protocol. The Biochemical analysis was also performed using via assessment of Malondialdehyde (MDA) level, reduced Glutathione (GSH) Reagent, Catalase Activity and SOD activity. All the tested compounds showed good anticonvulsant activity in maximal electroshock (MES) induced convulsion bioassay protocol. Derivative 2-[3-chloro-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl) propanoic acid (NL 2(3)) was found to be more active than other two derivatives. The results indicated azetidine nucleus can be a promising scaffold for development of molecules targeting neurological diseases.

EXPERIMANTAL:

Dataset:

Molecules 2-[3-chloro-2-(4-chlorophenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(2)), 2-[3-chloro-2-(4-methoxyphenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(3)), 2-[3-chloro-2-(3-nitrophenyl)-4-oxoazetidin-1-yl]-3-(1H-indol-3-yl)propanoic acid (NL2(4)) was taken from our previously reported research paper.[3]

Biological Activity:

The biological activity was performed in Department of Pharmacology, Channabasweshwar Pharmacy College (Degree), Latur. The protocols for the bioactivity were approved by IAEC, Channabasweshwar Pharmacy College (Degree), Latur via approval number CPCSEA/CBPL/AH/56. The Animals which are utilised for the activities are distributed in to the following groups

Animal: Rat (200 – 250 gm)

Group I: Control group Tween 80 in 0.9 % sodium chloride solutions

Group II: Standard group Phenytoin

Group IX: Test group NL2(2)

Group X: Test group NL2(3)

Group XI: Test group NL2(4)

Acute Oral Toxicity Study

Acute oral toxicity study was performed according to the OECD test guidelines 423- Acute toxic class method. Young healthy adult Swiss albino mice weighing between 18-22 g body weights, were divided into 12 groups of 3 animals test drug. Animals were housed in groups (3 animals /cage) in a well ventilated polypropylene cage. A 12-h light/12-h dark artificial photo period was maintained. A room temperature of 22°C (± 3°) and relative humidity of 50–80 % were maintained in the room. Animals had free access to pelleted feed and reverse osmosis purified water ad libitum. Test drug was administered once orally via gastric intubation to 3 h fasted mice. A specified dose of 100, 250, 500, 750, 1000 and 1500 mg/kg body weight of mice was administered orally as a single dose. The acute toxic symptoms and the behavioral changes produced by the test compounds were observed continuously for 4 h periods at Treatment Body weight (g) Day 0 Day 7 Day 14. [4-7]

Anti-Convulsant Activity Using Maximal Electroshock (MES) Induced Convulsion Test In Rat

The preliminary evaluation of the synthesized compounds NL2(2), NL2(3) and NL2(4) evaluated for anticonvulsant activity by the use of predictable animal models. [8-9] The MES seizure models represent the animal seizure models, most widely used in the search for new anticonvulsants. The anticonvulsant activity was performed on albino rats weighing about 180-220 gms are divided into twelve groups of six rats each. The test compounds NL2(2), NL2(3) and NL2(4) at concentration 100mg/kg dose with respect to standard drug phenytoin, was administered by i.p. route in rats. 60 min prior to the start of the experiments. After 1 hour they were subjected to a electric shock of 150 mA by electro-convulsometer by ear electrodes for 0.2 seconds and The postponement and incidence of Tonic Hind Limb Extension (THLE) and mortality rate has under observation for 15 min. Animals in which extensor response was eliminated were taken as protected rats. [10-11], the animal behavioral experimental data were statistically analyzed by one-way ANOVA followed by Dunnett's test using Graph Pad Prism software. A value of $P < 0.05$ was measured as statistical significance. All the values characterize standard error of the mean (SEM) expressed as mean ± SEM.

Biochemical estimation

***Malondialdehyde (MDA) level*:**

Lipid peroxidation was evaluated by measuring TBARS and was demonstrated in respect of malondialdehyde (MDA) content. Thiobarbituric acid-reactive substance content was measured in a medium containing To100 µL of tissue homogenate (10%), 15 µL of 8.1% SDS, 60 µL of acetic acid buffer (2.5 M, pH 3.4) and 115 µL of 0.81% thiobarbituric acid. The mixture was heated at 95 °C for 120min in a water bath. After cooling to room temperature, absorbance was determined in the supernatant at 532 nm. The content of malonaldehyde (MDA), articulated as n moles formed per milligram of protein in the tissue, was deliberate using the formula:

$$\text{Concentration} = A \times (V/E) \times P$$

Where, A is the volume of solution, E is extinction coefficient ($1.56 \times 105 cm^{-1}$) and P is the protein content of tissue calculated as milligram of protein per gram of tissue.

The test compounds NL2 (2), NL2(3) and NL2(4) were administered in the form of suspension made up of 0.5%. Tween 80 in 0.9 % sodium chloride solutions. The test compounds (100mg/kg). standard drug (Wagle, et al., 2009) was administered- intraperitoneal. Pentylenetetrazole (80mg/kg) dissolved in 0.9% sodium chloride solution was administered in the posterior midline of the mice and the onset and severity of convulsions was noted for the control group. The test group was administered with the selected compounds 30 minutes prior to the subcutaneous administration of PTZ. The absence or presence of an period of clonic convulsions was taken as the end point. The data were analyzed by one-way ANOVA followed by

Dunnett's test using Graph pad prism software. A value of $P < 0.05$ was examined as statistical significance. All values were reported as standard error of mean designated as ± SEM. [12-13]

Estimation of reduced Glutathione (GSH) Reagent:

0.3 M disodium hydrogen phosphate was perpreared via addition of 4.26g of anhydrous disodium hydrogen Phosphate was dissolved in 100ml distilled water and 7.92 mg of DTNB was dissolved in 20 ml of 1% citric acid to prepare 1% DTNB. The GSH content was estimated using method of [14], the tissue homogenate was mixed with TCA (10%w/v) in ratio 1:1 ratio. The tubes were centrifuged at 1000g for 10min at 4^0C. 0.5ml of the supernatant obtained was mixed with 2 ml of 0.3 M disodium hydrogen phosphate. Then 0.25ml of 0.01 M freshly prepared DTNB dissolved in 1% w/v citric acid was added and absorbance was measured spectrophotometrically at 412nm. A standard curve was plotted using 5-50µM of reduced form of glutathione and results were expressed as µmol of reduced glutathione per mg of protein as shown in table no1.

Table No. 1: Standard curve of GSH

Sample	Mean OD	GSH (µmol of reduced glutathione per mg of protein)
Standard 1	0.030	50
Standard 2	0.080	40
Standard 3	0.120	30
Standard 4	0.140	20
Standard 5	0.170	10
Standard 6	0.200	05

Catalase Activity:

Pipette 25 µL of samples or appropriate standards into duplicate wells in the plate. Pipette 25 µL of Assay Buffer into duplicate wells as the Zero standard. Add 25 µL of the supplied H_2O_2 Reagent to every well using a repeater pipet. Incubate at room temperature for 30 minutes. Add 25 µL of the supplied Substrate to every well using a repeater pipet. Initiate the reaction by adding 25 µL of the prepared HRP Reagent to every well using a repeater pipet. Incubate at room temperature for 15 minutes. Read the optical density at 560 nm +/- 20 nm (acceptable Range 540-580 nm). A standard curve was plotted results were expressed as U/ml of Catalase as shown in table no. 2.

Table No. 2: Standard curve of Catalase

Sample	Mean OD	Catalase (U/ml)
Standard 1	0.090	5.0
Standard 2	0.334	2.5
Standard 3	0.799	1.25
Standard 4	1.203	0.625
Standard 5	1.493	0.313
Standard 6	1.635	0.156

SOD activity:

Pipette 10 µL of samples or appropriate standards into identical wells in the plate. Pipette 10 µL of Assay Buffer into matched as wells as the Zero standard. Add 50 µL of the Substrate Preparation to every well using a repeater pipette. Add 25 µL of the Xanthine Oxidase Preparation to each well using a repeater pipet. Incubate at room temperature for 20 minutes. Read the optical density at 450 nm [15-16]. A standard curve was plotted results were expressed as U/ml of SOD as shown in table no3.

Table No 3: Standard curve of SOD

Sample	Mean OD	SOD (U/ml)
Standard 1	0.07	4
Standard 2	0.11	2
Standard 3	0.17	1
Standard 4	0.28	0.5
Standard 5	0.37	0.25
Standard 6	0.50	0.125
Standard 7	0.98	0.0625

RESULT AND DISCUSSION:

Acute Oral Toxicity Study

Animals treated with the newly synthesized compounds were free of any toxicity as per acceptable range given by the OECD guideline no. 423 and no mortality was found up to 1500 mg/kg, which indicates that the lethal dose of the compounds is above 1500 mg/kg body weight in mice and that the compounds can be considered to be safe and can be developed in future. *In vivo* acute oral toxicity study and gross behavioral studies of the newly synthesized compounds is shown in Table no 4 and 5.

Table No 4: Acute Oral Toxicity Study

Treatment	Treatment Body weight (g)		
	0 Day	7 Day	14 th day
NL 2(2)	20.46±0.11	21.33±0.374	22.23±0.66
NL 2(3)	20.46±0.11	20.96±0.66	22.13±0.76
NL 2(4)	20.16±0.11	20.96±0.60	22.03±0.63

Values expressed in mean ± SEM; n=3

Table No 5: *In vivo* acute oral toxicity study and gross behavioral

Group			Additional Observation								Behavioral observation					
	Toxicity	No. of Deaths	Chang	Lacrimation	Eyes	Salivation	Diarrhea	Respiration	Straub Tail	Pilo Erection	Convulsion	Normal	Motor Activity	Stereotypy	Sedation	Tremors	Hypnosis	Muscle Spasm
NL 2(2)	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N
NL 2(3)	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N
NL 2(4)	N	N	N	N	N	N	N	N	N	N	N	Y	N	N	N	N	N	N

Anticonvulsant activity of synthetic compounds by MES induced model:

The maximal electroshock induced convulsion in rats represents grandmal type of epilepsy. The tonic extensor phase is selectively eliminated by the drugs active in generalized tonic Clonic seizure. The most outstanding action of phenytoin showed abolition of tonic extensor phase of MES seizure many drugs that rise the brain content of Gama amino butyric acid (GABA) have shown anticonvulsant activity against seizures induced by MES[17]. There are various molecular mechanisms in which drugs can block seizure spread and or elevate seizure threshold. Indeed, efforts to correlate the anticonvulsant profiles of antiepileptic drugs with specific mechanisms of action expose certain notable trends. MES induced tonic extension can be choked by drugs such as phenytoin, carbamazepine,lamotrigine, felbamate and valproate that inhibit voltage dependent Na+ channels [18]. The extract produced significant decrease in the duration of hind limb tonic extension (HLTE) encouraged by maximal electroshock. NL2(2) NL2(3) to NL2(4) in a dose of 100 mg/kg significantly reduced the duration of HLTE and reduced the mortality. The standard antiepileptic drug phenytoin (20 mg/kg *i.p.*) significantly reduced the duration of HLTE of MES-induced convulsion and completely abolished the various phases of convulsion. The synthesized compounds from series NL2(2) NL2(3) to NL2(4) had performed for activity against the tonic seizure with decreased mean duration of tonic hind leg extension. Reduction in the duration of tonic hind limb extension of MES induced convulsion indicated NL2(2) NL2(3) to NL2(4) has anticonvulsant activity as shown in table no 6.

Table No. 6: Anticonvulsant activity of synthesized compounds

Treatment (dose, mg/kg, i.p)	Maximal electroshock seizure test		
	Mean duration of tonic hindleg extension (THLE) ± SEM (s)	No. of animals recovered	Protection against mortality (%)
Control	09.49 ± 1.07	3/6	**50.00**
Phenytoin (20)	Absence of extension	6/6	**100.00**
NL 2(2)	08.14 ± 0.43	3/6	**50.00**
NL 2(3)	02.21 ± 0.81	6/6	**100.00**
NL 2(4)	13.00 ± 0.79	1/6	**16.66**
Values are expressed in mean ± SEM, where n = 6. *P<0.05, P < 0.001;P<0.01, compared with vehicle treated group			

Biochemical estimation

Antioxidant Enzymes of the brain homogenate of the anticonvulsant activity of synthetic compounds by MES induced model.

Malondialdehyde (MDA) level:

Free radicals have been suggested to be the most commonly candidates responsible for producing the neuronal changes mediating the behavioral deficiency in neurodegenerative. Antioxidants are effective in rodent models of epilepsy, stroke and Alzheimer's disease [19]. The administration of NL2 (2), NL2(3) and NL2(4) at doses of 100mg/kg ip increased the SOD , GSH and CAT levels, and decreased lipid peroxidation in brain tissue which may indicate the antioxidant and free-radical scavenging property of of all synthesized compounds. The effect of synthesized compounds was showed significant inhibition of lipid peroxidation in rat brain tissue as compared to control group. The synthesized compound NL 2(3) at the concentration 100 mg/kg *i.p.*dose showed significant (p<0.001) inhibition in malondialdehyde content compared to control group..Therefore the effect NL 2(3) on oxidative stress in MES induced convulsion was evaluated. Rat represented with NL 2(3) in 100 mg/kg IP dose showed significant decrease in malondialdehyde content in rats brain compared with the control group as shown in table no 7

Table No.7:- Effect of synthesized compounds on Lipid peroxidation level in rat brain tissue in MES induced model.

Experimental group	OD	Lipid peroxidation *n* moles of MDA/mg of protein	Decrease in MDA (%)
Control	1.557	0.998	
Phenytoin (20)	0.764	0.489 ± 0.012**	51.10
NL 2(2)	1.016	0.651 ± 0.004**	35.10
NL 2(3)	0.758	0.485 ± 0.001***	51.50
NL 2(4)	1.164	0.746 ± 0.002*	25.40

All values expressed as mean±SEM; n=6 mice in each group, by one-way ANOVA followed by Dunneet's Multiple Comparison Test (compared with control group) *p<0.05, **p<0.01, and ***p<0.001.

Estimation of reduced Glutathione (GSH):

Glutathione is an antioxidant within the free radicals and prevents the generation of Hydroxy radicals, which are most toxic form of free radicals [19]. The decreased the level of reduced glutathione in control group rat seen in the present study indicates that there was an increased generation of free radicals and that the reduced glutathione was depleted during process of contesting oxidative stress [20]. NL 2(3) in 100 mg/kg dose showed significant increases the GSH level in rat brain as shown in table no 8.

Table No. 8:- Effect of synthesized compounds on glutathione (GSH) level in rat brain tissue.

Experimental group	OD	Glutathione µmoles/mg of protein
Normal	0.020	55.39± 1.68
Control	0.040	49.67± 2.08
Phenytoin (20)	0.025	53.96 ± 1.91***
NL 2(2)	0.031	52.24± 1.18**
NL 2(3)	0.023	54.53 ± 1.21***
NL 2(4)	0.046	47.95± 1.64*

All values expressed as mean±SEM; n=6 mice in each group, by one-way ANOVA followed by Dunneet's Multiple Comparison Test (compared with control group) *p<0.05, **p<0.01, and ***p<0.00

Catalase Activity:

Samples are liquefied in the provided Assay Buffer solution and added to the wells of a half area clear plate. H_2O_2 is added to each well and the plate incubated at room temperature for 30 minutes. The Substrate is added, followed by diluted horseradish *peroxidase* and incubated at 37^0c for 15 minutes. The HRP reacts with the substrate in the presence of hydrogen peroxide to change the colourless substrate into a pink-coloured product. The coloured product is interpreted at 560 nm. Increasing levels of *Catalase* in the samples causes depletion in hydrogen peroxide concentration and a reduction in pink product. The action of the *catalase* in the sample is measured after making a suitable correction for any dilution, using software available with most plate readers. The results are represented in terms of units of *catalase* activity per ml. The improper balance between ROMs (Reactive Oxygen Metabolites) and antioxidant defenses results in "oxidative stress", which deregulates the cellular functions leading to various pathological conditions including cancer [21]. ROMs over production induced by different exogenous and endogenous mechanism may exhaust the antioxidant system of cells and contribute to a number of destructive processes and diseases, including cancer [22]. SOD, CAT constitutes a mutually supportive team of defense against ROS. The present study revealed a marked decline in the levels of these antioxidant enzymes in induced control rat, especially due to the oxidative stress. The free radical scavenging system, superoxide dismutase and *catalase* are present in all oxygen metabolizing cells and their function is to provide a defense against the potentially damaging reactive of superoxide and hydrogen peroxide [23]. NL 2(3) in 100 mg/kg dose showed significant increases the catalase level in rat brain as shown in table no 9.

Table No. 9. Effect of synthesized compounds on Catalase level in rat brain tissue.

Samples	Reading	Catalase (U/ml)
Normal control	0.78	2.10 ± 0.07
Control (induced group)	0.87	1.81 ± 0.04
Phenytoin (20)	0.81	2.01 ± 0.06**
NL 2(2)	0.87	1.81 ± 0.02**
NL 2(3)	0.89	1.75 ± 0.04***
NL 2(4)	0.92	1.65 ± 0.04***

All values expressed as mean±SEM; n=6 mice in each group, by one-way ANOVA followed by Dunneet's Multiple Comparison Test (compared with control group) *p<0.05, **p<0.01, and ***p<0.001

SOD activity:

A bovine erythrocyte SOD standard is provided to produce a standard curve for the assay and all samples should be read off of the standard curve. Samples are diluted in our specially colored Assay Buffer and added to the wells. The Substrate is added followed by *Xanthine Oxidase* Reagent and incubated at 37^0c for 20 minutes. The *Xanthine Oxidase* generates superoxide in the presence of oxygen, which converts a colourless substrate in the Detection Reagent into a yellow colored product. The coloured product is read at 450 nm. Increasing levels of SOD in the samples causes depletion in superoxide concentration and a reduction in yellow product. The activity of the SOD in the sample is calculated after making a suitable modification for any dilution, using software available with most plate readers. The results are represented in terms of units of SOD activity per ml. SOD and Catalase are involved in the clearance of superoxide and hydrogen peroxide (H2O2). SOD catalyses the diminution of superoxide into H2O2 which is eliminated by CAT. Further, it has been reported that a decrease in SOD activity in MES induced group may be due to loss of Mn2+ and mitochondria, leading to a decrease in total SOD activity in brain [24]. The synthesized compound NL 2(2) 100 mg/kg dose restores antioxidant enzymes such as SOD, CAT. The lowering of lipid peroxidation, an increase in levels of SOD and catalase in all treated group indicates its potential as an inhibitor of intracellular oxidative stress. Antioxidant enzymes such as SOD and CAT can directly counter the oxidant attack and may protect cells against LPO and DNA damage. SOD inhibits. OH production [25-27]. NL 2(3) 100 mg/kg dose showed significant increases the SOD level in rat brain as shown in table no 10.

Table No.10:- Effect of synthesized compounds on SOD level in rat brain tissue.

Samples	Reading	SOD (U/ml)
Normal	0.210	2.16 ± 0.09
Control	0.340	1.26 ± 0.01
Phenytoin (20)	0.230	2.02 ± 0.04**
NL 2(2)	0.390	0.92 ± 0.01**
NL 2(3)	0.240	1.95 ± 0.02***

NL 2(4)	0.380	0.99 ± 0.03*

All values expressed as mean±SEM; n=6 mice in each group, by one-way ANOVA followed by Dunneet's Multiple Comparison Test (compared with control group) *p<0.05, **p<0.01, and ***p<0.001.

CONCLUSION

The maximal electroshock induced convulsion in animals represents grandmal type of epilepsy. The tonic extensor phase is selectively eliminated by the drugs effective in unspecified tonic clonic seizure. The most excellent action of phenytoin showed abolition of tonic extensor phase of Maximal Electro-Shock seizure many drugs that increase the brain content of Gama amino butyric acid (GABA) have illustrate anticonvulsant activity against seizures induced by MES. There are several molecular mechanisms through which drugs can block seizure spread and or raise seizure threshold. The synthesized molecules caused significant decrease in the duration of hind limb tonic extension (HLTE) induced by maximal electroshock. NL2(2), NL2(3) and NL2(4) dose of 100 mg/kg significantly reduced the duration of HLTE and reduced the mortality. The standard antiepileptic drug phenytoin (20 mg/kg *i.p.*) significantly reduced the duration of HLTE of MES-induced convulsion and completely abolished the various phases of convulsion.

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