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FULL PAPER

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Synthesis and radioligand-binding assay of 2,5-disubstituted thiadiazoles and evaluation of their anticonvulsant activities

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Abstract

In this study, a number of 2,5-disubstituted 1,3,4-thiadiazoles were synthesized using an appropriate synthetic route, and their anticonvulsant activity was determined by the maximal electroshock seizure (MES) test and their neurotoxicity was evaluated by the rotarod test. Additionally, their hypnotic activity was tested using the pentobarbital-induced sleep test. Compounds **7** (ED_{50} = 1.14 and 2.72 μmol/kg in the MES and sleep tests, respectively) and **11** (ED_{50} = 0.65 and 2.70 μmol/kg in the MES and sleep tests, respectively) were the most potent ones in the sleep test and anticonvulsant test, showing a comparable activity with diazepam as the reference drug. The results of in vivo studies, especially the antagonistic effects of flumazenil, and also the radioligand-binding assay confirmed the involvement of benzodiazepine (BZD) receptors in the anticonvulsant and hypnotic activity of compounds **7** and **11**. Finally, the docking study of compound **11** in the BZD-binding site of the $GABA_A$ (gamma-aminobutyric acid) receptor confirmed the possible binding of the compound to the BZD receptors. We concluded that the novel 1,3,4-thiadiazole derivatives with appropriate substitution at positions 2 and 5 of the heterocyclic ring had a good affinity to BZD receptors and showed significant efficacy in the pharmacological tests.

KEYWORDS

1,3,4-thiadiazole, anticonvulsant agents, benzodiazepine receptors, flumazenil, radioligand-binding assay

1 | INTRODUCTION

Epilepsy is a neurological disorder that affects approximately 60 million people around the world.[1,2] Epilepsy is characterized by epileptic seizures due to abnormal nerve cell activity and neuronal discharge, which are accepted as pathogenesis of epileptic seizures.[3] Brain injury, tumors, infections, stroke, and birth defects are known as causes of epilepsy. However, for most patients, the main causes remain unknown.[4] The currently available anticonvulsant drugs can only suppress the symptoms of epilepsy and called "symptomatic" agents. Also, most of these existing drugs are effective in less than 70% of patients and suffer from inadequate potency, toxicity, and a variety of side effects.[5] Although medication is known as the only treatment for epilepsy, many patients do not respond to monotherapy and need polytherapy with multiple anticonvulsants agents.[6] These limitations precisely explain the need for further investigations to find new and effective anticonvulsant agents.

Numerous therapeutic agents have been approved for clinical usage as anticonvulsants that belong to different chemical structures including hydantoins, barbiturates, benzodiazepines (BZDs), succinimides, valproate, imides, oxazolidinediones, sulfonamides, and gamma-aminobutyric acid (GABA) analogs.[7] BZDs are an important class of anticonvulsant agents with a great activity against generalized tonic–clonic and partial seizures, which act through binding to the $GABA_A$ receptors. This binding increases the frequency of chloride ion channel opening, which facilitates the inhibitory effects of GABA. However, these types of medicines have several adverse effects such as sedation, memory impairment, and development of tolerance.[8] Considering the fact that most of the clinically used BZDs have negative effects on memory, finding novel anticonvulsant agents as new ligands for the BZD-binding site of the GABA receptor, with less effect on memory, has been an interesting field of drug design in the recent decade.[9,10]

Many five-membered heterocyclic compounds such as triazoles,[11,12] oxadiazoles,[13] pyrrolidines, and pyrazoles[14] have been reported for their anticonvulsant and antidepressant activities. Particularly, thiadiazole derivatives have been widely used as anticonvulsant and antidepressant agents. Thiadiazole is a versatile five-membered heterocyclic compound with four isomeric structures (1,2,3-thiadiazole, 1,2,4-thiadiazole, 1,2,5-thiadiazole, and 1,3,4-thiadiazole) that are present in several biologically active natural products, medicinal agents, and commercial drugs.[15–17] Among these isomers, 1,3,4-thiadiazoles have a very well-established role as pharmacologically significant scaffolds that display a broad spectrum of activities such as antimicrobial, anti-inflammatory, antitumor, antioxidant, antitubercular, antiviral as well as enzymes and human platelet aggregation inhibitors.[18–22] In addition, a large number of 1,3,4-thiadiazole containing compounds have been synthesized and evaluated as anticonvulsant agents.[23–25]

As a part of our ongoing research program to design new anticonvulsant agents,[26–29] herein we reported the design and synthesis of several 2,5-disubstituted 1,3,4-thiadiazoles (**1–14**). The design of the newly synthesized compounds was inspired by the structures of well-known anticonvulsant drugs such as acetazolamide, diazepam, and phenytoin (Figure 1) by the attachment of the benzylthio-1,3,4-thiadiazole core to the aryl urea structure. The anticonvulsant activity of the novel derivatives was evaluated by the maximal electroshock seizure (MES) test. We also investigated the hypnotic effects of the compounds. Furthermore, to determine the mode of anticonvulsant action of the synthesized compounds, the effect of flumazenil, a BZD antagonist, on the anticonvulsant activity of the compounds was evaluated.

2 | RESULTS AND DISCUSSION

2.1 | Chemistry

The target compounds (**1–14**) were synthesized through the synthetic route outlined in Scheme 1. The pathway started from the reaction of thiosemicarbazide (**a**) with carbon disulfide in ethanol, which led to the synthesis of 5-amino-1,3,4-thiadiazole-2-thiol (**b**). In the next step, 5-amino-1,3,4-thiadiazole-2-thiol (**b**) was reacted with benzyl bromide derivatives, to afford the appropriate 2-amino-5-benzylthio-1,3,4-thiadiazoles (**c1–5**). Finally, in the condensation reaction of phenyl isocyanate derivatives with the intermediate compounds (**c1–5**) in dry dichloromethane, the final derivatives (**1–14**) were obtained in good yields (75–85%).

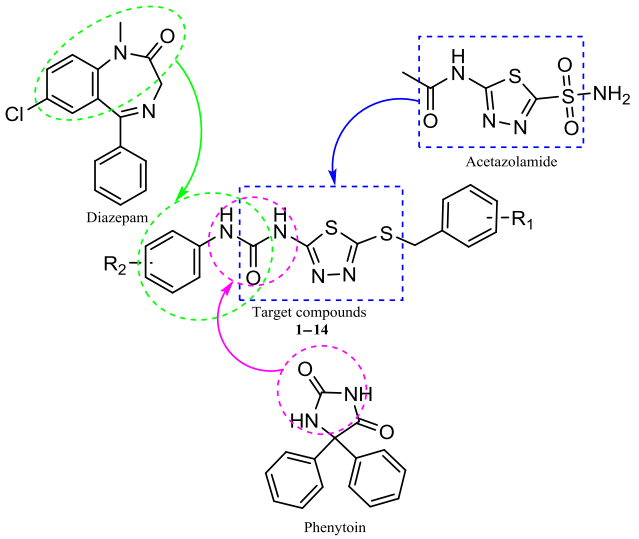


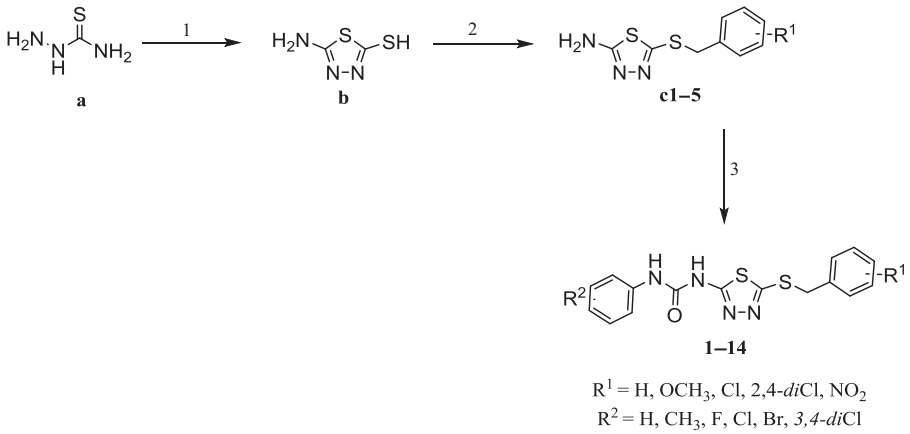
FIGURE 1 Structures of acetazolamide, diazepam, phenytoin, and the target compounds

2.2 | Pharmacology

2.2.1 | Anticonvulsant activity

The most adopted animal model, MES, is employed to screen the new anticonvulsants agents. MES is the preclinical test that is developed to identify the effective compounds against generalized seizures of the tonic–clonic (grand mal) type.[30] To determine the anticonvulsant activity of the synthesized compounds, the ability of the compounds to protect mice against convulsion induced by maximal electroshock was evaluated. The results were compared with diazepam, a BZD agonist, as the standard anticonvulsant drug that was effective in the MES test. The synthesized compounds, diazepam or vehicle, were administered 30 min before the induction of electroshock (60 Hz, 50 mA, and 0.2 s). After that time, the occurrences of hindlimb tonic extension (HLTE) were observed in the MES model.

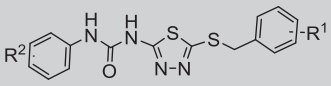
The anticonvulsant activities of the synthesized compounds are depicted in Table 1. The alteration of substituents on the phenylurea and benzylthio rings on positions 2 and 5 of the 1,3,4-thiadiazole moiety (R^2 and R^1, respectively) had a significant effect on the potency of the compounds against MES-induced seizures. On the basis of the substituents on the attached 5-benzylthio warhead (R^1), the designed compounds could be categorized into four groups: The unsubstituted (**1–6**), 3-methoxy- (**7–9**), chloro- (**10** and **11**), and 3-nitro-substituted



SCHEME 1 The synthesis of compounds **1–14**. Reagents and conditions: (1) (i) CS_2, Na_2CO_3, EtOH, ref, 8 hr; (ii) H_2O, HCl; (2) (i) H_2O, KOH, r.t., 15 min; (ii) EtOH, benzyl bromide derivatives, rt, 30 min; (3) phenyl isocyanate derivatives, dry CH_2Cl_2, r.t., overnight

derivatives (**12–14**). In each series, substituents on the 2-phenylurea ring were altered to optimize the anticonvulsant activity. In the MES test, the unsubstituted derivative (**1**) showed significant anticonvulsant activity, and alteration of substitutions on the 2-phenylurea (R^2 = CH_3, Cl, F, and 2,4-*di*Cl) decreased the anticonvulsant activity of compounds **2–4** and **6**. However, compound **5** with R^2 = 2-Br was the most potent compound in this series (ED_{50} = 1.05 μmol/kg). In the 3-methoxy-containing derivatives (**7–9**), all the compounds had moderate-to-good activity. However, attachment of 4-methyl on the 2-phenylurea warhead (**8**) decreased the anticonvulsant activity in the MES test. Among 3-nitro-containing compounds, the unsubstituted phenylurea derivative (**12**) exhibited an interesting anticonvulsant activity in the MES test. However, attachment of 4-methyl and 2-bromo on the 2-phenylurea warhead decreased the anticonvulsant activity of compounds **13** and **14**. In all four groups, the best results were seen by compound **11**, with 2,4-dichloro substitution on the benzylthio ring and 4-fluoro group on the phenylurea warhead in the MES test (ED_{50} = 0.65 μmol/kg), which showed a comparable activity with diazepam (ED_{50} = 0.43 μmol/kg). Furthermore, compounds **5** with R^1 = H and R^2 = 2-Br and **7** with R^1 = 3-OCH_3 and R^2 = H also showed a relatively strong anticonvulsant activity in the MES test (ED_{50} = 1.05 μmol/kg). On the basis of the structure–activity analysis, it could be

TABLE 1 The in vivo anticonvulsant effects of target compounds **1–14**



Compound	R^1	R^2	Sleep test ED_{50} (95% CI; μmol/kg)	MES test ED_{50} (95% CI; μmol/kg)
1	H	H	6.48 (4.52–9.30)	2.36 (1.22–4.54)
2	H	4-CH_3	10.11 (8.14–12.55)	3.36 (0.89–12.58)
3	H	4-F	9.91 (6.09–16.13)	3.14 (2.19–4.49)
4	H	4-Cl	13.14 (9.47–18.24)	3.53 (1.13–11.01)
5	H	2-Br	2.85 (2.10–3.88)	1.05 (0.37–2.94)
6	H	3,4-*di*Cl	12.66 (8.83–18.15)	3.38 (2.19–5.24)
7	3-OCH_3	H	2.72 (1.96–3.77)	1.14 (0.25–5.12)
8	3-OCH_3	4-CH_3	10.71 (0.76–7.50)	3.59 (1.40–9.22)
9	3-OCH_3	2-Br	3.42 (2.20–5.32)	1.27 (0.16–9.90)
10	3-Cl	4-Cl	4.10 (3.25–5.17)	2.36 (0.75–7.42)
11	2,4-*di*Cl	4-F	2.70 (2.16–3.38)	0.65 (0.23–1.82)
12	3-NO_2	H	2.61 (1.40–4.85)	1.15 (0.41–3.20)
13	3-NO_2	4-CH_3	6.53 (4.00–10.65)	2.44 (1.20–4.94)
14	3-NO_2	2-Br	9.86 (5.62–17.28)	2.98 (1.93–4.61)
Diazepam	–	–	1.86 (1.03–2.15)	0.43 (0.24–0.77)

Abbreviation: 95% CI, 95% confidence interval; MES, maximal electroshock seizure.

concluded that chloro substitution on the benzylthio ring and bromo substitution on the phenylurea warhead had a favorable impact on the anticonvulsant activity of the compounds. However, further structural modifications on the scaffold might lead to the discovery of more potent anticonvulsant agents.

2.2.2 | Rotarod test

The neurotoxicity of the compounds was assessed by the rotarod test. The most potent compounds (**7** and **11**) as anticonvulsants were selected for the rotarod test. TD_{50} values of compounds **7** and **11** were 29.68 (13.64–64.58) μmol/kg and 20.85 (11.39–38.18) μmol/kg, respectively. Furthermore, the calculated protective index (PI; the ratio between TD_{50} and ED_{50}) for anticonvulsant effects of these two compounds (**7** and **11**) in the MES test was 26.03 and 32.07, respectively.

2.2.3 | Pentobarbital-induced sleep test

To assess the hypnotic effects of the compounds, a pentobarbital-induced sleep test in mice was performed. Different doses of compounds were injected intraperitoneally (ip), and then animals received sodium pentobarbital at a dose of 40 mg/kg. Each mouse was observed for 120 min after the injection and the time of losing the righting reflex was recorded. The results were depicted as ED_{50} values and compared with diazepam (Table 1). On the basis of the results, it is observed that all the synthesized compounds had higher ED_{50} values than diazepam, which means that all of the newly synthesized derivatives had lower potency as hypnotic agents than the reference drug. Among the synthesized derivatives, compounds **7**, **11**, and **12** showed higher potency as hypnotic agents.

2.2.4 | Study on the possible mechanism of action

Effect of flumazenil on anticonvulsant activity of the compounds

To understand the possible mechanism of anticonvulsant activity of the most potent compounds **7** and **11**, we checked if flumazenil would be able to antagonize the effect of the compounds in the MES test.[25,31] Flumazenil is a well-known BZD antagonist, administrated at a dose of 10 mg/kg, 15 min before the induction of convulsion. According to the results, in the MES test, the anticonvulsant effects of **7** and **11** were completely inhibited (100%) by flumazenil at the dose of 40 mg/kg. These results confirmed that the anticonvulsant activity of compounds **7** and **11** was antagonized with flumazenil, which established the involvement of BZD receptors in reported effects. Furthermore, in the sleep test, flumazenil at 10 mg/kg dose significantly reduced the hypnotic effects of compounds **7** and **11**.

Radioligand-binding assay

The binding study by radioligands is a very powerful tool in the study of receptors and their ligands. In this type of study, the receptors are evaluated biochemically and physiologically, and their pharmacological properties are determined. The radioligand receptor binding assay using [^{3}H]flumazenil is a common method to evaluate the affinity of newly synthesized BZD ligands to the receptor in two basic types of experiments: saturation and competition studies. Saturation studies are used to determine the affinity of [^{3}H]flumazenil radioligand to a receptor, whereas, in competition studies, the affinity of nonradioactive ligands to receptors is measured.[32,33] Encouraged by the acceptable activity of compounds **7** and **11**, the competitive radioligand-binding assay is conducted to understand the mechanisms behind the anticonvulsant activity of these types of derivatives at the receptor level. In this assay, [^{3}H]flumazenil was used as the specific ligand for estimating the affinity of the compounds to the BZD receptor. The results showed that compounds **7** and **11** have an affinity to the BZD receptor in nanomolar concentrations (IC_{50} = 0.31 and 0.44 nM and K_i values = 0.18 and 0.26 nM, respectively), which were comparable with the affinity of diazepam to the BZD receptor (IC_{50} = 0.30 nM and K_i value = 0.17 nM). These results confirmed that the anticonvulsant activity of **7** and **11** was mediated by BZD receptors (Table 2).

2.3 | Molecular docking study

To evaluate the binding affinities and interactions of the most active compound (**11**) to the BZD-binding pocket of $GABA_A$ receptor, the docking study was examined by AutoDock 4.2.1 software[34] and the interactions were visualized by Discovery Studio software. The structure of $GABA_A$ receptor with diazepam (PDB ID: 6HUP) was used and the docking procedure was conducted as previously described.[35] As seen in Figure 2, hydrogen bonds with Met 261 and Leu 285 residues through urea moiety were formed. The 4-F phenyl ring formed π–sulfur interaction with Met 236 and Met 286 residues. The 2,4-*di*Cl phenyl ring made π–π interaction with the aromatic moiety of Phe 289, which was similar to the binding mode of diazepam's phenyl ring. Furthermore, this compound made other hydrophobic interactions (van der Waals interactions) with Thr 237, Thr 265, and Leu 269 residues. To summarize, according to the molecular docking simulation of compound **11** in the BZD-binding site of the $GABA_A$ receptor, the results were in accordance with the experimental results.

TABLE 2 The binding affinity of compounds **7** and **11** to the benzodiazepine receptor

Compound	IC_{50} (95% CI; nM)	K_i (nM)
7	0.31 (0.13–0.75)	0.18
11	0.44 (0.14–1.38)	0.26
Diazepam	0.30 (0.18–0.51)	0.174

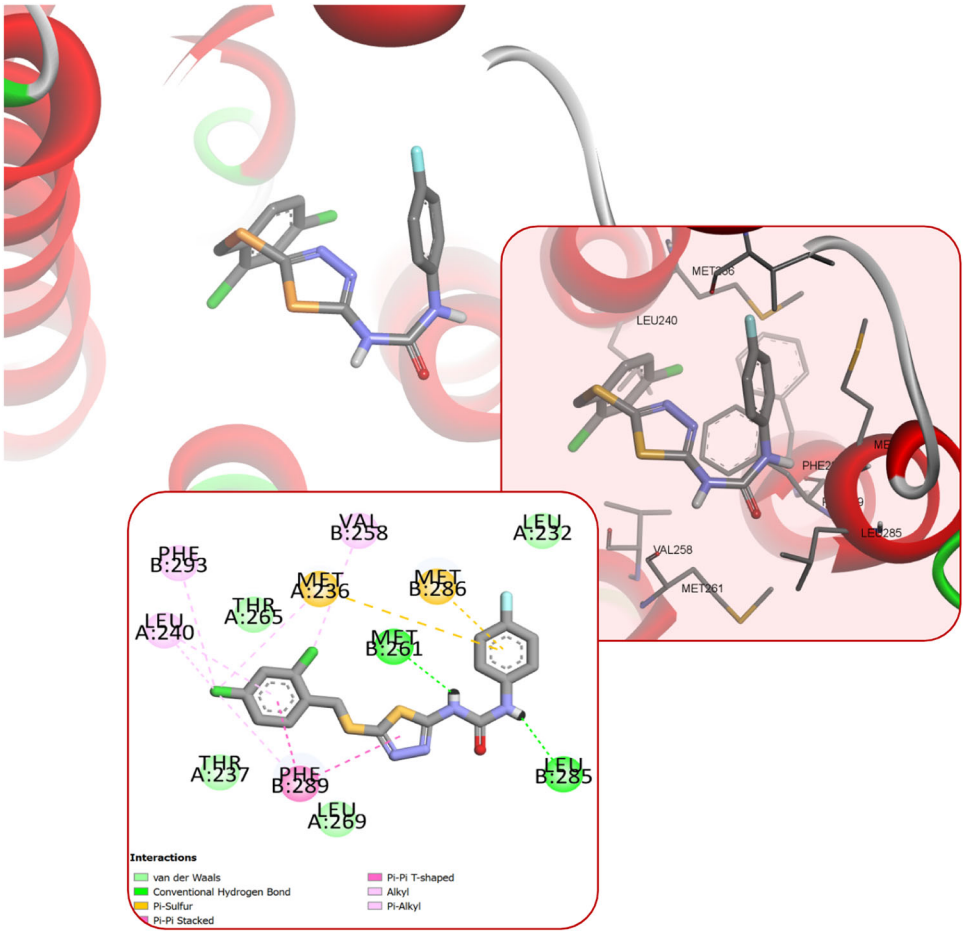


FIGURE 2 Binding mode of compound **11** with $GABA_A$ receptor

3 | CONCLUSIONS

A series of 2,5-disubstituted 1,3,4-thiadiazoles was synthesized to explore prospective anticonvulsant agents. The synthesized compounds were evaluated in the animal model of epilepsy, the MES test. The results depicted that the anticonvulsant activity was strongly associated with the type and the position of the substituents attached to the 2-phenylurea and 5-benzylthio rings of 1,3,4-thiadiazole moiety. The results of this investigation indicated that 1,3,4-thiadiazole having 2,4-dichloro substitutions on 5-benzylthio ring and 4-fluoro group on 2-phenylurea moiety (compound **11**) exhibited a promising anticonvulsant activity in the MES test, comparable to diazepam. Also, this compound showed a lower hypnotic effect in the sleep test as compared with diazepam. Additionally, the ability of flumazenil to antagonize the effects of the compounds and radioligand-binding assay confirmed that the anticonvulsant activity of the two selected compounds **7** and **11** resulted from the direct modulation of the BZD receptor. The neurotoxicity and PI of these compounds were also measured. In addition, in silico studies by using molecular docking simulations showed that the prototype compound could act as BZD receptor agonist.

4 | EXPERIMENTAL

4.1 | Chemistry

4.1.1 | General

All nuclear magnetic resonance (NMR) experiments (^{1}H and ^{13}C NMR spectra) were carried out on Bruker 500 MHz at 20°C in dimethyl sulfoxide (DMSO)-d_6 or $CDCl_3$ as the solvents, using tetramethylsilane as an internal standard, and chemical shifts were reported in δ parts per million (ppm). Melting points were determined with a Kofler hot-plate microscope apparatus and were uncorrected. Infrared (IR) spectroscopy was performed using a Nicolet FT-IR Magna 550 spectrograph (KBr disks). Elemental analysis was recorded by a Perkin Elmer 2400 (automatic elemental analyzer). Commercially available reagents and solvents used for the synthesis were purchased from Merck and Sigma-Aldrich companies without further purification. The progress of reactions and the purity of synthesized compounds were checked by thin-layer chromatography on silica gel 250 mm, F254 plastic sheets. The spots were monitored under ultraviolet irradiation (λ = 254 nm). Mass spectra were obtained at ionization potential of

70 eV using HP Agilent Technologies 5937. The purity of the final compounds was determined by the high-performance liquid chromatographic (HPLC) analysis; the HPLC column (Pronaos EP-C18; Changhai, China, 250 × 4.6 mm, inner diameter 5 µm) was employed for all analyses. The data acquisition was performed using ChemStation software (Agilent Technologies). The mobile phase was composed of KH_2PO_4 buffer (20 mM, pH 3.5) and methanol (20:80, v/v). The isocratic elution mode was used for detection at a flow rate of 1.0 ml/min and with the detector wavelength set at 272 nm (Agilent 1200; Agilent Technologies).

The original spectra of the investigated compounds are provided as Supporting Information, together with their InChI codes and some biological activity data.

4.1.2 | General procedure for the synthesis of 5-amino-1,3,4-thiadiazole-2-thiol (b)

To an ethanol solution of sodium carbonate (4.5 mmol) and thiosemicarbazide (5 mmol) (**a**), carbon disulfide (1 ml) was added dropwise. The reaction mixture was heated under reflux for 8 hr. The solid product obtained after the evaporation of the reaction mixture was dissolved in HCl and filtered and washed with water.

4.1.3 | General procedure for the synthesis of 2-amino-5-(benzylthio)-1,3,4-thiadiazol (c1–5)

Benzyl bromide derivatives (5.2 mmol) were slowly added to the solution of 5-amino-1,3,4-thiadiazole-2-thiol (5 mmol) (**b**) and KOH (5.3 mmol) in ethanol (3.5 ml) at 0°C. The reaction mixture was stirred at room temperature for 30 min and then diluted with cold water. The obtained solid was filtered, washed with water, and then crystallized from ethanol.

4.1.4 | General procedure for the synthesis of 1-(5-(benzylthio)-1,3,4-thiadiazol-2-yl)-3-phenylurea derivatives (1–14)

To a mixture of 2-amino-5-(benzylthio)-1,3,4-thiadiazol (**c1**–**5**) (0.45 mmol) in 1 ml of dry CH_2Cl_2, phenyl isocyanate derivatives (0.3 mmol) dissolved in 1 ml dry CH_2Cl_2 were added drop-by-drop. The reaction mixture was stirred overnight. After the consumption of reagents, the obtained solid was filtered and washed with CH_2Cl_2.

*1-[5-(Benzylthio)-1,3,4-thiadiazol-2-yl]-3-phenylurea (**1**)*

Yield: 85%; HPLC (purity: 99%); mp 209–211°C; IR (KBr, cm^{-1}): 3,365 (N–H), 3,227 (N–H), 1,721 (C═O), 1,599, 1,547, and 1,314. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.44 (s, 2H), 7.04 (t, 1H, J = 7.5 Hz), 7.25–7.34 (m, 5H), 7.39 (d, 2H, J = 7.5 Hz), 7.45 (d, 2H, J = 8.0 Hz), 9.02 (s, 1H), and 10.97 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.2, 118.3 (2C), 122.5, 126.9, 127.9 (2C), 128.3 (2C), 128.4 (2C), 136.2, 137.7, 150.9, 156.1, and 160.1. Anal. calcd. for $C_{16}H_{14}N_4OS_2$: C, 56.12; H, 4.12; N, 16.36. Found: C, 56.31; H, 4.04; N, 16.14; mass spectroscopy (MS) electrospray ionization (ESI) m/z 343.5 [M+H].

*1-[5-(Benzylthio)-1,3,4-thiadiazol-2-yl]-3-(p-tolyl)urea (**2**)*

Yield: 81%; HPLC (purity: 96%); mp 238–240°C; IR (KBr, cm^{-1}): 3,381 (N–H), 3,217 (N–H), 1,726 (C=O), 1,593, 1,548, and 1,317. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.24 (s, 3H), 4.43 (s, 2H), 7.12 (d, 2H, J = 8.0 Hz), 7.29–7.24 (m, 1H), 7.30–7.34 (m, 4H), 7.39 (d, 2H, J = 8.2 Hz), 8.91 (s, 1H), and 10.93 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 19.8, 37.2, 118.4 (2C), 126.9, 127.9 (2C), 128.4 (2C), 128.7 (2C), 131.5, 135.1, 136.2, 150.7, 156.0, and 160.0. Anal. calcd. for $C_{17}H_{16}N_4OS_2$: C, 57.28; H, 4.52; N, 15.72. Found: C, 57.41; H, 4.37; N, 15.81; MS (ESI) m/z 357.2 [M+H].

*1-[5-(Benzylthio)-1,3,4-thiadiazol-2-yl]-3-(4-fluorophenyl)urea (**3**)*

Yield: 79%; HPLC (purity: 100%); mp 249–251°C; IR (KBr, cm^{-1}): 3,385 (N–H), 3,221 (N–H), 1,712 (C═O), 1,589, 1,556, and 1,321. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.43 (s, 2H), 7.14 (t, 2H, J = 8.7 Hz), 7.27–7.24 (m, 1H), 7.31 (t, 2H, J = 8.1 Hz), 7.39 (d, 2H, J = 8.1 Hz), 7.46–7.48 (m, 2H), 9.06 (s, 1H), and 11.03 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 36.3, 114.1 (d, 2C, J = 20.0 Hz), 122.8 (d, 2C, J = 8.0 Hz), 125.3, 126.3 (2C), 127.1 (2C), 134.2, 135.6 (d, J = 3.1 Hz), 150.0, 154.1, 158.4 (d, J = 251.8 Hz), and 160.8. Anal. calcd. for $C_{16}H_{13}FN_4OS_2$: C, 53.32; H, 3.64; N, 15.54. Found: C, 53.47; H, 3.31; N, 15.74; MS (ESI) m/z 361.7 [M+H].

*1-[5-(Benzylthio)-1,3,4-thiadiazol-2-yl]-3-(4-chlorophenyl)urea (**4**)*

Yield: 75%; HPLC (purity: 97%); mp 264–266°C; IR (KBr, cm^{-1}): 3,374 (N–H), 3,227 (N–H), 1,727 (C═O), 1,594, 1,548, and 1,307. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.44 (s, 2H), 7.26 (t, 1H, J = 7.3 Hz), 7.32 (t, 2H, J = 7.6 Hz), 7.37–7.34 (m, 2H), 7.39 (d, 2H, J = 7.0 Hz), 7.52–7.45 (m, 2H), 9.20 (s, 1H), and 11.07 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.1, 119.3, 119.9 (2C), 126.1, 126.9, 127.9 (2C), 128.0, 128.1 (2C), 128.3 (2C), 136.2, 136.8, and 157.5. Anal. calcd. for $C_{16}H_{13}ClN_4OS_2$: C, 50.99; H, 3.48; N, 14.87. Found: C, 51.72; H, 3.59; N, 14.54; MS (ESI) m/z 377.5 [M+H].

*1-[5-(Benzylthio)-1,3,4-thiadiazol-2-yl]-3-(2-bromophenyl)urea (**5**)*

Yield: 78%; HPLC (purity: 95%); mp 197–199°C; IR (KBr, cm^{-1}): 3,343 (N–H), 3,215 (N–H), 1,708 (C═O), 1,587, 1,531, and 1,320. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.45 (s, 2H), 7.06 (t, 1H, J = 7.7 Hz), 7.26 (t, 1H, J = 6.6 Hz), 7.32 (t, 2H, J = 7.9 Hz), 7.41–7.34 (m, 3H), 7.65 (d, 1H, J = 8.0 Hz), 7.99 (d, 1H, J = 8.2 Hz), 8.56 (s, 1H), and 11.70 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.1, 113.3, 122.1, 124.9, 126.9, 127.7, 127.9 (2C), 128.4 (2C), 132.1, 135.2, 136.2, 150.5, 156.6, and 159.7. Anal. calcd. for $C_{16}H_{13}BrN_4OS_2$: C, 45.61; H, 3.11; N, 13.30. Found: C, 45.32; H, 3.28; N, 13.57; MS (ESI) m/z 422.3 [M+H].

*1-[5-(Benzylthio)-1,3,4-thiadiazol-2-yl]-3-(3,4-dichlorophenyl) urea (**6**)*

Yield: 75%; HPLC (purity: 99%); mp 255–257°C; IR (KBr, cm^{-1}): 3,376 (N–H), 3,205 (N–H), 1,732 (C═O), 1,591, 1,543, and 1,303. ^{1}H NMR

(DMSO-d_6, 500 MHz) δ: 4.44 (s, 2H), 7.26 (t, 1H, J = 7.3 Hz), 7.32 (t, 2H, J = 8.0 Hz), 7.42–7.38 (m, 3H), 7.54 (d, 1H, J = 8.7 Hz), 7.86 (s, 1H), 9.40 (s, 1H), and 11.29 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.0, 118.3, 119.4, 123.8, 126.9, 127.9 (2C), 128.3 (2C), 130.0, 130.5, 136.1, 138.1, 152.0, 156.0, and 160.6. Anal. calcd. for $C_{16}H_{12}Cl_2N_4OS_2$: C, 46.72; H, 2.94; N, 13.62. Found: C, 46.58; H, 2.69; N, 13.95; MS (ESI) m/z 412.2 [M+H].

*1-{5-[(3-Methoxybenzyl)thio]-1,3,4-thiadiazol-2-yl}-3-phenylurea (**7**)*

Yield: 80%; HPLC (purity: 99%); mp 195–197°C; IR (KBr, cm^{-1}): 3,365 (N–H), 3,212 (N–H), 1,720 (C═O), 1,599, 1,544, and 1,315. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.72 (s, 3H), 4.41 (s, 2H), 6.87–6.80 (m, 1H), 6.99–6.93 (m, 2H), 7.04 (t, 1H, J = 7.4 Hz), 7.24 (t, 1H, J = 8.1 Hz), 7.31 (t, 2H, J = 7.4 Hz), 7.46 (d, 2H, J = 8.0 Hz), 9.14 (s, 1H), and 11.03 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.2, 54.4, 112.4, 114.0, 118.3 (2C), 120.5, 122.5, 128.3 (2C), 128.9, 129.0, 137.7, 151.2, 156.1, 158.7, and 160.1. Anal. calcd. for $C_{17}H_{16}N_4O_2S_2$: C, 54.82; H, 4.33; N, 15.04. Found: C, 54.59; H, 4.52; N, 15.27; MS (ESI) m/z 373.2 [M+H].

*1-{5-[(3-Methoxybenzyl)thio]-1,3,4-thiadiazol-2-yl}-3-(*p*-tolyl) urea (**8**)*

Yield: 78%; HPLC (purity: 98%); mp 210–212°C; IR (KBr, cm^{-1}): 3,381 (N–H), 3,224 (N–H), 1,722 (C═O), 1,587, 1,534, and 1,314. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.24 (s, 3H), 3.72 (s, 3H), 4.40 (s, 2H), 6.84–6.82 (m, 1H), 6.96–6.94 (m, 2H), 7.11 (d, 2H, J = 8.0 Hz), 7.24 (t, 1H, J = 7.8 Hz), 7.33 (d, 2H, J = 8.0 Hz), 9.06 (s, 1H), and 11.00 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 19.8, 37.2, 54.4, 112.5, 114.0, 115.4, 118.3, 120.5, 125.4, 128.7 (2C), 129.0, 131.5, 135.1, 137.7, 156.0, 158.7, and 160.2. Anal. calcd. for $C_{18}H_{18}N_4O_2S_2$: C, 55.94; H, 4.69; N, 14.50. Found: C, 55.75; H, 4.84; N, 14.73; MS (ESI) m/z 387.5 [M+H].

*1-(2-Bromophenyl)-3-{5-[(3-methoxybenzyl)thio]-1,3,4-thiadiazol-2-yl}urea (**9**)*

Yield: 75%; HPLC (purity: 96%); mp 186–188°C; IR (KBr, cm^{-1}): 3,352 (N–H), 3,204 (N–H), 1,705 (C═O), 1,581, 1,531, and 1,317. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 3.71 (s, 3H), 4.41 (s, 2H), 6.83 (d, 1H, J = 7.1 Hz), 6.97–6.94 (m, 2H), 7.06 (t, 1H, J = 7.4 Hz), 7.24 (t, 1H, J = 7.8 Hz), 7.36 (t, 1H, J = 7.4 Hz), 7.64 (d, 1H, J = 8.0 Hz), 7.97 (d, 1H, J = 8.2 Hz), 8.61 (s, 1H), and 11.75 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.2, 54.4, 112.5, 113.5, 114.0, 120.5, 122.3, 124.9, 127.6, 129.0, 132.1, 135.2, 137.7, 150.7, 156.5, 158.7, and 159.8. Anal. calcd. for $C_{17}H_{15}BrN_4O_2S_2$: C, 45.24; H, 3.35; N, 12.41. Found: C, 45.51; H, 3.12; N, 12.73; MS (ESI) m/z 452.5 [M+H].

*1-{5-[(3-Chlorobenzyl)thio]-1,3,4-thiadiazol-2-yl}-3-(4-chlorophenyl) urea (**10**)*

Yield: 77%; HPLC (purity: 99%); mp 267–269°C; IR (KBr, cm^{-1}): 3,375 (N–H), 3,202 (N–H), 1,715 (C═O), 1,582, 1,547, and 1,317. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.42 (s, 2H), 7.20 (d, 1H, J = 7.5 Hz), 7.35–7.22 (m, 4H), 7.54–7.46 (m, 3H), 8.77 (s, 1H), and 11.36 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 38.0, 118.7 (2C), 126.5, 126.5, 126.8, 127.2, 127.3 (2C), 128.4, 132.9, 137.0, 137.5, 153.0, 154.5, and 166.2. Anal. calcd. for $C_{16}H_{12}Cl_2N_4OS_2$: C, 46.72; H, 2.94; N, 13.62. Found: C, 46.94; H, 2.62; N, 13.81; MS (ESI) m/z 412.3 [M+H].

*1-{5-[(2,4-Dichlorobenzyl)thio]-1,3,4-thiadiazol-2-yl}-3-(4-fluorophenyl)urea (**11**)*

Yield: 79%; HPLC (purity: 100%); mp 228–230°C; IR (KBr, cm^{-1}): 3,378 (N–H), 3,219 (N–H), 1,702 (C═O), 1,595, 1,553, and 1,323. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.48 (s, 2H), 7.15 (t, 2H, J = 8.7 Hz), 7.40 (dd, 1H, J = 8.2, 2.2 Hz), 7.50–7.42 (m, 3H), 7.66 (d, 1H, J = 2.3 Hz), 9.06 (s, 1H), and 11.08 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 34.7, 114.8 (d, 2C, J = 22.2 Hz), 120.3 (d, 2C, J = 7.8 Hz), 126.9 (2C), 128.4 (2C), 132.0, 132.6, 133.0, 133.7, 134.0 (d, J = 3.3 Hz), 155.0, and 157.5 (d, J = 236.7 Hz). Anal. calcd. for $C_{16}H_{11}Cl_2FN_4OS_2$: C, 44.76; H, 2.58; N, 13.05. Found: C, 44.58; H, 2.81; N, 13.21; MS (ESI) m/z 430.4 [M+H].

*1-{5-[(3-Nitrobenzyl)thio]-1,3,4-thiadiazol-2-yl}-3-phenylurea (**12**)*

Yield: 80%; HPLC (purity: 95%); mp 201–203°C; IR (KBr, cm^{-1}): 3,347 (N–H), 3,221 (N–H), 1,719 (C═O), 1,598, 1,544, and 1,315. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.47 (s, 2H), 6.99 (t, 1H, J = 7.0 Hz), 7.27 (t, 2H, J = 6.9 Hz), 7.44 (d, 2H, J = 6.8 Hz), 7.48 (t, 1H, J = 7.7 Hz), 7.63 (d, 1H, J = 7.7 Hz), 8.13 (d, 1H, J = 7.7 Hz), 8.30 (s, 1H), 8.89 (s, 1H), and 10.92 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 37.4, 117.0 (2C), 120.3, 120.7, 121.4, 126.6 (2C), 127.2, 132.2, 136.1, 136.7, 146.0, 152.0, 153.5, and 165.2. Anal. calcd. for $C_{16}H_{13}N_5O_3S_2$: C, 49.60; H, 3.38; N, 18.08. Found: C, 49.87; H, 3.12; N, 18.27; MS (ESI) m/z 388.1 [M+H].

*1-{5-[(3-Nitrobenzyl)thio]-1,3,4-thiadiazol-2-yl}-3-(*p*-tolyl)urea (**13**)*

Yield: 75%; HPLC (purity: 96%); mp 223–225°C; IR (KBr, cm^{-1}): 3,381 (N–H), 3,225 (N–H), 1,722 (C═O), 1,587, 1,534, and 1,314. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 2.24 (s, 3H), 4.59 (s, 2H), 7.11 (d, 2H, J = 8.1 Hz), 7.33 (d, 2H, J = 8.1 Hz), 7.62 (t, 1H, J = 8.0 Hz), 7.84 (d, 1H, J = 7.5 Hz), 8.11 (d, 1H, J = 8.3 Hz), 8.28 (s, 1H), 8.96 (s, 1H), and 10.97 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 19.7, 36.0, 118.4 (2C), 121.7, 123.0, 128.7 (2C), 129.3, 131.5, 135.0, 135.0, 139.2, 147.1, 151.0, 155.2, and 160.4. Anal. calcd. for $C_{17}H_{15}N_5O_3S_2$: C, 50.86; H, 3.77; N, 17.45. Found: C, 50.52; H, 3.97; N, 17.61; MS (ESI) m/z 402.4 [M+H].

*1-(2-Bromophenyl)-3-{5-[(3-nitrobenzyl)thio]-1,3,4-thiadiazol-2-yl} urea (**14**)*

Yield: 77%; HPLC (purity: 99%); mp 205–207°C; IR (KBr, cm^{-1}): 3,397 (N–H), 3,229 (N–H), 1,755 (C═O), 1,593, 1,546, and 1,325. ^{1}H NMR (DMSO-d_6, 500 MHz) δ: 4.46 (s, 2H), 7.15 (t, 1H, J = 7.6 Hz), 7.42 (t, 1H, J = 7.6 Hz), 7.49–7.46 (m, 2H), 7.57 (d, 1H, J = 7.8 Hz), 7.62 (d, 1H, J = 7.7 Hz), 8.11 (d, 1H, J = 7.7 Hz), 8.30 (s, 1H), 8.81 (s, 1H), and 11.11 (s, 1H). ^{13}C NMR (DMSO-d_6, 125 MHz): 35.9, 113.6, 121.8, 122.9, 123.9, 124.7, 127.4, 129.2, 131.9, 135.0, 136.2, 138.9, 146.94, 150.0, 155.9, and 159.9. Anal. calcd. for $C_{16}H_{12}BrN_5O_3S_2$: C, 41.21; H, 2.59; N, 15.02. Found: C, 41.54; H, 2.31; N, 15.27; MS (ESI) m/z 466.3 [M+H].

4.2 | Pharmacological assays

4.2.1 | Anticonvulsant activity

Animals and drugs

Adult male NMRI mice (Pasteur Institute, Iran), weighing 18–25 g, were kept in colony cages. Mice were housed under standardized conditions in a temperature-controlled animal facility (22 ± 2°C) and 12-hr light/dark cycle. They were allowed to acclimatize to the animal facility for 1 week before the experiments. All experimental animals had free access to food and water except during the experiment. After 7 days of adaptation to laboratory conditions, animals were divided into experimental groups randomly (each comprised of 8 mice). For the rest of the experiments, we used each mouse just one time for the experiments. Diazepam (Darou Pakhsh Co.), the synthesized compounds, and flumazenil (Hoffmann-La Roche) were suspended in a solution of carboxymethylcellulose (1%) and Tween 80 (0.5%) in distilled water. The compounds and diazepam were injected 30 min before the induction of convulsion and flumazenil was injected 15 min before the compounds and diazepam. This study was run on the basis of protocols approved by the institutional animal care committee, and the experimenters did their best to minimize the number of animals used in the study. First, the in vitro test was run; ethical approval had been granted after obtaining positive results in the in vitro assays.

Maximal electroshock-induced seizure test

The ability of the targeted compounds **1–14** to prevent MES-induced seizures was evaluated in mice. The compounds were injected (ip) at doses of 2.5, 5, 10, and 20 mg/kg. The occurrence of HLTE in mice was assessed by applying maximal electroshock with a 60-Hz alternating current of 50-mA intensity delivered for 0.2 s via ear electrodes. The MES was applied 30 min after (ip) administration of the compounds and diazepam. Mice were observed for 60 s for the incidence of HLTE. The results were recorded as the number of animals protected/number of animals tested.[36]

Pentobarbital-induced sleep test

Hypnotic effects of the compounds were assessed by the pentobarbital-induced sleep test. All the compounds were injected ip. After the pretreatment with different doses of compounds, animals were given sodium pentobarbital at a dose of 40 mg/kg. After pentobarbital injection, each mouse was observed for the onset of sleep (loss of righting reflex). The time between loss (sleep onset) and recovery of the righting reflex was measured. Animals were observed for 120 min after the injection.[37]

Rotarod test

The rotarod test was used to investigate the motor coordination of the subjects. The various doses of compounds **7** and **11** were injected ip; after 30 min, the animals were placed on a rotating straight rod at a speed of 6 rpm for 1 min. In this experiment, if the mouse fell three times during the 1-min trial, it would be considered as an animal with neurotoxicity. The number of animals that were not able to maintain on the rod during 1 min was recorded. The toxic dose values (TD_{50}) were measured for each novel compound.

4.2.2 | Radioligand receptor binding assay

The compounds were evaluated for their in vitro affinity to BZD receptor by the radioligand receptor binding assay. All of the binding assays were performed in triplicates. Male Sprague Dawley rats with weights of 200–250 g were anesthetized with isoflurane and then were killed. The cortical membrane tissue of the brain of the rats was collected and homogenized in 20 ml of ice-cold Tris-HCl buffer. The homogenate was centrifuged and the resulting supernatant was ultracentrifuged (35,000*g* for 25 min), followed by three centrifugations and resuspension cycles. The final pellet was resuspended in 30ml Tris-HCl buffer (50 mM, pH 7.4). The amount of protein was estimated in the membrane preparation by the Bradford method using bovine serum albumin as a standard. There are two fundamental types of receptor binding experiments: saturation and competition. For the saturation experiment, eight different concentrations of [^{3}H]flumazenil were incubated with the membrane preparation at 30°C for 35 min. The total binding (TB; receptor + radioligand), nonspecific binding (NSB; receptor + radioligand + excess diazepam), and specific binding (SB; TB-NSB) were measured at various radioligand concentrations. NSB was determined in parallel assays performed in the presence of 100 μM diazepam. The amount of radioligand required to saturate the receptors was used to determine the receptor binding affinity of [^{3}H]flumazenil (K_d), and the BZD receptor density (B_{max}) was measured in the saturation study based on the nonlinear regression analysis (K_d = 1.41 ± 0.4126 nM, B_{max} = 0.728 ± 0.109 pmol/mg). In the competition experiment, the affinity (K_i) of the synthesized ligands and diazepam (K_i) was determined in comparison with [^{3}H]flumazenil as a standard antagonist of BZD receptors. The rat brain protein (100 μg) in Tris-HCl buffer (50 mM, pH 7.4) was incubated with 8.6×10^{-5} nM of [^{3}H]flumazenil and increasing concentrations of the synthesized ligands (5 mM–50 pM) for 35 min at 30°C. At the end of the incubation, the reaction mixture was centrifuged and the radioactivity of pellet was counted, adding Ultima Gold cocktail (Perkin Elmer), using liquid scintillation counting (Triathler Multilabel Tester; Hidex, Finland). The concentration of ligands that inhibits the binding of [^{3}H]flumazenil by 50% is the IC_{50} value. The data were analyzed using GraphPad Prism software. To determine IC_{50} values, the data were fitted to the equation by using nonlinear regression analysis, competitive binding. NSB was determined in parallel assays performed in the presence of 100 μM diazepam.[38,39]

4.2.3 | Statistical analysis

In this study, results were described as significant when $p < .05$. In MES, pentobarbital-induced sleep, and rotarod tests, the ED_{50} and TD_{50} values were measured by the linear regression analysis of the log dose–response curve and presented as mean with 95% confidence intervals. To identify the IC_{50} values of in vitro studies, we used the nonlinear regression

analysis of competitive binding results and we reported the results as mean with 95% confidence intervals. All statistical analyses were carried out by the GraphPad Prism software (San Diego, CA; version 5.0).

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CONFLICTS OF INTERESTS

The authors declare that there are no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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