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Synthesis and biological evaluation of novel 4,5-bisindolyl-1,2,4-triazol-3-ones as glycogen synthase kinase-3β inhibitors and neuroprotective agents

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A series of novel 4,5-bisindolyl-1,2,4-triazol-3-ones were designed, prepared and evaluated for their glycogen synthase kinase (GSK)-3β inhibitory activities. Compounds exhibited favorable inhibitory potency towards GSK-3β kinase at the molecular level and in cells indicated by significantly reducing GSK-3β substrate Tau phosphorylation at Ser396 in primary neurons showing the inhibition of cellular GSK-3β. In an *in vitro* model of neuronal injury, compounds **6b**, **6d** and **6f** prevented glutamate-induced neuronal death which was closely associated with cerebral ischemic stroke. Preliminary structure-activity relationship was examined and showed that different substituents on the indole ring had significant influences on the GSK-3β inhibitory potency. These findings may provide new insights into the development of novel GSK-3β inhibitors as neuroprotective agents.

1. Introduction

Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine protein kinase that mediates the addition of phosphate molecules onto serine and threonine amino acid residues which was identified in the late 1970s (Embi et al. 1980; Welsh and Proud 1993). In mammals, GSK-3 is encoded by two known genes, GSK-3α and GSK-3β, both of which share high homology at the catalytic domain but significantly differ in their N-terminal domain (Woodgett 1990). Phosphorylation of a protein by GSK-3 usually inhibits the activity of its downstream target. GSK-3 is active in a number of central intracellular signaling pathways, including cellular proliferation, migration, glucose regulation, and apoptosis (Ali et al. 2001; Eldar-Finkelman 2002; Grimes Jope 2001). Inhibition of GSK-3β may provide therapy approaches for several diseases such as type II diabetes (Diabetes mellitus type 2), Alzheimer's disease, inflammation, cancer, and bipolar disorder (Castro Martinez 2005; Hall et al. 2002; Jope et al. 2007; Kim and Kimmel 2000; Klein and Melton 1996; Wagman and Nuss 2001). Accordingly, it is extremely popular to search for novel potent and selective GSK-3β inhibitors. In recent years, several kinds of bisindolylmaleimides (Fig. 1) have been identified as potent GSK-3β inhibitors. However, the selectivity of these compounds towards GSK-3β homologous enzymes such as protein kinase C (PKC) and cyclin-dependent kinases (CDKs) is poor, which limits their clinical application (Hers et al. 1999, Zhang et al. 2003). Further study indicated that some 4,5-bisindolylpyrazolones with a pyrazolone (5-membered heterocycle), which can keep the hydrogen-bonding interactions with the hinge of enzyme (Asp133 and Val135), as a replacement for the maleimide cycle of bisindolylmaleimides showed potent and selective GSK-3β inhibitory activities (Chen et al. 2011). These facts prompted us to design and synthesize a series of novel 4,5-bisindolyl-1,2,4-triazol-3-ones, using a struc-

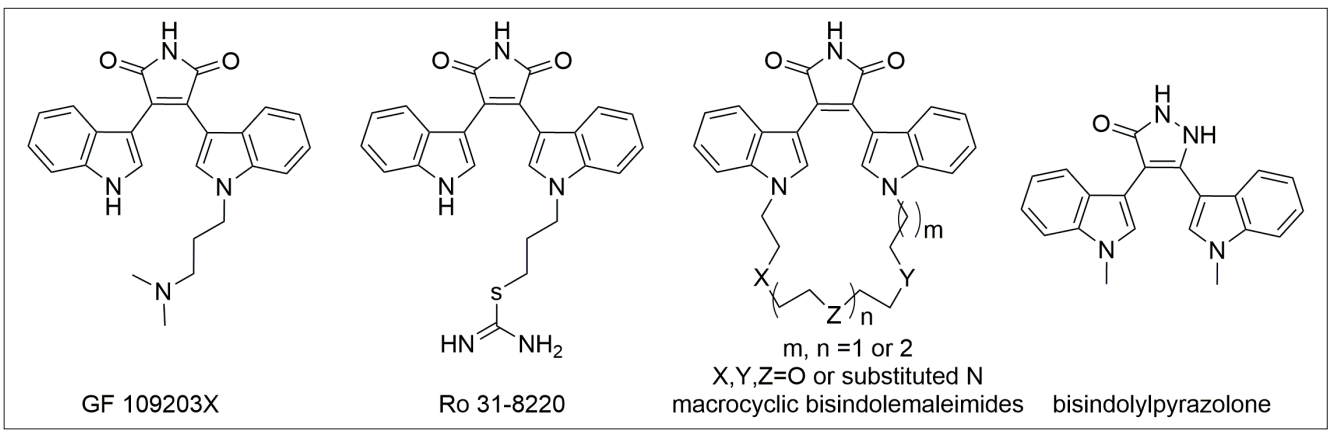


Fig. 1: Bisindolylmaleimide and bisindolylpyrazolone GSK-3β inhibitors

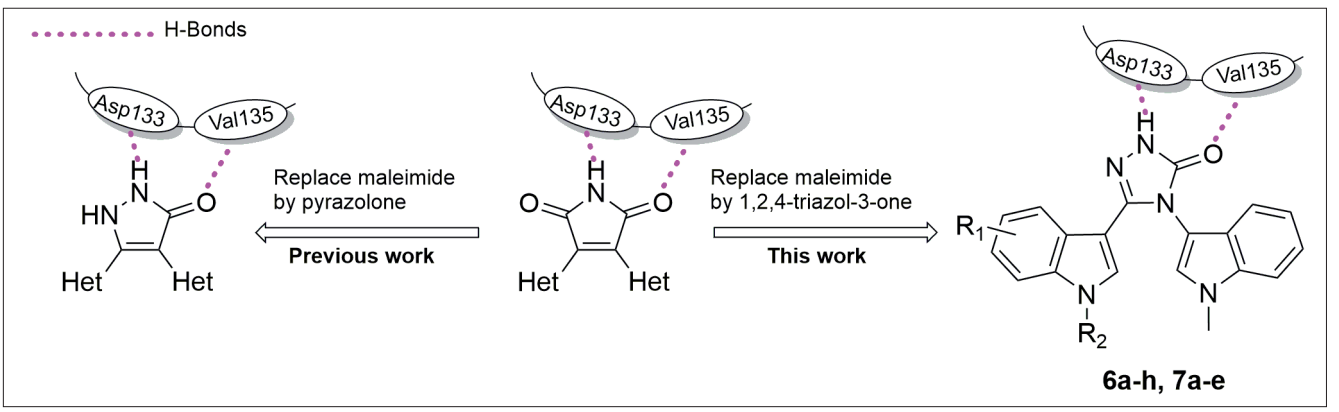


Fig. 2: Alternative GSK-3β inhibitor scaffolds with a pyrazolone or 1,2,4-triazol-3-one (5-membered heterocycle) as a replacement for the maleimide core.

ture-based design, to find more potent and selective GSK-3β inhibitors as neuroprotective agents (Fig. 2). In addition, their structure-activity relationship and an in silico molecular modeling study are also presented.

2. Investigations, results and discussion

2.1. Chemistry

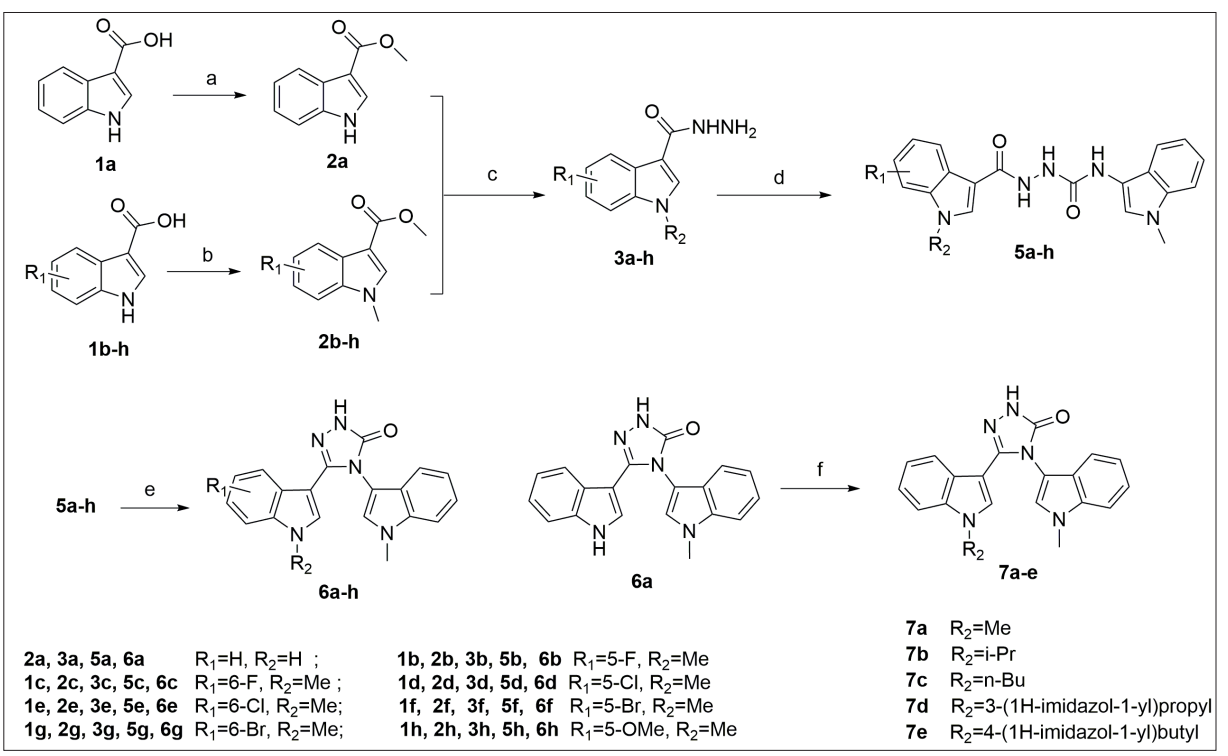
The synthetic route of compounds **6a-h** and **7a-e** is illustrated in Scheme 1. Esterification of indole-3-carboxylic acid **1a** with methanol in the presence of concentrated sulfuric acid at refluxing temperature produce a compound **2a**. Indole-3-carboxylic acid derivatives **1b-h** were methylated by iodomethane using NaH as a base in dry DMF to obtain intermediates **2b-h**. Reaction of **2a-h** with hydrazine hydrate afforded intermediates **3a-h**. Condensation of **3a-h** with 3-isocyanato-1-methyl-1H-indole **4** in dry THF afforded intermediates **5a-h**, which were cyclized to afford **6a-h** in the presence of an excess amount of trimethylsilyl trifluoromethanesulfonate (TMSOTf) and triethylamine in dry DMF. Compounds **7a-e** were obtained by N-alkylation of **6a** with different alkyl halides using NaH as a base in dry DMF.

2.2. Biological activity and molecular modeling

2.2.1. Enzymatic activity

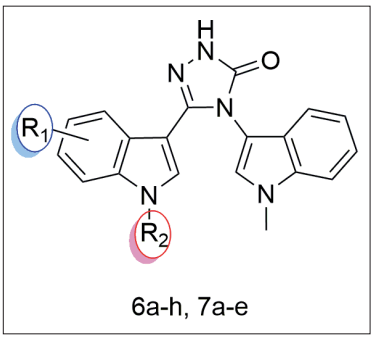
The GSK-3β inhibitory potency of all compounds was examined. A well-known kinase inhibitor staurosporine was used as the positive control. The results are presented in Table 1.

As shown in Table 1, among the tested compounds, **6b**, **6d** and **6f** exhibited favorable inhibitory potency towards GSK-3β. Comparing the inhibitory activity of **6b-h** with **7a** revealed that different substituents (R_1) on the indole ring could affect the potency. Introduction of a fluorine (**6b**, IC_{50} = 1.88 μM), chlorine (**6d**, IC_{50} = 0.61 μM) or bromine (**6f**, IC_{50} = 0.35 μM) atom at the 5-position of the indole ring led to an obvious enhancement of the activity as compared to **7a**. Replacement of the 5-halogen with a methoxy resulted in a significant loss of activity (**6h**, IC_{50} = 6.2 μM). A halogen atom at the 6-position of the indole ring (**6c**, **6e**, **6g**) was not tolerated for the activity. Introduction of a hydrophobic alkyl (**7a**, **7b**, **7c**) or hydrophilic side chain (**7d**, **7e**) at N^1-position of the indole ring (R_2) did not lead to an increase in GSK-3β inhibitory activity as compared to **6a**, which suggested that they may not effectively form hydrophobic or hydrogen-bonding interactions with the protein, respectively.



Scheme 1: Synthetic route to compounds **6a-h** and **7a-e**. Reagents and conditions: (a) concentrated sulfuric acid, CH_3OH; (b) NaH, iodomethane, DMF ; (c) $N_2H_4 \cdot H_2O$; (d) 3-isocyanato-1-methyl-1H-indole, THF; (e) TMSOTf, triethylamine, THF; (f) NaH, DMF, R_2X.

Table 1: GSK-3β inhibitory activities of target compounds



Compd.	R_1	R_2	IC_{50} (μM) ± SEa or Inhibition% at ~ 1 μg/ml
Staurosporine			0.032±0.00
6a	H	H	18.15
6b	5-F	CH_3	1.88±0.33
6c	6-F	CH_3	7.50
6d	5-Cl	CH_3	0.61±0.08
6e	6-Cl	CH_3	25.39
6f	5-Br	CH_3	0.35±0.02
6g	6-Br	CH_3	22.07
6h	5-OMe	CH_3	6.2 ± 0.88
7a	H	CH_3	9.97
7b	H	i-Pr	5.79
7c	H	n-Bu	5.82
7d	H	3-(1H-imidazol-1-yl)propyl	10.45
7e	H	3-(1H-imidazol-1-yl)butyl	11.15

[a] SE: standard error mean.

2.2.2. *Cellular activity*

It has been reported that GSK-3β inhibition displays neuroprotective effects in cerebral ischemia and improves neuronal survival. Cerebral ischemia is characterized by excess glutamate release, low oxygen and glucose supply, insufficient nutrient supply in the brain which lead to neuronal death finally (Leng Chuang 2006; Leng et al. 2008; Nonaka and Chuang 1998; Ye et al. 2015a, b). Therefore, compounds **6b**, **6d** and **6f** with favorable GSK-3β inhibitory activities were further investigated for their *in vitro* neuroprotective effects on neurotoxicity induced by glutamate. The results are shown in Table 2.

Table 2: Neuroprotective effects of selected compounds on glutamate-induced neurotoxicity in primary rat cerebellar granule neuronal cells (CGCs)

Compd.	Cell viability[a] (%)		
	0.1 μM	1 μM	5 μM
6b	78.5±2.5	83.3±4.7	64.8±4.3
6d	70.7±4.7	72.2±4.3	105.1±1.8
6f	91.9±3.9	74.8±2.1	71.2±5.4

a: Data were expressed as means ± SD, n=3.

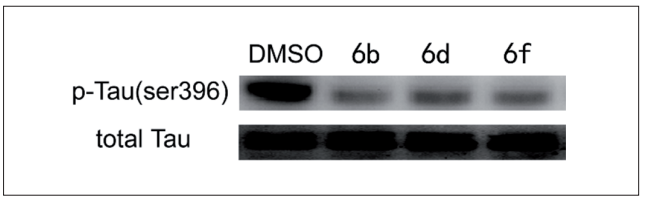


Fig. 3. Effects of GSK-3β inhibitors on Tau phosphorylation in primary rat cerebellar granule neuronal cells (CGCs). The phosphorylated Tau (Ser396) and total Tau protein were determined by Western blotting. Compounds (5 μM) show inhibition of Tau (Ser396) phosphorylation in neurons.

2.3. *Molecular modeling study*

Docking analysis was carried out by using the LigandFit module (Discovery Studio, version 2.5; Accelrys, San Diego, CA, USA, 2008) to examine possible binding modes of compound **6f** with GSK-3β. The X-ray crystal structure of GSK-3β (PDB ID: 1Q3D) (Bertrand et al. 2003) was used for the docking calculation. Compound **6f** can tightly occupy ATP binding site of GSK-3β kinase (Fig. 4). NH and carbonyl group in 1,2,4-triazol-3-one ring of **6f** can form a tight interaction with the hinge backbone Asp133 and Val135 in of GSK-3β via the hydrogen bonding network as expected. In addition, the phenyl group of Tyr134 interacts with the indole ring of compound **7f** through π–π stacking. The bromine atom of compound **6f** inserts into the small hydrophobic pocket embraced by Cys199, Leu132, Val110 and Met101 which seems to explain that the inhibitory activity of **6f** is obviously higher than **7a**.

2.4. *In silico prediction of blood brain barrier permeability*

Apart from potent GSK-3β inhibitory activity, the ability to permeate blood brain barrier (BBB) is also important for a promising neuroprotective agent (Di et al. 2013; Gabathuler 2010; Pardridge 2003). Therefore, the prediction of BBB permeability of compounds **6b**, **6d** and **6f** was carried out using ADMET analysis program within the Discovery Studio 2.5 software package. The predicted results suggested that these GSK-3β inhibitors may have the ability to penetrate BBB (Table 3) as all the predicted compounds lay in the ellipses of 95% and 99% blood brain barrier (BBB) confidence region (Figure 5).

Table 3: Results of blood-brain barrier (BBB) penetration predictions

Compd.	ADMET_BBB	ADMET_BBB Level
6b	0.161	High
6d	0.303	High
6f	0.329	High

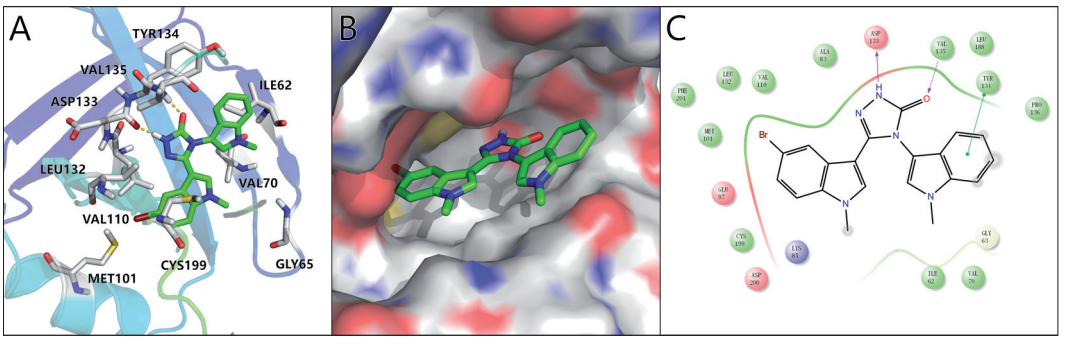


Fig. 4: Docking of **6f** to GSK-3β crystal structure. (a) compound **6f** (green carbons) bound to GSK-3β; (b) compound **6f** bound to GSK-3β after surfacing receptor; (c) Protein-ligand interaction diagram (hydrogen bonds indicated by magenta arrows; aromatic stacking interactions by green vectors).

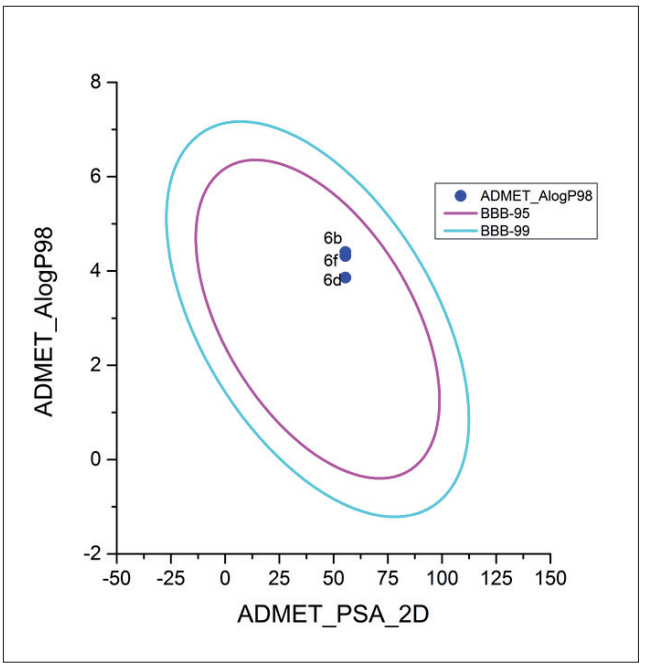


Fig. 5: ADMET_BBB plot of compounds **6b**, **6d** and **6f**

2.5. *Conclusions*

In summary, a novel series of 4,5-bisindolyl-1,2,4-triazol-3-ones were synthesized and biologically evaluated as GSK-3β inhibitors and neuroprotective agents. Among them, compounds **6b**, **6d** and **6f** exhibited favorable inhibitory potency against GSK-3β, which can prevent glutamate-induced neuronal death in an *in vitro* neuronal injury model. Preliminary structure-activity relationship (SAR) and molecular modeling studies provide information that could be useful for further studies.

3. Experimental

Melting points were determined with a BÜCHI Melting Point B-450 apparatus (Büchi Labortechnik, Flawil, Switzerland) and are uncorrected. 1H NMR spectra were recorded on a 500 MHz (chemical shifts are expressed as δ values relative to TMS as internal standard). ESI (positive) was recorded on an Esquire-LC-00075 spectrometer. Thin layer chromatography (TLC) was carried out using plate silica gel F254 Merck. All reactions were monitored by TLC. All reagents were obtained from commercial sources and used without further purification unless stated. THF was distilled from sodium-benzophenone. DMF was distilled from calcium hydride. Preparation methods and physicochemical properties for compounds **4** were reported by Rigby and Burke (2006).

3.1. Synthesis of compounds 2a – 2h

3.1.1. Methyl 1H-indole-3-carboxylate (2a)

A mixture of indole-3-carboxylic acid (2.0 g, 12.4 mmol), concentrated sulfuric acid (1 mL) and anhydrous methanol (25 mL) was refluxed for 16 h. After cooling, part of the methanol was concentrated under reduced pressure. The resulted mixture was neutralized with a saturated solution of $NaHCO_3$ and then extracted with ethyl acetate (50 mL × 3). The organic phase was combined, dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel to afford **2a** as a white solid, mp: 146-148 °C, yield 93.0%. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 8.86 (s, 1H), 8.23-8.15 (m, 1H), 7.90 (d, *J* = 3.0 Hz, 1H), 7.44-7.36 (m, 1H), 7.31-7.22 (m, 2H), 3.93 (s, 3H).

3.1.2. General procedure for the preparation of 2b-h

60% NaH (0.91 g, 22.8 mmol) was added in portions to a solution of **1b-h** (11.2 mmol) in dry DMF (35 mL) at 0-5 °C. After addition, the mixture was stirred for 30 min. Iodomethane (33.6 mmol) was then added at 0-5 °C and stirred for 2 h at room temperature. The mixture was then poured into H_2O (120 mL) and the resulting solution was extracted with ethyl acetate (50 mL × 3). The organic phase was combined and washed with brine (150 mL × 3), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The obtained residue was purified by flash column chromatography on silica gel to afford **2b-h**.

3.1.3. Methyl 5-fluoro-1-methyl-1H-indole-3-carboxylate **(2b)**

According to the general method, the reaction of 5-fluoro-1H-indole-3-carboxylic acid with iodomethane afforded **2b** in 89.3% yield as a white solid, mp: 83-84 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.82 (dd, *J* = 9.6, 2.6 Hz, 1H), 7.78 (s, 1H), 7.27-7.23 (m, 1H), 7.03 (td, *J* = 9.0, 2.6 Hz, 1H), 3.91 (s, 3H), 3.82 (s, 3H).

3.1.4. Methyl 6-fluoro-1-methyl-1H-indole-3-carboxylate **(2c)**

According to the general method, the reaction of 6-fluoro-1H-indole-3-carboxylic acid with iodomethane afforded **2c** in 91.3% yield as a white solid, mp: 116-118 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.11-8.09 (m, 1H), 7.75 (s, 1H), 7.08-6.98 (m, 2H), 3.91 (s, 3H), 3.78 (s, 3H).

3.1.5. Methyl 5-chloro-1-methyl-1H-indole-3-carboxylate **(2d)**

According to the general method, the reaction of 5-chloro-1H-indole-3-carboxylic acid with iodomethane afforded **2d** in 93.0% yield as a light yellow solid, mp: 96-98 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.14 (t, *J* = 1.4 Hz, 1H), 7.78 (s, 1H), 7.26-7.24 (m, 2H), 3.92 (s, 3H), 3.82 (s, 3H).

3.1.6. Methyl 6-chloro-1-methyl-1H-indole-3-carboxylate **(2e)**

According to the general method, the reaction of 6-chloro-1H-indole-3-carboxylic acid with iodomethane afforded **2e** in 65.2% yield as a light yellow solid, mp: 100-103 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.08 (d, *J* = 8.5 Hz, 1H), 7.76 (s, 1H), 7.35 (d, *J* = 1.8 Hz, 1H), 7.25 (dd, *J* = 8.5, 1.8 Hz, 1H), 3.91 (s, 3H), 3.81 (s, 3H).

3.1.7. Methyl 5-bromo-1-methyl-1H-indole-3-carboxylate **(2f)**

According to the general method, the reaction of 5-bromo-1H-indole-3-carboxylic acid with iodomethane afforded **2f** in 92.4% yield as a white solid, mp: 101-102 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.32 (d, *J* = 1.9 Hz, 1H), 7.78 (s, 1H), 7.40 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.23 (d, *J* = 8.7 Hz, 1H), 3.93 (s, 3H), 3.84 (s, 3H).

3.1.8. Methyl 6-bromo-1-methyl-1H-indole-3-carboxylate **(2g)**

According to the general method, the reaction of 6-bromo-1H-indole-3-carboxylic acid with iodomethane afforded **2g** in 83.2% yield as a light yellow solid, mp: 106-107 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 8.04 (d, *J* = 8.5 Hz, 1H), 7.75 (s, 1H), 7.52 (d, *J* = 1.7 Hz, 1H), 7.38 (dd, *J* = 8.5, 1.7 Hz, 1H), 3.91 (s, 3H), 3.81 (s, 3H).

3.1.9. Methyl 5-methoxy-1-methyl-1H-indole-3-carboxylate **(2h)**

According to the general method, the reaction of 5-methoxy-1H-indole-3-carboxylic acid with iodomethane afforded **2h** in 94.2% yield as a white solid, mp: 92-94 °C. ^{1}H NMR (500 MHz, chloroform-*d*) δ 7.73 (s, 1H), 7.67 (d, *J* = 2.5 Hz, 1H), 7.24 (d, *J* = 8.8 Hz, 1H), 6.95 (dd, *J* = 8.8, 2.5 Hz, 1H), 3.92 (s, 3H), 3.91(s, 3H), 3.82 (s, 3H).

3.2. Synthesis of compounds 3a – 3h

3.2.1. General procedure for the preparation of 3a-3h

A mixture of **2a-h** (8.6 mmol) and 85% hydrazine hydrate (30 mL) was refluxed for 1 h. After cooling, the mixture was isolated by filtration, washed with a small amount of water and dried to afford **3a-h**.

3.2.2. 1H-indole-3-carbohydrazide **(3a)**

According to the general method, the reaction of **2a** with 85% hydrazine hydrate afforded **3a** in 78.7% yield, mp: 224-226 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 11.50 (s, 1H), 9.14 (s, 1H), 8.13 (d, *J* = 7.8 Hz, 1H), 7.96 (d, *J* = 2.9 Hz, 1H), 7.42 (d, *J* = 8.0 Hz, 1H), 7.18-7.06 (m, 2H), 4.37 (s, 2H).

3.2.3. 5-Fluoro-1-methyl-1H-indole-3-carbohydrazide **(3b)**

According to the general method, the reaction of **2b** with 85% hydrazine hydrate afforded **3b** in 76.0% yield, mp: 219-221 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.18 (s, 1H), 7.99 (s, 1H), 7.82 (dd, *J* = 10.2, 2.7 Hz, 1H), 7.53-7.50 (m, 1H), 7.08 (td, *J* = 9.1, 2.7 Hz, 1H), 4.39 (s, 2H), 3.82 (s, 3H).

3.2.4. 6-Fluoro-1-methyl-1H-indole-3-carbohydrazide **(3c)**

According to the general method, the reaction of **2c** with 85% hydrazine hydrate afforded **3c** in 71.0% yield, mp: 188-190 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.18 (s, 1H), 8.15-8.08 (m, 1H), 7.92 (s, 1H), 7.38 (dd, *J* = 10.1, 2.4 Hz, 1H), 7.05-6.96 (m, 1H), 4.36 (s, 2H), 3.79 (s, 3H).

3.2.5. 5-Chloro-1-methyl-1H-indole-3-carbohydrazide **(3d)**

According to the general method, the reaction of **2d** with 85% hydrazine hydrate afforded **3d** in 65.0% yield, mp: 219-221°C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.22 (s, 1H), 8.14 (d, *J* = 2.1 Hz, 1H), 8.00 (s, 1H), 7.53 (d, *J* = 8.7 Hz, 1H), 7.23 (dd, *J* = 8.7, 2.1 Hz, 1H), 4.34 (s, 2H), 3.82 (s, 3H).

3.2.6. 6-Chloro-1-methyl-1H-indole-3-carbohydrazide **(3e)**

According to the general method, the reaction of **2e** with 85% hydrazine hydrate afforded **3e** in 76.0% yield, mp: 211-212 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (s, 1H), 8.12 (d, *J* = 8.5 Hz, 1H), 7.95 (s, 1H), 7.64 (d, *J* = 1.9 Hz, 1H), 7.16 (dd, *J* = 8.5, 1.9 Hz, 1H), 4.33 (s, 2H), 3.81 (s, 3H).

3.2.7. 5-Bromo-1-methyl-1H-indole-3-carbohydrazide **(3f)**

According to the general method, the reaction of **2f** with 85% hydrazine hydrate afforded **3f** in 69.0% yield, mp: 231-233 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.23 (s, 1H), 8.29 (d, *J* = 2.1 Hz, 1H), 7.97 (s, 1H), 7.49 (d, *J* = 8.7 Hz, 1H), 7.35 (dd, *J* = 8.7, 2.1 Hz, 1H), 4.33 (s, 2H), 3.82 (s, 3H).

3.2.8. 6-Bromo-1-methyl-1H-indole-3-carbohydrazide **(3g)**

According to the general method, the reaction of **2g** with 85% hydrazine hydrate afforded **3g** in 72.0% yield, mp: 212-214 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.21 (s, 1H), 8.12 (d, *J* = 8.5 Hz, 1H), 7.94 (s, 1H), 7.64 (d, *J* = 1.9 Hz, 1H), 7.17 (dd, *J* = 8.5, 1.9 Hz, 1H), 4.37 (s, 2H), 3.81 (s, 3H)

3.2.9. 5-Methoxy-1-methyl-1H-indole-3-carbohydrazide **(3h)**

According to the general method, the reaction of **2h** with 85% hydrazine hydrate afforded **3h** in 71.0% yield, mp: 167-169 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.11 (s, 1H), 7.87 (s, 1H), 7.64 (d, *J* = 2.5 Hz, 1H), 7.38 (d, *J* = 8.9 Hz, 1H), 6.85 (dd, *J* = 8.9, 2.5 Hz, 1H), 4.37 (s, 2H), 3.78 (s, 6H).

3.3. Synthesis of compounds 5a – 5h

3.3.1. General procedure for the preparation of 5a-5h

Compound **4** (0.29 g, 1.7 mmol) in dry THF (5 mL) was added dropwise to a mixture of **3a-h** (1.7 mmol) in dry THF (20 mL) at 0-5 °C. After addition, the mixture was stirred for 2 h at room temperature. The mixture was then filtered and dried to afford **5a-h**.

3.3.2. 2-(1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl)hydrazine-1-carboxamide **(5a)**

According to the general method, the reaction of **3a** with **4** afforded **5a** in 85.8% yield as a off-white solid, mp: 218-220 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.67 (s, 1H), 9.78 (s, 1H), 8.64 (s, 1H), 8.19-8.15 (m, 2H), 7.95 (s, 1H), 7.59 (d, *J* = 8.0 Hz, 1H), 7.47 (d, *J* = 8.0 Hz, 1H), 7.44 (s, 1H), 7.39 (d, *J* = 8.0 Hz, 1H), 7.20-7.13 (m, 3H), 7.04 (t, *J* = 7.5 Hz, 1H), 3.74 (s, 3H).

3.3.3. 2-(5-Fluoro-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5b)**

According to the general method, the reaction of **3b** with **4** afforded **5b** in 63.8% yield as an off-white solid, mp: 224-226 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.84 (s, 1H), 8.65 (s, 1H), 8.19 (s, 1H), 8.00-7.89 (m, 2H), 7.72-7.28 (m, 4H), 7.25-6.95 (m, 3H), 3.87 (s, 3H), 3.75 (s, 3H).

3.3.4. 2-(6-Fluoro-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5c)**

According to the general method, the reaction of **3c** with **4** afforded **5c** in 77.8% yield as an off-white solid, mp: 215-217 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.83 (s, 1H), 8.64 (s, 1H), 8.16-8.09 (m, 2H), 7.96 (s, 1H), 7.58 (d, *J* = 7.9 Hz, 1H), 7.46-7.42 (m, 2H), 7.39 (d, *J* = 8.3 Hz, 1H), 7.16 (t, *J* = 7.6 Hz, 1H), 7.08-7.01 (m, 2H), 3.84 (s, 3H), 3.74 (s, 3H).

3.3.5. 2-(5-Chloro-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5d)**

According to the general method, the reaction of **3d** with **4** afforded **5d** in 62.2% yield as an off-white solid, mp: 233-235 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 9.86 (s, 1H), 8.63 (s, 1H), 8.16 (s, 1H), 8.14 (s, 1H), 7.97 (s, 1H), 7.58 (t, *J* = 7.2 Hz, 2H), 7.43(s, 1H) ,7.39 (d, *J* = 8.2 Hz 1H), 7.27 (d, *J* = 8.4 Hz, 1H), 7.15 (t, *J* = 7.5 Hz, 1H), 7.03 (d, *J* = 7.4 Hz, 1H), 3.87 (s, 3H), 3.74 (s, 3H).

3.3.6. 2-(6-Chloro-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5e)**

According to the general method, the reaction of **3e** with **4** afforded **5e** in 54.7% yield as an off-white solid, mp: 223-224 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.83 (s, 1H), 8.61 (s, 1H), 8.17-8.11 (m, 2H), 7.95 (s, 1H), 7.69 (s, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.43 (s, 1H), 7.39 (d, *J* = 8.3 Hz, 1H), 7.24 -7.12 (m, 2H), 7.03 (t, *J* = 7.5 Hz, 1H), 3.86 (s, 3H), 3.74 (s, 3H).

3.3.7. 2-(5-Bromo-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5f)**

According to the general method, the reaction of **3f** with **4** afforded **5f** in 77.6% yield as an off-white solid, mp: 230-232 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.85 (s, 1H), 8.61 (s, 1H), 8.31 (s, 1H), 8.15 (s, 1H), 7.96 (s, 1H), 7.60-7.51 (m, 2H), 7.43 (s, 1H), 7.42-7.35 (m, 2H), 7.16 (t, *J* = 7.7 Hz, 1H), 7.03 (t, *J* = 7.5 Hz, 1H), 3.87 (s, 3H), 3.74 (s, 3H).

3.3.8. 2-(6-Bromo-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5g)**

According to the general method, the reaction of **3g** with **4** afforded **5g** in 63.3% yield as an off-white solid, mp: 225-226 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.84 (s, 1H), 8.61 (s, 1H), 8.17-8.11 (m, 2H), 7.95 (s, 1H), 7.69 (d, *J* = 1.8 Hz, 1H), 7.57 (d, *J* = 8.0 Hz, 1H), 7.43 (s, 1H), 7.38 (d, *J* = 8.3 Hz,1H), 7.21 (dd, *J* = 8.6, 1.9 Hz, 1H), 7.18-7.11 (m, 1H), 7.08-6.99 (m, 1H), 3.86 (s, 3H), 3.74 (s, 3H).

3.3.9. 2-(5-Methoxy-1-methyl-1H-indole-3-carbonyl)-N-(1-methyl-1H-indol-3-yl) hydrazine-1-carboxamide **(5h)**

According to the general method, the reaction of **3h** with **4** afforded **5h** in 86.9% yield as an off-white solid, mp: 203-205 °C. ^{1}H NMR (500 MHz, DMSO-d_6) δ 9.78 (s, 1H), 8.54 (s, 1H), 8.16 (d, *J* = 7.9 Hz, 1H), 8.11 (s, 1H), 7.88 (s, 1H), 7.53 (d, *J* = 8.3 Hz, 1H), 7.37 (s, 1H), 7.30 (d, *J* = 8.9 Hz, 1H), 7.28 – 7.21 (m, 1H), 7.22 – 7.15 (m, 1H), 7.07 (d, *J* = 2.5 Hz, 1H), 6.80 (dd, *J* = 8.9, 2.4 Hz, 1H), 3.87 (s, 3H), 3.78 (s, 3H), 3.70 (s, 3H).

3.4. Synthesis of compounds 6a – 6h

3.4.1. General procedure for the preparation of 6a-6h

Trimethylsilyl trifluoromethanesulfonate (3.0 mmol) and dry triethylamine (4.0 mmol) were added to a solution of **5a-h** (0.5 mmol) in dry DMF (10 mL). The reaction mixture was stirred for 4 h at 110 °C. After cooling, the mixture was poured into water (100 mL) and extracted with ethyl acetate (50 mL × 3). The organic phase was combined, washed with brine (150 mL × 3), dried over Na_2SO_4 and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate (1:2, v/v) as eluent to afford **6a-h**.

3.4.2. 5-(1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6a)**

According to the general method, the reaction of **5a** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6a** in 78.6% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, Chloroform-*d*) δ 11.90 (s, 1H), 11.16 (d, *J* = 1.9 Hz, 1H), 8.19 (d, *J* = 7.5 Hz, 1H), 7.72 (s, 1H), 7.60 (d, *J* = 8.4 Hz, 1H), 7.38 (d, *J* = 7.5 Hz, 1H), 7.33 -7.20 (m, 2H), 7.19-7.10 (m, 2H), 7.07 (t, *J* = 7.3 Hz, 1H), 6.59 (d, *J* = 2.8 Hz, 1H), 3.90 (s, 3H).

3.4.3. 5-(5-Fluoro-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6b)**

According to the general method, the reaction of **5b** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6b** in 60.4% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.92 (s, 1H), 7.81 (dd, *J* = 10.1, 2.6 Hz, 1H), 7.70 (s, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.47-7.44 (m, 1H), 7.25 (t, *J* = 7.7 Hz, 1H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.11 (dd, *J* = 9.1, 2.4 Hz, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.71 (s, 1H), 3.90 (s, 3H), 3.54 (s, 3H). HRMS (ESI) m/z [M + H]$^+$ for $C_{20}H_{17}N_5OF$ calcd 362.1412, found 362.1410.

3.4.4. 5-(6-Fluoro-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6c)**

According to the general method, the reaction of **5c** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6c** in 67.3% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.93 (s, 1H), 8.15-8.12 (m, 1H), 7.70 (s, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.33 (dd, *J* = 10.0, 2.1 Hz, 1H), 7.25 (t, *J* = 7.6 Hz, 1H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.08-7.00 (m, 2H), 6.65 (s, 1H), 3.90 (s, 3H), 3.50 (s, 3H).

3.4.5. 5-(5-Chloro-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6d)**

According to the general method, the reaction of **5d** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6d** in 63.2% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.94 (s, 1H), 8.13 (d, *J* = 1.4 Hz, 1H), 7.70 (s, 1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.48 (d, *J* = 8.7 Hz, 1H), 7.29-7.22 (m, 2H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.05 (t, *J* = 7.4 Hz, 1H), 6.71 (s, 1H), 3.90 (s, 3H), 3.54 (s, 3H). HRMS (ESI) m/z [M + H]$^+$ for $C_{20}H_{17}N_5OCl$ calcd 378.1116, found 378.1104.

3.4.6. 5-(6-Chloro-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6e)**

According to the general method, the reaction of **5e** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6e** in 70.4% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.95 (s, 1H), 8.12 (d, *J* = 8.6 Hz, 1H), 7.70 (s, 1H), 7.62-7.58 (m, 2H), 7.25 (t, *J* = 7.7 Hz, 1H), 7.22-7.16 (m, 2H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.69 (s, 1H), 3.90 (s, 3H), 3.53 (s, 3H).

3.4.7. 5-(5-Bromo-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6f)**

According to the general method, the reaction of **5f** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6f** in 81.3% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.95 (s, 1H), 8.29 (d, *J* = 1.9 Hz, 1H), 7.71 (s, 1H), 7.61 (d, *J* = 8.4 Hz, 1H), 7.43 (d, *J* = 8.7 Hz, 1H), 7.37 (dd, *J* = 8.7, 1.9 Hz, 1H), 7.26 (t, *J* = 7.7 Hz,1H), 7.20 (d, *J* = 7.9 Hz, 1H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.70 (s, 1H), 3.90 (s, 3H), 3.53 (s, 3H). HRMS (ESI) m/z [M + H]$^+$ for $C_{20}H_{17}N_5OBr$ calcd 422.0611, found 422.0596.

3.4.8. 5-(6-Bromo-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6g)**

According to the general method, the reaction of **5g** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6g** in 80.2% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.95 (s, 1H), 8.07(d, *J* = 8.6 Hz, 1H), 7.72 (d, *J* =1.4Hz, 1H), 7.70 (s,1H), 7.60 (d, *J* = 8.3 Hz, 1H), 7.30 (dd, *J* = 8.6,1.4 Hz, 1H), 7.25 (t, *J* =7.6 Hz, 1H), 7.19 (d, *J* = 7.9 Hz, 1H), 7.05 (t, *J* = 7.4 Hz, 1H), 6.67 (s, 1H), 3.90 (s, 3H), 3.53 (s, 3H).

3.4.9. 5-(5-Methoxy-1-methyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(6h)**

According to the general method, the reaction of **5h** with trimethylsilyl trifluoromethanesulfonate and dry triethylamine provided **6h** in 58.9% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.86 (s, 1H), 7.66 (s, 1H), 7.59 (d, *J* = 8.3 Hz, 1H), 7.54 (d, *J* = 2.5 Hz, 1H), 7.31 (d, *J* = 8.9 Hz, 1H), 7.26-7.19 (m, 2H), 7.05 (t, *J* = 7.5 Hz, 1H), 6.84 (dd, *J* = 8.9, 2.5 Hz, 1H), 6.67 (s, 1H), 3.88 (s, 3H), 3.70 (s, 3H), 3.50 (s, 3H). HRMS (ESI) m/z [M + H]$^+$ for $C_{21}H_{20}N_5O_2$ calcd 374.1612, found 374.1602.

3.5. Synthesis of compounds 7a – 7h

3.5.1. 4,5-Bis(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(7a)**

60% NaH (0.23 mmol) was added to a solution of **6a** (0.23 mmol) in dry DMF (15 mL) at 0-5 °C. After stirring for 15 min at room temperature. Iodomethane (0.34 mmol) was then added and stirred for 2 h at room temperature. The mixture was then poured into H_2O (120 mL) and the resulting solution was extracted with ethyl acetate (60 mL × 3). The organic phase was combined, washed with brine (180 mL × 3), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The obtained residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate/MeOH (5:10:1, v/v) as eluent to afford **7a** as a white solid, mp: >250 °C, yield 5.3%. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.25 (s, 1H), 8.20 (d, *J* = 7.5 Hz, 1H), 7.72 (s, 1H), 7.61 (d, *J* = 8.3 Hz, 1H), 7.37 (d, *J* = 7.4 Hz, 1H), 7.30-7.21 (m, 2H), 7.20-7.11 (m, 2H), 7.06 (t, *J* = 7.4 Hz, 1H), 6.57 (s, 1H), 3.90 (s, 3H), 3.53 (s, 3H).

3.5.2. General procedure for the preparation of 7b and 7c

60% NaH (0.23 mmol) was added to a solution of **6a** (0.23 mmol) in dry DMF (15 mL) at 0-5 °C. After stirring for 15 min at room temperature. 2-Bromopropane or 1-bromobutane (0.34 mmol) was added and stirred for 5 h at 45 °C. The mixture was then poured into H_2O (120 mL) and the resulting solution was extracted with ethyl acetate (60 mL × 3). The organic phase was combined, washed with brine (180 mL × 3), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate/MeOH (5:10:1, v/v) as eluent to afford **7b** or **7c**.

3.5.3. 5-(1-Isopropyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(7b)**

According to the general method, the reaction of **6a** with 2-bromopropane provided **7b** in 11.9% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.17 (s, 1H), 8.22 (d, *J* = 6.5 Hz, 1H), 7.74 (s, 1H), 7.61 (d, *J* = 8.3 Hz, 1H), 7.37 (d, *J* = 6.5 Hz, 1H), 7.26 (t, *J* = 7.6 Hz, 1H), 7.23-7.14 (m, 3H), 7.06(t, *J* = 7.4 Hz, 1H), 6.57 (d, *J* = 2.5 Hz, 1H), 4.53-4.45 (m, 1H), 3.90 (s, 3H), 1.47(s, 3H), 1.45 (s, 3H).

3.5.4. 5-(1-Butyl-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(7c)**

According to the general method, the reaction of **6a** with 1-bromobutane provided **7c** in 18.5% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 7.58 (d, *J* = 7.6 Hz, 1H), 7.16 (s, 1H), 7.03 (d, *J* = 8.3 Hz, 1H), 6.88 (d, *J* = 8.0 Hz, 1H), 6.70-6.57(m, 3H), 6.54 (d, *J* = 7.9 Hz, 1H), 6.44 (t, *J* = 7.5 Hz, 1H), 5.98 (s, 1H), 3.32-3.28 (m, 5H), 1.45-1.38 (m, 2H), 1.26 -1.21 (m, 2H), 0.83 (t, J=7.0 Hz, 3H).

3.5.5. General procedure for the preparation of 7d and 7e

60% NaH (0.23 mmol) was added to a solution of **6a** (0.23 mmol) in dry DMF (15 mL) at 0-5°C. After stirring for 15 min at room temperature. 1-(3-chloropropyl)-1H-imidazole or 1-(4-chlorobutyl)-1H-imidazole (0.34 mmol) was added and stirred for 12 h at 60-65 °C. After cooling, the mixture was poured into H_2O (120 mL) and the resulting solution was extracted with ethyl acetate (60 mL × 3). The organic phase was combined, washed with brine (180 mL× 3), dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash column chromatography on silica gel using petroleum ether/ethyl acetate/MeOH (5:10:1, v/v) as eluent to afford **7d** or **7e**.

3.5.6. 5-(1-(3-(1H-imidazol-1-yl)propyl)-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(7d)**

According to the general method, the reaction of **6a** with 1-(3-chloropropyl)-1H-imidazole provided **7d** in 23.1% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.69 (s, 1H), 8.18 (d, *J* = 7.9 Hz, 1H), 8.11 (s, 1H), 7.71 (d, *J* = 2.6 Hz, 1H), 7.54 (d, *J* = 8.2 Hz, 1H), 7.49-7.40 (m, 2H), 7.26-7.24 (m, 2H), 7.20-7.18 (m, 2H), 7.13 (s, 1H), 7.03 (t, *J* = 7.5 Hz, 1H), 6.61 (s, 1H), 4.22 (t, *J* = 6.7 Hz, 2H), 3.88 (t, *J* = 6.6 Hz, 2H), 3.52 (s, 3H), 2.39-2.26 (m, 2H).

3.5.7. 5-(1-(4-(1H-imidazol-1-yl)butyl)-1H-indol-3-yl)-4-(1-methyl-1H-indol-3-yl)-2,4-dihydro-3H-1,2,4-triazol-3-one **(7e)**

According to the general method, the reaction of **6a** with 1-(4-chlorobutyl)-1H-imidazole provided **7e** in 17.0% yield as a white solid, mp: >250 °C. ^{1}H NMR (500 MHz, DMSO-*d6*) δ 11.33 (s, 1H), 8.17 (d, *J* = 7.4 Hz, 1H), 7.74-7.69 (m, 2H), 7.61 (d, *J* = 8.3 Hz, 1H), 7.38 (d, *J* = 7.6 Hz, 1H), 7.28-7.23 (m, 2H), 7.20-7.14 (m, 3H), 7.06 (t, *J* = 7.3 Hz, 1H), 6.92 (s, 1H), 6.57 (s, 1H), 4.09 (t, *J* = 6.4 Hz, 2H), 3.94-3.86 (m, 5H), 1.90-1.82 (m, 2H), 1.80-1.73 (m, 2H).

3.6. Biological activity assay

Cell culture medium and supplements were obtained from Invitrogen (Carlsbad, CA, USA). Glutamate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Primary antibodies used for Western blot analysis were: mouse anti-phospho-Tau (Ser396) (1:1000), mouse anti-Tau (1:1000), all from Cell Signaling Technology (Danvers, MA, USA). Secondary antibodies for Western blot analysis were: goat anti-mouse IgG (1:5,000, Jackson ImmunoResearch, West Grove, PA, USA). SuperSignal West Dura Substrate for chemiluminescent detection was purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). All other chemicals were obtained from Sigma-Aldrich unless otherwise stated.

3.7. Kinase assay

The GSK-3β kinase assay was carried out with the Invitrogen Z-LYTE™ Kinase Assay kit, with a final enzyme concentration of 50 nM. All reactions were carried out in triplicate, blank values were subtracted, and the GSK-3β activity was expressed in pico-moles of phosphate incorporated in CREB per minute or in percent-age of maximal activity. The IC_{50} (concentration at which a 50% of enzyme inhibition is shown) values are gathered.

3.8. Primary rat cerebellar granule neuron cultures

Cerebellar granule neuronal cells (CGCs) were isolated from 8-day old Sprague Dawley rat pups as described previously (Pang et al. 2016). Cerebella were collected and placed in ice-cold Hank's balanced salt solution (HBSS) (Invitrogen). After removal of the meninges, the cerebellas were dispersed into the same buffer containing 0.25% trypsin (Invitrogen) and digested for 15 min at 37 °C. Trypsin digestion was stopped by adding a two-fold volume of DMEM (Invitrogen), supplemented with 10% FBS (Invitrogen) and 0.1 mg/ml DNase I (Sigma-Aldrich). After gentle trituration, digested tissues were centrifuged at 1000× rpm for 5 min. The cell pellets were resuspended in the complete Neurobasal culture medium (Invitrogen) supplemented with 2% B27 (Invitrogen) and 0.5 mM GlutaMax (Invitrogen). After filtration through a 70 µm cell strainer (BD Falcon, Vernon Hills, IL, USA), cells were plated at a density of 1×10^6 cells/ml onto poly-l-lysine coated 96-well or 6-well plates (Becton Dickinson and Company, Franklin Lakes, NJ, USA). Cultures were incubated in a humidified atmosphere of 5% CO2-95% air at 37°C. Cytosine arabinofuranoside (10 µM) was added to the cultures 24 h after plating to arrest the growth of glia cells. Cultures 6-8 days *in vitro* were used in this study. Immunocytochemical validation with anti-MAP2 antibody and DAPI revealed that more than 95% of the cells in our cultures system were neurons at the time of experiment.

3.9. MTT assay

The CGCs were pretreated with compounds for 24 h and then incubated with 200 µM glutamate for another 24 h. Cell viability was evaluated by incubating with 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) for 4 h under 5% CO_2/95% air at 37 °C. Active mitochondrial dehydrogenases of living cells cause cleavage and reduction of soluble yellow MTT dye to the insoluble purple formazan, which was extracted in dimethyl sulfoxide (DMSO). Media were replaced with 100 µl DMSO, and the optical density was measured at 570 nM in a BD plate-reader.

3.10. Western blotting

To determine phospho-Tau (Ser396) and total Tau protein blots, cells were lysed in Tris-Glycine SDS lysis buffer at indicated time point after compounds addition, and the lysates were boiled for 10 min. The extracted whole-cell proteins were separated by electrophoresis on 10% SDS-PAGE gels and transferred onto nitrocellulose

membranes. The membranes were blocked for 1 h in casein-based blocking buffer and incubated overnight at 4 °C with the primary antibody followed by washing and exposure to the secondary antibody for 30 min at room temperature. The membranes were exposed to SuperSignal West Dura Substrate for chemiluminescent detection.

3.11. Statistical analysis

Statistical significance was determined using GraphPad Prism 5 Software (GraphPad Software, San Diego, CA). Multiple group comparisons were performed by one-way ANOVA followed by Newman-Keuls post test. Differences were considered statistically significant at $P < 0.05$. Values are expressed as the mean ± SD.

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