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Synthesis and biological evaluation of (4*H*-1,2,4-triazol-4-yl)isoquinoline derivatives as selective glycine transporter 1 inhibitors

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ABSTRACT

To identify novel glycine transporter 1(GlyT1) inhibitors with greater selectivity relative to GlyT2 and improved aqueous solubility, we synthesized a series of 4*H*-1,2,4-triazole derivatives with heteroaromatic rings at the 4-position and investigated their structure–activity relationships. Replacement of the 2-fluorophenyl group of lead compound **5** with various aromatic groups led to the identification of 5-(3-biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)isoquinoline (**15**) with 38-fold selectivity between GlyT1 and GlyT2. **15** also showed improved aqueous solubility and in vivo efficacy on (+)-HA966-induced hyperlocomotion in mice over the lead compound.

1. Introduction

In the central nervous system (CNS), specific sodium/chloride-dependent transporters regulate glycine levels in the synapse. Glycine activity here is terminated by reuptake via two high-affinity glycine transporters: glycine transporters 1 (GlyT1) and 2 (GlyT2).[1,2] GlyT1 has widespread distribution in forebrain areas such as the cortex and hippocampus. GlyT1 expressions is observed in brain areas rich in the *N*-methyl-D-aspartate (NMDA) receptors, and is believed to modulate the NMDA receptor function. In contrast, expression of GlyT2 is limited to the spinal cord, brain stem, and cerebellum, and GlyT2 is therefore thought to control the function of the strychnine-sensitive glycine receptor.

The hypofunction of the NMDA receptors is believed to be associated with a number of human diseases. For example, the functional deterioration of this receptor may have a role in inducing schizophrenia,[3] with clinical studies demonstrating that positive, negative, and cognitive symptoms in schizophrenic patients can be ameliorated with administration of glycine, D-serine (glycine site agonist of the NMDA receptors), and sarcosine (*N*-methyl glycine, weak GlyT1 inhibitor) in conjunction with conventional therapy.[4] These previous findings suggest that potentiating NMDA receptor function may be useful in treating schizophrenia.

In addition to its above association with disease induction, the NMDA receptors are also known to be associated with memory formation and learning.[5] Activation of the NMDA receptors is involved in long-term potentiation (LTP), which is considered a mechanism of memory formation and learning at the neuronal level.[6] In addition, improvement of the NMDA receptor function via glycine-binding sites rescues neurons from falling victim to the cellular mechanisms thought to underlie age-related deficits in learning and memory.[7] Taken together, these previous findings strongly suggest that memory and cognitive dysfunction may be ameliorated by activation of the NMDA receptors. As such, medications which inhibiting the activity of GlyT1 and thereby activating the function of the NMDA receptors may be useful as therapeutic agents for treating schizophrenia, dementia, and related disorders.

A previous study identified *N*-[(3*R*)-3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine (**1**), an analogue of sarcosine, as a glycine transporter inhibitor (Fig. 1),[8] with preclinical in vivo studies in rodents further demonstrating **1** to have efficacy similar to that of clozapine in prepulse inhibition (PPI) and latent inhibition (LI) mouse models.[9,10] In light of these findings, a number of sarcosine analogues have been subsequently synthesized and reported in the literature.[2,11–14] However, pharmaceutical companies have recently launched new efforts to investigate non-substrate-based

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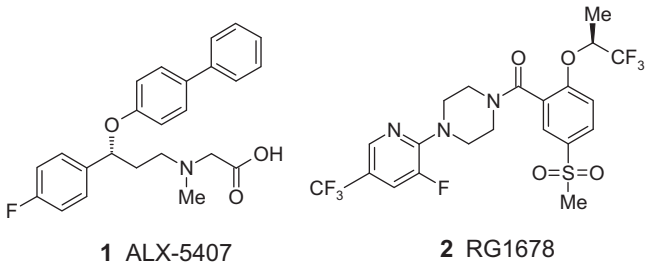


Figure 1. Structures of ALX-5407 (**1**) and RG1678 (**2**).

inhibitors, with a wide variety of compounds subsequently being disclosed.[2,11–14] Among these compounds, [4-(3-fluoro-5-trifluoromethylpyridin-2-yl)piperazin-1-yl][5-methanesulfonyl-2-((*S*)-2,2,2-trifluoro-1-methylethoxy)phenyl]methanone (**2**) was found to exert a beneficial effect in schizophrenic patients in a recent phase II clinical trial.[15]

Our team has also contributed to this field of study with our recent report on a novel series of GlyT1 inhibitors derived from the high-throughput screening (HTS) hit, 3-biphenyl-4-yl-4-(2-methoxyphenyl)-5-methyl-*4H*-1,2,4-triazole (**3**) with a novel structure (Fig. 2).[16] We have established the structure–activity relationships (SARs) in this series of GlyT1 inhibitors, demonstrating that the 3-bipheny-4-yl-4-phenyl-*4H*-1,2,4-triazole moiety is an essential core for this novel class of non-sarcosine-derived GlyT1 inhibitors. Among the compounds prepared previously, **4** showed good oral bioavailability and brain permeability. Compound **4** also effectively antagonized (+)-3-amino-1-hydroxypyrrolid-2-one [(+)-HA966]-induced hyperlocomotion, ameliorating learning impairment in the passive avoidance task in mice. However, despite this attractive profile, the selectivity of **4** between GlyT1 and GlyT2 was moderate, and its aqueous solubility was extremely poor, precluding further preclinical development of the compound and its related analogues (Table 1).

Here, we report our efforts to improve the GlyT2/GlyT1 selectivity and aqueous solubility of this series of GlyT1 inhibitors by replacing the 2-fluorophenyl moiety of **4** with various heteroaromatic rings.

2. Chemistry

The 1,2,4-triazole derivatives were prepared as shown in Schemes 1–5. Scheme 1 describes the synthesis of 1,2,4-triazole derivatives **8**, **10–18**, **23**, and **24**. Commercially available phenylbenzoyl hydrazide (**6**) was first acylated, followed by cyclization using $POCl_3$ to obtain the common intermediate **7**. Derivatives **8**, **10–18**, **23**, and **24** were then obtained by addition-elimination reactions of **7** with various aromatic amines under acidic conditions. *N*-Me indole derivative **9** was synthesized by methylation of **8** with MeI under basic conditions (Scheme 2).

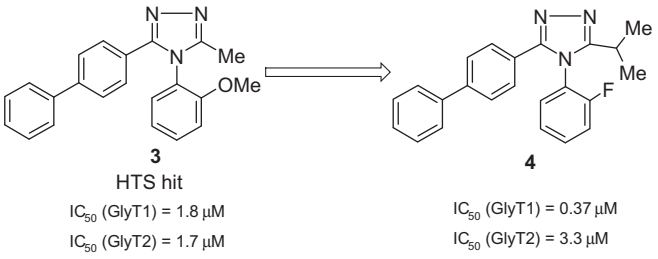
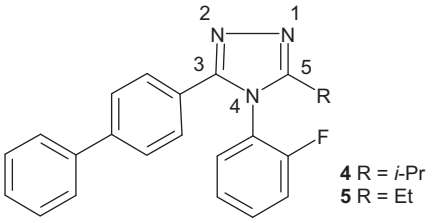


Figure 2. In our previous work, modification of HTS hit **3** led to identification of compound **4**.

Table 1
In vitro activity and solubility of 1,2,4-triazole derivertives **4** and **5**



Compd	rGlyT1 IC_{50} (μM)[a]	rGlyT2 IC_{50} (μM)[a]	Solubility pH 6.8 (μM)
4	0.37 ± 0.020	3.3 ± 0.053	<1
5	0.22 ± 0.050	0.23 ± 0.092	<1

[a] Values are means ± standard error for three experiments.

We were unable to obtain the isoquinoline derivative **19** from reaction with intermediate **7** and 1-aminoisoquinoline (**25**) under acidic conditions, likely due to the low nucleophilicity of **25**. Compound **19** was thus synthesized via the reaction shown in Scheme 3. Briefly, **25** was first reacted with propionyl chloride to produce amide **26,** and then was treated with Lawesson's reagent to give thioamide (**27**). This compound was then methylated in aqueous methanol to produce thioimidate (**28**), which was coupled with acyl hydrazide (**6**) under acidic conditions to give the desired 1,2,4-triazole derivative **19**.

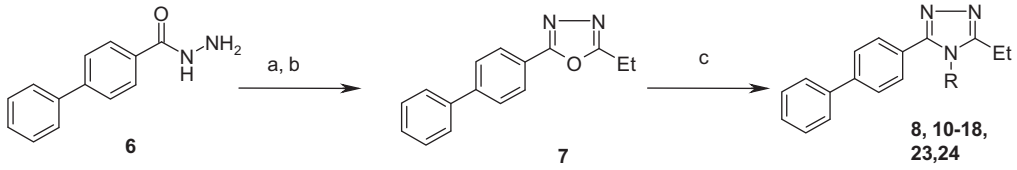
Scheme 4 shows the preparation of the isoquinoline analogues **20** and **21**. Compound **15** was first converted into *N*-oxide (**20**) using *m*-chloroperbenzoic acid (*m*CPBA), followed by the heating in Ac_2O to give the 1-acetoxy derivative **29**. Isoquinoline-1-one (**21**) was then obtained by hydrolysis of **29**. Lastly, 1,2,3,4-tetrahydroisoquinoline analogue **22** was obtained by the reduction of the corresponding isoquinoline (**15**) using $NaBH_3CN$ in acetic acid (Scheme 5).

3. Result and discussion

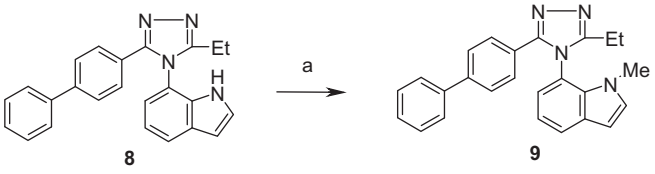
GlyT1 and GlyT2 inhibitory activities were assessed according to reported protocols utilizing [^{3}H]glycine uptake into rat C6 glioma cells[17] and rat brainstem cells, respectively. In our previous SAR study,[16] we reported that while introduction of an isopropyl group at the 5-position of 1,2,4-triazole effectively improved selectivity between GlyT1 and GlyT2 inhibition, this modification also reduced aqueous solubility. Given these previous findings, we conducted an SAR study with compound **5** as the lead to improve both selectivity and solubility.

Our previous examination of the SAR using a substituted phenyl group found that ortho substituted phenyl groups at the 4-positon of triazoles were well tolerated, exerting potent GlyT1 inhibitory activities. However, we have never investigated the SAR using heteroaromatic rings, particularly with bicyclic rings such as indoles and quinolines. Working under the belief that these nitrogen-containing structures would not only modulate affinities for both GlyTs, but also improve physiochemical properties, we decided to incorporate hetero-bicyclic rings into the compounds to hopefully obtain GlyT1-selective compounds with improved solubility over currently available compounds.

We first investigated the SAR of compounds following introduction of an indole, indazole, and benzimidazole (Table 2). Replacement of the 2-fluorophenyl moiety with an indole resulted in reduced activity (**8**, IC_{50} = 22 μM), and while introduction of a methyl group at the 1-position of the indole showed better activity (**9**, IC_{50} = 3.0 μM), its activity was 14-fold less potent than that of compound **5**. We also examined other nitrogen-containing 5,6-membered heterocyclic rings, such as indazole and benzimid-



Scheme 1. Reagents and conditions: (a) EtCOCl, Et_3N, DMF; (b) $POCl_3$, 100 °C, (c) R-NH_2, *p*-TsOH·H_2O, 160–200 °C.

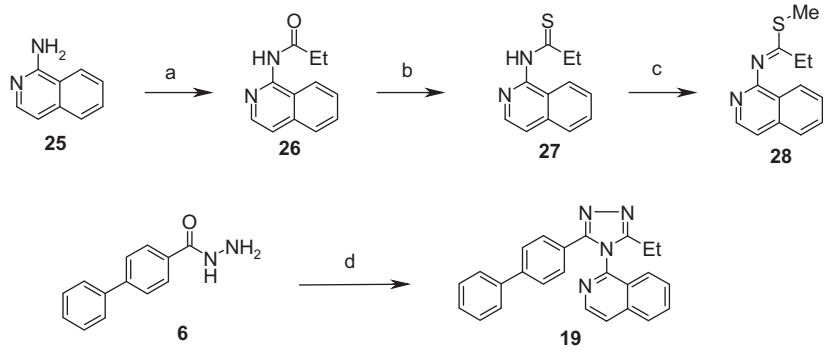


Scheme 2. Reagents: (a) MeI, KOH, DMF.

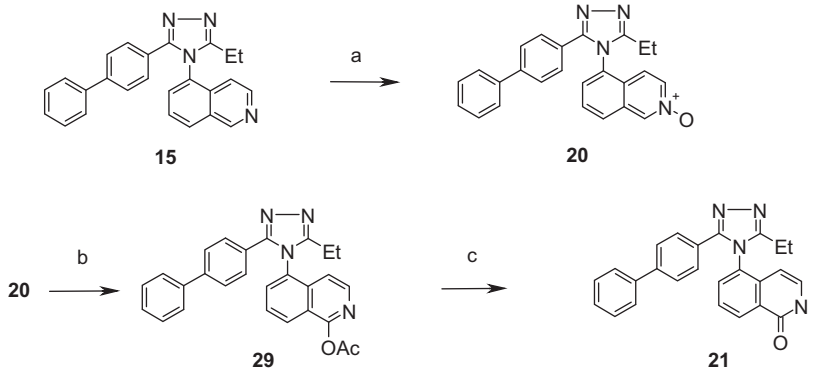
azole, but found the in vitro potencies of these compounds to be weak, similar to that for indole derivatives (IC_{50} = 2.8–7.5 μM).

We next turned our attention to the SAR of 6,6-membered heterocyclic derivatives such as quinolines. We evaluated the quinoline and isoquinoline derivatives shown in Table 3, examining all N-substitution patterns. Interestingly, all quinoline and isoquinoline derivatives retained their GlyT1 inhibitory activity, and the introduction of a nitrogen atom into the 1–4 positions (see the positions on structure **13**) was found to be particularly effective for GlyT1 inhibitory activity. Of the compounds examined, the quinoline-8-yl (**13**) and isoquinoline-5-yl (**15**) analogues showed potent GlyT1 inhibitory activity comparable to that of compound **5** (**13**, IC_{50} = 0.30 μM; **15**, IC_{50} = 0.37 μM). Perhaps more noteworthy, however, is that **15** showed weak GlyT2 inhibitory activity (IC_{50} = 14 μM) and a selectivity ratio (GlyT2/GlyT1) of 38-fold, values much higher than observed with **5**.

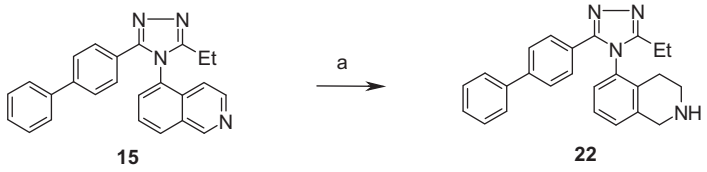
In light of our finding that the isoquinoline-5-yl fragment is useful for improving selectivity (GlyT2/GlyT1), we next synthesized



Scheme 3. Reagents and conditions: (a) EtCOCl, pyridine, THF; (b) Lawesson's reagent, pyridine, 100 °C; (c) MeI, NaOH, EtOH; (d) **28**, *p*-TsOH·H_2O, xylene, 130 °C.

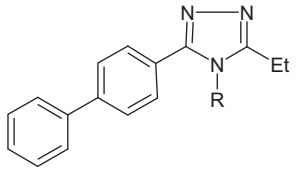


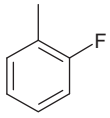
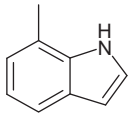
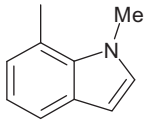
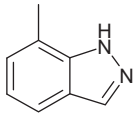
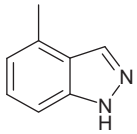
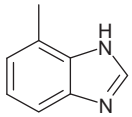
Scheme 4. Reagents and conditions: (a) *m*CPBA, CH_2Cl_2; (b) Ac_2O, 120 °C; (c) NaOH, MeOH.



Scheme 5. Reagents: (a) $NaBH_3CN$, AcOH.

Table 2
In vitro GlyT1 inhibitory activities of 1,2,4-triazole derivatives **5**, **8**–**12**



Compd	R	rGlyT1 IC_{50}[a] (μM)
5		0.22 ± 0.050
8		22 ± 3.5
9		3.0[b]
10		5.6[b]
11		2.8[b]
12		7.5 ± 0.80

[a] Values are means ± standard error for three experiments.
[b] Values are means for two experiments.

derivatives of **15** as described in Table 4—attempts which unfortunately resulted in loss of activity. For example, compounds **20** and **21**, both containing oxygen atoms, showed complete loss of GlyT1 inhibitory activity, as did tetrahydroisoquinoline and pyridine analogues **22–24**. These results suggest that either or both the aromaticity of isoquinoline or the conformational restrictions are essential to retaining GlyT1 inhibitory activity.

Given our successful improvement of selectivity for GlyT1 over GlyT2 utilizing the isoquinoline moiety, we next evaluated the aqueous solubility of **15** and compared it with the solubility of compounds **4** and **5** (Table 5). Compound **15** showed higher solubility than either **4** or **5** in both pH 1.2 and 6.8 aqueous solutions. The related analogues **13**, **14**, and **16** also showed improved solubility in both pH 1.2 and 6.8 aqueous solutions, probably due to the contribution of basic and hydrophilic properties of the quinoline and isoquinoline moiety.

Lastly, we assessed the in vivo efficacy of **15** on (+)-HA966 (an antagonist for Gly binding sites on the NMDA receptors)[18] -induced hyperlocomotion in monoamine-depleted mice (Table 5). Compound **15** showed 82% inhibition of hyperlocomotion at a dose of 3 mg/kg orally (po).[19] This attenuation of the locomotor activity indicates that **15** increases the extracellular level of glycine, which indirectly displaces the (+)-HA966 from Gly binding sites on the NMDA receptors. Considering that compound **4** showed 70% inhibition at a dose of 10 mg/kg intraperitoneally (ip), the in vivo efficacy of **15** was judged to be much improved over that of **4**. The improved solubility may have contributed to the potent in vivo efficacy of **15**.

4. Conclusion

Through the iterative processes of synthesis and biological evaluation starting from lead compound **5**, we demonstrated that replacement of the 2-fluorophenyl group of **5** with a heteroaromatic group such as quinoline or isoquinoline maintained GlyT1 inhibitory activity. From these studies, we successfully derived isoquinoline derivative **15**, which showed 38-fold selectivity and improved aqueous solubility over lead compound **5**. Compound **15** also reversed (+)-HA966-induced hyperlocomotion at 3 mg/kg po, which was better in vivo activity than obtained with **4**. Further pharmaceutical characterization of **15** is ongoing.

5. Experiment

5.1. Chemistry

Uncorrected melting points (Mps) were determined using BÜCHI B-545 or Yanaco MP-500D micro melting apparatuses. ^{1}H NMR spectra were recorded on a JEOL JMN-LA-300 or JMN-EX-400, and the chemical shifts were expressed in δ (ppm) values with trimethylsilane as an internal reference (s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, and br = broad peak). Mass spectra (MS) were recorded on a Hitachi M-80 or JEOL JMS-LX2000 spectrometer. Elemental analyses were performed using a Yanaco MT-5 (C, H, N), Elementar Vario EL III (C, H, N), and Dionex DX-500 (S, halogene) and were within ±0.4% of theoretical values.

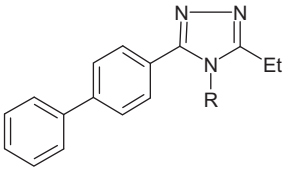
5.1.1. 2-(Biphenyl-4-yl)-5-ethyl-1,3,4-oxadiazole (7)

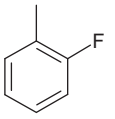
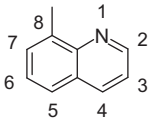
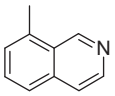
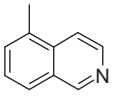
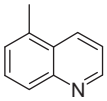
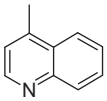
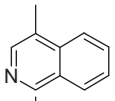
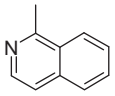
Propionyl chloride (4.37 g, 47.2 mmol) at 0 °C was added to a solution of biphenyl-4-carboxylic hydrazide (**6**, 10.0 g, 47.2 mmol) and triethylamine (4.96 g, 49.1 mmol) in *N*,*N*-dimethylformamide (DMF) (200 mL), and the mixture was stirred at room temperature for 2 h before being concentrated in vacuo. EtOAc and water was then added, and the mixture was further stirred for 10 min. The precipitate was collected by filtration and washed with water and EtOAc, and the wet solid was dried in vacuo to give *N*'-propionylbiphenyl-4-carbohydrazide (8.20 g, 65%) as a colorless powder. A solution of *N*'-propionylbiphenyl-4-carbohydrazide (11.5 g, 42.9 mmol) in phosphorus oxychloride (50 mL) was stirred at 100 °C for 1 h. After cooling at room temperature, the mixture was concentrated in vacuo, and the residue was then partitioned between EtOAc and water. The organic layer was washed with brine, dried over $MgSO_4$, and concentrated in vacuo, while the residue was purified using column chromatography on silica gel (hexane/EtOAc = 3/1) to give **7** (8.21 g, 97%) as a white solid. ^{1}H NMR (DMSO-d_6) δ 1.46 (3H, t, J = 7.5 Hz), 2.98 (2H, q, J = 7.5 Hz), 7.35–7.52 (3H, m), 7.61–7.66 (2H, m), 7.71 (2H, dt, J = 2.0, 8.6 Hz), 8.10 (2H, dt, J = 1.8, 8.6 Hz); MS (FAB) m/z 251 $[M+H]^+$.

5.1.2. 5-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)isoquinoline (15)

To the mixture of **7** (500 mg, 2.0 mmol) and 5-aminoisoquinoline (651 mg, 4.52 mmol) was added *p*-toluenesulfonic acid monohydrate (50 mg, 0.26 mmol), and the resultant mixture was stirred at 160 °C for 24 h. After cooling at room temperature, the mixture was purified using column chromatography on silica gel ($CHCl_3$/MeOH = 98/2) to give crude **15** as a brown solid. The solid was recrystallized from EtOAc to give **15** (300 mg, 40%) as a colorless powder. Mp: 228–230 °C; ^{1}H NMR (DMSO-d_6) δ 1.09 (3H, t, J = 7.6 Hz), 2.33–2.50 (2H, m), 7.11 (1H, d, J = 5.8 Hz), 7.31–7.42 (5H, m), 7.53–7.58 (4H, m), 7.89–7.91 (1H, t, J = 7.9 Hz), 8.17 (1H, d, J = 7.4 Hz), 8.42 (1H, d, J = 8.3 Hz), 8.54 (1H, d, J = 6.3 Hz), 9.51

Table 3
In vitro GlyT1 inhibitory activities of 1,2,4-triazole derivatives of **5**, **13**–**16**



Compd	R	rGlyT1 IC_{50}[a] (μM)	rGlyT2 IC_{50}[a] (μM)	Selectivity (rGlyT2/rGlyT1)
5		0.22 ± 0.050	0.23 ± 0.092	1.0
13		0.30 ± 0.060	0.38 ± 0.11	1.3
14		0.80 ± 0.021	4.2 ± 0.87	5.3
15		0.37 ± 0.012	14 ± 5.3	38
16		0.82 ± 0.17	2.7 ± 0.47	3.3
17		1.7 ± 0.033	ND[b]	ND[b]
18		3.7 ± 0.52	ND[b]	ND[b]
19		1.4 ± 0.10	ND[b]	ND[b]

[a] Values are means ± standard error for three experiments.
[b] ND, not determined.

(1H, s); MS (FAB) *m/z* 377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4$: C, 79.76; H, 5.35; N, 14.88. Found: C, 79.73; H, 5.30; N, 14.85.

Compounds **8**, **10–14, 16–18**, **23** and **24** were prepared by a method similar to that described for **15**. Yields refer to the final substitution step.

5.1.3. 7-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-1*H*-indole (8) (35%)

Mp: 254–256 °C; ^{1}H NMR (DMSO-d_6) δ 1.11 (3H, t, J = 7.4 Hz), 2.38–2.44 (2H, q, J = 7.4 Hz), 6.60 (1H, s), 7.12–7.16 (2H, m), 7.31–7.44 (6H, m), 7.52–7.62 (4H, m), 7.73–7.78 (1H, m), 11.5 (1H, s); MS (FAB) *m/z* 365 $[M+H]^+$; Anal. Calcd for $C_{24}H_{20}N_4$: C, 79.10; H, 5.53; N, 15.37. Found: C, 79.17; H, 5.54; N, 15.38.

5.1.4. 7-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-1*H*-indazole (10) (47%)

Mp: 294–296 °C; ^{1}H NMR (DMSO-d_6) δ 1.12 (3H, t, J = 7.5 Hz), 2.43–2.49 (2H, m), 7.27 (1H, t, J = 7.8 Hz), 7.32–7.43 (5H, m), 7.49 (1H, d, J = 7.0 Hz), 7.55–7.61 (4H, m), 8.00 (1H, d, J = 8.0 Hz), 8.26 (1H, s), 13.5 (1H, s); MS (FAB) *m/z* 366 $[M+H]^+$; Anal. Calcd for $C_{23}H_{19}N_5$: C, 75.59; H, 5.24; N, 19.16. Found: C, 75.62; H, 5.31; N, 19.18.

5.1.5. 4-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-1*H*-indazole (11) (22%)

Mp: 164–167 °C; ^{1}H NMR (DMSO-d_6) δ 1.11 (3H, t, J = 7.5 Hz), 2.51–2.57 (2H, m), 7.28–7.45 (6H, m), 7.48–7.64 (5H, m), 7.76 (1H, d, J = 8.0 Hz), 7.83 (1H, s), 13.50–13.57 (1H, brs); MS (FAB) *m/z* 366 $[M+H]^+$; Anal. Calcd for $C_{23}H_{19}N_5$: C, 75.59; H, 5.24; N, 19.16. Found: C, 75.65; H, 5.15; N, 19.13.

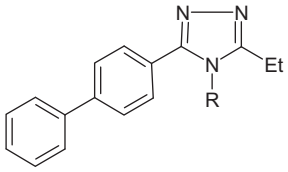
5.1.6. 7-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-1*H*-benzimidazole (12) (46%)

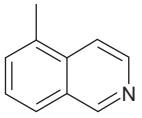
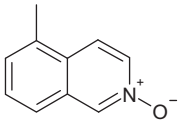
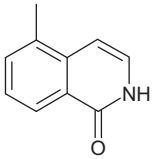
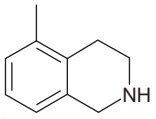
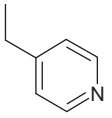
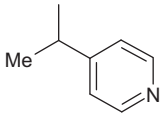
Mp: >300 °C; ^{1}H NMR (DMSO-d_6) δ 1.10 (3H, t, J = 7.6 Hz), 2.41–2.48 (2H, m), 7.26 (1H, d, J = 7.3 Hz), 7.32–7.47 (6H, m), 7.55 (2H, d, J = 8.3 Hz), 7.60 (2H, d, J = 7.8 Hz), 7.75 (1H, d, J = 8.3 Hz), 8.32 (1H, s), 12.9 (1H, s); MS (FAB) *m/z* 366 $[M+H]^+$; Anal. Calcd for $C_{23}H_{19}N_5$: C, 75.59; H, 5.24; N, 19.16. Found: C, 75.65; H, 5.14; N, 19.17.

5.1.7. 8-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)quinoline (13) (79%)

Mp: 205–207 °C; ^{1}H NMR ($CDCl_3$) δ 1.21 (3H, t, J = 7.5 Hz), 2.39–2.66 (2H, m), 7.21–7.65 (12H, m), 8.01 (1H, dd, J = 2.2, 7.3 Hz), 8.28 (1H, dd, J = 1.8, 7.3 Hz), 8.92 (1H, dd, J = 1.8, 2.2 Hz); MS (FAB) *m/z*

Table 4
In vitro GlyT1 inhibitory activities of 1,2,4-triazole derivatives of **15**, **20**–**24**



Compd	R	rGlyT1 IC_{50}[a] (μM)
15		0.37 ± 0.012
20		>10 (28%) [b]
21		>10 (16%) [b]
22		>10 (25%) [b]
23		>10 (15%) [b]
24		>10 (36%) [b]

[a] Values are means ± standard error for three experiments.
[b] % Inhibition at 10 μM.

Table 5
Aqueous solubility and effects on (+)-HA966 induced hyperlocomotion for 1,2,4-triazole derivertives **4**, **5**, and **13**–**16**

Compd	Solubility pH 1.2 (μM)	Solubility pH 6.8 (μM)	(+)-HA966-induced hyperlocomotion in mice inhibition[a] (%)
4	5	<1	70% @ 10 mg / kg ip
5	96	<1	ND[b]
13	>100	23	ND[b]
14	>100	11	ND[b]
15	>100	5	82% @ 3 mg/kg po
16	>100	9	ND[b]

[a] n = 16 experiments.
[b] ND, not determined.

377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4$: C, 79.76; H, 5.35; N, 14.88. Found: C, 79.73; H, 5.35; N, 14.91.

5.1.8. 8-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)isoquinoline (14) (22%)

Mp: 197–198 °C; ^{1}H NMR (DMSO-d_6) δ 1.11 (3H, t, J = 7.6 Hz), 2.38–2.55 (2H, m), 7.30–7.43 (5H, m), 7.52–7.60 (4H, m), 7.98–8.07 (3H, m), 8.24–8.28 (1H, m), 8.59–8.63 (2H, m); MS (FAB) m/z 377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4$: C, 79.76; H, 5.35; N, 14.88. Found: C, 79.91; H, 5.44; N, 14.83.

5.1.9. 5-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)- quinoline (16) (18%)

Mp: 202–203 °C; ^{1}H NMR (DMSO-d_6) δ 1.09 (3H, t, J = 7.6 Hz), 2.33–2.51 (2H, m), 7.30–7.42 (5H, m), 7.53–7.59 (5H, m), 7.65–7.68 (1H, m), 7.96–7.80 (2H, m), 8.26–8.31 (1H, m), 9.00 (1H, dd, J = 3.9, 1.4 Hz); MS (FAB) m/z 377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4$: C, 79.76; H, 5.35; N, 14.88. Found: C, 79.58; H, 5.37; N, 14.76.

5.1.10. 4-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)- quinoline (17) (32%)

Mp: 189–191 °C; ^{1}H NMR (DMSO-d_6) δ 1.10 (3H, t, J = 7.8 Hz), 2.35–2.52 (2H, m), 7.32–7.35 (2H, m), 7.38–7.42 (4H, m), 7.55–7.59 (4H, m), 7.66 (1H, t, J = 7.3 Hz), 7.87 (1H, t, J = 7.3 Hz), 7.96 (1H, d, J = 4.4 Hz), 8.20 (1H, d, J = 8.3 Hz), 9.16 (1H, d, J = 7.8 Hz); MS (FAB) m/z 377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4.0.2H_2O$: C, 79.01; H, 5.41; N, 14.74. Found: C, 78.86; H, 5.66; N, 14.51.

5.1.11. 4-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)- isoquinoline (18) (29%)

Mp: 209–210 °C; ^{1}H NMR (DMSO-d_6) δ 1.10 (3H, t, J = 7.8 Hz), 2.35–2.52 (2H, m), 7.29–7.35 (2H, m), 7.38–7.42 (4H, m), 7.54–7.59 (4H, m), 7.79–7.89 (2H, m), 8.35 (1H, d, J = 7.8 Hz), 8.85 (1H, s), 9.57 (1H, s); MS (FAB) m/z 377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4$: C, 79.76; H, 5.35; N, 14.88. Found: C, 79.83; H, 5.41; N, 15.09.

5.1.12. 4-[(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-methyl]pyridine (23) (48%)

Mp: 205–206 °C; ^{1}H NMR (DMSO-d_6) δ 1.24 (3H, t, J = 7.3 Hz), 2.65 (2H, q, J = 7.3 Hz), 5.40 (2H, s), 6.99 (2H, d, J = 5.9 Hz), 7.39 (1H, t, J = 5.4 Hz), 7.48 (2H, t, J = 7.8 Hz), 7.62 (2H, d, J = 8.3 Hz), 7.70 (2H, d, J = 6.8 Hz), 7.78 (2H, d, J = 8.3 Hz), 8.53 (2H, d, J = 5.3 Hz); MS (FAB) m/z 377 $[M+H]^+$; Anal. Calcd for $C_{22}H_{20}N_4$: C, 77.62; H, 5.92; N, 16.46. Found: C, 77.50; H, 5.87; N, 16.47.

5.1.13. 4-[1-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-ethyl]pyridine (24) (6%)

Mp: 175–176 °C; ^{1}H NMR (DMSO-d_6) δ 1.17 (3H, t, J = 7.8 Hz), 1.82 (3H, d, J = 6.5 Hz), 2.23–2.34 (1H, m), 2.55–2.66 (1H, m), 5.61 (1H, q, J = 7.3 Hz), 7.09 (2H, d, J = 6.4 Hz), 7.40 (1H, t, J = 7.3 Hz), 7.46–7.55 (4H, m), 7.71 (2H, d, J = 7.4 Hz), 7.79 (2H, d, J = 8.3 Hz), 8.56 (2H, d, J = 5.9 Hz); MS (FAB) m/z 355 $[M+H]^+$; Anal. Calcd for $C_{23}H_{22}N_4.0.2H_2O$: C, 77.15; H, 6.31; N, 15.65. Found: C, 77.35; H, 6.15; N, 15.37.

5.1.14. 7-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-1-methyl-1*H*-indole (9)

CH_3I (0.023 ml, 0.37 mmol) and K_2CO_3 (65 mg, 0.47 mmol) were added to a solution of **8** (110 mg, 0.30 mmol) in DMF (5.0 mL), and the mixture was stirred at room temperature. After 3 h, a second batch of CH_3I (0.015 ml, 0.24 mmol) and KOH (30 mg, 0.54 mmol) was added, and the mixture was stirred for 19 h. The reaction mixture was partitioned between EtOAc and water, and the organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was removed via evaporation, and the residue was purified using column chromatography on silica gel ($CHCl_3$/MeOH = 98/2) to give crude **9**. The crude solid was recrystallized from diisopropyl ether to give **9** (74 mg, 65%) as a colorless powder. Mp: 185–186 °C; ^{1}H NMR (DMSO-d_6) δ 1.16 (3H, t, J = 7.6 Hz), 2.45–2.51 (2H, m), 3.16 (3H, s), 6.57 (1H, d, J = 2.9 Hz), 7.22 (1H, t, J = 7.6 Hz), 7.32–7.37 (3H, m), 7.39–7.44 (2H, m), 7.46–7.50 (2H, m), 7.57–7.64 (4H, m), 7.79 (1H, d, J = 7.3 Hz); MS (FAB) m/z 378 $[M+H]^+$; Anal. Calcd for $C_{25}H_{22}N_4$: C, 79.34; H, 5.86; N, 14.80. Found: C, 79.37; H, 6.03; N, 14.72.

5.1.15. 1-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)isoquinoline (19)

Propionyl chloride (3.34 ml, 38.5 mmol) solution in THF (20 ml) at 0 °C was added to a solution of 1-aminoisoquinoline (**25**, 5.04 g, 35.0 mmol) in pyridine (100 ml), and the mixture was stirred for 11 h at room temperature. MeOH (50 ml) was then, and the solution was concentrated in vacuo. The residue was then partitioned between EtOAc and water, and the organic layer was separated and dried over anhydrous Na_2SO_4. The solvent was removed via evaporation to give *N*-isoquinolin-1-ylpropanamide (**26**, 5.22 g, 74%).

Lawesson's reagent (1.70 g, 4.2 mmol) was added to a suspension of **26** (1.40 g, 7.0 mmol) in pyridine (30 ml), and the mixture was stirred at 100 °C for 2 h. After cooling at room temperature, the reaction mixture was partitioned between EtOAc and water, and the organic layer was separated and dried over anhydrous $MgSO_4$. The solvent was removed via evaporation, and the residue was purified using column chromatography on silica gel (hexane/EtOAc = 1/1) to give *N*-isoquinolin-1-ylpropanethioamide (**27**, 1.39 g, 92%) as a pale-yellow solid.

CH_3I (1.36 g, 9.57 mmol) and 1 M NaOH solution (13 ml) were added to a solution of **27** (1.38 g, 6.38 mmol) in EtOH (20 ml), and the resultant mixture was stirred at room temperature for 0.5 h. The mixture was then evaporated in vacuo, and the residue was partitioned between EtOAc and water. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The residue was purified using column chromatography on silica gel (hexane/EtOAc = 2/1) to give methyl *N*-isoquinolin-1-ylpropanimidothioate (**28**, 1.29 g, 88%) as a pale-yellow oil. A mixture of **28** (500 mg, 2.17 mmol), **6** (424 mg, 2.0 mmol), *p*-toluenesulfonic acid monohydrate (35 mg, 0.18 mmol), and xylene (5 mL) was stirred at 130 °C for 4 h. After it was cooled at room temperature, the residue was purified using column chromatography on silica gel ($CHCl_3$/MeOH = 20/1) to give a crude solid, which was then purified by column chromatography on Al_2O_3 ($CHCl_3$) to give **19** (20 mg, 3%) as a brown solid. Mp: 179–181 °C; ^{1}H NMR ($CDCl_3$) δ 1.21 (3H, t, J = 7.5 Hz), 2.59 (2H, q, J = 7.5 Hz), 7.26–7.46 (10H, m), 7.55 (1H, t, J = 7.7 Hz), 7.74 (1H, t, J = 7.7 Hz), 7.88 (1H, d, J = 5.9 Hz), 7.95 (1H, d, J = 8.1 Hz), 8.61 (1H, d, J = 5.8 Hz); MS (FAB) m/z 377 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4$: C, 79.76; H, 5.35; N, 14.88. Found: C, 79.99; H, 5.41; N, 15.12.

5.1.16. 5-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)isoquinoline 2-oxide (20)

*m*CPBA (1.80 g, 8.0 mmol) was added to a solution of **15** (1.95 g, 5.2 mmol) in CH_2Cl_2 (20 mL), and the mixture was stirred at room temperature for 6 days. K_2CO_3 was then added, and the solution was subjected to extraction with $CHCl_3$-MeOH (10:1). The organic layer was dried over anhydrous $MgSO_4$ and concentrated in vacuo, and the residue was purified using column chromatography on silica gel ($CHCl_3$/MeOH = 20/1) to give **20** (1.42 g, 70%) as a white solid. Mp: 287–289 °C; ^{1}H NMR (DMSO-d_6) δ 1.12 (3H, t, J = 7.6 Hz), 2.40–2.55 (2H, m), 7.21 (1H, d, J = 7.2 Hz), 7.31–7.44 (5H, m), 7.56–7.62 (4H, m), 7.84–7.88 (1H, m), 7.90–7.94 (1H, m), 8.09–8.14 (2H, m), 9.13 (1H, d, J = 1.2 Hz); MS (FAB) m/z 393 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4O$: C, 76.51; H, 5.14; N, 14.28. Found: C, 76.58; H, 5.16; N, 14.56.

5.1.17. 5-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)isoquinolin-1(2H)-one (21)

A solution of **20** (1.01 g, 2.57 mmol) in acetic anhydride (15 ml) was stirred at 120 °C for 14 h. The reaction mixture was then evaporated, and the residue was partitioned between EtOAc and K_2CO_3 solution. The organic layer was separated, washed with brine, and dried over anhydrous $MgSO_4$. The solvent was removed by evaporation, and the residue was purified using column chromatography on silica gel ($CHCl_3$/MeOH = 20/1) to give **29**. 1 M NaOH solution (5 ml) was added to a solution of **29** in MeOH (5 ml), and the mixture was stirred for 10 min. The reaction mixture was concentrated in vacuo, and the residue was purified using column chromatography on Al_2O_3 ($CHCl_3$/MeOH = 20/1) to obtain a solid, which was then washed with hexane/EtOAc (1:2) to give **21** (730 mg, 72%) as a pale-yellow solid. Mp: 257–259 °C; ^{1}H NMR (DMSO-d_6) δ 1.13 (3H, t, J = 7.5 Hz), 2.33–2.50 (2H, m), 5.71 (1H, d, J = 7.3 Hz), 7.23 (1H, t, J = 6.0 Hz), 7.32–7.45 (5H, m), 7.60–8.02 (5H, m), 7.99 (1H, d, J = 7.5 Hz), 8.40 (1H, d, J = 8.2 Hz), 11.57 (1H, d, J = 5.5 Hz); MS (FAB) m/z 393 $[M+H]^+$; Anal. Calcd for $C_{25}H_{20}N_4O.0.2H_2O$: C, 75.81; H, 5.19; N, 14.15. Found: C, 75.86; H, 5.17; N, 14.17.

5.1.18. 5-(3-Biphenyl-4-yl-5-ethyl-4*H*-1,2,4-triazol-4-yl)-1,2,3,4-tetrahydroisoquinoline (22)

$NaBH_3CN$ (500 mg. 7.96 mmol) was added to a solution of **15** (520 mg, 1.38 mmol) in acetic acid (20 ml), and the mixture was stirred at room temperature for 2 h and partitioned between EtOAc and K_2CO_3 solution. The organic layer was separated, washed with $NaHCO_3$ (aqueous) and brine, and dried over anhydrous $MgSO_4$. The solvent was removed via evaporation, and the residue was purified using column chromatography on silica gel ($CHCl_3$/MeOH = 10/1) to give crude **22**. The crude product was washed with hexane/EtOAc (2:1) to give **22** (276 mg, 53%) as a white solid. Mp: 155–156 °C; ^{1}H NMR ($CDCl_3$) δ 1.27 (3H, t, J = 7.5 Hz), 2.16 (2H, t, J = 5.7 Hz), 2.56 (2H, q, J = 7.5 Hz), 2.97 (2H, t, J = 5.8 Hz), 4.07 (2H, s), 7.15–7.56 (12H, m); MS (FAB) m/z 381 $[M+H]^+$; Anal. Calcd for $C_{25}H_{24}N_4$: C, 78.92; H, 6.36; N, 14.73. Found: C, 78.87; H, 6.47; N, 14.72.

5.2. Aqueous solubility

Small volumes of the DMSO solutions of test compounds were diluted to 130 μM by adding the aqueous buffer solutions of pH1.2 and pH6.8. After incubation at 25 °C for 20 h, precipitates were separated by filtration. The solubility was determined by HPLC analysis of each filtrate.

5.3. Biology

5.3.1. [^{3}H]Glycine uptake assay (GlyT1)

Gomenza et al. previously reported the expression of rat GlyT1 in C6 cells.[17] In the present study, C6 cells were maintained in growth medium (D-MEM containing 10% fetal calf serum) at 37 °C in humidified air with 5% CO_2. Two days before the [^{3}H]glycine uptake experiments, C6 cells were plated at a density of 2×10^4 cells per well in a 96-well white CulturPlate (PerkinElmer Inc.). The assays were performed at 37 °C in 100 μL of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer containing NaCl (150 mmol/L), KCl (5 mmol/L), $MgCl_2$ (1 mmol/L), $CaCl_2$ (1 mmol/L), HEPES (10 mmol/L), D-glucose (10 mmol/L), and L-alanine (5 mmol/L) at pH 7.4. The growth medium was removed, and after being washed with HEPES buffer, the cells were incubated for 30 min with 50 μL of HEPES buffer containing the test compounds.

Uptake was initiated by adding 50 μL of 0.167 mmol/L [^{3}H]glycine (53.3 Ci/mmol). Non-specific uptake was detected using 3 mmol/L sarcosine. After incubating for 10 min at 37 °C, the wells were aspirated and washed three times with ice-cold phosphate-buffered saline (PBS). After solubilizing the cells with 17 μL of 0.1 mol/L NaOH, 100 μL of scintillant (Micro Scint PS; PerkinElmer Inc.) was added to the wells, and the plate was counted in a Top Count (Hewlett-Packard Company; Palo Alto, CA, USA). We evaluated sarcosine in parallel as a reference GlyT1 inhibitor. The drug concentrations required to inhibit 50% of the specific [^{3}H]glycine

uptake (IC_{50}) were obtained using nonlinear regression analysis and SAS software (SAS Institute Inc.; Cary, NC, USA), with the mean ± SEM obtained from three independent experiments.

5.3.2. [^{3}H]Glycine uptake assay (GlyT2)

A primary cell culture of rat brain was prepared for a [^{3}H]glycine uptake assay. Pregnant rats (Wistar at 17 days' gestation; Japan SLC, Shizuoka, Japan) were euthanized under ether anesthesia by exsanguination through severing the carotid artery. The fetuses were then obtained and their brainstems isolated. After digestion by papain, brainstem cells were dispersed into the culture medium and plated at a density of 5.0×10^4 cells per well in 96-well white plates coated with poly-l-lysine, at which point the plates were incubated for 14–21 days in a CO_2 incubator (37 °C, 5% CO_2).

On the day of the assay, after being washed with assay buffer (150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgCl_2$, 1 mmol/L $CaCl_2$, 10 mmol/L glucose, 5 mmol/L l-Alanine, 3 mmol/L sarcosine, and 10 mmol/L HEPES, pH 7.4), each well was filled with 100 μL of assay buffer and preincubated at 37 °C. After 10 min preincubation, the incubation buffer was replaced with reaction mixture containing the test compounds and approximately 0.3 μM [^{3}H]glycine, the plate was then incubated for 10 min at 37 °C, and the reaction was stopped by washing the cells four times with ice-cold buffer. After solubilizing the cells with 17 μL of 0.1 mol/L NaOH, 100 μL of scintillant was added and the plate was counted using Top Count (Hewlett-Packard Company; Palo Alto, CA, USA). In each experiment, specific [^{3}H]glycine uptake was estimated based on the uptake inhibition of 3 mmol/L glycine. The drug concentrations required to inhibit 50% of the specific [^{3}H]glycine uptake (IC_{50}) were obtained using nonlinear regression analysis and SAS software (SAS Institute Inc.; Cary, NC, USA). We evaluated glycine in parallel as the reference, and the mean ± SEM from three independent experiments gave the final inhibition values.

5.3.3. (+)-HA966-induced enhancement of locomotor activity in mice

The method reported previously[20] was used to conduct the experiment, with some modification.

- Animal: male ICR mice (Japan SLC; age 5–7 weeks)
- Drug: reserpine (Apoplon injection, 1 mg/ml; Daiichi Pharmaceuticals Co., Ltd., Tokyo, Japan), (+)-HA966, a-methyl-para-tyrosine methyl ester (Sigma, Inc.)
- Equipment: Supermex (Muromachi Machine)
- Methods:
  1) Pharmaceutical drug-treated groups were defined as follows, with 16 mice per group: [artificial cerebrospinal fluid (ACSF) + Vehicle] group, [(+)-HA966 80 μg/mouse (intracerebroventricular) + Vehicle] group, [(+)-HA966 80 μg/mouse (intracerebroventricular) + test compound] group
  2) Nineteen hours before (+)-HA966 administration, reserpine (10 mg/kg) was administered intraperitoneally.
  3) Thirty minutes before (+)-HA966 administration, a-methyl-para-tyrosine methyl ester (250 mg/kg) was administered intraperitoneally.
  4) Twenty minutes before (+)-HA966 administration, a test compound was administered orally or intraperitoneally.
  5) (+)-HA966 was acutely administered bilaterally into the lateral ventricule (free-handed using two-step needle). Immediately after administration, each animal was placed in the measuring cage of an activity measurement apparatus.
  6) Activity per 90 min was measured immediately upon placing the animal in the cage.
  7) The integral value of locomotor activity per 90 min was selected as the data, and the inhibition(%) of hyperlocomotion with the test compounds was determined as follows:

$$100 \times (\{\text{activity of } [(+) - \text{HA966} + \text{Vehicle}]\text{group} - \text{activity of } (\text{ACSF} + \text{Vehicle})\text{group}\} - \{\text{activity of } [(+) - \text{HA966} + \text{test compound}]\text{group} - \text{activity of}(\text{ACSF} + \text{Vehicle})\text{group}\})/\{\text{activity of } [(+) - \text{HA966} + \text{Vehicle}]\text{group} - \text{activity of}(\text{ACSF} + \text{Vehicle})\text{group}\}.$$

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