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From molecular design to antiepileptic evaluation: sulfonamide–pyrazole derivatives as promising neuroactive agents

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Neuroinflammation, oxidative stress, and glutamate-mediated excitotoxicity are central pathological mechanisms underlying epileptogenesis and seizure propagation. In the present study, a series of rationally designed sulfonamide pyrazole derivatives was evaluated for their anticonvulsant potential using pentylenetetrazol (PTZ)- and pilocarpine-induced seizure models in mice. Among the tested compounds, compound **6d** emerged as the most promising candidate, exhibiting superior anticonvulsant efficacy. In the PTZ model, compound **6d** afforded 90% seizure protection with complete survival, outperforming the reference drug sodium valproate. In the pilocarpine-induced status epilepticus model, compound **6d** significantly prolonged seizure onset latency, markedly suppressed seizure severity (reducing Racine scores by >80%), and ensured 100% survival. Mechanistic investigations revealed that compound **6d** exerted pronounced neuroprotective effects in hippocampal tissue by significantly attenuating oxidative stress (malondialdehyde and nitrite levels), neuroinflammation (TNF- α and IL-6), and excitotoxicity (glutamate levels), with greater efficacy than valproate. Importantly, sub-chronic oral administration of compound **6d** did not induce detectable hepatic, renal, or cardiac toxicity, indicating a favorable preliminary safety profile. Collectively, these findings identify compound **6d** as a promising lead anticonvulsant agent with multimodal neuroprotective actions and support its further preclinical development as a potential disease-modifying therapy for epilepsy.

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1 Introduction

Epilepsy is a prevalent chronic neurological disorder affecting over 50 million people worldwide, representing nearly 1% of the global population.¹ It is second only to stroke in prevalence among neurological conditions and ranks as one of the most

significant contributors to the worldwide burden of brain disorders. Beyond its hallmark seizures, epilepsy carries profound health, social, and economic consequences, placing a heavy strain on patients, caregivers, and healthcare systems.² Although epilepsy has been recognized for centuries, significant progress in its understanding, diagnosis, and treatment has only occurred in recent decades. Despite the availability of numerous antiepileptic drugs (AEDs), approximately one-third of patients remain refractory to treatment, continuing to experience uncontrolled seizures and highlighting the urgent need for more effective therapeutic strategies.

The management of epilepsy predominantly relies on long-term pharmacotherapy, with surgical interventions reserved for select patients in whom medications fail to provide adequate seizure control.³ Conventional antiepileptic drugs (AEDs), including phenytoin (**I**) and carbamazepine (**II**), act *via* diverse mechanisms to suppress neuronal hyperexcitability and prevent seizure recurrence. Despite their widespread clinical use, these agents often demonstrate limited efficacy in drug-resistant populations and are associated with undesirable adverse effects, underscoring the need for safer and more effective therapeutic options,^{4,5} (Fig. 1).

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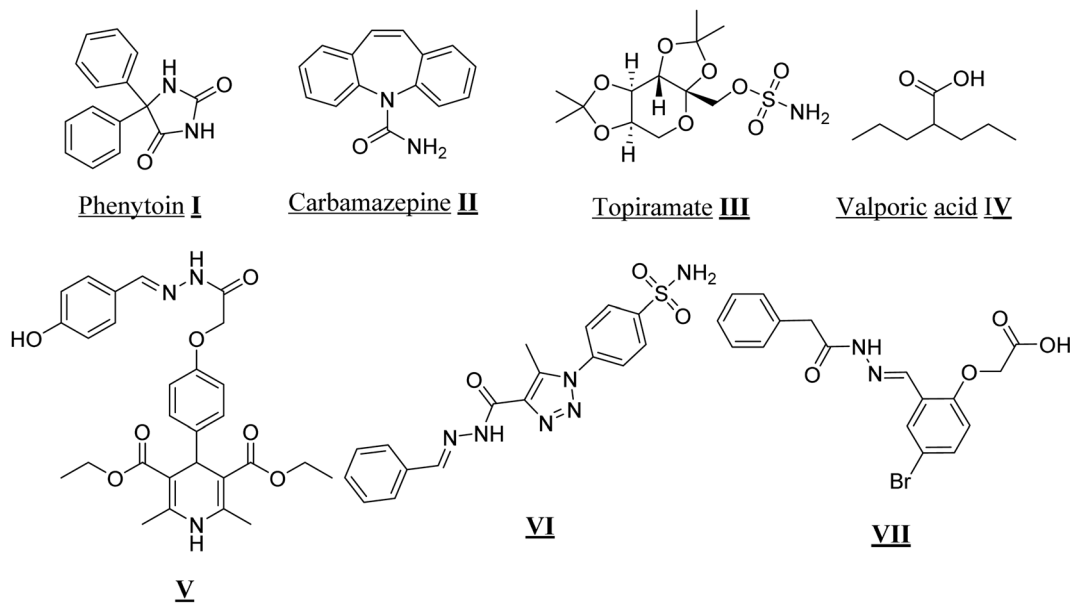


Fig. 1 Diagram of marketed compounds I–IV, and previously reported compounds V–VII, as antiepileptic candidates.

Sulfamate-substituted drugs, such as topiramate (**III**), have shown efficacy across multiple seizure types, representing an important advancement in clinical management. More recently developed AEDs, including valproic acid (**IV**), levetiracetam, and lamotrigine, have further expanded the therapeutic landscape, however, many of these agents still produce adverse effects such as hepatotoxicity, hirsutism, nausea, and weight gain.⁶ Furthermore, drug resistance and neurotoxicity remain significant barriers to optimal seizure control,⁷ highlighting the persistent need for novel anticonvulsant agents with enhanced efficacy and improved safety profiles (Fig. 1).

In contemporary medicinal chemistry, efforts are increasingly directed toward the rational design of compounds with greater target selectivity and optimized pharmacological properties. Mechanistically, AEDs primarily exert their effects by potentiation of GABAergic inhibitory transmission, suppression of glutamatergic excitatory signaling, or modulation of voltage-gated sodium and T-type calcium channels.⁸ Such targeted approaches are essential for developing next-generation antiepileptic agents capable of overcoming the limitations of existing therapies.

Recent literature suggests that the mechanisms of action of many currently available antiepileptic drugs (AEDs) remain incompletely understood. While some AEDs do not exhibit specific receptor-binding interactions, others act through diverse and sometimes poorly defined pathways.^{9,10} This lack of selectivity and clarity in mechanism can contribute to suboptimal efficacy and undesirable side effects, underscoring the urgent need for the development of novel therapeutic agents with precise molecular targets and improved safety profiles. Consequently, contemporary medicinal chemistry research has increasingly focused on designing new anticonvulsant compounds that not only provide better seizure

control but also overcome the limitations associated with existing therapies.¹¹

Inflammation, the body's natural defense response to harmful stimuli such as trauma, infection, or chemical irritants, has been recognized as a key contributor to the pathophysiology of epilepsy.¹² Clinically, inflammatory processes manifest as heat, swelling, redness, pain, and impaired tissue function.¹³ At the molecular level, inflammation involves the activation and migration of leukocytes, increased vascular permeability, and the release of various pro-inflammatory mediators. Critical enzymes, including cyclooxygenases (COX) and lipoxygenases, drive the production of prostaglandins, leukotrienes, and reactive oxygen species (ROS), which collectively propagate and amplify the inflammatory cascade.¹⁴ Understanding these pathways highlights the potential for designing multifunctional compounds that combine anticonvulsant and anti-inflammatory activities, offering a promising strategy for more effective epilepsy treatment.

Hybrid molecules, which integrate multiple pharmacophoric units into a single chemical scaffold, have attracted considerable interest in drug discovery due to their potential for enhanced biological activity and multi-target interactions. Within this framework, hydrazone derivatives have emerged as promising candidates, particularly when combined with benzene sulfonamide and pyrazole moieties through molecular hybridization and bioisosteric modifications. This approach not only enhances synthetic accessibility but also broadens the pharmacological profile of the resulting compounds.¹⁵

Importantly, several hydrazone-based derivatives containing structurally distinct moieties **V–VII** have demonstrated significant anticonvulsant activity through diverse mechanisms of action, highlighting their relevance in the ongoing search for novel antiepileptic agents.^{16–18}



By strategically incorporating benzenesulfonamide cores with flexible hydrogen-bonding or cyclic hydrazide linkers, along with pyrazole and additional aromatic substituents, these hybrid molecules are designed to optimize target interactions, enhance binding affinity, and provide multifunctional activity, making them promising candidates for next-generation anti-convulsant therapy (Fig. 1).

2 Rational and design

Building upon our previously published work on anti-inflammatory scaffolds, the current study extends this framework through a rational repurposing strategy aimed at exploring their antiepileptic potential. As illustrated in the referenced Fig. 2, the design concept was derived from earlier reported benzenesulfonamide-based derivatives, which were strategically modified *via* molecular hybridization and bioisosteric replacement to enhance their neuroactive profile. Specifically, the previously reported anti-inflammatory pharmacophores were integrated with hydrogen-bonding linkers or cyclic hydrazide

moieties, alongside the incorporation of a pyrazole ring and additional aromatic substitutions to improve hydrophobic and binding interactions within the target site. Furthermore, bioisosteric transformation, including the replacement of the triazole ring with a pyrazole scaffold and the introduction of isatin-based moieties, was employed to optimize biological activity. This rational evolution from our prior study highlights the structural continuity while emphasizing the novelty of the current design, ultimately leading to the identification of promising candidates **6b–d**, **7a**, **7e**, and **7f** with dual anti-inflammatory and anticonvulsant potential, as supported by *in vivo* and biochemical evaluations.

3 Bioinformatics study

3.1 Identification of overlapping targets and hub genes between active compound and anti-epilepsy-related targets

A total of 1853 anti-epilepsy-associated targets were retrieved from the GeneCards and DisGeNET databases. These targets were intersected with the top 100 predicted targets for each

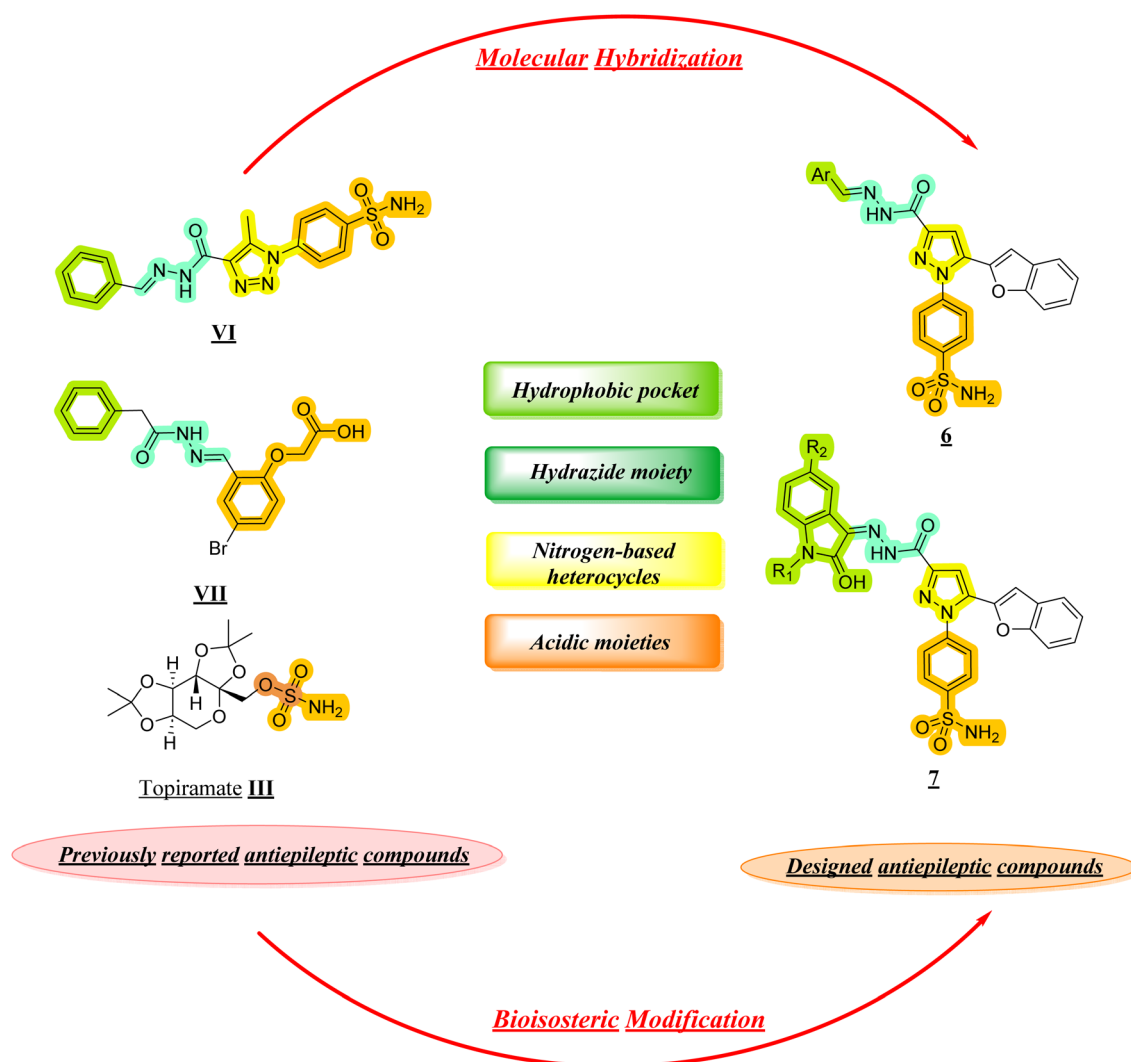


Fig. 2 Rational design strategy of dual antiepileptic and anti-inflammatory agents **6b–d**, **7a**, **7e**, and **7f**.



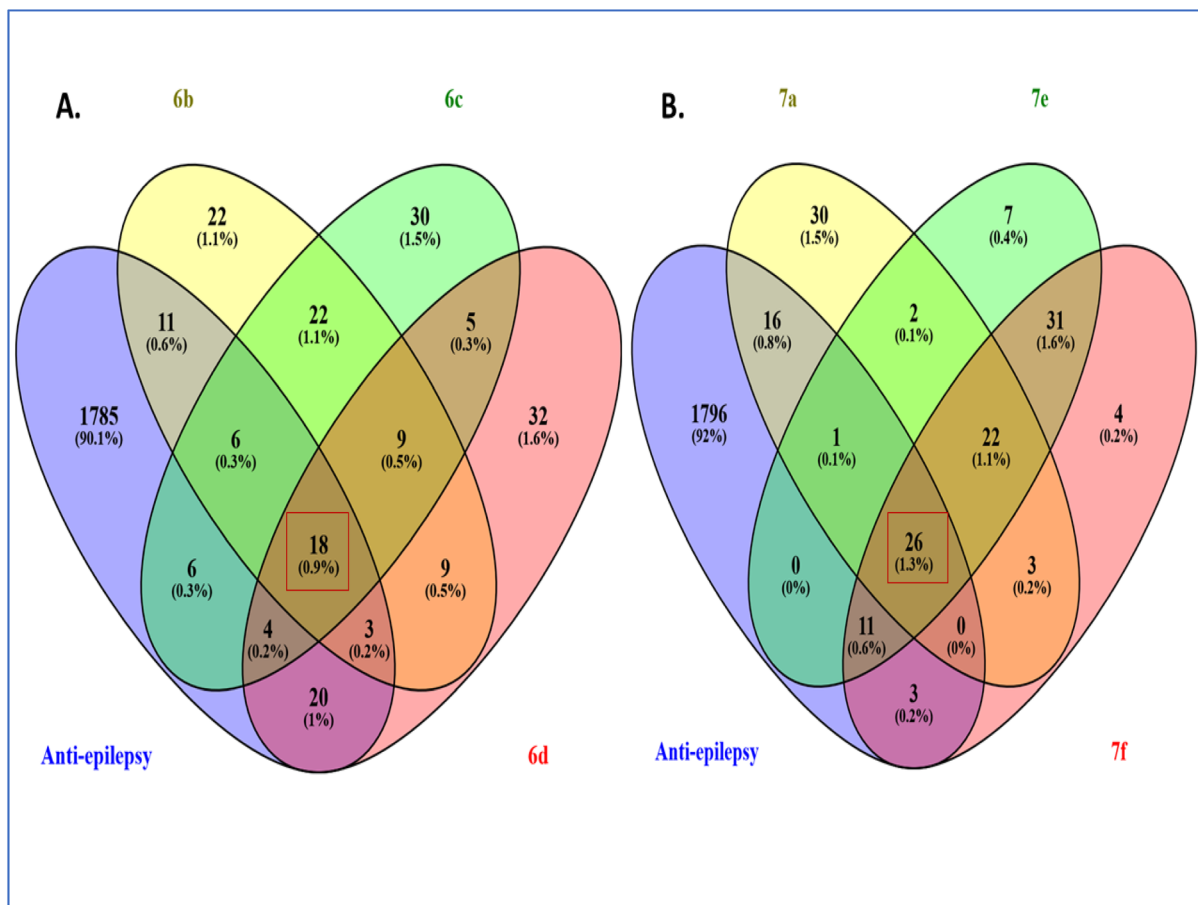


Fig. 3 Overlap between compounds **6b–d**, **7a**, **7e**, and **7f**, with anti-epilepsy-associated targets. (A) Venn diagram illustrating the overlapping targets between compounds **6b–6d** and anti-epilepsy-associated target genes. (B) Common targets shared between compounds **7a**, **7e**, **7f** and anti-epilepsy-associated targets.

group of compounds **6b–d**, **7a** and **7e**, **7f**, resulting in 18 and 26 overlapping genes, respectively, as illustrated in the Venn diagrams (Fig. 3A and B).

3.2 Integrated anti-epileptic network analysis of predicted compound targets

The KEGG pathway enrichment analysis was performed to explore anti-epilepsy-related signaling pathways potentially targeted by the investigated compounds. As shown in Table 1,

compounds **6b**, **6c**, and **6d** were significantly enriched in pathways related to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor resistance, vascular endothelial growth factor (VEGF) signaling pathway, erythroblastic oncogene B (ErbB) signaling pathway, and Ras-associated protein-1 (Rap1) signaling pathway. These pathways exhibited high fold enrichment values and statistically significant false discovery rate (FDRs), indicating a strong involvement of receptor-mediated and intracellular signaling mechanisms.

Table 1 Functional enrichment analysis of predicted targets associated with the antiepileptic potential of the investigated compounds (**6b**, **6c**, **6d**)^a

Enrichment FDR	<i>n</i> Genes	Pathway genes	Fold enrichment	Pathways
9.1×10^{-14}	8	79	130.8	EGFR tyrosine kinase inhibitor resistance
9.2×10^{-9}	5	59	109.5	VEGF signaling pathway
1.7×10^{-11}	7	84	107.7	ErbB signaling pathway
2.1×10^{-9}	7	211	42.9	Rap1 signaling pathway

^a Pathway genes represent the total number of genes annotated in the corresponding KEGG pathway. Fold enrichment reflects the degree of overrepresentation of target genes within each pathway. FDR denotes the false discovery rate. FDR, false discovery rate; *n*Genes, number of enriched genes, EGFR, epidermal growth factor receptor; ErbB, erythroblastic oncogene B; VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor-1; Rap1, Ras-associated protein-1.



Table 2 The KEGG enrichment of anti-epileptic-related target genes for compounds (7a, 7e, 7f)^a

Enrichment FDR	nGenes	Pathway genes	Fold enrichment	Pathways
7.0×10^{-15}	9	79	101.9	EGFR tyrosine kinase inhibitor resistance
9.0×10^{-15}	9	84	95.8	ErbB signaling pathway
6.8×10^{-10}	6	59	91	VEGF signaling pathway
4.5×10^{-10}	7	109	57.4	HIF-1 signaling pathway
4.3×10^{-13}	10	211	42.4	Rap1 signaling pathway
1.8×10^{-12}	11	362	27.2	PI3K-Akt signaling pathway
8.0×10^{-9}	8	300	23.9	MAPK signaling pathway

^a Pathway genes represent the total number of genes annotated in the corresponding KEGG pathway. Fold enrichment reflects the degree of overrepresentation of target genes within each pathway. FDR denotes the false discovery rate. FDR, false discovery rate; nGenes, number of enriched genes, EGFR, epidermal growth factor receptor; ErbB, erythroblastic oncogene B; VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor-1; Rap1, Ras-associated protein-1; PI3K-Akt, phosphoinositide 3-kinase/protein kinase B; MAPK, mitogen-activated protein kinase; EGFR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor.

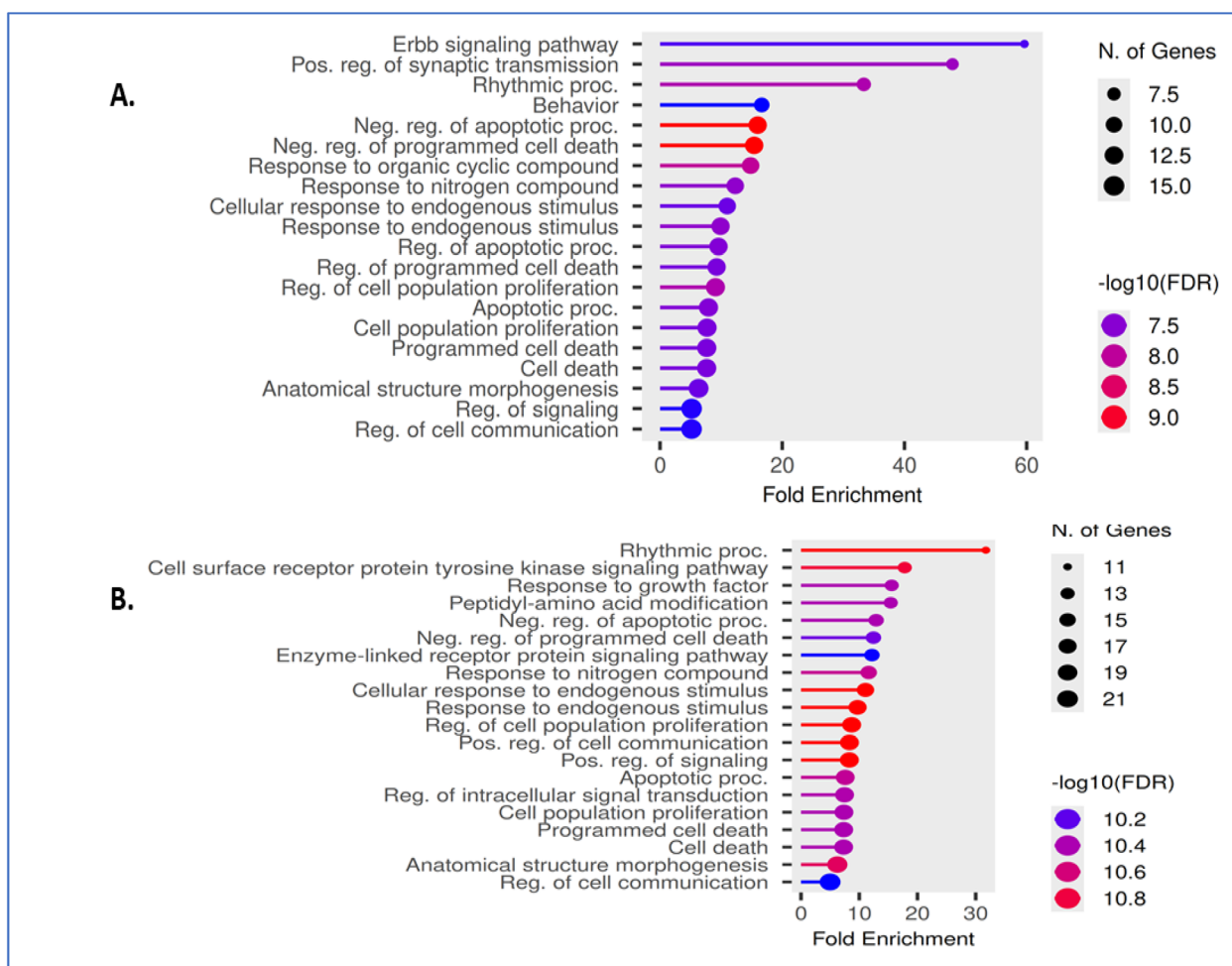


Fig. 4 Gene ontology (GO) biological process enrichment analysis of compounds evaluated for potential antiepileptic activity. (A) Compounds 6b, 6c, and 6d, showing enrichment in biological processes related to regulation of apoptosis, programmed cell death, and cellular signaling. (B) Compounds 7a, 7e, and 7f, enriched in receptor-mediated signaling pathways, rhythmic processes, and regulation of cell communication. Nodes represent enriched pathways; node size indicates enrichment strength. Edges show shared genes/functional overlap. Clusters highlight coordinated immune and ECM-related signaling. Dot size represents the number of genes, color indicates $-\log_{10}(\text{FDR})$, and the x-axis denotes fold enrichment. Analysis was performed using Shiny GO.



In Table 2, compounds **7a**, **7e**, and **7f** demonstrated enrichment in multiple kinase-associated pathways, including EGFR tyrosine kinase inhibitor resistance, ErbB signaling pathway, VEGF signaling pathway, hypoxia-inducible factor-1 (HIF-1) signaling pathway, Rap1 signaling pathway, phosphoinositide 3-kinase/protein kinase B (PI3K-Akt) signaling pathway, and mitogen-activated protein kinase (MAPK) signaling pathway. Notably, these pathways showed higher gene counts and broader pathway coverage compared with compounds **6b–6d**.

As shown in Fig. 4A, gene ontology (GO) biological process enrichment analysis of compounds were predicted to influence processes including the regulation of apoptosis, programmed cell death, cell communication, receptor-mediated signaling, and rhythmic processes. Such these processes are directly relevant to neuroprotection and seizure modulation, implying that these compounds could counteract neuronal hyperexcitability associated with epilepsy.

In Fig. 4B, compounds **7a**, **7e**, and **7f** exhibited prominent enrichment in biological processes associated with cell surface receptor protein tyrosine kinase signaling, enzyme-linked receptor protein signaling pathway, and rhythmic process. Moreover, enrichment was observed in processes related to regulation of cell communication, apoptotic signaling, and cell death, suggesting modulation of receptor-mediated signaling and neuronal synchronization.

To evaluate the potential antiepileptic relevance of the investigated compounds, gene ontology (GO) molecular function enrichment analysis was conducted using Shiny GO. Compounds were grouped as **6b**, **6c**, and **6d** (Fig. 5A). These compounds demonstrated significant enrichment in molecular functions associated with G protein-coupled adenosine receptor activity, histone deacetylase activity, and phosphotransferase activity transferring phosphorus-containing groups. Additional enrichment was observed in protein kinase activity and ATP binding, indicating involvement in signaling and regulatory processes relevant to neuronal

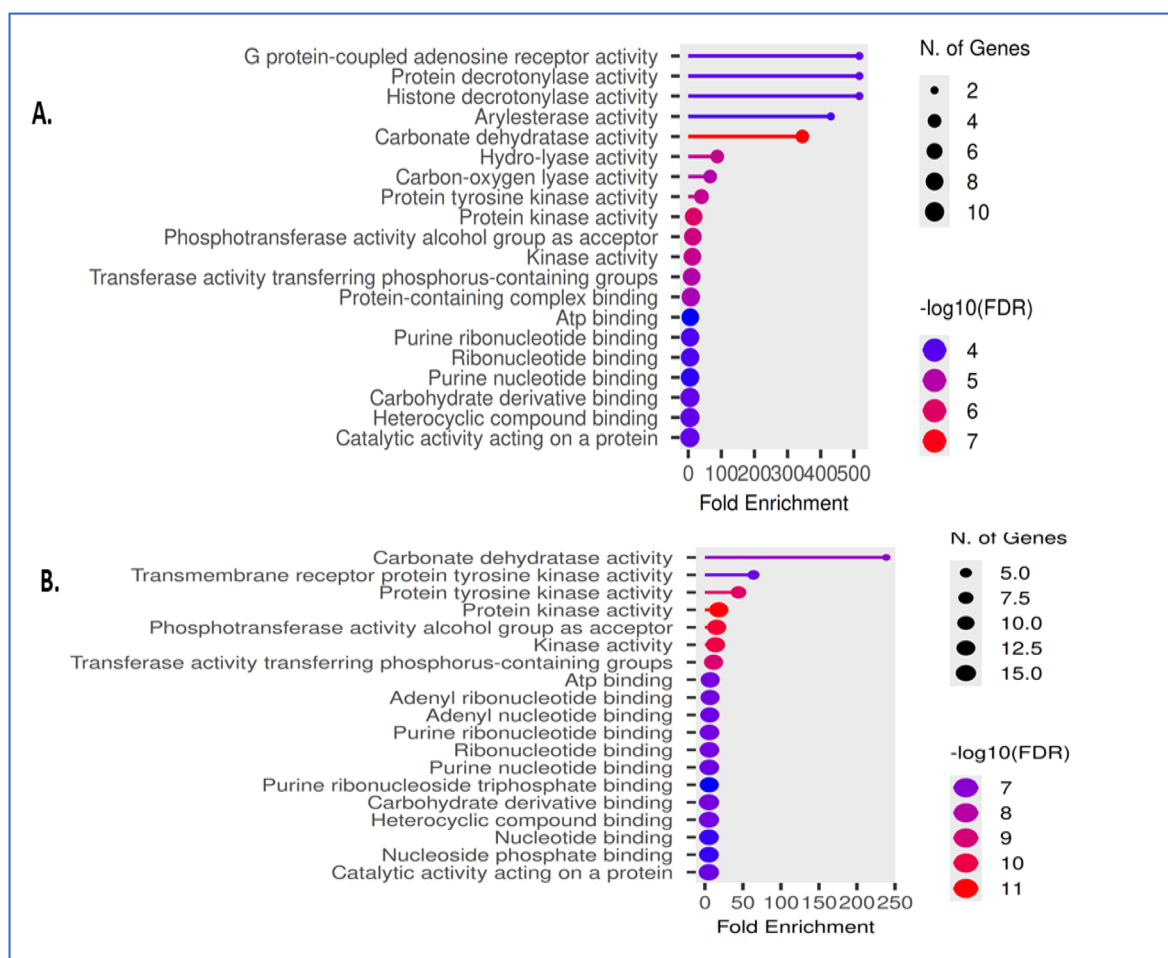


Fig. 5 Gene ontology (GO) molecular function enrichment analysis of compounds evaluated for potential antiepileptic activity. (A) GO molecular function network for compounds **6b**, **6c**, **6d**. (B) GO molecular function network for compounds **7a**, **7e**, **7f**. Nodes represent enriched pathways; node size indicates enrichment strength. Edges show shared genes/functional overlap. Dot size represents the number of genes, color indicates $-\log_{10}(\text{FDR})$, and the x-axis shows fold enrichment. Analysis was performed using Shiny GO.



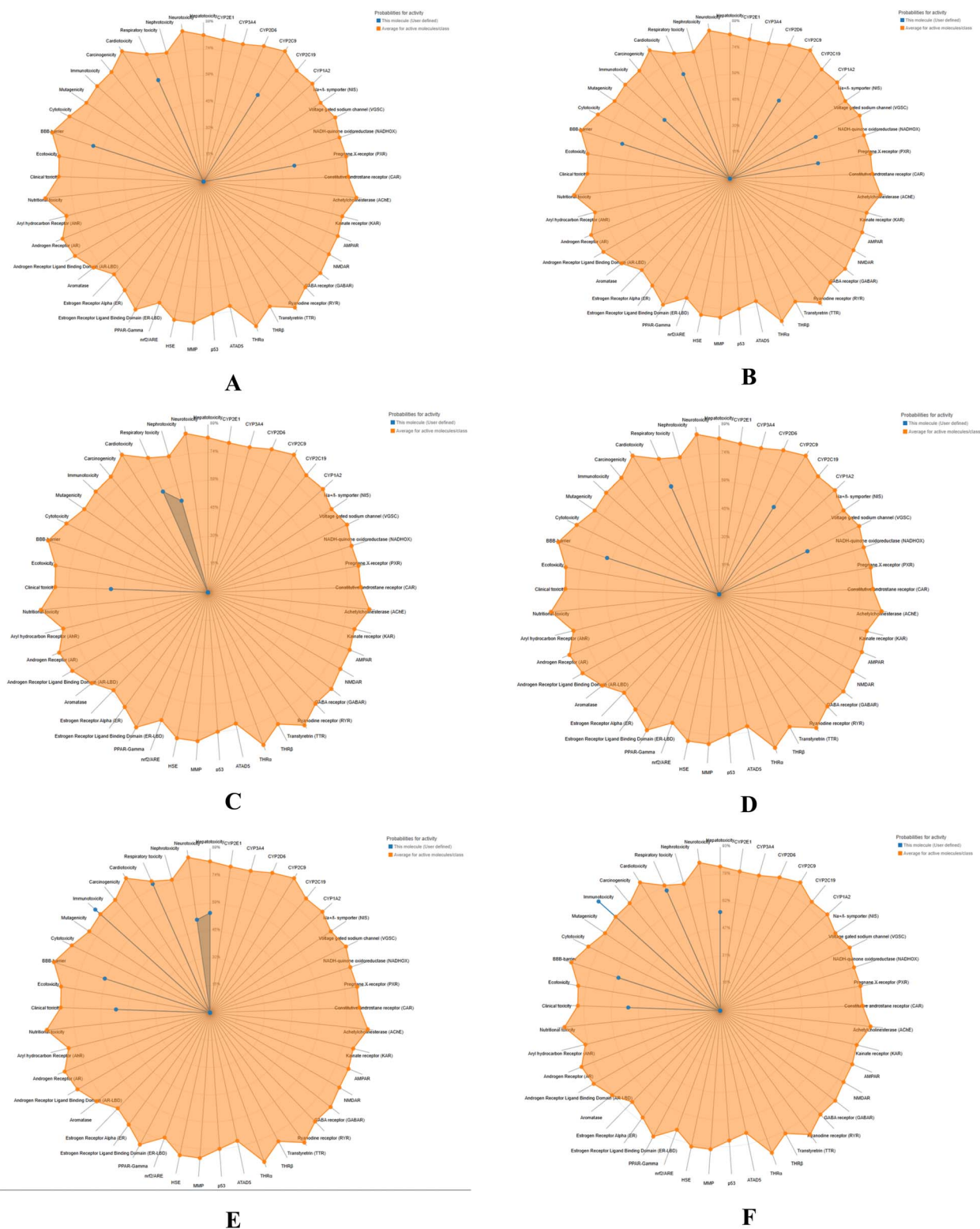


Fig. 6 Toxicity radar of the designed compounds predicted using the ProTox-3.0 software 6b (A), 6c (B), 6d (C), 7a (D), 7e (E), and 7f (F).

Pathophysiology of Neuroinflammation

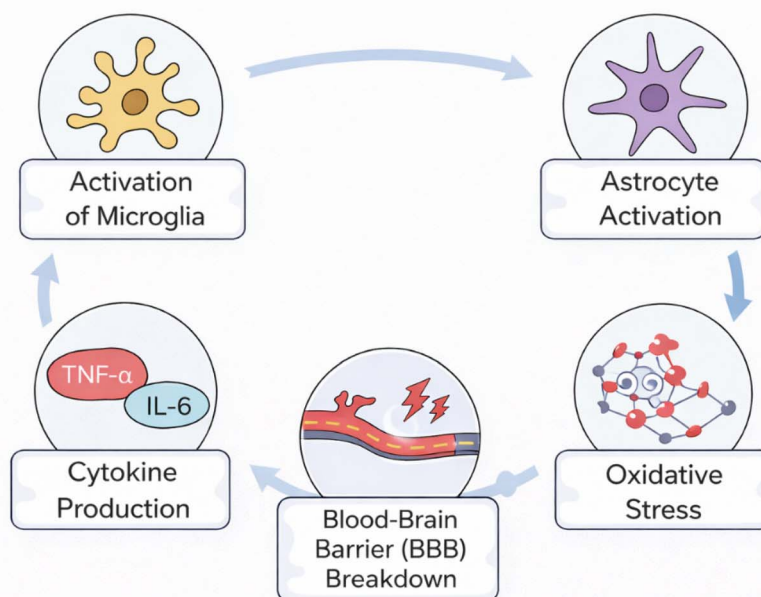


Fig. 7 Neuroinflammatory and oxidative stress cascade in epilepsy.

excitability and **7a**, **7e**, and **7f** (Fig. 5B). Compounds were predominantly enriched in transmembrane receptor protein kinase activity, protein tyrosine kinase activity, and phosphotransferase activity, along with purine ribonucleotide binding and other nucleotide-related functions. These pathways showed comparatively higher fold enrichment values, suggesting strong engagement of phosphorylation-dependent signaling mechanisms.

4 Predicted toxicological profile

ProTox-3.0 *in silico* predictions indicated that compounds **6b–d**, **7a**, **7e**, and **7f** belong to toxicity class VI, with estimated LD₅₀ values ranging from approximately 500 to 3000 mg kg⁻¹. These results suggest a low risk of acute oral toxicity and a wide therapeutic safety margin. Consistently, toxicity radar chart profiling and active toxicity cluster analyses confirmed the overall low toxicological risk of the designed compounds (Fig. 6 and S1).

5 Pathophysiology of neuroinflammation

Seizure-induced neuronal hyperexcitability disrupts the integrity of the blood–brain barrier (BBB), leading to increased permeability.¹⁹ This process is initiated primarily by glutamate-mediated activation of NMDA receptors. BBB

dysfunction is accompanied by altered expression of ATP-binding cassette transporters, particularly P-glycoprotein (P-gp), which is markedly upregulated following seizure activity both at the BBB and within brain tissue. P-gp actively effluxes antiepileptic drugs from the brain, thereby reducing their effective intracerebral concentrations.²⁰ Mechanistic studies reveal that glutamate-induced P-gp overexpression is mediated through NMDA receptor activation and downstream cyclooxygenase-2 (COX-2) signaling, and that pharmacological inhibition of COX-2 or blockade of EP1 receptors can attenuate seizure-induced P-gp upregulation.²¹ These findings collectively highlight COX-2 as a key regulator of BBB dysfunction and a critical contributor to pharmacoresistance in epilepsy (Fig. 7).

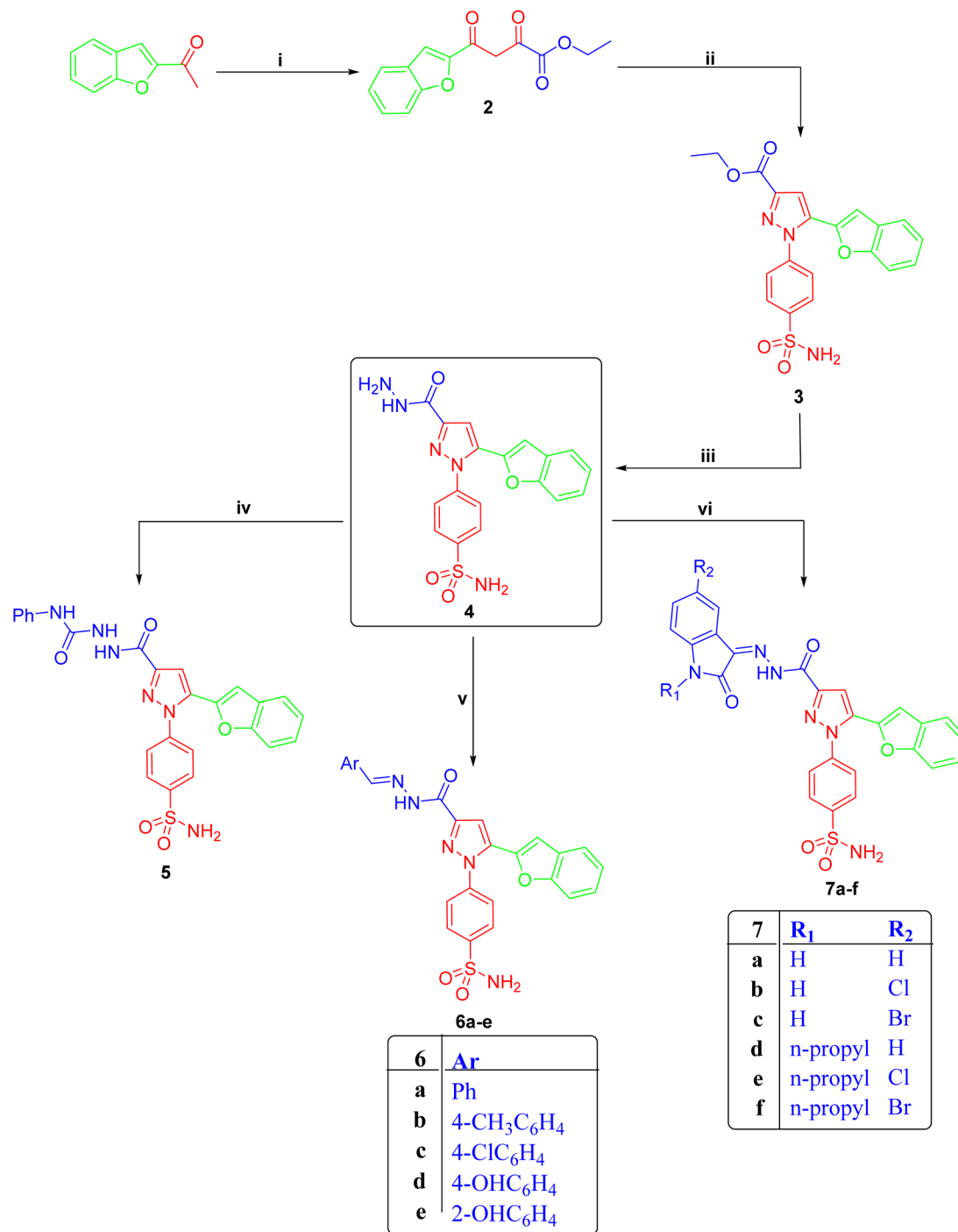
6 Results and discussion

6.1 Chemistry

The design and synthesis of the target compounds **6b–d**, **7a**, **7e**, and **7f** were carried out following the synthetic pathways and experimental protocols described in our earlier study²² (SI).

Scheme: reagents and conditions: (i) (COOEt)₂/NaOC₂H₅/EtOH/0 °C; (ii) *p*-SO₂NH₂C₆H₄NHNH₂/EtOH, AcOH/reflux 3 h; (iii) N₂H₄·H₂O/reflux 3 h; (iv) phenyl isothiocyanate/EtOH reflux 1 h, (v) EtOH/few drops AcOH/reflux 4 h, (vi) EtOH, few drops AcOH/reflux 4 h.



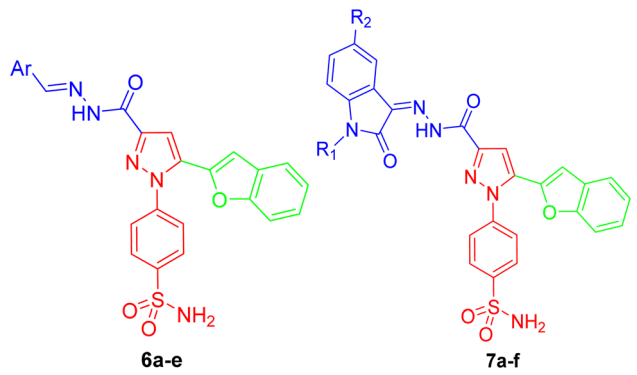


7 Biological study

Guided by the mechanistic insights into the self-perpetuating pathological cycle of neuroinflammation, oxidative stress, and neuronal hyperexcitability in epilepsy, our design strategy prioritized the evaluation of the most potent candidates **6b–d**, **7a**, **7e**, and **7f**, which demonstrated pronounced COX-2 inhibitory activity. These selected compounds were subsequently investigated for their

anticonvulsant efficacy by targeting key molecular and cellular pathways involved in seizure initiation and propagation. The underlying rationale is that pharmacological inhibition of COX-2 may attenuate the neuroinflammatory cascade by suppressing pro-inflammatory cytokine release, mitigating oxidative stress, and stabilizing neuronal excitability, thereby conferring dual therapeutic benefits as an anti-inflammatory and an antiepileptic agent.



Table 3 *In vitro* inhibitory activity of designed compounds **6b–d**, **7a**, **7e**, and **7f** toward COX-2 enzymes


Compound	X	R ₁	R ₂	COX-2 IC ₅₀ (μM)	SI
Celecoxib	—	—	—	0.04 ± 0.002	322.30
6b	4-CH ₃ C ₆ H ₅	—	—	0.07 ± 0.006	103.18
6c	4-ClC ₆ H ₅	—	—	0.08 ± 0.006	84.00
6d	4-OHC ₆ H ₅	—	—	0.05 ± 0.006	103.12
7a	—	H	H	0.05 ± 0.006	104.70
7e	—	<i>n</i> -Propyl	Cl	0.08 ± 0.006	103.60
7f	—	<i>n</i> -Propyl	Br	0.07 ± 0.006	102.27

8 Anti-inflammatory assay

8.1 *In vitro* COX-2 assay²²

In line with our previously published findings, compounds **6b–d**, **7a**, **7e**, and **7f** showed potent and selective inhibitory activity against the COX-2 isozyme, with IC₅₀ values of 0.07–0.09 μM, comparable to or exceeding those of celecoxib (Table 3).

8.2 *In vivo* assay of most potent compound **6d**²²

Compound **6d** exhibited the most pronounced anti-inflammatory effect, producing the greatest reduction in paw thickness at the 5 h time point (45.61% inhibition) and demonstrating the fastest onset of action, with a marked inhibition of 76.33% within the first hour compared with the carrageenan-treated group.

Table 4 Paw thickness difference at hourly intervals (and percentage of inhibition) and the percentage of paw weight increase^a

	Paw thickness difference at hourly intervals (mm) (percentage of inhibition (%))					Paw weight increase (%)
	1st hour	2nd hour	3rd hour	4th hour	5th hour	
Control	−0.07 ± 0.07	−0.12 ± 0.08	−0.18 ± 0.09	−0.18 ± 0.09	−0.18 ± 0.08	0.99 ± 1.59 ^b
Carrageenan	1.35 ± 0.14 ^b	2.63 ± 0.15 ^b	3.4 ± 0.13 ^b	3.78 ± 0.17 ^b	4.66 ± 0.14 ^b	77.46 ± 4.03 ^b
Celecoxib	0.98 ± 0.11 ^b (27.39%)	1.45 ± 0.15 ^{b,c} (44.96%)	1.72 ± 0.09 ^{b,c} (49.26%)	2.01 ± 0.19 ^{b,c} (46.85%)	2.37 ± 0.12 ^{b,c} (49.02%)	27.5 ± 4.17 ^{b,c}
6d	0.32 ± 0.11 ^{b,c,d,e} (76.33%)	1.29 ± 0.16 ^{b,c} (50.86%)	1.63 ± 0.14 ^{b,c} (52.16%)	2.05 ± 0.2 ^{b,c} (45.79%)	2.53 ± 0.33 ^{b,c} (45.61%)	26.54 ± 3.38 ^{b,c}

^a The data are presented as mean ± SD and were analyzed using two-way ANOVA (for paw thickness difference) or one-way ANOVA (for paw weight increase percentage), followed by Tukey's multiple comparisons test; *n* = 6. ^b Significantly different from control group at *p* < 0.05. ^c Significantly different from carrageenan group at *p* < 0.05. ^d Significantly different from celecoxib group at *p* < 0.05. ^e Significantly different from mefenamic acid group at *p* < 0.05.

Table 5 Protective efficacy, relative potency, and mortality rates of test compounds in the PTZ-induced seizure model^a

	Protection (%)	Relative protection (%)	24 h mortality (%)
PTZ	0%	—	80%
Valproic acid	60%	—	30%
6b	60%	100%	20%
6c	70%	116.67%	20%
6d	90%	150%	0%
7a	50%	83.33%	50%
7e	50%	83.33%	50%
7f	30%	50%	60%

^a Seizure protection was calculated as the percentage of animals that did not develop tonic-clonic seizures. Relative protection values were expressed as percentages compared to sodium valproate (protection test/protection valproate × 100). *n* = 10. Mortality was recorded within 24 hours after PTZ challenge. Data are expressed as percentages.

Furthermore, compound **6d** achieved the highest suppression of paw edema, as reflected by inhibition of the percentage increase in paw weight (65.74%), outperforming all other tested compounds. Notably, it was the only compound that showed no statistically significant difference when compared with both reference drugs (Table 4).

9 Anti-epileptic assay

9.1 Anticonvulsant activity in the PTZ-induced seizure model

As shown in Table 5, PTZ administration induced generalized tonic-clonic seizures in all control mice, yielding 0% seizure protection and 80% mortality, confirming the model's severity. Pretreatment with valproic acid (300 mg kg^{−1}) significantly improved seizure outcomes, producing 60% protection and reducing mortality to 30%. This finding is in line with its known anticonvulsant efficacy.^{23,24} The test compounds showed substantially greater activity. The most efficacious were **6d**, **6c**, and **6b**, which afforded 90%, 70%, and 60% protection, respectively. Notably, **6d** completely abolished mortality (0%), while **6c** and **6b** limited deaths to 20% (*versus* 30% in the valproate group). When expressed relative to valproate, these correspond to protective potencies of 150%, 116.7%, and 100%,



Table 6 Effects of valproic acid and test compounds on seizure onset, severity, and survival in the pilocarpine-induced seizure model^a

	Onset (min)	Seizure severity				24 h survival (%)
		30 min	60 min	90 min	120 min	
Pilocarpine	6.89 ± 0.93	3.6 ± 0.52	4 ± 0.47	4.6 ± 0.52	4.78 ± 0.44	40
Valproic acid	15.58 ± 1.01 ^b	2.8 ± 0.63	2.8 ± 0.42 ^b	2.4 ± 0.52 ^b	2.5 ± 0.53 ^b	70
6b	13.31 ± 0.97 ^{b,c}	2.9 ± 0.32	2.8 ± 0.42 ^b	2.5 ± 0.71 ^b	2.9 ± 0.88 ^b	80
6c	16.87 ± 0.86 ^b	2.7 ± 0.67	2.6 ± 0.52 ^b	2.4 ± 0.52 ^b	2.4 ± 0.52 ^b	80
6d	20.94 ± 1.06 ^{b,c}	2.2 ± 0.42 ^b	2.2 ± 0.42 ^b	1.3 ± 0.48 ^{b,c}	0.8 ± 0.42 ^{b,c}	100
7a	11.5 ± 0.72 ^{b,c}	2.9 ± 0.57	3.2 ± 0.79	3.5 ± 0.53 ^{b,c}	3.4 ± 0.84 ^b	50
7e	10.72 ± 1.03 ^{b,c}	3 ± 0.67	3.3 ± 0.48	3.7 ± 0.67 ^c	3.56 ± 0.73 ^{b,c}	40
7f	9.67 ± 1.4 ^{b,c}	3.4 ± 0.52	3.7 ± 0.67	3.67 ± 0.71 ^c	3.89 ± 0.78 ^c	40

^a Data are presented as mean ± SD or percentages for survival rates. Statistical analysis was performed by one-way ANOVA for seizure onset and two-way ANOVA for seizure severity, followed by Tukey's post hoc test. ^b Indicates significant difference *versus* pilocarpine group ($P < 0.05$), while. ^c Indicates significance *versus* valproic acid group ($P < 0.05$).

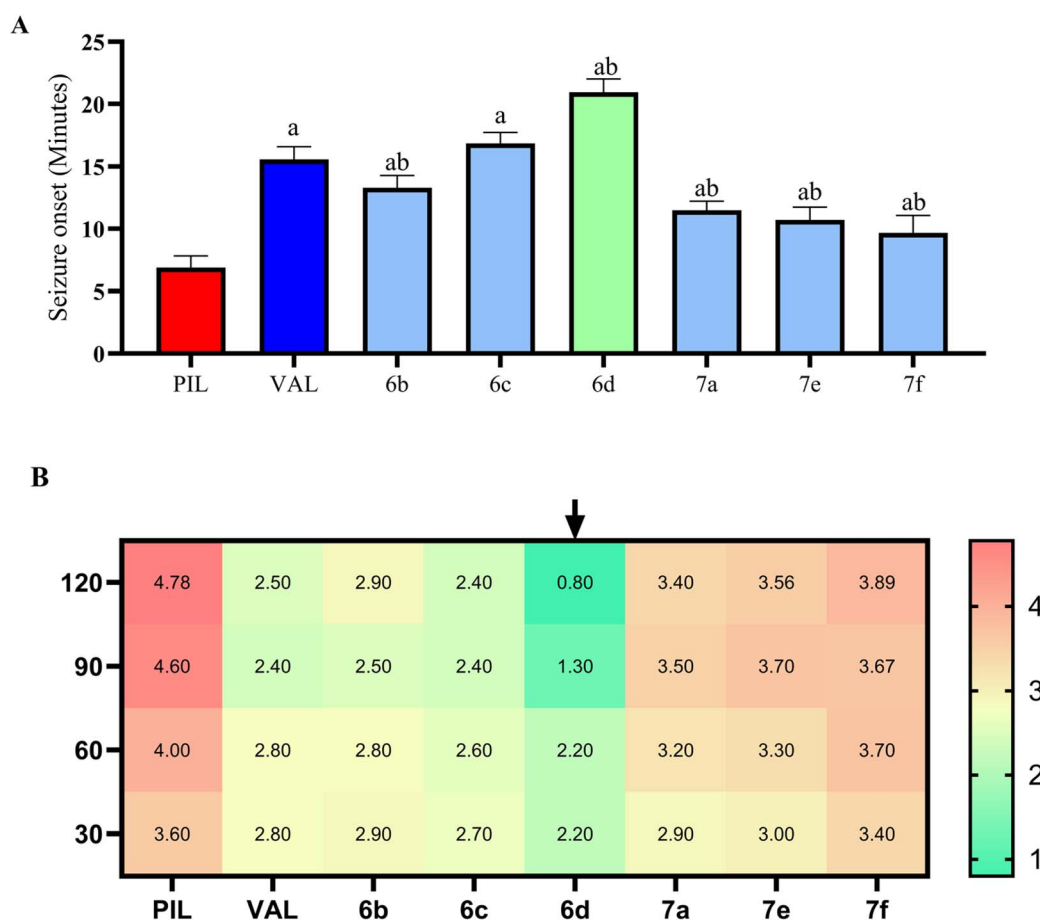


Fig. 8 Effects of the investigated compounds on seizure onset latency and seizure intensity in the pilocarpine-induced seizure model. Panel (A) illustrates the time to onset of stage 3 seizures (in minutes) following pilocarpine administration across the different experimental groups. Panel (B) presents a heat-map visualization of seizure severity progression, expressed as Racine scores, recorded at the indicated time intervals over the 120 min monitoring period. Lower seizure intensities are depicted by green color gradients, whereas higher severity levels are indicated by darker red gradients. Data are shown as group mean values ($n = 6$), with detailed variability and statistical outcomes provided in Table 4. Seizure onset latency was analyzed using one-way ANOVA, while seizure severity over time was assessed using two-way ANOVA, followed by Tukey's post hoc multiple comparisons test. Superscript letters indicate statistically significant differences at $p < 0.05$: a, compared with the pilocarpine control group; b, compared with the valproic acid group. Abbreviations: PIL, pilocarpine control; VPA, sodium valproate.

respectively, highlighting their superior anticonvulsant efficacy profiles. Moderate efficacy was observed with **7a** and **7e** (each 50% protection, 50% mortality; 83.33% relative potency),

whereas **7f** was the least active (30% protection, 60% mortality; 50% relative potency). Overall, the compounds can be ranked in the terms of potency as follows: **6d** > **6c** > **6b** > **7a** = **7e** > **7f**.



9.2 Evaluation of seizure onset, severity, and survival in the pilocarpine-induced convulsion model

Table 6 summarizes the outcomes of the pilocarpine model. In control mice, pilocarpine injection triggered rapid seizures: latency was only 6.89 min (6.893 ± 0.30 min, mean \pm SEM) (Fig. 8A) and severity escalated to a Racine score of 4.78 by 120 min (Fig. 8B), with only 40% survival. Pretreatment with valproic acid significantly delayed seizure onset to 15.58 ± 1.01 min (an increase of 125.94% relative to control) (mean difference [MD] vs. pilocarpine = 8.68 min, 95% CI 7.26 to 10.10; $p < 0.0001$) (Fig. 8A) and reduced the final Racine score to 2.5 (a 47.67% decrease compared to control) (120 min: MD = 2.28 Racine units, 95% CI 1.25 to 3.31; $p < 0.0001$) (Fig. 8B). This yielded a 70% survival rate, demonstrating moderate protection.

The test compounds differed markedly. **6d** was the most effective: it extended the seizure latency to 20.94 ± 1.06 min (203.82% longer than control, 34.47% longer than valproate) (vs. pilocarpine: MD = 14.05 min, 95% CI 12.63 to 15.47; $p < 0.0001$; vs. valproate: MD = 5.37 min, 95% CI 3.95 to 6.79; $p < 0.0001$) (Fig. 8A) and strongly suppressed seizure progression. At 120 min, its Racine score was only 0.8 ± 0.42 (about an 83.26% reduction vs. control, versus 47.67% for valproate) (120 min vs. pilocarpine: MD = 3.98 Racine units, 95% CI 2.95 to 5.01; $p < 0.0001$; vs. valproate: MD = 1.70, 95% CI 0.70 to 2.70; $p < 0.0001$) (Fig. 8B). Notably, **6d** ensured 100% survival. Compound **6c** also provided robust protection: onset was delayed to 16.87 ± 0.86 min (+144.71% vs. control) (vs. pilocarpine: MD = 9.98 min, 95% CI 8.56 to 11.39; $p < 0.0001$; vs. valproate: MD = 1.29 min, 95% CI -0.12 to 2.71; $p = 0.0991$), final severity was 2.4 ± 0.52 (49.77% reduction) (120 min vs. pilocarpine: MD = 2.38 Racine units, 95% CI 1.35 to 3.41; $p < 0.0001$; vs. valproate: MD = 0.10, 95% CI -0.90 to 1.10; $p > 0.9999$), and 80% of mice survived. **6b** showed intermediate activity: latency 13.31 ± 0.97 min (+93.09%) (vs. pilocarpine: MD

= 6.42 min, 95% CI 5.00 to 7.83; $p < 0.0001$; vs. valproate: MD = -2.27 min, 95% CI -3.68 to -0.85 ; $p = 0.0001$), final score 2.9 ± 0.88 (39.3% reduction) (120 min vs. pilocarpine: MD = 1.88 Racine units, 95% CI 0.85 to 2.91; $p < 0.0001$; vs. valproate: MD = -0.40 , 95% CI -1.40 to 0.60; $p = 0.9998$), and 80% survival. In contrast, **7a**, **7e**, and **7f** were less efficacious. They produced smaller delays in onset (on the order of +40–67% vs. control) (all significantly longer than pilocarpine, $p < 0.0001$) and only partial seizure control. Their final Racine scores remained high (>3.4), and survival was only 40–50%. In fact, **7e** and **7f** showed significantly higher severity scores than the valproate group at later time points, indicating lower protection (**7e** vs. valproate: 90 min, MD = 1.30, 95% CI 0.30 to 2.30, $p = 0.0006$; 120 min, MD = 1.06, 95% CI 0.03 to 2.09, $p = 0.0364$; **7f** vs. valproate: 90 min, MD = 1.27, 95% CI 0.24 to 2.30, $p = 0.0017$; 120 min, MD = 1.39, 95% CI 0.36 to 2.42, $p = 0.0002$).

Overall, **6d** clearly outperformed valproate and the other compounds in the pilocarpine model. It not only delayed seizures and reduced their severity to near-normal levels, but also prevented mortality. Its superiority over valproate was statistically significant for seizure latency and for seizure severity at 90- and 120 min. Compound **6c** performed comparably to valproate in many respects, with no significant difference from valproate in seizure latency (adjusted $p = 0.0991$) or final Racine score at 120 min ($p > 0.9999$), and **6b** was somewhat less effective but still markedly better than the other compounds, **7a**, **7e**, and **7f**. These results underscore **6d** > **6c** > **6b** as the leading candidates, with **6d** showing a multi-fold benefit over valproate in latency extension and seizure suppression.

9.3 Structure–activity relationship analysis based on *in vivo* anticonvulsant models

9.3.1 Impact of phenyl ring substitution (series 6). • Electronic effects: the introduction of a methyl group **6b** at the

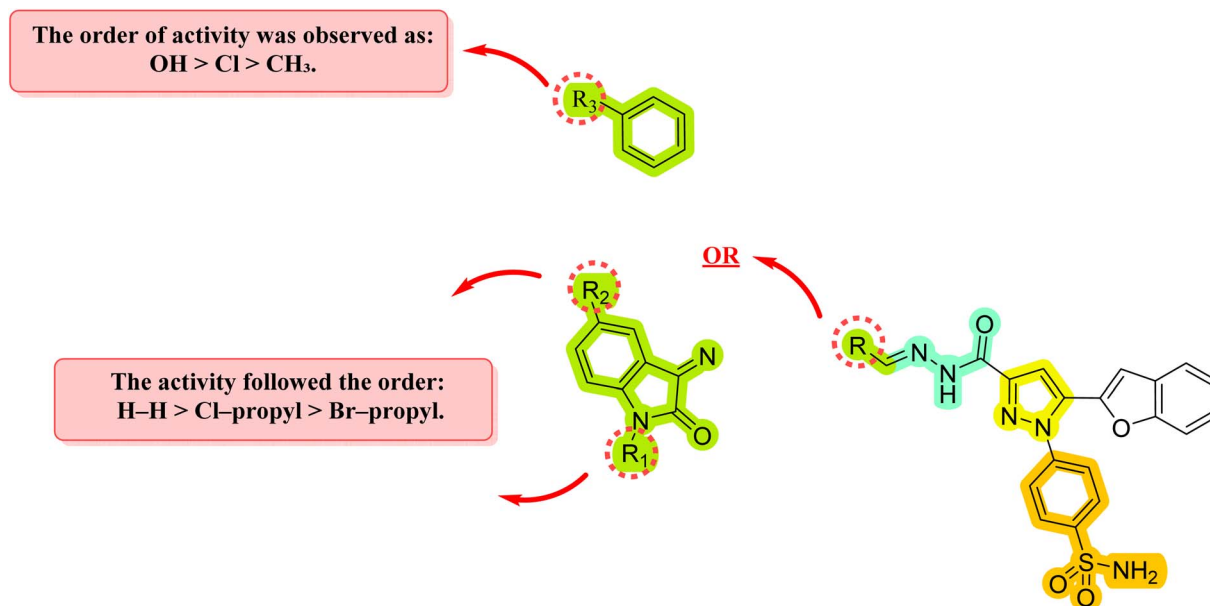


Fig. 9 SAR investigation of the designed compounds **6b–d**, **7a**, **7e**, and **7f** in different *in vivo* seizure models.



phenyl ring, which exerts a positive inductive effect, resulted in a reduction in potency. This suggests that increasing electron density on the aryl moiety may be unfavorable for target binding.

- Halogenation: conversely, substitution with a chloro atom **6c**, which possesses a strong negative inductive effect and lipophilic character, led to a marked enhancement in activity. This improvement likely stems from improved hydrophobic interactions within the binding pocket.

- Hydrogen-bonding potential: the highest anticonvulsant efficacy was achieved with the hydroxyl-substituted analog **6d**. This indicates that the presence of a strong hydrogen-bond donor (HBD) is a critical structural requirement, potentially facilitating key dipole-dipole interactions with the biological target that stabilize the ligand-receptor complex (Fig. 9).

9.3.2 Evaluation of the isatin scaffold (series 7).

- Scaffold morphing: replacement of the phenyl moiety with a more rigid, bicyclic isatin (indoline-2,3-dione) scaffold **7a**, **7e**, and **7f** yielded a distinct SAR trend. The unsubstituted isatin derivative **7a** maintained moderate activity, highlighting the bioisosteric potential of the indole-based system.

- *N*-alkylation and halogenation: interestingly, the combination of halogen substitution (chloro or bromo) on the isatin nucleus with *N*-alkylation *via* a propyl group (**7e** and **7f**) resulted in a significant loss of potency (Fig. 9).

9.4 Impact of test compounds on pilocarpine-induced hippocampal alterations

Oxidative stress and neuroinflammation are key pathological drivers of seizure progression and neuronal injury. Seizure activity is associated with excessive generation of reactive oxygen and nitrogen species, which destabilize neuronal membranes and mitochondria, leading to increased lipid peroxidation and nitric oxide production.^{25–27} At the same time,

excessive glutamatergic neurotransmission amplifies excitotoxicity and overwhelms endogenous antioxidant defenses.^{28,29} Neuroinflammatory cascades further contribute to neuronal damage by upregulating cytokines such as TNF- α and IL-6, which perpetuate injury.^{30–32} These interdependent mechanisms underscore the importance of developing therapeutic strategies that simultaneously counter oxidative stress, excitotoxicity, and inflammation. Based on its superior anticonvulsant profile, compound **6d** was selected for detailed biochemical analysis in hippocampal tissue.

As shown in Fig. 10, hippocampal MDA and nitrite (markers of lipid peroxidation and nitrosative stress) were dramatically elevated after pilocarpine (MDA: +242.86%; nitrite: +171.38%) (CON vs. PIL: MDA MD = -3.075, 95% CI -3.517 to -2.633, $p < 0.0001$; nitrite MD = -151.3, 95% CI -167.0 to -135.6, $p < 0.0001$). Valproic acid significantly blunted this oxidative surge: MDA was reduced by 41.07% and nitrite by 36.72% compared to the pilocarpine group. Compound **6d** produced even greater antioxidant effects. **6d** lowered MDA by 53.57% vs. pilocarpine and nitrite by 51.48% (PIL vs. **6d**: MDA MD = 2.326, 95% CI 1.883 to 2.768, $p < 0.0001$; nitrite MD = 123.3, 95% CI 107.7 to 139.0, $p < 0.0001$). Relative to valproate, these represent additional improvements of 21.21% (MDA) and 23.31% (nitrite) (VAL vs. **6d**: MDA MD = 0.5426, 95% CI 0.1003 to 0.9849, $p = 0.0129$; nitrite MD = 35.35, 95% CI 19.66 to 51.03, $p < 0.0001$). Thus, **6d** more effectively neutralized seizure-induced oxidative damage than valproate.

Pilocarpine also greatly upregulated hippocampal inflammatory markers. TNF- α rose 250.09%, IL-6 by 717.04%, and glutamate by 207.81% relative to controls (CON vs. PIL: TNF- α MD = -213.8, 95% CI -228.9 to -198.6, $p < 0.0001$; IL-6 MD = -165.7, 95% CI -176.6 to -154.9, $p < 0.0001$; glutamate MD = -26.30, 95% CI -28.32 to -24.27, $p < 0.0001$). Valproate pretreatment significantly attenuated these increases (TNF- α by 32.89%, IL-6 by 45.44%, and glutamate by 37.85% vs.

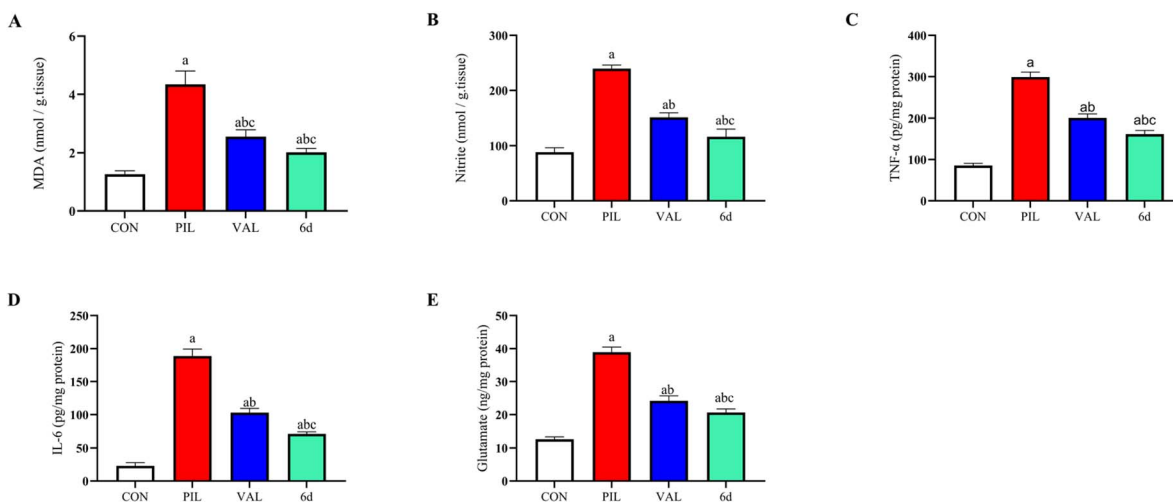


Fig. 10 Effects of experimental compound **6d** on hippocampal oxidative stress, inflammation, and excitotoxicity markers. Data (mean \pm SD, $n = 6$) were analyzed using one-way ANOVA, followed by Tukey's post hoc test for multiple comparisons. Statistically significant differences are marked as: ^a for comparisons with the normal control group ($P < 0.05$), ^b for comparisons with the pilocarpine group ($P < 0.05$), and ^c for comparisons with the valproic acid group ($P < 0.05$). Abbreviations: CON, normal control; PIL, pilocarpine control; VPA, sodium valproate.

pilocarpine) (PIL vs. VAL: TNF- α MD = 98.44, 95% CI 83.30 to 113.6, $p < 0.0001$; IL-6 MD = 85.82, 95% CI 74.99 to 96.66, $p < 0.0001$; glutamate MD = 14.74, 95% CI 12.71 to 16.77, $p < 0.0001$). Again, **6d** outperformed valproate on all measures. With **6d**, significantly lowering TNF- α by 46.03% from pilocarpine (19.57% further reduction beyond valproate), IL-6 by 62.25% relative to pilocarpine (30.81% extra to valproate), and glutamate by 46.99% vs. pilocarpine (14.7% vs. valproate) (PIL vs. **6d**: TNF- α MD = 137.7, 95% CI 122.6 to 152.9, $p < 0.0001$; IL-6 MD = 117.6, 95% CI 106.7 to 128.4, $p < 0.0001$; glutamate MD = 18.30, 95% CI 16.27 to 20.33, $p < 0.0001$; VAL vs. **6d**: TNF- α MD = 39.30, 95% CI 24.16 to 54.44, $p < 0.0001$; IL-6 MD = 31.75, 95% CI 20.92 to 42.59, $p < 0.0001$; glutamate MD = 3.558, 95% CI 1.529 to 5.586, $p = 0.0005$). In practical terms, **6d** brought cytokines and excitatory glutamate much closer to control levels than valproate did. These data demonstrate that **6d** exerts strong anti-inflammatory and anti-excitotoxic actions in the hippocampus.

The epileptogenic process is driven by a complex interplay between oxidative stress, excitotoxic neurotransmission, and inflammatory signaling within vulnerable brain regions. In the present investigation, pilocarpine challenge elicited a marked increase in hippocampal oxidative burden, as evidenced by significant elevations in MDA and nitrite levels. MDA, a well-recognized indicator of seizure-associated lipid peroxidation,^{25,27} reflects extensive oxidative damage to neuronal membranes, while the concomitant rise in nitrite suggests excessive nitric oxide production and subsequent formation of reactive nitrogen species, including peroxynitrite, which are known to impair mitochondrial function and promote neuronal loss.³³ Pretreatment with compound **6d** markedly attenuated these oxidative alterations, indicating a pronounced antioxidant capacity that may contribute to preservation of neuronal integrity and mitigation of free radical-mediated neurotoxicity.

Beyond oxidative stress, pilocarpine-induced seizures were accompanied by a substantial enhancement of glutamatergic activity, a central feature of excitotoxic injury. Pathological accumulation of extracellular glutamate leads to sustained activation of postsynaptic glutamate receptors, resulting in excessive calcium influx, mitochondrial dysfunction, and progressive neuronal degeneration.³⁴ The test compound **6d** significantly reduced hippocampal glutamate levels. This modulation of glutamatergic signaling suggests an important role in restoring the excitatory-inhibitory balance and limiting glutamate-driven excitotoxic cascades.

In parallel, pilocarpine provoked a pronounced neuro-inflammatory response, characterized by marked upregulation of the proinflammatory cytokines TNF- α and IL-6. These mediators are critically involved in blood-brain barrier dysfunction, enhanced neuronal excitability, and the maintenance of recurrent seizure activity.³⁰ Pretreatment with compound **6d** significantly suppressed the pilocarpine-induced cytokine surge. Collectively, these findings underscore the ability of compound **6d** to counteract oxidative stress, glutamate-mediated excitotoxicity, and neuroinflammation, three interrelated processes that drive epileptogenesis.

The reduction in pro-inflammatory cytokines such as TNF- α and IL-6 observed after treatment with compound **6d** suggests suppression of neuroinflammatory signaling in hippocampal tissue. Neuroinflammation is known to enhance neuronal excitability and facilitate seizure propagation. Therefore, attenuation of these inflammatory mediators may contribute to the delayed seizure onset and reduced seizure severity observed in both PTZ and pilocarpine models.

9.5 Toxicity and safety evaluation

A comprehensive serum biochemical panel was performed to evaluate the subchronic systemic safety of the lead compound **6d** following once-daily oral administration for 14 consecutive days. Across all evaluated parameters, biochemical values remained within physiological limits and did not differ significantly from those of the normal control group, indicating good overall tolerability under the applied dosing conditions (Table 7).

Assessment of hepatic safety was based on the determination of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are widely accepted indicators of hepatocellular membrane integrity and enzyme leakage. Sub-chronic exposure to compound **6d** did not produce any significant alterations in ALT or AST activities compared with control animals, suggesting preservation of normal liver function at the tested dose. These findings are particularly relevant given the established sensitivity of these enzymes as early biomarkers of drug-induced hepatic stress in preclinical investigations.^{35,36}

Renal safety was assessed by measuring serum urea and creatinine concentrations, parameters that reflect glomerular filtration efficiency and renal handling capacity. Following 14 days of treatment with **6d**, both markers remained comparable to control values and showed no statistically significant changes. This finding indicates that subchronic administration

Table 7 Toxicological biochemical parameters for liver, kidney, and cardiac function^a

	ALT (IU L ⁻¹)	AST (IU L ⁻¹)	Serum urea (mg dL ⁻¹)	Serum creatinine (mg dL ⁻¹)	CK-MB (Pg mL ⁻¹)	Troponin T (Pg mL ⁻¹)
Control	76.16 ± 5.27	88.22 ± 3.04	0.45 ± 0.02	21.69 ± 1.15	5.49 ± 0.31	20.66 ± 0.82
6d	78.12 ± 8.53	83.98 ± 4.73	0.46 ± 0.03	22.82 ± 1.57	5.4 ± 0.26	19.32 ± 1.61

^a Data (mean ± SD, $n = 6$) were analyzed using unpaired Student's t -test. No statistically significant differences were observed across the groups for any of the evaluated parameters ($P > 0.05$).



a reference ligand, with redocking producing RMSD values of 0 Å for both upper and lower limits, confirming the reliability of the protocol (Fig. S2).

Subsequently, compound **6d** was docked to assess its ability to mimic or enhance the binding interactions of the reference drug and the antiepileptic drug valproic acid (**VI**), nine binding poses were generated, and the best pose was selected, yielding a best docking score of $-10.3 \text{ kcal mol}^{-1}$. The comparative docking results supported the anticonvulsant potential of compound **6d** and provide a hypothesis for the potential anti-convulsant mechanism, suggesting possible modulation of calcium channel activity.

The reference drug, valproic acid, was found to form hydrogen-bonding interactions *via* its carboxylate moiety with key amino acid residues in the active site of the voltage-gated sodium/calcium channel, including Thr29, Val289, and Arg119. In a comparable manner, the most potent compound **6d** established hydrogen-bond interactions with Arg119 and Val289. Additionally, the hydrazide linker of compound **6d** interacted with Tyr161 and Arg163 *via* its carbonyl group, while an additional hydrogen bond was formed with Tyr193 through the pyrazole moiety. The benzofuran moiety engaged in π - π stacking interactions with Tyr227. Finally, the sulfonamide group formed hydrogen bonds with Ser230 and Trp247, thereby stabilizing the ligand within the binding pocket (Fig. 11).

11 Conclusion

Neuroinflammation, oxidative stress, and glutamate-mediated excitotoxicity are recognized as key pathological correlates of epileptogenesis and seizure propagation. In the present study, compound **6d** emerged as a potent anticonvulsant lead with a pronounced multimodal neuroprotective profile. *In vivo* evaluations demonstrated superior efficacy; **6d** afforded 90% protection in the pentylenetetrazol (PTZ) model and significantly delayed seizure onset while attenuating severity in the pilocarpine-induced model, achieving 100% survival.

Parallel to its behavioral effects, compound **6d** elicited significant biochemical shifts within hippocampal tissue. Treatment led to a marked reduction in oxidative stress markers (MDA and nitrite), pro-inflammatory cytokines (TNF- α and IL-6), and glutamate levels. While these results indicate a suppression of neuroinflammatory and excitotoxic pathways, these biochemical changes should be interpreted as supportive evidence of the compound's broad neuroprotective influence rather than a demonstrated causal mechanism for its antiseizure activity. The robust pharmacological profile of **6d** may be partially attributed to its hydroxyl substituent, which molecular modeling suggests facilitates essential hydrogen-bonding interactions within the target binding pocket. These interactions likely stabilize the ligand-target complex, potentially contributing to both the termination of seizure activity and the concomitant modulation of downstream inflammatory markers.

Furthermore, repeated oral administration of **6d** revealed a favorable preliminary safety profile, with no detectable toxicity in hepatic, renal, or cardiac tissues. In summary, while **6d**

robustly attenuates hippocampal neuroinflammation and oxidative stress in tandem with its anticonvulsant action, further investigation is warranted to delineate the specific mechanistic hierarchy and determine whether these anti-inflammatory effects are a primary driver or a secondary consequence of seizure suppression.

Within the SAR framework, compound **6d** demonstrated a marked enhancement in anticonvulsant potency and neuroprotective efficacy compared to its analogues, suggesting a critical role of its specific structural features. In particular, the presence of the hydroxyl substituent appears to be a key determinant of activity, potentially enabling additional hydrogen-bonding interactions within the target binding site, as supported by molecular modeling analysis. This structural attribute may contribute to improved target affinity and, consequently, superior biological performance. Furthermore, the ability of compound **6d** to significantly reduce oxidative stress markers, pro-inflammatory cytokines, and glutamate levels indicates that such structural modifications not only enhance anticonvulsant activity but also promote modulation of neuroinflammatory and excitotoxic pathways.

12 Experimental

12.1 Synthesis of pyrazole sulfonamide **6b-d**, **7a**, **7e**, and **7f** (ref. 22)

Compound **2**, ethyl 4-(benzofuran-2-yl)-2,4-dioxobutanoate, was reacted with 4-aminosulfonylphenylhydrazine in an ethanol/acetic acid medium to give ethyl 5-(benzofuran-2-yl)-1-(4-sulfamoylphenyl)-1H-pyrazole-3-carboxylate **3**. Treatment of ester **3** with hydrazine hydrate under reflux conditions afforded the corresponding hydrazide derivative, 4-(5-(benzofuran-2-yl)-3-(hydrazinecarbonyl)-1H-pyrazol-1-yl)benzenesulfonamide **4**. Further refluxing of compound **4** underwent condensation with a series of substituted aldehydes or isatins in refluxing ethanol containing a catalytic amount of acetic acid, leading to the formation of the target compounds **6b-d** and **7a**, **7e**, **7f** (SI).

The HPLC purity results were included in the SI file.

12.2 *In silico* and biological studies⁴³⁻⁴⁵

Detailed methodologies for molecular docking, and bioinformatics analyses, together with the experimental protocols for the *in vivo* anti-inflammatory, anticonvulsant, and analgesic models, ELISA measurements, and toxicological studies, are described in the SI.

13 Limitations of the study

Despite the promising findings, several limitations should be acknowledged. First, the *in vivo* anticonvulsant models employed in this study, while widely accepted, may not fully recapitulate the complexity and heterogeneity of human epilepsy, which could limit the direct translational relevance of the results. Second, the molecular docking analysis provides only a static representation of ligand-target interactions and does not account for protein flexibility, solvent effects, or



dynamic conformational changes, which may influence binding affinity and accuracy of the predicted interactions. In addition, the sample size used in the biological evaluation was relatively limited, which may affect the statistical robustness of the observed effects. Furthermore, although the designed compounds demonstrated promising anticonvulsant activity, potential off-target effects and long-term safety profiles were not fully explored in the current study.

Conflicts of interest

The authors report no conflicts of interest related to this work.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d6ra01165a>.

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