Journal Pre-proof

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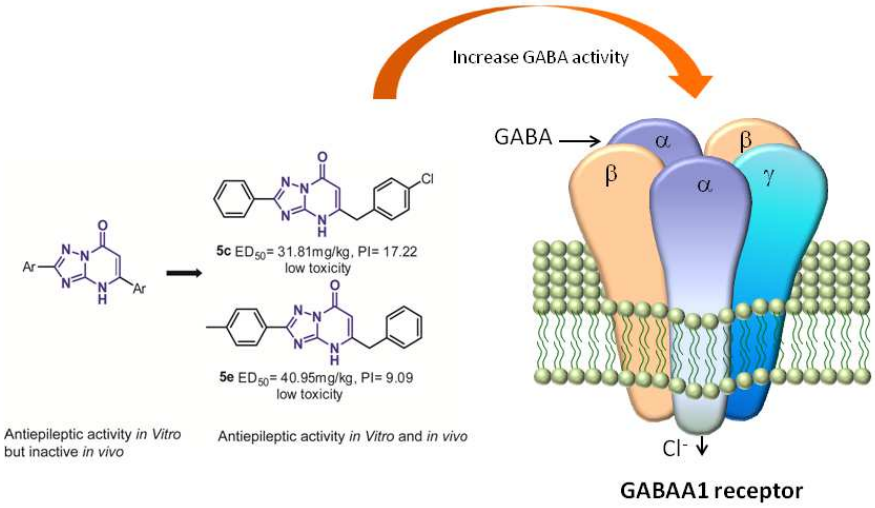
Graph Abstract



Thirty compounds were synthesized based on the structure of 1,2,4-triazopyrimidione. The biological tests showed that compounds 5-(4-chlorobenzyl)-2-phenyl-4H-[1,2,4]-triazole[1,5-a] pyrimidine-7-one (**5c**) and 5-benzyl-2-p-toluene-4H-[1,2,4] triazopyrimidine-7-one (**5e**) exhibited very pronounced anticonvulsant activity and lower neurotoxicity. Moreover, these compounds had specific effects on $GABA_{A1}$ receptor targets as positive modulators.

TITLE PAGE

Discovery of [1,2,4]-triazolo [1,5-a]pyrimidine-7(4H)-one derivatives as positive modulators of $GABA_{A1}$ receptor with potent anticonvulsant activity and low toxicity

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Abstract

In searching for more effective and safer antiepileptic drugs, a series of 2,5-disubstituted [1,2,4]-triazolo[1,5-a]pyrimidine-7(4H)-one derivatives were designed and synthesized. Spontaneous Ca^{2+} oscillations (SCOs) of cortical neurons were used for *in vitro* phenotypic screening. Maximal electroshock test (MES) and pentylenetetrazole (PTZ) test were used to access their anticonvulsant activity, and rotarod test was used to estimate their neurotoxicity. The active compounds in *in vitro* model are specifically effective in pentylenetetrazole (PTZ)-induced epilepsy model but not maximal electroshock (MES) model, more importantly with lower neurotoxicity as compared to commonly used drugs. Among them, compound **5c** and **5e** showed significant anticonvulsant activities in PTZ-induced epilepsy model with ED_{50} values at 31.81 mg/kg and 40.95 mg/kg, respectively. These compounds have improved neurotoxicity with protective index ($PI=TD_{50}/ED_{50}$) values at 17.22 and 9.09, respectively. Finally we demonstrated that compound **5c** and **5e** mainly acted on $GABA_A$ receptor as positive modulators but not sodium channels. Thus the present study has provided potential candidates for further investigation in epilepsy.

Keywords: Epilepsy, anticonvulsant, PTZ, GABA

Abbreviations: SCOs,spontaneous Ca^{2+} oscillations; MES, Maximal electroshock test; PTZ, pentylenetetrazole; SUDEP, sudden unexpected death in epilepsy; AEDs, antiepileptic drugs; 4-AP, 4-Aminopyridine; GABA, gamma-aminobutylic acid; VGSCs, voltage-gated sodium channels; TMS, tetramethylsilane; DIV, days in vitro;FLIPR, Fluorescent Imaging Plate Reader; PI, protective index.

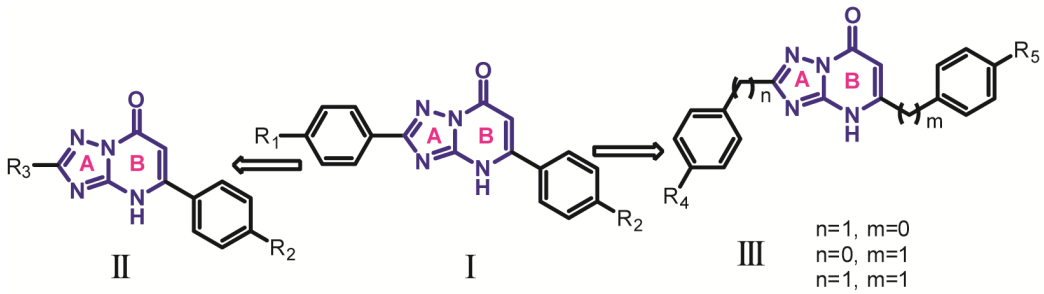
1. Introduction

Epilepsy is one of the most common and serious neurological disorders. It affects approximately 1-2% of the population worldwide [1], and each year more than 1 in 1000 people with epilepsy die from sudden unexpected death in epilepsy (SUDEP) [2]. Although the current drugs provide adequate seizure control, approximately 30% of patients are still poorly treated with the available antiepileptic drugs (AEDs) [3]. Moreover, many AEDs have serious side effects, and lifelong medication may be required. Toxicity, intolerance, lack of efficacy and either unknown or not fully understood mechanism(s) of action are the limitations of the current AEDs [4]. Therefore, it is necessary and imperative to find more effective and safer AEDs, especially with clear action mechanism.

In our previous study [5], series of 2,5-diaryl-[1,2,4]triazolo[1,5-a]pyrimidin-7(4H)-one derivatives were synthesized and tested for their anticonvulsant activity *in vitro*. Compound **Ia** and **Ib** showed good activity with IC_{50} values of 2.35 μM and 3.21 μM, respectively, and pyrimidine-7(4H)-one motif was found to be the necessary "active core" of antiepileptic activity among these compounds. However, the two compounds did not show any effects *in vivo* including the maximal electroshock test (MES) and pentylenetetrazole (PTZ) test. Tracking back to these compounds' structures, we found that the whole molecular structures of compound Ia and Ib look like a large π-conjugate plane. This caused the poor solubility in water (about 0.0143 mg/mL) and organic solvents even in DMSO (about 3mg/mL at 25℃), a solvent commonly used for dissolving compounds in animal models.

To search for compounds with desired therapeutic properties and lower neurotoxicity based on the core structures of compounds **Ia** and **Ib**, we used the strategy to reduce the planarity of

active compound **Ia** and **Ib** to improve the solubility of their analogues in solvents such as DMSO. As shown in **Figure 1**, in order to break the large planar conjugate system of compounds **Ia** and **Ib**, compound **II** (the alkyl chain instead of phenyl ring connecting with ring A of compounds **Ia** and **Ib** such as compound **8** in scheme 1) and compound **III** (analogues of compounds **Ia** and **Ib** through inserting a carbon between the phenyl ring and 1,2,4-tiazolo[1,5-a] pyrimidin-7(4H)-one (ring A and B) such as compound **5-7** in scheme 1) were designed and synthesized. The molecule structure of compound **5-8** is not a large π-conjugate plane any more, which was confirmed by the single crystal structure of compound **5c** in **Figure 2**. As we expected, all of these compounds showed better solubility in DMSO and water (the solubility of compounds **5-8** in DMSO and water is 10-20 times and 5-10 times than that of compounds **Ia** and **Ib,** respectively) and some of them possessed a better anticonvulsant activity *in vitro*. Their structures were characterized using ^{1}H, ^{13}C NMR, HRMS and X-ray single crystal diffraction. Their anticonvulsant activities and neurotoxicity *in vivo* were evaluated by MES and PTZ test and rotarod test. In the meanwhile, the possible mechanism of action was also explored.



Ia: R_1=-H, R_2=-Cl, IC_{50}=2.35 μM; **Ib:** R_1=-CH_3, R_2=-H, IC_{50}=3.21 μM;

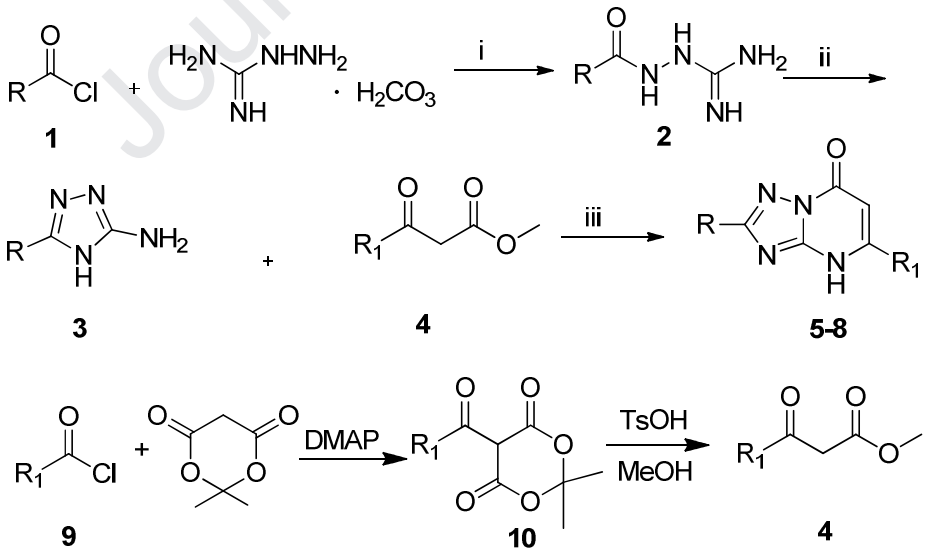
II: R_3=-Alkyl, R_2=H or Cl; **III:** R_4, R_5=-H, -F, -CH_3 or –Cl

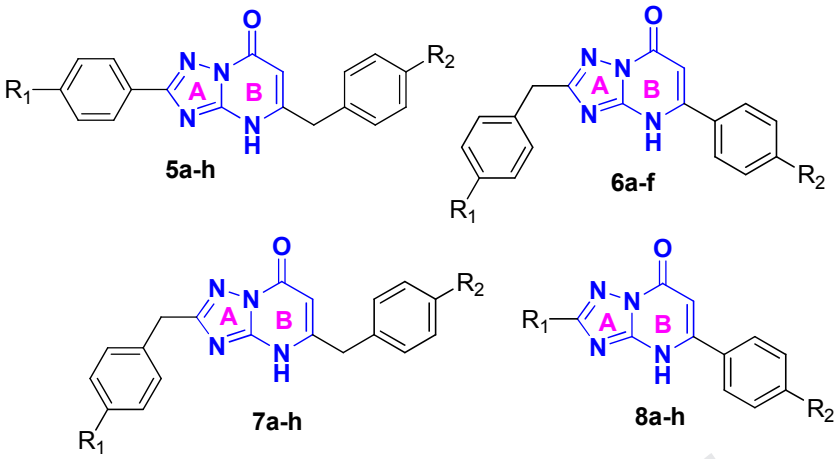
Figure 1： The modification strategy based on active compounds **Ia** and **Ib**

2. Results and discussion

2.1 Chemistry

Compounds were prepared as outlined in **Scheme 1**. Acyl chloride derivatives **1** reacted with aminoguanidine bicarbonate at room temperature using 1,4-dioxane as solvent in the presence of catalytic tetrabutylammonium bromide (TBAB) to afford the hydrazide derivatives **2**. Derivatives **3** (3-amino-1, 2, 4-triazole) was prepared by refluxing compound **2** in water for 12 h, the yield of the two steps is 75-90%. Compound **3** reacted with methyl acetoacetate derivatives **4** to afford **5-8** in n-butanol in the presence of 10 mol% *p*-toluenesulfonic acid (*p*-TsOH) with the yield of 45-85%. The methyl acetoacetate derivatives **4** was prepared by the method reported by Yamamoto [6], acyl chlorides **9** reacted with Meldrum's acid to afford the intermediate **10**, compound **4** was prepared by refluxing of compound **10** in MeOH in the presence of catalytic amount of *p*-TsOH.





Reagents and conditions: i: TBAB, dioxane, rt, 12 h; ii: water, 100□, 12 h; iii: *n*-BuOH, *p*-TsOH, 125□, 24 h.

5a: R_1= -H, R_2= -H; **5b**: R_1= -H, R_2= -4-F; **5c**: R_1= -H, R_2= -4-Cl; **5d**: R_1= -H, R_2= -4-CH_3; **5e**: R_1= -4-CH_3, R_2= -H; **5f**: R_1= -4-CH_3, R_2= -4-F; **5g**: R_1= -4-CH_3, R_2= -4-Cl; **5h**: R_1= -4-CH_3, R_2= -4-CH_3;

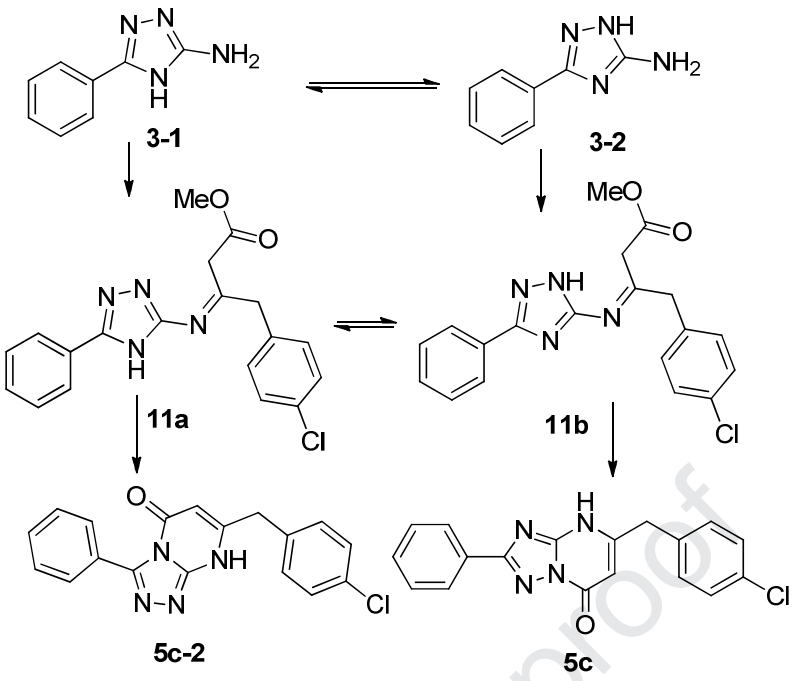
6a: R_1= -H, R_2= -H; **6b**: R_1= -H, R_2= -4-Cl; **6c**: R_1= -4-F, R_2= -H; **6d**: R_1= -4-Cl, R_2= -H; **6e**: R_1= -4-CH_3, R_2= -H; **6f**: R_1= -4-CH_3, R_2= -4-Cl;

7a: R_1= -H, R_2= -H; **7b**: R_1= -H, R_2= -4-Cl; **7c**: R_1= -H, R_2= -4-CH_3; **7d**: R_1= -4-F, R_2= -H; **7e**: R_1= -4-F, R_2= -4-Cl; **7f**: R_1= -4-F, R_2= -4-CH_3;**7g**: R_1= -4-Cl, R_2= -H; **7h**: R_1= -4-CH_3, R_2= -H;

8a: R_1= -$(CH_2)_3CH_3$, R_2= -H; **8b**: R_1= -$(CH_2)_3CH_3$, R_2= -4-Cl; **8c**: R_1= -$(CH_2)_4CH_3$, R_2= -H; **8d**: R_1= -$(CH_2)_4CH_3$, R_2= -4-Cl; **8e**: R_1= -$(CH_2)_5CH_3$, R_2= -H; **8f**: R_1= -$(CH_2)_5CH_3$, R_2= -4-Cl; **8g**: R_1= -$(CH_2)_6CH_3$, R_2= -H; **8h**: R_1= -$(CH_2)_6CH_3$, R_2= -4-Cl;

Scheme 1: Synthetic routes of compounds **5-8**

Taking the synthesis of compound **5c** as an example, as shown in the Scheme 2, compound **3** has two possible resonance structures **3-1** and **3-2** that reacted with compound **4** to afford two possible intermediate **11a** and **11b**. The resonance structure **11a** and **11b** was possibly cyclized to afford pyrimidine-(4H)-one **5c** and **5c-2**, respectively. The structure of the compound was determined as **5c** by X-ray single crystal diffraction (**Figure 2**), which indicates that **11b** is the major transition state in the cyclization reaction of **3** and **4**.



Scheme 2: Possible mechanism of synthesis of compound **5c**

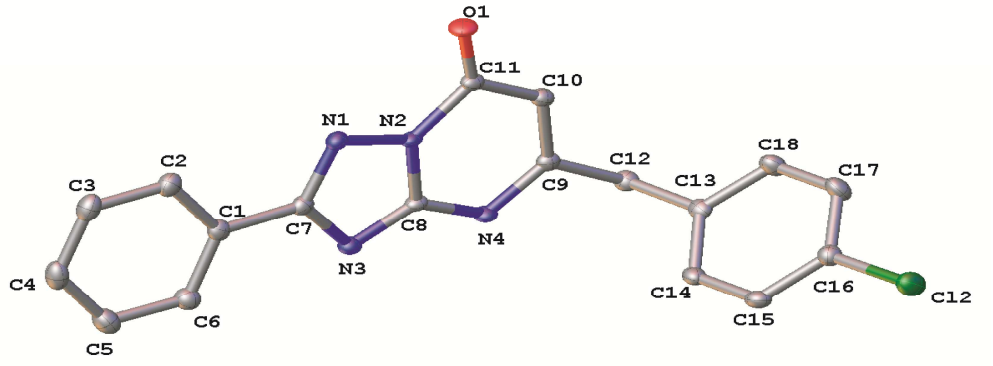


Figure 2：X-ray single crystal diffraction of compound **5c**

2.2 Biological evaluations

2.2.1 Anticonvulsant activity in vitro

Primary cultured neocortical neurons form neuronal networks and display spontaneous Ca^{2+} oscillations (SCOs). These SCOs occur simultaneously with action potential generation. SCO activity is dependent on the balanced excitatory/inhibitory neuronal inputs. 4-Aminopyridine (4-AP) is able to induce hyper-excitability in neurons and has been routinely used to make *in vitro* seizurogenic model widely for antiepileptic activity test [7]. In the present study, totally 30 compounds were tested in 4-AP-induced SCO model in primary

cultured neocortical neurons. As shown in Supplementary data, these compounds **5c**, **5e**, **6a-6f**, **7e-7h**, **8d** and **8f** displayed inhibitory effects on the 4-AP-induced hyper-excitability model expressed as intracellular calcium ion concussion in neurons. Through a continuous assay procedure, different concentrations of compound **5c**, **5e**, **6a**, **6c-6f**, **7e-7h**, **8d** and **8f** were tested. As shown in Table 1, the IC_{50} values ranged from 0.33 μM to 8.25 μM. Among these compounds, **5c** and **5e** displayed more potent anticonvulsant activity (IC_{50} 0.33 μM and 0.45 μM, respectively) significantly above the positive control carbamazepine (IC_{50} 35 μM).

Table 1. IC_{50} values of **13** active compounds *in vitro*

Compounds	4-AP inducedhyper-excitability Model（IC_{50}，μM)）
5c	0.452
5e	0.334
6a	1.31
6c	2.52
6d	1.83
6e	1.15
6f	5.44
7e	3.65
7f	3.02
7g	4.28
7h	2.47
8d	8.25
8f	8.72

2.2.2 Anticonvulsant activity in vivo

2.2.2.1 Phase I preliminary evaluation of anticonvulsant activity in mice - MES and sc-PTZ tests

All the compounds tested in *in vitro* model were further screened for their anticonvulsant activity in mice by using the popular acceptable seizure models- Maximal electroshock(MES) test and subcutaneous pentylenetetrazol (sc-PTZ) test [8, 9]. In this study, DMSO was used as solvents, which has been frequently used to dissolve compounds in screenings for anticonvulsant compounds. As shown in Table 2, some of the test compounds

were active on the sc-PTZ model, but all of the compounds were inactive in the MES model. Interestingly, the results from *in vivo* PTZ model are very consistent with that from *in vitro* model, which indicates that the active compounds *in vitro* could be confirmed in sc-PTZ test specifically. The results demonstrated that the cellular model *in vitro* is suitable for preliminary screening of AEDs. At the lower dose 30 mg/kg, only **5c**, **5e**, **6c**, **6d** and **6e** were able to decrease the seizure behaviors; when the dose was increased to 100 mg/kg, more compounds displayed anti-seizure effects including **5c**, **5e**, **5g**, **6a**, **6c**, **6d**, **6e**, **7b** and **7h** (see supplementary data); and at the dose of 300 mg/kg, most compounds showed protection except **8a-b**, **8g-h** against the sc-PTZ-induced seizure model (see supplementary data).

Table 2 Phase I preliminary evaluation of anticonvulsant activity in mice on MES and sc-PTZ models

Compounds	MES (mg/kg)			SC-PTZ (mg/kg)		
	30	100	300	30	100	300
5c	0/3	0/3	***1/3***	***3/3***	***3/3***	***3/3***
5e	0/3	0/3	0/3	***3/3***	***3/3***	***3/3***
6a	0/3	0/3	0/3	***1/3***	***2/3***	***3/3***
6c	0/3	0/3	0/3	***2/3***	***2/3***	***33***
6d	0/3	0/3	***1/3***	***2/3***	***2/3***	***3/3***
6e	0/3	0/3	0/3	***2/3***	***2/3***	***3/3***
6f	0/3	0/3	***1/3***	0/3	0/3	***2/3***
7e	0/3	0/3	0/3	0/3	***1/3***	***3/3***
7f	0/3	0/3	0/3	0/3	***1/3***	***2/3***
7g	0/3	0/3	0/3	0/3	***1/3***	***2/3***
7h	0/3	0/3	***1/3***	***1/3***	***2/3***	***3/3***
8d	0/3	0/3	***1/3***	***1/3***	***1/3***	***2/3***
8f	0/3	0/3	0/3	***1/3***	***1/3***	***3/3***

Note: All drugs were administered by the intraperitoneal (ip) route. The effective compounds were shown in positive animal number out of the total animal number (n=3) and displayed in bold italic

2.2.2.2 Phase II quantitative anticonvulsant evaluation in Mice using sc-PTZ test

Based on the result of preliminary screening, compound **5c**, **5e**, **6c**, **6d** and **6e** were further

moved to phase II trials to quantify their anticonvulsant activity (ED_{50}) using sc-PTZ test and neurotoxicity (TD_{50}) using rotarod test in mice. Results of the quantitative test for selected compounds, standard tool drug Carbamazepine, Phenytoin, Phenobarbital and Valproate are shown in Table 3. Among them, compound **5c** and **5e** gave an ED_{50} value of 31.81 mg/kg, 40.95 mg/kg, and a TD_{50} value of 547.89 mg/kg, 372.39 mg/kg, respectively. Both compounds displayed higher protective index (PI) value-17.22 and 9.09 than the four tool drugs. Compound **5c** and **5e** showed nearly equipotent activity, much higher than carbamazepine, phenytoin and valproate, and slightly weaker than phenobarbital. The neurotoxicity of the compound **5c** and **5e**, especially **5c** (PI=17.22) is much weaker than the four standard drugs. Compound **6c**, **6d** and **6e** showed moderate activity with ED_{50} value of 71.63 mg/kg, 61.09 mg/kg and 65.79 mg/kg, and a TD_{50} value of 261.35 mg/kg, 210.71 mg/kg and 280.53 mg/kg, respectively. The toxicity of them is much weaker than phenytoin, valproate and carbamazepine, and higher than phenobarbital.

Table 3 Phase II quantitative anticonvulsant evaluation in Mice using PTZ test

Compounds	[a]ED_{50} (mg/kg) (sc-PTZ)	[b]TD_{50}（mg/kg）	[c]PI
5c	**31.81 [e](17.66-56.62)**	**547.89（400.86-746.44）**	**17.22**
5e	40.95 (24.54-68.07)	372.39（216.27-639.73）	9.09
6c	71.63(37.94-135.8)	261.35（153.81-442.58）	3.67
6d	61.09(33.50-111.43)	210.71（126.47-350.75）	3.45
6e	65.79(35.70-122.7)	280.53（168.26-466.65）	4.26
Phenobarbital[d]	13.2(5.8-15.9)	69 (62.8-72.9)	5.20

Valproate[d]	149(123-177)	426 (369-450)	2.90
Phenytoin[d]	>300	65.5 (52.5-72.9)	<0.22
Carbamazepine[d]	>100	71.6 (45.9-135)	<0.76

a) Dose measured in mg/kg. b) Minimal neurotoxicity was determined using the rotarod test after test compounds were administered for 30min. c) $PI=TD_{50}/ED_{50}$. d) Data from Ucar et al., 1998. [10] e) 95% confidence limits.

*2.3 Mechanism study of compound **5c** and **5e***

In searching for potential antiepileptic compounds, it is crucial to precisely define their molecular mechanism of action. A clear molecular mechanism of anticonvulsant action would significantly facilitate the process of designing potential antiepileptic drugs based on the present discovery like the 1,2,4-triazole skeleton structure. However, this has been difficult because many AEDs have multiple molecular targets [11].

Encouraged by the promising data generated in the present study, we initiated studies aiming to understand the molecular mechanism of these active compounds. Due to the selective activity of these compounds in PTZ model, $GABA_{A1}$ receptor would be the first candidate of the epilepsy targets to be considered. We have ever done some molecular docking study for these compounds on $GABA_{A1}$ receptor [5], which also indicated the possible binding of these compounds on $GABA_{A1}$ receptor. Therefore we tested the effects of these compounds on $GABA_{A1}$ receptor. In the meanwhile, these compounds were also tested on voltage-gated sodium channels.

2.3.1 Effect on the $GABA_{A1}$ receptor

GABA is the main inhibitory neurotransmitter substance in the brain, and is widely implicated in epilepsy. Inhibition of GABAergic neurotransmission has been shown to promote and facilitate seizures, and enhancement of GABAergic neurotransmission is known

to inhibit or attenuate seizures [12]. PTZ has been reported to produce seizures by inhibiting gamma-aminobutylic acid (GABA) neurotransmission [13]. The present study found that compounds **5c** and **5e** specifically attenuated PTZ-induced seizures but not MES-induced seizures in mice, which indicated that these compounds are very likely involved in the GABAergic neurotransmission process. Therefore, we further determined whether $GABA_{A1}$ receptors are involved in the antiepileptic effect of compounds **5c** and **5e**. By using membrane potential FLIPR assay [14], when compound **5c** and **5e** were added directly to the cells, they could not induce any activation of the $GABA_{A1}$ receptor (data not shown), however, in the presence of GABA (0.2 μM), it was found that compounds **5c** and **5e** could increase GABA-induced activation of $GABA_{A1}$ receptor (**Figure 3**, **A and C**) in a dose-dependent manner with EC_{50} value of 3.08 ± 1.08 μM and 12.02 ± 1.29 μM, respectively (**Figure 3, B and D**). The dose response curves were fitted with Hill equation by Origin 6.0 software.

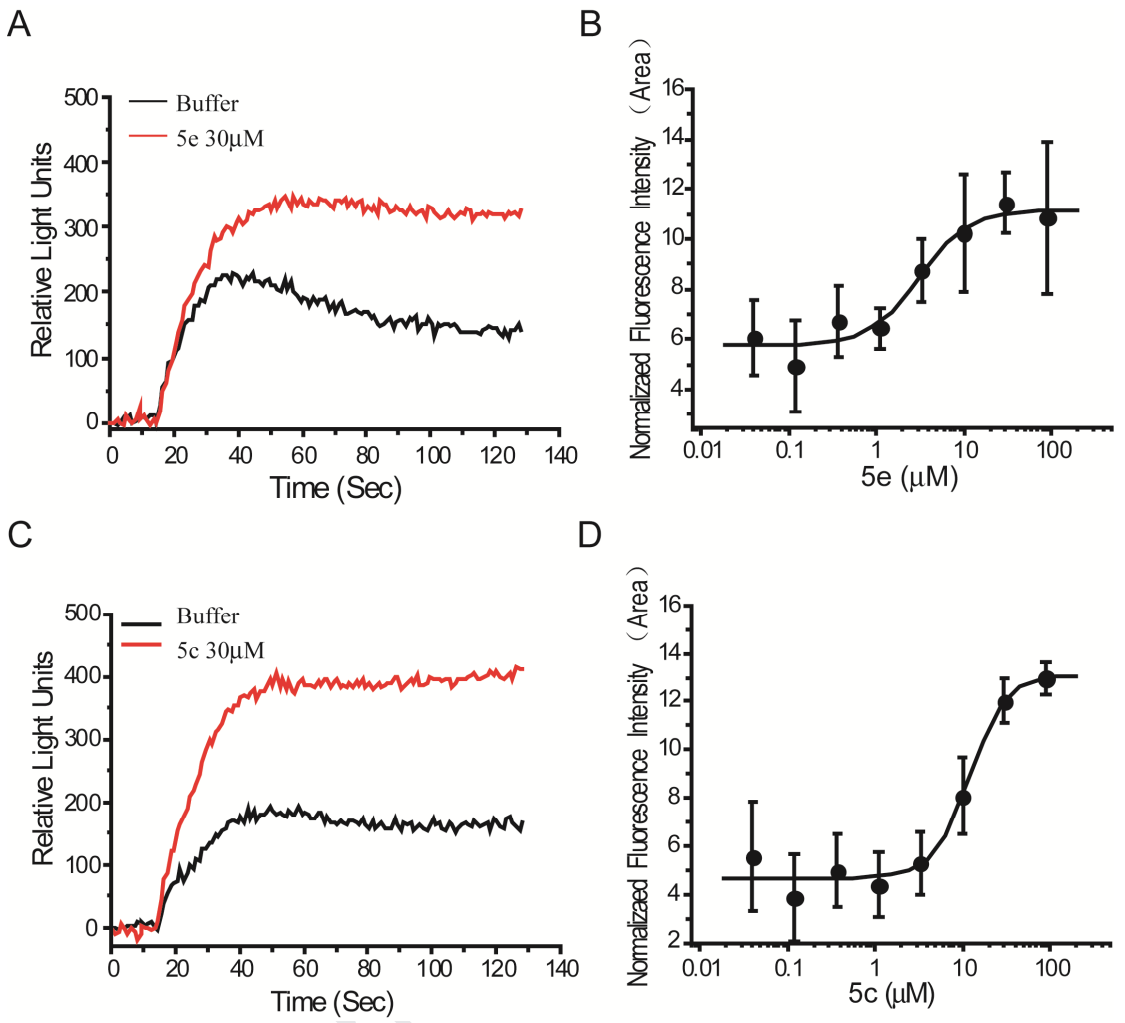


Figure 3: Dose-response curves of compounds **5c** and **5e** on $GABA_{A1}$ receptor in the presence of GABA (0.2 μM)

These data indicated that the two compounds are likely positive allosteric modulators of GABA receptor. Therefore, the effects of **5e** and **5c** on GABA-induced dose-response activation of $GABA_{A1}$ receptor were further studied. GABA-induced dose-response activation of $GABA_{A1}$ receptor was shown in **Figure 4 (A and B)** (Black filled circles, EC_{50}=0.25±0.03 μM). The effect of **5e** and **5c** at 30 μM on GABA EC_{50} was shown in A and B (Red filled circles), respectively. In the presence of **5e** 30 μM, GABA-induced signal was increased but EC_{50} of GABA was not altered significantly (GABA EC_{50}=0.24±0.04 μM). However, compound **5c** (30 μM) not only increased GABA-induced signal, but also leftward-shifted

EC_{50} curve of GABA (EC_{50}=0.20±0.04 μM). The results indicate that compounds **5c** and **5e** might be allosterically modulating the function of GABA receptors.

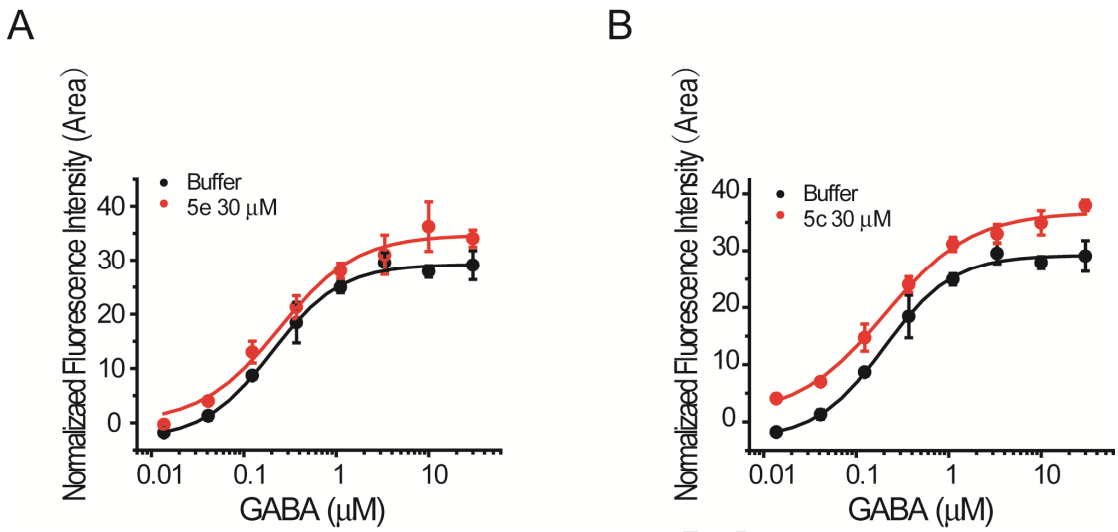


Figure 4. The effect of **5e** and **5c** on GABA-induced dose response curve of $GABA_A$ receptor

2.3.2 Effect on the voltage-gated sodium channels (VGSCs)

Furthermore, voltage-gated sodium channels are also playing important roles in the generation of epilepsy [15]. To determine whether these compounds affected VGSCs, the electrophysiological study of compound **5c** and **5e** on VGSCs was carried out. At 10μM, the two compounds did not show any effect on neuronal sodium channels (data not shown). Further a full dose response curve for one of them (compound **5e**) was conducted. As shown in **Figure 5**, only compound **5e** at 100 μM showed some extent of blockade effect on VGSCs.

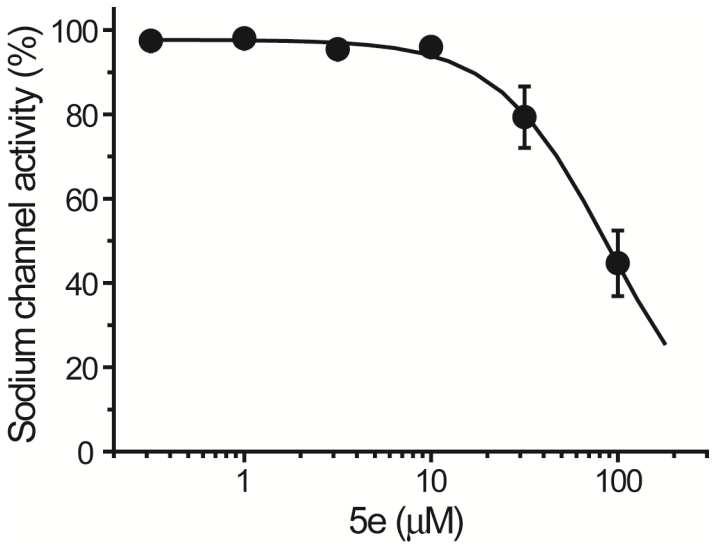


Figure 5. Effect of compound **5e** on voltage-gated sodium channels in neurons

3. Conclusion

In conclusion, a series of anticonvulsants **[**1,2,4]-triazolo [1,5-a]pyrimidine-7(4H)-one derivatives have been designed and synthesized. Two compounds **5c** and **5e** exhibited more potent anticonvulsant activity as compared to commonly used drugs. More importantly, these compounds displayed a clear action mechanism on GABAergic neurotransmission with a very lower neurotoxicity and a higher protective index. These properties are very critical for the compounds to be explored as potential drug candidates for further investigations.

4. Experimental Sections

4.1 Chemistry

All of the solvents and materials used in the present study were analytical grade. General ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker-500 (Bruker bioscience, Billerica, MA, USA), and chemical shifts were given in ppm relative to tetramethylsilane (TMS). Mass spectra were measured on an UPLC-Q/TOF Xevo G2-XS (Waters, USA). All chemical shifts are reported as d (ppm) values. Splitting patterns are designated as follows: s: singlet; d: doublet; t: triplet; q: quartet; and m: multiplet.

*4.1.1 Synthesis of 3-amino-1,2,4-triazole derivatives (**3**)[5]*

To a cooled suspension of aminoguanidine carbonate (20.0mmol), catalytic tetrabutylammonium bromide (TBAB) (1.0 mmol) in dioxane (50 mL) was added acyl chloride derivatives **1** (10.0 mmol) dropwise at 0□ over 0.5 h, after that, the reaction mixture was allowed to warm to room temperature and stirred for 12 h at room temperature. The resulting mixture was concentrated under vacuum to give a residue, which was dissolved in water (30 mL) and adjusted to pH 12 with sodium hydroxide. The precipitate was collected by

filtration to gain intermediate 2 which was used for next step without further purification, and intermediate **2** was suspended in water (100mL), the resulting mixture was stirred overnight at 100□. The reaction was cooled down to room temperature; the product was precipitated and collected by filtration and dried in an oven at 40□ under reduced pressure to afford compound **3**. The yield is in the range of 75-90%.

*4.1.2 Synthesis of methyl acetoacetate derivatives **(4)**[6]*

A solution of an acid chloride (26 mmol) in dry CH_2Cl_2 (15 mL) was added dropwise with stirring over a period of 1h to a solution of Meldrum's acid (26 mmol) and pyridine (65 mmol) in dry CH_2Cl_2 (50 mL) in an ice bath. The temperature was maintained for an additional 1h, and then the reaction mixture was allowed to warm to room temperature and stirred at room temperature for 16h. After the resulting mixture was washed with 3 mol/L HCl (3×100 mL) and water (50 mL) sequentially, the organic layer was separated, dried over $MgSO_4$ and concentrated under reduced pressure. The residue was dissolved in methanol (50 mL) and the resulting solution was heated to reflux for 6 h. After the solvent was evaporated under vacuum, the residue was purified by flash column using PE/EA=10:1 as the eluent to afford compound **4**. The yield is around 85-93%.

*4.1.3 Synthesis of [1,2,4]triazolo[1,5-a]pyrimidin -7-one derivatives **(5-8)**[5]*

A mixture of compound **3** (5.7 mmol), compound **4** (11.5 mmol) and p-TsOH (1.5 mmol) in n-BuOH (15 mL) was stirred at 125 □ for 24 h - 48 h. After the volatile was evaporated under vacuum, the residue was purified by flash column using DCM/Methanol=20:1 as the eluent to afford the compound **5-8** with yield in the range of 45-85%.

*4.1.3.1. 5-Benzyl-2-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5a**)*

White solid, yield 85%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.53 (s, 1H), 8.14 (d, *J*= 6.15 Hz, 2H), 7.56(d, *J*= 6.7Hz, 3H), 7.44 (d, *J*= 7.35 Hz, 2H), 7.39 (d, *J*= 7.7 Hz, 2H), 7.32 (d, *J*= 7.0 Hz, 1H), 5.88 (s, 1H), 3.99 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 37.93, 98.67, 126.55 (2C), 127.04, 128.62 (2C), 128.84 (2C), 128.95 (2C), 130.04, 130.28, 136.42, 151.39, 153.64, 155.81, 160.90. HRMS (ESI$^+$) m/z calcd for $C_{18}H_{15}N_4O$ $[M + H]^+$ 303.1246, found 303.1249. Anal. Calcd for $C_{18}H_{14}N_4O$; C, 71.51; H, 4.67; N, 18.53. Found: C, 71.41; H, 4.45; N, 18.43.

*4.1.3.2. 5-(4-Fluoro-benzyl)-2-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5b**)*

White solid, yield 82%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.49 (s, 1H), 8.12-8.1(m, 2H), 7.56-7.52(m, 3H), 7.47-7.44 (m, 2H), 7.20 (t, *J*= 8.9 Hz, 2H), 5.86(s, 1H), 3.96(s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 37.06, 98.65, 115.27, 115.44, 126.55 (2C), 128.62 (2C), 128.84 (2C), 130.29, 130.91, 130.97, 132.58, 151.40, 153.52, 155.81, 160.35, 160.88, 162.28. HRMS (ESI$^+$) m/z calcd for $C_{18}H_{14}N_4OF$ $[M + H]^+$ 321.1152, found 321.1150. Anal. Calcd for $C_{18}H_{13}N_4OF$; C, 67.49; H, 4.09; N, 17.49, . Found: C, 67.32; H, 4.22; N, 17.69.

*4.1.3.3. 5-(4-Chloro-benzyl)-2-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5c**)*

White solid, yield 84%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.46 (s, 1H), 8.08(d, *J*= 7.8Hz, 2H), 7.51(d, *J*= 5.4Hz, 3H), 7.4 (s, 4H), 5.9 (s, 1H), 3.93 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 37.70, 99.31, 127.05 (2C), 129.05 (2C), 129.35 (2C), 130.41, 130.81, 131.35 (2C), 132.30, 135.96, 151.40, 153.14, 155.79, 160.35, 160.89. HRMS (ESI$^+$) m/z calcd for

$C_{18}H_{14}N_4OCl$ $[M + H]^+$ 337.0856, found 337.0857. Anal. Calcd for $C_{18}H_{13}N_4OCl$; C, 64.20; H, 3.89; N, 16.64. Found: C, 64.36; H, 3.51; N, 16.86.

*4.1.3.4. 5-(4-Methyl-benzyl)-2-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5d**)*

White solid, yield 79%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.47 (s, 1H), 8.12-8.1 (dd, *J*=, 2H), 7.55-7.49(m, 3H), 7.29 (d, *J*= 7.9 Hz, 2H), 7.16 (d, *J*= 7.9, 2H), 5.82 (s, 1H), 3.90 (s, 2H), 2.28 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 20.60, 37.56, 98.50, 126.55 (2C), 129.85 (4C), 129.17 (2C), 130.04, 130.28, 133.31, 136.19, 151.38, 153.95, 155.81, 160.89. HRMS (ESI^+) m/z calcd for $C_{19}H_{17}N_4O$ $[M + H]^+$ 317.1402, found 317.1403. Anal. Calcd for $C_{19}H_{16}N_4O$; C, 72.13; H, 5.10; N, 17.71. Found: C, 72.16; H, 5.14; N, 17.89.

*4.1.3.5. 5-Benzyl-2-p-tolyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5e**)*

White solid, yield 75%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.45 (s, 1H), 7.98(d, *J*= 8.1 Hz, 2H), 7.40-7.26(m, 7H), 5.83 (s, 1H), 3.95 (s, 2H), 2.36 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 20.60, 37.56, 98.50, 126.55 (2C), 129.85 (4C), 129.17 (2C), 130.04, 130.28, 133.31, 136.19, 151.38, 153.95, 155.81, 160.89. HRMS (ESI^+) m/z calcd for $C_{19}H_{17}N_4O$ $[M + H]^+$ 317.1402, found 317.1404. Anal. Calcd for $C_{19}H_{16}N_4O$; C, 72.13; H, 5.10; N, 17.71. Found: C, 72.19; H, 5.14; N, 17.61.

*4.1.3.6. 5-(4-Fluoro-benzyl)-2-p-tolyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5f**)*

White solid, yield 77%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.44 (s, 1H), 7.98 (d, *J*= 8.1Hz, 2H), 7.44 (t, *J*=5.7 Hz, 2H), 7.32 (d, *J*=8.0 Hz, 2H), 7.18 (t, *J*=8.8 Hz, 2H), 5.84 (s, 1H), 3.94 (s, 2H), 2.36 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 21.43, 37.50, 98.08, 115.72, 115.89, 126.96 (2C), 127.72, 129.86 (2C), 131.37 (2C), 133.06, 140.47, 151.78, 153.85, 155.81, 156.24, 160.78, 162.72. HRMS (ESI$^+$) m/z calcd for $C_{19}H_{16}N_4OF$ $[M + H]^+$ 335.1308, found 335.1305. Anal. Calcd for $C_{19}H_{15}N_4OF$; C, 68.25; H, 4.52; N, 16.76. Found: C, 68.47; H, 4.22; N, 16.84.

*4.1.3.7. 5-(4-Chloro-benzyl)-2-p-tolyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5g**)*

White solid, yield 79%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.42 (s, 1H), 7.96 (d, *J*= 8.1 Hz, 2H), 7.39 (s, 4H), 7.30 (d, *J*=8.1Hz, 2H), 7.18 (t, *J*=8.8Hz, 2H), 5.83 (s, 1H), 3.92 (s, 2H), 2.34 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 21.43, 37.62, 99.23, 126.96 (2C), 127.70, 129.00 (2C), 129.86 (2C), 131.29 (2C), 132.23, 135.94, 140.47, 151.77, 153.43, 156.22, 161.40. HRMS (ESI$^+$) m/z calcd for $C_{19}H_{16}N_4OCl$ $[M + H]^+$ 351.1013, found 351.1014. Anal. Calcd for $C_{19}H_{15}N_4OCl$; C, 65.05; H, 4.31; N, 15.97. Found: C, 65.31; H, 4.06; N, 15.96.

*4.1.3.8. 5-(4-Methyl-benzyl)-2-p-tolyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**5h**)*

White solid, yield 45%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.43 (s, 1H), 7.99 (d, *J*= 8.1Hz, 2H), 7.33 (d, *J*=8.1 Hz, 2H), 7.28 (d, *J*=8.0 Hz, 2H), 7.16 (d, *J*=7.9 Hz, 2H), 5.80 (s, 1H), 3.90 (s, 2H), 2.37 (s, 3H), 2.28 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 21.05, 21.43, 37.97, 98.91, 126.96 (2C), 127.77, 129.29 (2C), 129.61 (2C), 129.86 (2C), 133.78, 136.62,

140.44, 151.76, 154.27, 156.24, 161.42. HRMS (ESI^+) m/z calcd for $C_{20}H_{19}N_4O$ $[M + H]^+$ 331.1559, found 331.1562. Anal. Calcd for $C_{20}H_{18}N_4O$; C, 72.71; H, 5.49; N, 16.96. Found: C, 72.58; H, 5.67; N, 16.90.

*4.1.3.9. 2-Benzyl-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**6a**)*

White solid, yield 83%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.5 (s, 1H), 7.87 (d, *J*= 6.7 Hz, 2H), 7.56-7.52(m, 3H), 7.37-7.32 (m, 4H), 7.26 (d, *J*=7.1 Hz, 1H), 6.33 (s, 1H), 4.12 (s, 2H); ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 34.45, 98.08, 127.09, 127.83 (2C), 128.89 (2C), 129.37 (3C), 129.48 (3C), 131.43, 133.37, 137.39, 151.70, 156.30. HRMS (ESI^+) m/z calcd for $C_{18}H_{15}N_4O$ $[M + H]^+$ 303.1246, found 303.1248. Anal. Calcd for $C_{18}H_{14}N_4O$; C, 71.51; H, 4.67; N, 18.53. Found: C, 71.64; H, 4.61; N, 18.46.

*4.1.3.10. 2-Benzyl-5-(4-chloro-phenyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**6b**)*

White solid, yield 80%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.61 (s, 1H), 7.92 (d, *J*= 8.0 Hz, 2H), 7.59 (d, *J*= 8.3 Hz, 2H), 7.37-7.32 (m, 4H), 7.26 (d, *J*=7.1 Hz, 1H), 6.40 (s, 1H), 4.13 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 33.59, 97.96, 126.68, 128.00, 128.42 (2C), 128.56, 128.67, 128.84 (2C), 128.97 (2C), 129.11 (2C), 135.63, 136.51, 151.08, 155.75. HRMS (ESI^+) m/z calcd for $C_{18}H_{14}N_4OCl$ $[M + H]^+$337.0856, found 337.0859. Anal. Calcd for $C_{18}H_{13}N_4OCl$; C, 64.20; H, 3.89; N, 16.64. Found: C, 64.02; H, 3.91; N, 16.80.

*4.1.3.11. 2-(4-Fluoro-benzyl)-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**6c**)*

Light yellow solid, yield 73%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.45 (s, 1H), 7.86 (s, 2H), 7.53 (s, 3H), 7.39 (s, 2H), 7.15 (s, 3H), 6.32 (s, 1H), 4.13 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 33.61, 98.03, 115.56, 115.50, 127.84 (4C), 129.37 (4C), 131.34, 131.41, 151.79, 156.30, 160.61, 162.54. HRMS (ESI$^+$) m/z calcd for $C_{18}H_{14}N_4OF$ $[M + H]^+$ 321.1152, found 321.1155. Anal. Calcd for $C_{18}H_{13}N_4OF$; C, 67.49; H, 4.09; N, 17.49. Found: C, 67.41; H, 4.11; N, 17.55.

*4.1.3.12. 2-(4-Chloro-benzyl)-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**6d**)*

White solid, yield 70%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.61 (s, 1H), 7.86 (s, 2H), 7.55 (s, 3H), 7.39 (s, 4H), 6.32 (s, 1H), 4.13 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 30.42, 97.59,125.45, 127.37,128.03 (2C), 128.31 (2C), 128.50 (2C), 128.89 (2C), 134.20, 137.66, 145.55, 149.91, 151.25, 155.76. HRMS (ESI$^+$) m/z calcd for $C_{18}H_{14}N_4OCl$ $[M + H]^+$337.0856, found 337.0857. Anal. Calcd for $C_{18}H_{13}N_4OCl$; C, 64.20; H, 3.89; N, 16.64. Found: C, 64.26; H, 3.85; N, 16.62.

*4.1.3.13. 2-(4-Methyl-benzyl)-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**6e**)*

White solid, yield 68%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.39 (s, 1H), 7.86 (s, 2H), 7.54 (s, 3H), 7.13 (s, 4H), 6.33 (s, 1H), 4.06 (s, 2H), 2.27 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 20.57, 33.49, 97.55, 125.47, 127.31 (2C), 128.02, 128.53, 128.83 (2C), 128.86 (2C), 128.93 (2C), 130.89, 133.75, 135.65, 151.19, 155.82. HRMS (ESI$^+$) m/z calcd for $C_{19}H_{17}N_4O$

$[M + H]^+$ 317.1402, found 317.1405. Anal. Calcd for $C_{19}H_{16}N_4O$; C, 72.13; H, 5.10; N, 17.71. Found: C, 72.19; H, 5.07; N, 17.68.

4.1.3.14. 5-(4-Chloro-phenyl)-2-(4-methyl-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one ***(6f)***

Light brown solid, yield 63%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.42 (s, 1H), 7.96 (d, *J*=8.1 Hz, 2H), 7.39(s, 4H), 7.30 (d, *J*=8.1 Hz, 2H), 5.83 (s, 1H), 3.92 (s, 2H), 2.34 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 20.59, 30.79, 97.97, 125.45, 128.04, 128.56, 128.84 (2C), 128.98 (2C), 129.12 (2C), 132.23, 135.59, 135.78, 136.15, 137.66, 151.04, 155.77. HRMS (ESI^+) m/z calcd for $C_{19}H_{16}N_4OCl$ $[M + H]^+$ 351.1013, found 351.1014. Anal. Calcd for $C_{19}H_{15}N_4OCl$; C, 65.05; H, 4.31; N, 15.97. Found: C, 65.11; H, 4.33; N, 15.89.

*4.1.3.15. 2,5-Dibenzyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7a**)*

White solid, yield 79%; 1H-NMR: (DMSO-d6, 500 MHz):δ 13.32 (s, 1H), 7.42-7.38 (m, 4H), 7.33- 7.29 (m, 4H), 7.24-7.21 (m, 2H), 5.81 (s, 1H), 4.04 (s, 2H), 3.96 (s, 2H). 13C-NMR: (DMSO-d6, 125 MHz):δ 34.40, 37.85, 98.46, 126.40, 126.99, 128.28 (2C), 128.60 (2C), 128.89 (4C), 136.46, 137.41, 150.95, 153.38, 155.73, 163.50. HRMS (ESI+) m/z calcd for $C_{19}H_{17}N_4O$ $[M + H]^+$ 317.1402, found 317.1403. Anal. Calcd for $C_{19}H_{16}N_4O$; C, 72.13; H, 5.10; N, 17.71. Found: C, 72.36; H, 5.16; N, 17.42.

*4.1.3.16. 2-Benzyl-5-(4-chloro-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7b**)*

White solid, yield 82%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.26 (s, 1H), 7.41-7.37 (m, 4H), 7.32- 7.28 (m, 4H), 7.24-7.21 (m, 1H), 5.81 (s, 1H), 4.04 (s, 2H), 3.96 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 34.38, 37.13, 98.62, 126.41, 128.28 (2C), 128.52 (2C), 128.89 (2C), 130.79 (2C), 131.73, 135.51, 137.36, 150.95, 152.86, 155.71, 163.45. HRMS (ESI^+) m/z calcd for $C_{19}H_{16}N_4OCl$ $[M + H]^+$ 351.1013, found 351.1016. Anal. Calcd for $C_{19}H_{15}N_4OCl$; C, 65.05; H, 4.31; N, 15.97. Found: C, 65.34; H, 4.16; N, 15.83.

*4.1.3.17. 2-Benzyl-5-(4-methyl-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7c**)*

White solid, yield 58%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.24 (s, 1H), 7.30 (s, 4H), 7.22 (s, 3H), 7.14 (s, 2H), 5.74 (s, 1H), 4.04 (s, 2H), 3.84 (s, 2H), 2.26 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 20.58, 34.38, 37.54, 98.31, 126.39, 128.28 (2C), 128.79 (2C), 128.88 (2C), 129.14 (2C), 133.37, 136.13, 137.40, 150.94, 153.74, 155.73, 163.44. HRMS (ESI^+) m/z calcd for $C_{20}H_{19}N_4O$ $[M + H]^+$ 331.1559, found 331.1563. Anal. Calcd for $C_{20}H_{18}N_4O$; C, 72.71; H, 5.49; N, 16.96. Found: C, 72.45; H, 5.36; N, 16.83.

*4.1.3.18. 5-Benzyl-2-(4-fluoro-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7d**)*

White solid, yield 67%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.28 (s, 1H), 7.34 (d, *J*= 5.9 Hz, 6H), 7.27 (s, 1H), 7.12 (d, *J*= 8.8 Hz, 2H), 5.78 (s, 1H), 4.05 (s, 2H), 3.9 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 33.48, 37.91, 98.50, 114.89, 115.06, 127.00, 128.59 (2C), 128.89 (2C), 130.79, 133.54, 136.46, 150.98, 153.41, 155.72, 160.01, 161.93, 163.33. HRMS

(ESI$^+$) m/z calcd for $C_{19}H_{16}N_4OF$ $[M + H]^+$ 335.1308, found 335.1309. Anal. Calcd for $C_{19}H_{15}N_4OF$; C, 68.25; H, 4.52; N, 16.76. Found: C, 68.28; H, 4.69; N, 16.56.

*4.1.3.19. 5-(4-Chloro-benzyl)-2-(4-fluoro-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7e**)*

Light yellow solid, yield 71%; ^{1}H-NMR: (DMSO-d_6, 500 MHz):δ 13.28 (s, 1H), 7.39-7.34 (m, 6H), 7.12 (t, J= 8.7, 2H), 5.81 (s, 1H), 4.04 (s, 2H), 3.9 (s, 2H); ^{13}C-NMR: (DMSO-d_6, 125 MHz):δ 33.95, 37.71, 99.15, 115.38, 115.55, 129.37 (2C), 131.28 (4C), 132.24, 134.00, 136.02, 151.47, 153.43, 156.20, 160.51, 162.44. HRMS (ESI$^+$) m/z calcd for $C_{19}H_{15}N_4OFCl$ $[M + H]^+$ 369.0918, found 369.0918. Anal. Calcd for $C_{19}H_{14}N_4OFCl$; C, 61.88; H, 3.83; N, 15.19. Found: C, 61.67; H, 3.73; N, 15.50.

*4.1.3.20. 2-(4-Fluoro-benzyl)-5-(4-methyl-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7f**)*

White solid, yield 65%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.25 (s, 1H), 7.35-7.34 (m, 2H), 7.24-7.22 (m, 2H), 7.14-7.10 (m, 4H), 5.75 (s, 1H), 4.04 (s, 2H), 3.85 (s, 2H), 2.26 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 21.08, 33.97, 38.05, 98.83, 115.38, 115.55, 129.29 (2C), 129.64 (2C), 131.27 (2C), 133.86, 134.04, 136.64, 151.46, 154.33, 156.22, 160.50, 162.43. HRMS (ESI$^+$) m/z calcd for $C_{20}H_{18}N_4OF$ $[M + H]^+$ 349.1465, found 349.1467. Anal. Calcd for $C_{20}H_{17}N_4OF$; C, 68.95; H, 4.92; N, 16.08. Found: C, 68.64; H, 4.90; N, 16.41.

*4.1.3.21. 5-Benzyl-2-(4-chloro-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7g**)*

White solid, yield 98%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.28 (s, 1H), 7.99 (s, 2H), 7.48 (d, *J*= 7.8 Hz, 2H), 7.41 (d, *J*= 8.2 Hz, 2H), 7.31 (d, *J*= 8.1 Hz, 2H), 7.12 (d, *J*= 7.4 Hz, 2H), 3.99 (s, 1H), 2.28 (s, 2H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 20.74, 30.38, 125.44 (3C), 128.08 (3C), 128.50 (3C), 130.70 (2C), 131.78, 134.16, 137.78, 145.35, 149.81, 151.39. HRMS (ESI$^+$) m/z calcd for $C_{19}H_{16}N_4OCl$ $[M + H]^+$ 351.1013, found 351.1015. Anal. Calcd for $C_{19}H_{15}N_4OCl$; C, 65.05; H, 4.31; N, 15.97. Found: C, 65.36; H, 4.32; N, 15.65.

*4.1.3.22. 5-Benzyl-2-(4-methyl-benzyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**7h**)*

White solid, yield 72%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.25 (s, 1H), 7.36-7.32 (m, 2H), 7.28-7.25 (m, 1H), 7.19 (d, *J*= 8.0Hz, 2H), 7.10 (d, *J*= 7.8Hz, 2H), 5.77 (s, 1H), 3.99 (s, 2H), 3.90 (s, 2H), 2.50 (t, *J*= 2Hz, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 20.57, 33.99, 37.90, 98.47, 126.98, 128.59 (2C), 128.74 (2C), 128.82 (2C), 128.88 (2C), 134.31, 135.40, 136.47, 150.92, 153.39, 155.73, 163.65. HRMS (ESI$^+$) m/z calcd for $C_{20}H_{19}N_4O$ $[M + H]^+$ 331.1559, found 331.1560. Anal. Calcd for $C_{20}H_{18}N_4O$; C, 72.71; H, 5.49; N, 16.96. Found: C, 72.51; H, 5.29; N, 17.36.

*4.1.3.23. 2-Butyl-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8a**)*

White solid, yield 78%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 7.84 (d, *J*= 3.6 Hz, 2H), 7.44 (d, *J*= 6.1 Hz, 3H), 6.3 (s, 1H), 2.67 (t, *J*= 7.5 Hz, 2H), 1.67-1.61 (m, 2H), 1.33-1.25 (m, 2H), 0.83 (t, *J*= 7.4 Hz, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 13.55, 21.53, 26.73, 28.77, 97.66,

127.21 (3C), 128.81 (4C), 130.69, 150.92, 155.83. HRMS (ESI^+) m/z calcd for $C_{15}H_{16}N_4ONa$ $[M + Na]^+$ 291.1222, found 291.1224. Anal. Calcd for $C_{15}H_{16}N_4O$; C, 67.15; H, 6.01; N, 20.88. Found: C, 67.36; H, 6.12; N, 20.56.

*4.1.3.24. 2-Butyl-5-(4-chloro-phenyl)-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8b**)*

White solid, yield 79%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 7.89 (d, *J*= 8.5 Hz, 2H), 7.46 (d, *J*= 8.5 Hz, 2H), 6.33 (s, 1H), 2.65 (t, *J*= 7.5 Hz, 2H), 1.66-1.60 (m, 2H), 1.31-1.24 (m, 2H), 0.82 (t, *J*= 7.4 Hz, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 13.52, 21.50, 26.37, 28.58, 97.92, 125.47, 128.00, 128.75 (2C), 128.92 (2C), 133.75, 135.29, 150.91, 155.83. HRMS (ESI^+) m/z calcd for $C_{15}H_{16}N_4OCl$ $[M + H]^+$ 325.0832, found 325.0840. Anal. Calcd for $C_{15}H_{15}N_4OCl$; C, 59.51; H, 4.99; N, 18.51. Found: C, 59.56; H, 4.90; N, 18.55.

*4.1.3.25. 2-Pentyl-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8c**)*

White solid, yield 75%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 7.94 (d, *J*= 3.6 Hz, 2H), 7.51 (d, J= 2.3 Hz, 3H), 6.33 (s, 1H), 2.72 (t, *J*= 7.5 Hz, 2H), 1.74 (t, *J*= 7.0 Hz, 2H), 1.33 (d, *J*= 3.4Hz, 4H), 0.89-0.84 (m, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 13.80, 21.76, 26.4, 27.06, 30.61, 97.51, 125.46, 127.18 (2C), 128.00, 128.78 (2C), 130.62, 134.17, 151.12, 155.90. HRMS (ESI^+) m/z calcd for $C_{16}H_{19}N_4O$ $[M + H]^+$ 283.1559, found 283.1559. Anal. Calcd for $C_{16}H_{18}N_4O$; C, 68.06; H, 6.43; N, 19.84. Found: C, 68.27; H, 6.21; N, 19.85.

*4.1.3.26. 5-(4-Chloro-phenyl)-2-pentyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8d**)*

White solid, yield 77%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 7.90 (d, *J*= 8.4 Hz, 2H), 7.48 (d, *J*= 8.4 Hz, 2H), 6.34 (s, 1H), 2.65 (t, *J*= 7.5 Hz, 2H), 2.32 (t, *J*= 7.5 Hz, 2H), 1.66 (t, *J*= 7.0 Hz, 2H), 1.51-1.47 (m, 2H), 1.18 (s, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 13.82, 20.73, 21.78, 26.99, 30.61, 97.18, 125.46, 128.01, 128.70 (2C), 128.87 (2C), 134.36, 135.06, 151.89, 155.20. HRMS (ESI$^+$) m/z calcd for $C_{16}H_{18}N_4OCl$ $[M + H]^+$ 317.1169, found 317.1165. Anal. Calcd for $C_{16}H_{17}N_4OCl$; C, 60.66; H, 5.41; N, 17.69. Found: C, 60.56; H, 5.31; N, 17.89.

*4.1.3.27. 2-Hexyl-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8e**)*

White solid, yield 73%; 1H-NMR: (DMSO-d6, 500 MHz): δ 7.90 (d, *J*= 5.8 Hz, 2H), 7.51 (d, *J*= 5.8 Hz, 3H), 6.37 (s, 1H), 2.73 (t, *J*= 7.4 Hz, 2H), 1.74-1.69 (m, 2H), 1.27 (d, *J*= 3.6 Hz, 6H), 0.85 (s, 3H). 13C-NMR: (DMSO-d6, 125 MHz): δ 13.89, 21.93, 26.66, 27.08, 28.10, 30.89, 97.63, 127.21 (2C), 128.81 (2C), 129.36, 130.68, 133.96, 146.65, 150.97, 155.85. HRMS (ESI$^+$) m/z calcd for $C_{17}H_{21}N_4O$ $[M + H]^+$ 297.1715, found 297.1719. Anal. Calcd for $C_{17}H_{20}N_4O$; C, 68.90; H, 6.80; N, 18.90. Found: C, 68.60; H, 6.91; N, 19.09.

*4.1.3.28. 5-(4-Chloro-phenyl)-2-hexyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8f**)*

White solid, yield 71%; 1H-NMR: (DMSO-d6, 500 MHz): δ 7.89 (d, *J*= 8.1 Hz, 2H), 7.48 (d, *J*= 8.1 Hz, 2H), 6.37 (s, 1H), 2.66 (t, *J*= 7.2 Hz, 2H), 1.65 (t, *J*= 6.7 Hz, 2H), 1.20 (s, 6H), 0.78 (s, 3H). 13C-NMR: (DMSO-d6, 125 MHz): δ 13.87, 21.91, 26.51, 26.78, 28.07, 30.87, 97.72, 128.74 (2C), 128.91 (2C), 133.93, 135.23, 151.19, 154.99, 155.92, 158.82. HRMS

(ESI$^+$) m/z calcd for $C_{17}H_{20}N_4OCl$ [M + H]$^+$ 331.1326, found 331.1329. Anal. Calcd for $C_{17}H_{19}N_4OCl$; C, 61.72; H, 5.79; N, 16.94. Found: C, 61.51; H, 5.70; N, 17.24.

*4.1.3.29. 2-Heptyl-5-phenyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8g**)*

White solid, yield 69%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 13.52 (s, 1H), 7.84 (d, *J*= 5.6 Hz, 2H), 7.44(d, *J*= 5.7 Hz, 3H), 6.3 (s, 1H), 2.66 (t, *J*= 7.5 Hz, 2H), 1.67-1.62 (m, 2H), 1.23-1.17 (m, 8H), 0.77 (t, *J*= 6.2 Hz, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 13.88, 22.02, 26.68, 27.05, 28.33 (2C), 31.09, 97.65, 125.47, 127.20 (2C), 127.99, 128.80 (2C), 130.68, 134.31, 150.91, 155.83. HRMS (ESI$^+$) m/z calcd for $C_{18}H_{23}N_4O$ [M + H]$^+$ 311.1872, found 311.1876. Anal. Calcd for $C_{18}H_{22}N_4O$; C, 69.65; H, 7.14; N, 18.05. Found: C, 69.59; H, 7.20; N, 18.05.

*4.1.3.30. 5-(4-Chloro-phenyl)-2-heptyl-4H-[1,2,4]triazolo[1,5-a]pyrimidin-7-one (**8h**)*

White solid, yield 74%; ^{1}H-NMR: (DMSO-d_6, 500 MHz): δ 7.90 (d, *J*= 8.4 Hz, 2H), 7.46 (d, *J*= 8.4 Hz, 2H), 6.3 (s, 1H), 2.64 (t, *J*= 7.4 Hz, 2H), 1.64 (t, *J*= 6.8 Hz, 2H), 1.22-1.17 (m, 8H), 0.76 (t, *J*= 6.4 Hz, 3H). ^{13}C-NMR: (DMSO-d_6, 125 MHz): δ 13.85, 21.99, 26.48, 26.69, 28.26, 28.32, 31.05, 97.95, 128.75 (2C), 128.92 (2C), 133.78, 135.31, 150.89, 154.97, 155.82, 158.62. HRMS (ESI$^+$) m/z calcd for $C_{18}H_{22}N_4OCl$ [M + H]$^+$ 345.1482, found 345.1487. Anal. Calcd for $C_{18}H_{21}N_4OCl$; C, 62.69; H, 6.14; N, 16.25. Found: C, 62.59; H, 6.04; N, 16.45.

4.2 Methods for biological evaluation

4.2.1 Anticonvulsant activity in vitro

4.2.1.1 Primary neocortical neuron culture

All animal experiments were carried out in accordance with the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) approved by the Animal Care and Use Committee at Institute of Materia Medica in Chinese Academy of Medical Sciences. Neocortical neurons were obtained from C57 BL/6 mice at embryonic day 16. Briefly, neocortical tissues were collected; meninges were stripped off; cortical tissues were minced by trituration with a Pasteur pipette, and treated with trypsin for 25 min at 37°C. The cells were then dissociated by two successive trituration and sedimentation steps in soybean trypsin inhibitor and DNase I-containing isolation buffer, centrifuged, and resuspended in neurobasal complete medium (neurobasal medium supplemented with 2% NS21, 1 mM L-glutamine, 1% HEPES with 5% fetal bovine serum), Cells were plated onto poly-L-lysine-coated clear-bottomed, black-wall, 96-well plates (Corning Life Sciences, Acton, MA) at a density of 5.6 × 105 cells/cm^2 and incubated at 37°C in a 5% CO_2 and 95% humidity atmosphere. A final concentration of cytosine arabinoside (ARA-C) (10 μM) was added to the culture medium after plating for 24-36 h to prevent the astrocytes proliferation. Culture medium was half-replaced on day 5 and 7 with serum-free growth medium containing Neurobasal medium supplemented with 2% NS21, 1% HEPES and 0.2 mM L-glutamine.

4.2.1.2. Intracellular Ca^{2+} oscillation determination

Neocortical neurons on 8-9 days *in vitro* (DIV) were used to investigate the effect of compounds on intracellular Ca^{2+} dynamics[16]. Briefly, the growth medium was removed and replaced with dye loading buffer (100 μL/well) containing 4 μM Fluo-8/AM and 0.5% BSA in Locke's buffer (in mM: HEPES 8.6, KCl 5.6, NaCl 154, D-glucose 5.6, $MgCl_2$ 1.0, $CaCl_2$ 2.3, and glycine 0.1, adjust pH to 7.4). After 45 min incubation in dye loading buffer, the neurons were washed four times with fresh Locke's buffer (200 μL/well) and placed in a Fluorescent Imaging Plate Reader (FLIPR^{Tetra}; Molecular Devices, Sunnyvale, CA) incubation chamber. Basal fluorescence levels were acquired in Locke's buffer for 5 min at a sampling rate of 1 Hz followed by an addition of vehicle or compounds using a programmable 96-channel pipetting robotic system, and the fluorescent signals were recorded for an additional 8 min. To investigate the influence of compounds on 4-AP -induced Ca^{2+} response, a second addition of 4-AP was made and the fluorescent signals were recorded for an additional 8 min.

The background fluorescence of the plate was determined from a sister well without Fluo-8/AM loading, and all the fluorescence signals were corrected by subtracting the background fluorescence. Data were presented as F/F0, where F is the fluorescent signal at different time point minus background fluorescence whereas F0 is the basal fluorescence minus the background fluorescence. To determine the compounds response on SCOs, the SCO frequency from an epoch of 300 s after addition of vehicle (0.1% DMSO) or compounds for 60 s was manually counted. All the data were presented as % vehicle. To determine the compounds response on 4-AP-induced Ca^{2+}

oscillation, the Ca^{2+} oscillation frequency was manually counted from an epoch of 300 s in the presence or absence of compounds after addition of 4-AP for 120 s. The data were presented as % of 4-AP. Events having F/F0 > 0.05 units were included in the analyses of SCO frequency.

4.2.2 Anticonvulsant activity in vivo

Neurotoxicity was assessed by rotarod test. The MES, sc-PTZ and rotarod test were carried out by the methods described in the antiepileptic drug development program (ADD) of the national institutes of Health following previously described testing procedures. All compounds dissolved in DMSO were evaluated for anticonvulsant activities with male KunMing mice (weight 18-20 g) obtained from Beijing Huafukang Bio-tech. Co, Ltd, PRC.

4.2.2.1 Maximal electroshock seizure

Seizures were elicited with a 60 Hz stimulating current at 50 mA intensity in mice. The current was applied via corneal electrodes for 0.2 s. Protection against the spread of MES-induced seizures was defined as the abolition of tonic maximal extension of the hind leg. After the administration of the compounds for 30 min, the activities were evaluated in MES test.

4.2.2.2 Pentylenetetrazole (PTZ)-induced seizure [17]

After the intraperitoneal administration of the compounds or vehicle controls for 30 min, animals were injected with PTZ (85 mg/kg) subcutaneously. 100% of the animals displayed clonic seizure behaviors after the PTZ treatment. The doses were calculated which prevented 50% of the treated animals from clonic convulsions (ED_{50}).

4.2.2.3 Neutotoxicity screening (Rotarod test)

At 30min after the administration of the compounds, the animals were tested on a 1-inch diameter, knurled plastic rod rotating at 6 rpm for 1min. Neurotoxicity was indicated by the inability of an animal to maintain equilibrium in each of three trials[18].

4.2.2.4 Protective index (PI)

The protective index for the investigated compounds was calculated by dividing a TD_{50} value, as determined in the rotarod test, by the ED_{50} value, as determined in the sc-PTZ test. The protective index is considered as a factor to judge the safety margin and tolerability of a compound between anticonvulsant doses and toxic doses[19].

4.2.2.5 $GABA_{A1}$ receptor test

Cell culture

T-REx™-CHO Cell Line was purchased from Thermofisher (R71807) and used to make stable cell line expressing the human $\alpha1\beta2\gamma2$ $GABA_A$ receptor subtype with Lipofectamine™ LTX Reagent with PLUS™ Reagent (Thermofisher, A12621). The stable cell line was cultured and maintained in DMEM and nutrient F-12 mixture supplemented with 10% FCS, Blasticidin 10 μg/ml, Hygromycin 300 μg/ml, Zeocin 100 μg/ml and Puromycin 1 μg/ml.

Compound Preparation

The compound stock was prepared in DMSO at 30 mM. On the experimental day, the drug was diluted to respective concentrations (5-fold) for FMP experiments.

Membrane potential FLIPR assay

The membrane potential FLIPR assay was adapted from the previous paper by Liu et al .

T-REx ™-CHO Cells expressing α1β2γ2 $GABA_A$ receptor were seeded at 12000 cells per well in 384-well poly-D-lysine-coated plates for FMP assay incubated overnight (16~20 h) at 37 ˚C and 5% CO_2. FMP blue dye was used (Molecular Devices, United States). The FMP assay protocol was adapted from the manufacturer's recommended protocol. Briefly, medium was removed; cells were loaded with FMP dye, 20 μL/well, for 30 min at room temperature in the dark. The compounds were tested from the maximal concentration 90 uM with 3-fold dilutions for 8 doses. Cell plates were loaded to FLIPR (Molecular Devices, United States). After establishing fluorescence baseline by 1 Hz scanning for 10 s, the GABA channels were activated by addition of GABA or the testing compounds 5 μL (5-fold), and fluorescence measurement was continued at 1 Hz for another 120 s. EC_{50} values were determined from dose-response curves for 8 distinct concentrations in quadruplicates. The concentration-response curves were fitted by Hill equation, according to the standard procedure.

4.2.2.6 Electrophysiological experiments on VGSCs

All the electrophysiological experiments were performed using EPC-10 patch clamp amplifier (HEKA Elektronik GmbH, Germany) as described by Cao [20] . Cultured neocortical neurons (5-7 DIVs) were bathed in external solution containing (in mM): NaCl 30, $CaCl_2$ 1.8, $MgCl_2$ 1, CsCl 5, glucose 25, HEPES 5, TEA-Cl 90, KCl 5 (pH adjusted to 7.4 with NaOH). Intracellular solution was (in mM): CsF 135, NaCl 10, HEPES 5 (pH adjusted to 7.2 with CsOH), pipette-tip resistances were 8-12 MΩ. Voltage errors were minimized using 80% series resistance compensation, and the capacitance artifact was cancelled using computer-controlled circuitry of the patch clamp amplifier. Experimental data were collected

and analyzed using Patchmaster (HEKA Electronics, Germany) and Graphpad 5.0 (GraphPad Software, San Diego, CA). Concentration-response curves were fitted using Hill equation: Inor= C+A/[1 + (compound /IC_{50})p], where Inor is normalized peak current, IC_{50} is the half maximal inhibitory concentration, and p is the slop factor. All data are presented as mean ± SEM, and n is the number of independent experimental cells [20].

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Highlights

- A series of [1,2,4]-triazolo[1,5-a]pyrimidine-7(4H)-one derivatives were designed and synthesized.
- These compounds exhibited specific anticonvulsant activity in *in vitro* and *in vivo* models.
- Among them, compound **5c** and **5e** displayed more pronounced activity and lower neurotoxicity compared to the commonly used drugs.
- Compound **5c** and **5e** selectively acted on $GABA_{A1}$ receptor as positive modulators.

Declaration of interest statement:

Authors declared that they have no conflicts of interest to this work submitted.