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ARTICLE TYPE

Discovery of 4-benzoylpiperidine and 3-(piperidin-4-yl)benzo[*d*]isoxazole derivatives as potential and selective GlyT1 inhibitors

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Regulation of glycine transporter 1 (GlyT1) activity is a currently investigated strategy in the drug discovery for schizophrenia. This study developed a series of new 4-benzoylpiperidine derivatives as GlyT1 inhibitors by bioisosteric replacement and mimic of the pyridine ring of RG1678. Among the 4-benzoylpiperidine derivatives, **23q** showed an IC_{50} of 30 nM. Preliminary optimization of the blood-brain barrier penetration led to the discovery of 3-(piperidin-4-yl)benzo[*d*]isoxazole derivatives. Both series showed good selectivity over GlyT2, D_1, D_2, D_3, 5-HT_{1A} and 5-HT_{2A} receptors. Moreover, behavioral testing showed **23q** (40 mg/kg, intragastric) can inhibit the hyperlocomotion induced by acute treatment of phencyclidine, and improve the impaired negative and cognitive symptoms in chronic phencyclidine-induced C57BL/6J mice. Interesting finding showed that 3-(piperidin-4-yl)benzo[*d*]isoxazole was a privileged scaffold of atypical antipsychotic agents but exhibited high selectivity and potency as GlyT1 inhibitors.

Introduction

Schizophrenia is a severe and chronic psychiatric illness, characterized by positive, negative and cognitive symptoms. Currently available drugs are effective in relief of the positive symptoms, but elicit weak or no improvement on the negative symptoms and cognitive impairments.[1] It is generally believed that the N-methyl-D-aspartate (NMDA) receptor hypofunction plays a critical role in the pathophysiology of schizophrenia and may associate with the development of negative symptoms and cognitive deficits in the illness.[2] Activating the NMDA receptor and restoring function of glutamatergic neurons may be an alternative therapeutic strategy for schizophrenia treatment.[3] Because the NMDA receptor is widely distributed in the brain and direct activation of the NMDA receptor would cause seizures and serious neurotoxic side effects,[4] efforts have turned to the auxiliary glycine binding site. Glycine is an obligatory co-agonist of the NMDA receptor, and elevated glycine levels in the synaptic cleft can promote activation of the NMDA receptor.[5] Glycine in the synaptic cleft can be uptaken into neurons or surrounding glia by the glycine transporter (GlyT). There are two types of glycine transporters in the brain: GlyT1, which is localized to the NMDA receptor area, and GlyT2, which is distributed around the glycinergic neurons.[6] Therefore, selective inhibition of GlyT1 could elevate glycine concentrations in the glutamatergic synapse and enhance NMDA receptor activity, thereby may be of potential therapeutic effects in schizophrenia.

So far, at least two series of GlyT1 inhibitors have been identified.[7] One is the sarcosine-derived GlyT1 inhibitor, such as **1**[8] and **2**.[9] However, some compounds of this series were discontinued in clinical trials because of undesired adverse effects. Another series is the non-sarcosine-based GlyT1 inhibitor, such as **3**,[10] **4**,[11] and **5**.[12] Among them, RG1678 demonstrated a beneficial effect in patients with schizophrenia characterized with predominant negative symptoms in a phase II proof-of-concept study[13] and it is currently in phase III clinical trial.

In our effort to find new GlyT1 inhibitors, we considered RG1678 as a potential starting point for further structural modification. A patent from Roche revealed some bioisosteric replacements of piperazine ring, among them pyrrolidine series was the most potent and well investigated (compound **6** with an IC_{50} = 90 nM) while piperidine ring did not exhibit good potency[14]. In this case, we considered that piperidine ring was an appropriate bioisostere of piperazine and when the piperazine ring was replaced with piperidine, some heteroatoms should be incorporated to compensate for the loss of binding energy like hydrogen bond. Based on this consideration, we first introduced a carbonyl to 4-benzylpiperidine of compound **7** and a series of 4-benzoylpiperidine derivatives were synthesized, and tested.

Results and discussion

Chemistry

All described target compounds were prepared by condensation of 2,5-disubstituted benzoic acids and corresponding piperidine moieties. The 2,5-disubstituted benzoic acids were performed as shown in Scheme 1 and 2. **8a–c** were commercially available or

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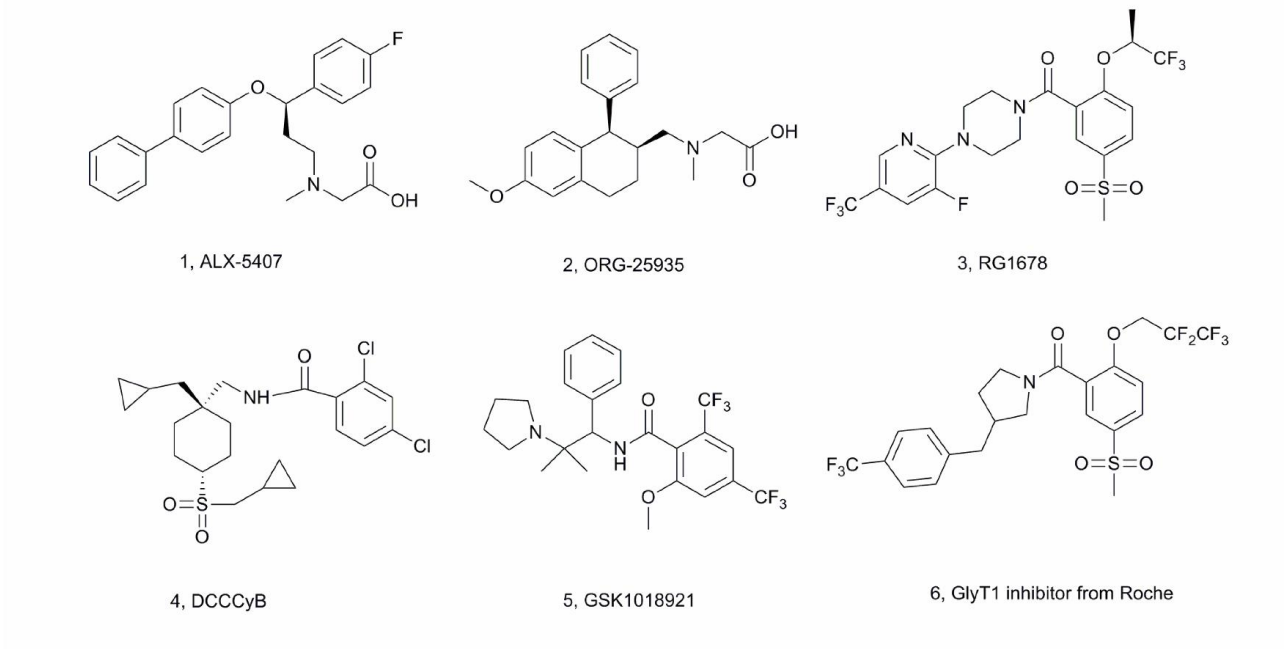

Fig. 1. Structures of GlyT1 inhibitors.

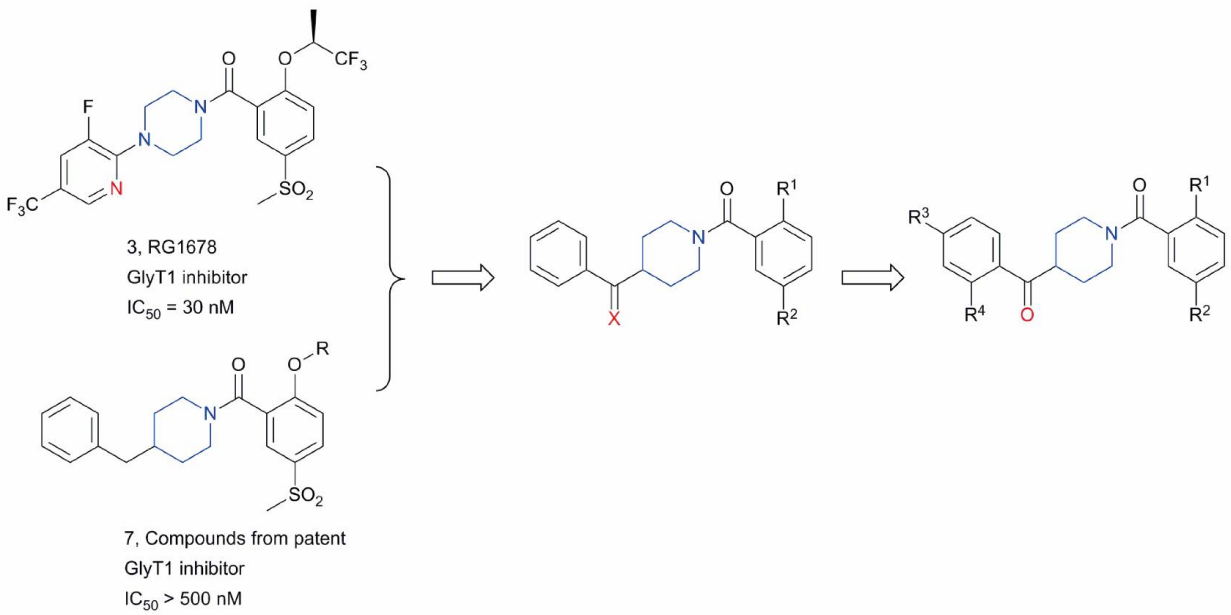

Fig. 2. Design of new GlyT1 inhibitors.

synthesized as listed in references.[10] The R^2 group preferred an electron-withdrawing groups (EWGs). So it was selected from nitro, methylsulfonyl, and N-methylsulfamoyl. First, 2-alkoxy-5-substituted benzoates **9a–h** were prepared by substitution reactions with alkyl halides or Mitsunobu reactions with alkyl alcohols in good yields. Then the substituted esters were hydrolyzed by sodium hydroxide to afford the corresponding acids **10a–h**. By reacting with triflic anhydride, the phenolic hydroxyl group of **8a–b** was converted into trifluoromethylsulfonyloxy group in 72–79% yields and then coupled with 4-fluorophenylboronic acid or substituted by some alkyl amines to yield **12a–b**. Further hydrolysis was performed to get **13a–b**. Alternatively, considering the electron-withdrawing groups of the phenyl ring, the alkoxy and alkyl amine group at position 2 was also introduced by 2-fluorobenzoic acid analogues following the synthetic route described in Scheme 2.

The synthesis of piperidine moieties is outlined in Scheme 3. Substituted bromobenzene was converted to a Grignard reagent and then reacted with Weinreb amide, resulting in **20a–b**. Following reflux in HCl (6N), **21a–b** were prepared. **21c** was

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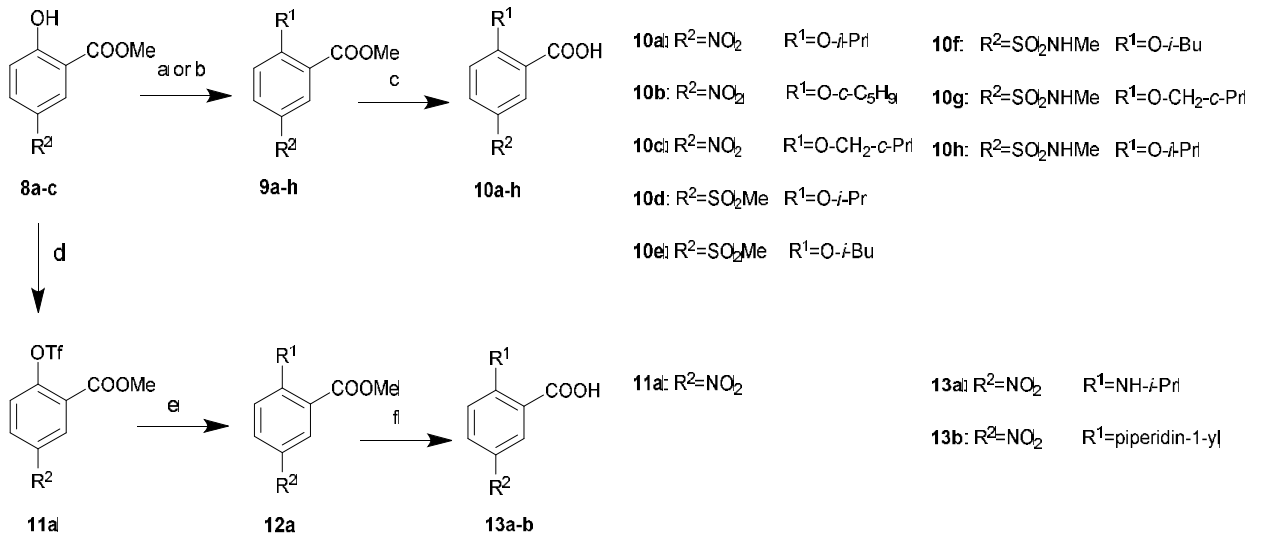
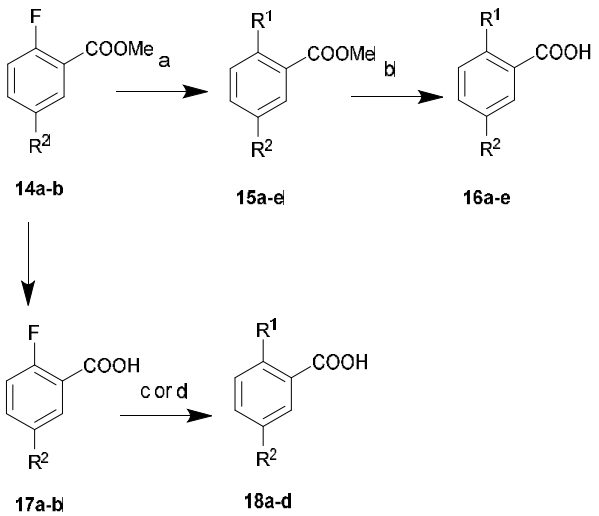


10a: $R^2=NO_2$ R^1=O-*i*-Pr
10b: $R^2=NO_2$ R^1=O-*c*-C_5H_9
10c: $R^2=NO_2$ R^1=O-CH_2-*c*-Pr
10d: $R^2=SO_2Me$ R^1=O-*i*-Pr
10e: $R^2=SO_2Me$ R^1=O-*i*-Bu
10f: $R^2=SO_2NHMe$ R^1=O-*i*-Bu
10g: $R^2=SO_2NHMe$ R^1=O-CH_2-*c*-Pr
10h: $R^2=SO_2NHMe$ R^1=O-*i*-Pr

11a: $R^2=NO_2$

13a: $R^2=NO_2$ R^1=NH-*i*-Pr
13b: $R^2=NO_2$ R^1=piperidin-1-yl

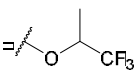
Scheme 1. Reagents and conditions: (a) alkyl bromide, K_2CO_3, DMF, 40–60 °C, overnight; (b) alkyl alcohol, DIAD, PPh_3, THF, rt, 24h; (c) NaOH, MeOH/THF/H_2O (1:1:1), 40 °C, 2 h; (d) triflic anhydride, Et_3N, CH_2Cl_2, 0 °C-rt, 20 min; (e) alkylamine, NMP, microwave, 210 °C, 5 min; (f) NaOH, MeOH/THF/H_2O (1:1:1), 60 °C, 2 h.



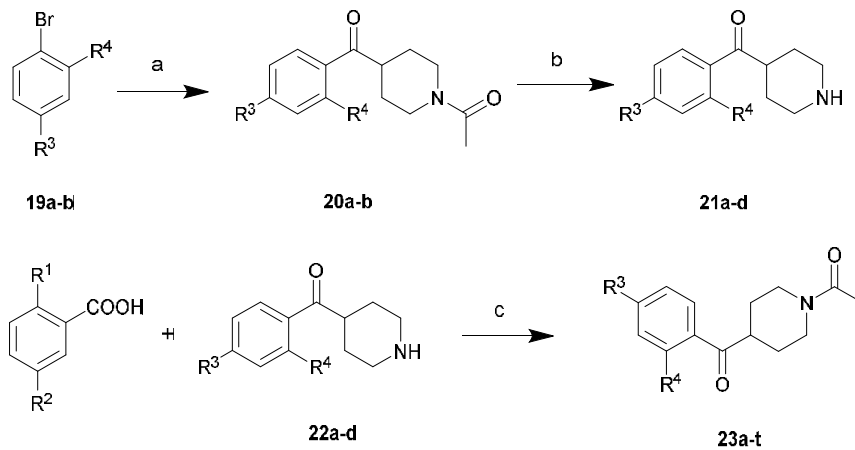
14a: $R^2=SO_2Me$
14b: $R^2=SO_2NHMe$

16a: $R^2=SO_2Me$ R^1=NH-*i*-Pr
16b: $R^2=SO_2Me$ R^1=piperidin-1-yl
16c: $R^2=SO_2NHMe$ R^1=NH-*i*-Pr
16d: $R^2=SO_2NHMe$ R^1=piperidin-1-yl
16e: $R^2=SO_2NHMe$ R^1=morpholino

17a: $R^2=SO_2Me$
17b: $R^2=SO_2NHMe$

18a: $R^2=SO_2Me$ R^1= 
18b: $R^2=SO_2Me$ $R^1=OCH_2CF_2CF_3$
18c: $R^2=SO_2NHMe$ $R^1=OCH_2CF_2CF_3$
18d: $R^2=SO_2NHMe$ R^1=S-*i*-Pr

Scheme 2. Reagents and conditions: (a) alkylamine, THF, 100 °C, microwave, 40 min; (b) NaOH (2N), THF, 60 °C, 2 h; (c) alkyl alcohol, *t*-BuOK, dry THF, rt-60 °C, 2 h; (d) propane-2-thiol, Cs_2CO_3, DMA, 100 °C, 3 h.



21a: R^3=F R^4=H
21b: $R^3=CF_3$ R^4=H
21c: $R^3=CF_3$ R^4=F
21d: R^3=F R^4=F

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Scheme 3. Reagents and conditions: (a) Mg, I_2, dry THF, 1-acetyl-N-methoxy-N-methylpiperidine-4-carboxamide, reflux to rt, overnight; (b) HCl (6N), reflux, 4h; (c) HATU, Et_3N, CH_2Cl_2, rt, overnight.

synthesized according to a previous study[15] and **21d** was commercially available. Finally, the target compounds were prepared in a single step by HATU-mediated condensation yields ranging from 57% to 85%.

In vitro biological evaluation and discussion

All final compounds were initially evaluated for their ability to inhibit GlyT1, and the results are summarized in Table 1 and Table 2. Considering the convenience of synthesis, we introduced a nitro group to R^2 position first and then explored the R^3 and R^4 group. The structure-activity relationship study (SAR) of RG1678 revealed that the aromatic ring of the left side preferred hydrophobic substituents, so we incorporated trifluoromethyl and fluoro to R^3 and R^4 positions. As shown in Table 1，for the R^4 group, fluoro was more potent than hydrogen (**23a** vs. **23b**, **23c** vs. **23d**, and **23e** vs. **23f**), while on the R^3 position, trifluoromethyl was less suitable than fluoro (**23a** vs. **23c** and **23b** vs. **23d**). For the R^1 group, introduction of some alkylaminos, such as **23g** and **23h**, led to great loss in GlyT1 activity. Though **23e** showed a slight increase of activity against **7,** the others did not meet our satisfaction. This result prompted us to optimize the substituents of the benzoic moiety.

As shown in Table 2, nitro was first replaced with another electron withdrawing N-methylsulfamoyl group while R^3 and R^4 were kept as the most potent fluoro. To our delight, the N-methylsulfamoyl series exhibited well tolerance on the R^1 group, and all alkoxy and alkylamino groups on R^1 position showed moderate activities. However, none of the compounds had a better performance with an IC_{50} value below 50 nM. Finally, we tested methylsulfonyl on R^2 position of benzoic moiety. The result was consistent with nitro series and alkoxy groups were more potent than alkylamino on R^1 position. Surprisingly, **23q** gave the most satisfactory result (IC_{50} = 30 nM). The various substituents at R^1 position of benzoic that were tolerated indicated that a lipophilic bulk pocket might exist at this position for the GlyT1 receptor.

In vivo pharmacokinetic study

With the identification of **23q** as the most potent GlyT1 inhibitor *in vitro*, the pharmacokinetic parameters of **23q** were evaluated in ICR mice. After oral administration of **23q** (10 mg/kg), blood samples and brain tissues were collected and analyzed at different time points. As shown in Table 3, **23q** had a short half-life ($T_{1/2}$ = 0.7 h), moderate plasma exposure ($AUC_{0-\infty}$ = 737 h*ng/mL) with a low brain exposure (C_{max} in brain = 26.3 ng/g), indicating a poor B/P ratio. However, the concentration in the brain (C_{max} in brain = 26.3 ng/g) was just above the IC_{50} value. This result promoted us to make structure modifications to improve the brain exposure.

Initial attempts to improve brain exposure

The low B/P ratio of **23q** indicated that it may be difficult to penetrate the Blood-Brain Barrier (BBB). Strategies to improve BBB permeability have been reviewed and the structure optimization methods include increasing lipophilicity, reducing hydrogen bond donors, increasing rigidity and lowering polar surface area (PSA).[16] Herein we used two parameters, CLogP and tPSA (topological PSA) calculated by ChembioDraw, to represent the lipophilicity and PSA.

As shown in Table 4，**23q** had a similar tPSA value with RG1678 while a slightly lower CLogP, which indicating that higher lipophilicity may be helpful to improve the B/P ratio. At the same time, we made a confirmation restriction of 4-benzoylpiperidine moiety, leading to the 3-(piperidin-4-yl)benzo[*d*]isoxazole derivatives. By this transformation, we kept the heteroatom in proper position, increased the rigidity and lipophilicity of molecule, but caused a slight adverse effect to tPSA. When **23q** was transformed to **24a**, a great loss in activity was also observed (IC_{50} = 185 nM). A scale of optimization was performed and the selected compounds summarized in Table 4 demonstrated that 3-(piperidin-4-yl)benzo[*d*]isoxazole derivatives had moderate to acceptable GlyT1 inhibiting activities (**24g** with an IC_{50} = 38 nM). But the initial optimization failed to reach a balance among the activity, CLogP and tPSA. As we expected, with a higher tPSA value, **24g** did not have better performance than **23q** *in vivo* pharmacokinetic study (data not shown).

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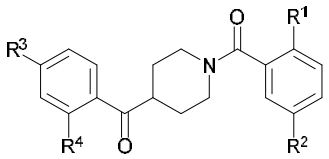
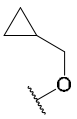
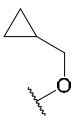
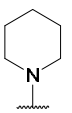
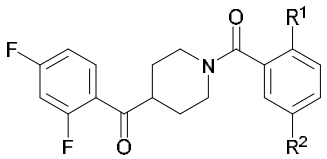
Table 1 *In vitro* biological evaluation of **23a-23h**.

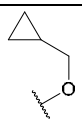
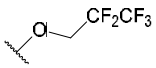
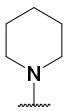
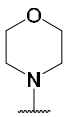
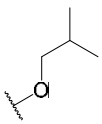
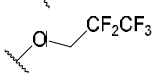
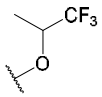
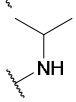
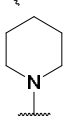


Compd	R^1	R^2	R^3	R^4	GlyT1 IC_{50} (nM) [a]
23a		nitro	F	F	471±54
23b		nitro	F	H	2790±40
23c		nitro	CF_3	F	1855±45
23d		nitro	CF_3	H	77.87%@10μM
23e		nitro	F	F	310±94
23f		nitro	F	H	461±165
23g		nitro	F	F	78.53%@10μM
23h		nitro	F	F	82.45%@10μM

[a] IC_{50} values were determined using [^{3}H] glycine and rat glioma C6 cell lines. Data are the mean ± SEM of at least three independent determinations.

Table 2 *In vitro* biological evaluation of **23i-23t**



Compd	R^1	R^2	GlyT1 IC_{50} (nM) [a]
23i		N-methylsulfamoyl	172±36
23j		N-methylsulfamoyl	143±7.25
23k		N-methylsulfamoyl	117±5.37
23l		N-methylsulfamoyl	184±14
23m		N-methylsulfamoyl	80±10.47
23n		N-methylsulfamoyl	115±32.9
23o		methylsulfonyl	58.02%@10μM
23p		methylsulfonyl	501±84.9
23q		methylsulfonyl	30±5
23r		methylsulfonyl	572±67
23s		methylsulfonyl	44.73%@10μM
23t		methylsulfonyl	955±101

[a] IC_{50} values were determined using [^{3}H] glycine and rat glioma C6 cell lines. Data are the mean ± SEM of at least three independent determinations.

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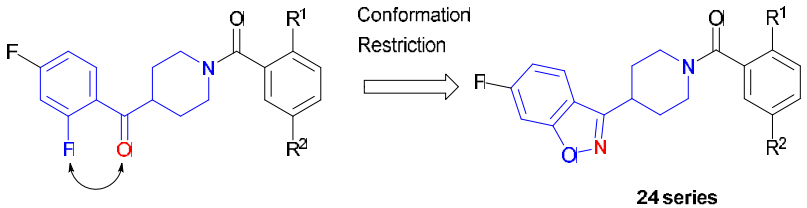
Table 3 Pharmacokinetics parameters of **23q** in ICR mice.

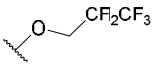
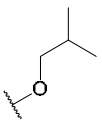
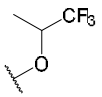
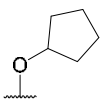
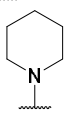
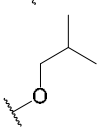
Compd	$T_{1/2}$ (h)	T_{max} (h)	$C_{max,p}$ [a] (ng/mL)	AUC_{0-t} (h*ng/mL)	$AUC_{0-\infty}$ (h*ng/mL)	$MRT_{0-\infty}$ (h)	$C_{max,b}$ [b] (ng/g)	B/P [c]
23q	0.7	0.5	955	728	737	0.91	26.3	0.03

[a] $C_{max,\,p} = C_{max}$ in plasma. [b] $C_{max,\,b} = C_{max}$ in brain. [c] $B/P = C_{max,\,b} / C_{max,\,p}$. n = 3 mice/group; male ICR mice (18–20g); 10 mg/kg, intragastric administration, formulated in 0.5% HMPC. PK parameters were calculated by non-compartmental model analysis on Phoenix WinNonlin 6.0 (Pharsight, Mountain View, CA).

Table 4 *In vitro* biological evaluation of 3-(piperidin-4-yl)benzo[*d*]isoxazole derivatives.



24 series

Compd	R^1	R^2	GlyT1 IC_{50} (nM) [a]	CLogP [b]	tPSA [c]
RG1678	-	-	30 [d]	2.99	79.28
23q	-	-	30±5	2.67	80.75
24a		methylsulfonyl	185±56	3.28	85.27
24b		methylsulfonyl	159±43	2.93	85.27
24c		methylsulfonyl	496±58.9	2.58	85.27
24d		nitro	89±7.8	4.19	102.94
24e		nitro	85±5.15	2.46	79.28
24f		N-methylsulfamoyl	61±6.83	2.92	97.3
24g		N-methylsulfamoyl	38±7.63	3.54	97.3

[a] IC_{50} values were determined using [^{3}H] glycine and rat glioma C6 cell lines. Data are the mean ± SEM of at least three independent determinations. [b] Data were calculated by ChemBioDraw. [c] Data were calculated by ChemBioDraw. [d] Data was selected from published reference .

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Table 5 Selectivity evaluation of **23** and **24** series.

Compd	GlyT1 IC_{50} (nM)	GlyT2 [a] @10μM	D_1 [b] @10μM	D_2 [b] @10μM	D_3 [b] @10μM	5-HT_{1A} [b] @10μM	5-HT_{2A} [b] @10μM	hERG [c] IC_{50} (μM)
23i	172±36	25.67%	19.73%	5.87%	25.14%	2.90%	0.66%	- [d]
23k	117±5.37	11.94%	7.62%	18.45%	14.43%	33.06%	8.84%	-
23m	80±10.47	14.92%	14.87%	6.20%	10.80%	5.80%	4.86%	-
23q	30±5	14.62%	16.33%	10.31%	15.29%	11.22%	5.21%	8.1
24b	159±43	8.65%	22.25%	24.24%	17.49%	3.91%	4.05%	-
24d	89±7.8	18.95%	23.37%	5.20%	21.51%	1.75%	31.55%	-
24f	61±6.83	19.70%	6.21%	3.94%	30.30%	24.01%	6.39%	-
24g	38±7.63	17.61%	8.02%	22.48%	25.81%	35.67%	16.51%	-

[a] GlyT2 inhibiting activity was examined with the cultured primary brain stem neurons. [b] D_1, D_2, D_3, 5-HT_{1A}, and 5-HT_{2A} were obtained from HEK293 stable-transfecting cell lines. [c] hERG channels were obtained from HEK293 stable-transfecting cell lines. [d] Not tested.

Selectivity evaluation of 23 and 24 series

Though **24** series was unsuccessful to improve the B/P ratio, we were surprised to find that the 3-(piperidin-4-yl)benzo[*d*]isoxazole moiety was a privileged scaffold that appears frequently in atypical antipsychotic agents[17] such as risperidone[18] and iloperidone,[19] which were dopamine and serotonin receptors dual antagonists. 4-Benzoylpiperidine moiety was also incorporated to discover multitarget ligands of aminergic receptors.[20] With this in mind, we assessed the selectivity of some selected compounds. Data were shown in Table 5. Both **23** and **24** series exhibited high selectivity over GlyT2, dopamine and serotonin receptors (IC_{50} > 10 μM), indicating that **23** and **24** series were selective GlyT1 inhibitors. On the other hand, the result demonstrated that 3-(piperidin-4-yl)benzo[*d*]isoxazole moiety was not only a privileged scaffold in dopamine hypothesis, but also well tolerated in NMDA hypofunction hypothesis.

In vivo behavioral tests on C57BL/6J mice

With the identification of **23q** as a potent and selective GlyT1 inhibitor, we assessed *in vivo* anti-psychotic activity using phencyclidine (PCP)-induced schizophrenia-like animal models. Considering the low B/P ratio, we assessed using higher doses (20, 40 mg/kg, intragastric).

Firstly, the effect of **23q** on the acute PCP-induced hyperlocomotor activity was examined as described previously.[21], **23q** alone did not change the mice's locomotor activity. Pretreatment of **23q** (40 mg/kg) attenuated PCP (5 mg/kg) - induced hyperlocomotion of mice, indicating that **23q** is a potential anti-psychotic drug *in vivo* (Figure 3).

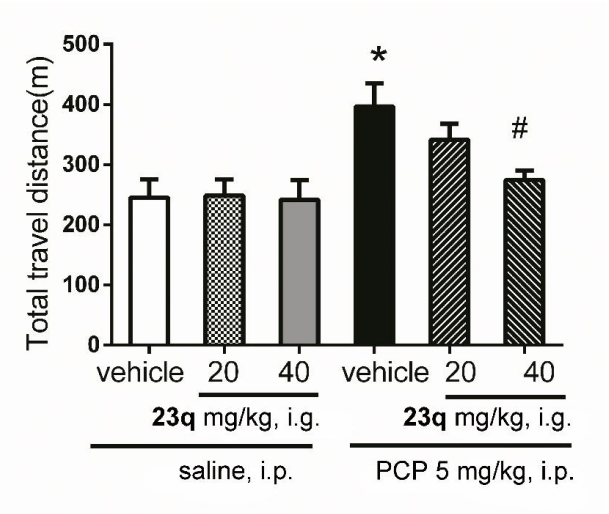

Fig. 3. Effects of **23q** on PCP-induced hyperlomotor activity in male C57BL/6J mice. The locomotor activity was indicated by the total travelling distance during 1 hour recording period after the injection of PCP (5 mg/kg). Vehicle: treated with saline without PCP or **23q**. PCP + vehicle: treated with PCP (5 mg/kg, i.p.). PCP + **23q**: treated with **23q** (20 mg/kg or 40 mg/kg, i.g.) for 60 min, then followed by the injection of PCP (5 mg/kg, i.p.). Data were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) and Dunnett's post-hoc test. *: $p < 0.05$, vs. vehicle; #: $p < 0.05$, vs. PCP + vehicle. n = 10.

We then examined whether **23q** could improve the social interaction and cognitive function. C57BL/6J mice received daily injections of PCP (10 mg/kg) for 2 weeks prior to administration of **23q** for an additional 2 weeks. Then social interaction and novel object recognition tests were performed. Methods are summarized in the experimental section.[21-23]

As shown in Figure 4，mice treated with PCP exhibited a social behavioral deficit in the social interaction test. Two weeks of treatment with **23q** (40 mg/kg) significantly restored the impaired social interaction in PCP-treated mice. Similarly, administration of **23q** (40 mg/kg) significantly improved the cognitive deficit in the chronic PCP-treated mice (Figure 5).

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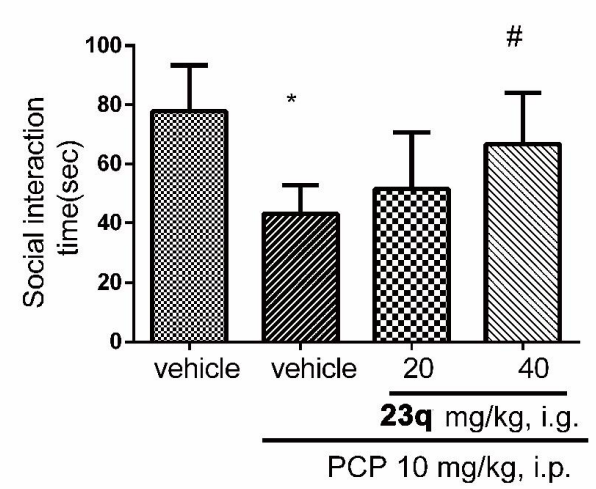

Fig. 4. Effects of **23q** on PCP-induced C57BL/6J mice in the social interaction test. Social interaction time was defined as sniffing at any part of the partner's body, grooming, following, or crawling over/under. Vehicle: treated with saline without PCP or **23q**. PCP + vehicle: treated with PCP (10 mg/kg, i.p.) once daily for 2 weeks and then saline for an additional 2 weeks. PCP + **23q**: treated with PCP (10 mg/kg, i.p.) once daily for 2 weeks and then **23q** (20 mg/kg or 40 mg/kg, i.g.) for another 2 weeks. Data were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) and Dunnett's post-hoc test. *: $p < 0.05$, *vs*. vehicle; #: $p < 0.05$, *vs*. PCP + vehicle. n = 10.

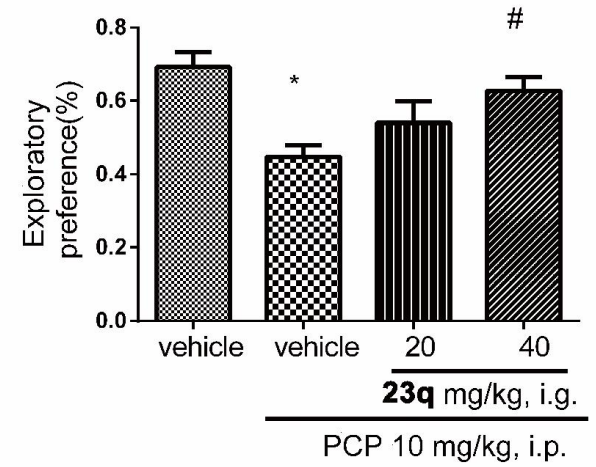

Fig. 5. Effects of **23q** on PCP-induced C57BL/6J mice in novel object recognition test. Exploratory preference was a ratio of the amount of time spent exploring the novel object over the total time spent exploring both familiar and novel objects. Vehicle: treated with saline without PCP or **23q**. PCP + vehicle: treated with PCP (10 mg/kg, i.p.) once daily for 2 weeks and then saline for an additional 2 weeks. PCP + **23q**: treated with PCP (10 mg/kg, i.p.) once daily for 2 weeks and then **23q** (20 mg/kg or 40 mg/kg, i.g.) for another 2 weeks. Data were expressed as mean ± SEM and analyzed using one-way analysis of variance (ANOVA) and Dunnett's post-hoc test. *: $p < 0.05$, *vs*. vehicle; #: $p < 0.05$, *vs*. PCP + vehicle. n = 10.

Conclusions

In summary, based on the bioisosteric replacement and mimic of nitrogen atom of pyridine ring of RG1678, we synthesized and evaluated a series of 4-benzoylpiperidine derivatives (**23a-t**). Among them **23q** was the most potent GlyT1 inhibitor *in vitro* with an IC_{50} value of 30 nM and showed high selectivity against GlyT2 and dopamine and serotonin receptors. Further *in vivo* study demonstrated that **23q** was effective on the chronic PCP-treated schizophrenia-like behavioral models with 40 mg/kg. The relatively high dose of **23q** was needed to achieve the anti-psychotic efficacy may attribute to the poor pharmacokinetic parameters and low B/P ratio. Preliminary optimization did not improve the pharmacokinetic parameters but led to the discovery of another potential and selective GlyT1 inhibitors: the 3-(piperidin-4-yl)benzo[*d*]isoxazole derivatives. Next we will make more structure modifications to improve the brain exposure. 4-Benzoylpiperidine and 3-(piperidin-4-yl)benzo[*d*]isoxazole, which were privileged scaffolds in atypical antipsychotic agents, exhibited high potency in GlyT1 inhibition. We suspect whether they may be potential templates to build multitarget ligands of GlyT1, dopamine and serotonin receptors.

Experimental Section

General

All reagents were purchased from commercial suppliers and used without further purification unless otherwise stated. Yields were not optimized. Microwave reactions were performed in a Biotage Initiator. Column chromatography was performed using pre-packed silica cartridges (from 4 to 40 g) from Bonna-Agela Technologies Inc. (Tianjin, China) and eluted with a CombiFlash® Rf 200 from Teledyne Isco. ^{1}H NMR and ^{13}C NMR spectra was recorded on a Bruker AC300 or a Bruker AC400 NMR spectrometer, using tetramethylsilane as an internal reference. Low-resolution mass spectra were determined on Agilent liquid-chromatography mass spectrometer system that consisted of an Agilent 1260 infinity LC coupled to Agilent 6120 Quadrupole mass spectrometer (electrospray positive ionization; ESI) using an Agilent ZORBAX 1.8 mm SB-C18 column (2.1×50 mm) with aqueous CH_3CN (30–90%) containing 0.05% formic acid monitored at 240 nm. High-resolution mass spectra were recorded on a Q-Tof Ultima Globe mass spectrometer (Micromass, Manchester, UK). HPLC analysis for all compounds tested in biological systems was performed on an Agilent 1200 series LC system (Agilent ChemStation, Agilent Eclipse XDB-C18, 5 μm, 4.6×150 mm, 30 °C, UV240 nm, 1.0 ml/min) with aqueous CH_3CN (35–90%) containing formic acid (0.05%) for 25 min. Experiments in pharmacokinetics study and behavioral tests with live animals were performed in compliance with the Guidelines for the Care and Use of Laboratory Animals (National Research Council, People's Republic of China, 1996). The animal protocols were approved by the Institutional Animal Care and Use Committees of Shanghai Institute of Materia Medica (SIMM) and Soochow University.

General synthetic procedure for benzoic acids and piperidine moieties.

Preparation of **10a-h**, **13a-b**, **16a-e**, **18a-d** and **21a-b** was summarized in Electronic Supplementary Information.

General synthetic procedure for 23a-23t, 24a-24g.

To a solution of benzoic acid moiety (1 equiv) in CH_2Cl_2 (15 mL) was added HATU (1.3 equiv) and the mixture was stirred at room temperature for 30 min. Then Et_3N (3 equiv) and piperidine moiety (1.2 equiv) were added subsequently and the reaction

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mixture was stirred at room temperature overnight. The mixture was diluted with CH_2Cl_2 (10 mL) and washed with water. The organic layer was dried over $MgSO_4$ and concentrated. The residue was purified by flash column chromatography to give the final compounds.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(2-isopropoxy-5-nitrophenyl)methanone (23a): **23a** was prepared from **10a** (0.1 g, 0.44 mmol) and **21d** (0.14 g, 0.53 mmol). Yellow solid (0.14 g, 76%). HPLC purity: 96.66%; ^{1}H NMR (500 MHz, CDCl3): δ 8.28 – 8.08 (m, 2H), 7.95 – 7.81 (m, 1H), 7.04 – 6.94 (m, 2H), 6.93 – 6.83 (m, 1H), 4.79 – 4.62 (m, 2H), 3.51 (tt, J = 13.3, 4.1 Hz, 1H), 3.44 – 3.30 (m, 1H), 3.23 – 2.91 (m, 2H), 2.13 – 2.02 (m, 1H), 1.94 – 1.53 (m, 3H), 1.48 – 1.35 ppm (m, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) 198.54, 166.83-164.88 (d, J = 245.7 Hz), 165.29, 163.05-161.02 (d, J = 255.8 Hz), 158.75, 140.92, 133.19-133.00 (m), 127.60, 126.26, 124.22, 121.39-121.29 (d, J = 12.6 Hz), 112.70, 112.37-112.20 (d, J = 21.4 Hz), 105.07-104.64 (d, J = 27.1 Hz), 72.19, 47.66, 46.64 (2C), 28.14 (2C), 21.85 (2C). HRMS (ESI): m/z $[M+H]^+$ calcd for $C_{22}H_{23}O_5N_2F_2$: 433.1575, found: 433.1564.

(4-(4-Fluorobenzoyl)piperidin-1-yl)(2-isopropoxy-5-nitrophenyl)methanone (23b): **23b** was prepared from **10a** (0.1 g, 0.44 mmol) and **21a** (0.11 g, 0.53 mmol). Light yellow solid (0.14 g, 77%). HPLC purity: 99.86%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.28 – 8.13 (m, 2H), 8.06 – 7.93 (m, 2H), 7.22 – 7.12 (m, 2H), 6.99 (t, J = 8.7 Hz, 1H), 4.83 – 4.67 (m, 2H), 3.61 – 3.45 (m, 2H), 3.26 – 2.98 (m, 2H), 2.10 – 1.66 (m, 4H), 1.52 – 1.35 ppm (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) 199.40, 166.20-164.92 (d, J = 193.3 Hz), 164.76, 158.31, 140.43, 131.48, 130.45-130.39 (d, J = 9.1 Hz, 2C), 127.14, 125.78, 123.75, 115.50, 111.90-111.72 (d, J = 27.2 Hz, 2C), 71.76, 46.04 (2C), 40.86, 27.97 (2C), 21.36 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{22}H_{24}O_5N_2F$: 415.1669, found:415.1658.

(4-(2-Fluoro-4-(trifluoromethyl)benzoyl)piperidin-1-yl)(2-isopropoxy-5-nitrophenyl)methanone (23c): **23c** was prepared from **10a** (0.1 g, 0.44 mmol) and **21c** (0.14 g, 0.53 mmol). Yellow solid (0.12 g, 57%). HPLC purity: 99.20%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.23 (dd, J = 9.2, 2.8 Hz, 1H), 8.15 (dd, J = 16.1, 2.8 Hz, 1H), 7.94 – 7.84 (m, 1H), 7.52 (d, J = 8.1 Hz, 1H), 7.43 (dd, J = 10.8, 6.4 Hz, 1H), 6.97 (dd, J = 9.2, 5.6 Hz, 1H), 4.80 – 4.61 (m, 2H), 3.57 – 3.45 (m, 1H), 3.45 – 3.33 (m, 1H), 3.22 – 2.94 (m, 2H), 2.12 – 2.02 (m, 1H), 1.92 – 1.81 (m, 1H), 1.79 – 1.59 (m, 2H), 1.48 – 1.33 ppm (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 199.29, 165.23, 161.82-159.29 (d, J = 255.5 Hz), 158.71, 140.99, 136.50-135.50 (m), 131.96-131.78 (m), 127.62, 126.21, 124.48-124.22 (d, J = 26.3 Hz), 123.95, 121.71, 128.10-121.23 (m), 114.50, 112.41-112.23 (m), 72.19, 47.92, 46.46 (2C), 27.88 (2C), 21.94 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{23}O_5N_2F_4$: 483.1543, found: 483.1533.

(1-(2-Isopropoxy-5-nitrobenzoyl)piperidin-4-yl)(4-(trifluoromethyl)phenyl)methanone (23d): **23d** was prepared from **10a** (0.1 g, 0.44 mmol) and **21b** (0.14 g, 0.53 mmol). Off-white solid (0.14 g, 69%). HPLC purity: 98.61%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.28 – 8.14 (m, 2H), 8.11 – 8.00 (m, 2H), 7.81 – 7.70 (m, 2H), 6.99 (t, J = 8.6 Hz, 1H), 4.82 – 4.67 (m, 2H), 3.62 – 3.51 (m, 2H), 3.27 – 3.01 (m, 2H), 2.12 – 1.69 (m, 4H), 1.52 – 1.36 ppm (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) 200.08, 164.81, 158.29, 140.44, 137.83, 134.24-134.03 (m), 128.11 (2C), 127.03, 126.78-122.09 (m), 125.95-125.82 (m, 2C), 125.46, 123.75, 111.92, 71.78, 45.95, 42.92 (2C), 27.76 (2C), 21.37 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{24}O_5N_2F_3$: 465.1637, found: 465.1625.

(1-(2-(Cyclopropylmethoxy)-5-nitrobenzoyl)piperidin-4-yl)(2,4-difluorophenyl)methanone (23e): **23e** was prepared from **10c** (0.1 g, 0.42 mmol) and **21d** (0.13 g, 0.51 mmol). Light yellow solid (0.15 g, 80%). HPLC purity: 98.09%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.36 – 8.14 (m, 2H), 7.94 – 7.78 (m, 1H), 7.04 – 6.84 (m, 3H), 4.87 – 4.59 (m, 1H), 4.05 – 3.85 (m, 2H), 3.53 (t, J = 13.7 Hz, 1H), 3.44 – 3.30 (m, 1H), 3.27 – 2.91 (m, 2H), 2.14 – 1.91 (m, 2H), 1.79 – 1.19 (m, 3H), 0.78 – 0.61 (m, 2H), 0.46 – 0.26 ppm (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.42, 167.13-164.57 (d, J = 258.6 Hz), 165.02, 163.19-160.75 (d, J = 246 Hz), 159.64, 141.27, 132.97, 127.10, 126.30, 124.16, 121.48-121.36 (d, J = 12.1 Hz), 112.66-112.45 (d, J = 21.2 Hz), 111.49 , 104.79 (m) , 74.34, 47.82, 46.78 (2C), 28.00 (2C), 9.86, 3.93 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{23}O_5N_2F_2$: 445.1575, found: 445.1568.

(1-(2-(Cyclopropylmethoxy)-5-nitrobenzoyl)piperidin-4-yl)(4-fluorophenyl)methanone (23f): **23f** was prepared from **10c** (0.1 g, 0.42 mmol) and **21a** (0.10 g, 0.51 mmol). Light yellow solid (0.13 g, 77%). HPLC purity: 99.71%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.28 – 8.14 (m, 2H), 8.03 – 7.94 (m, 2H), 7.20 – 7.13 (m, 2H), 6.96 (d, J = 8.0 Hz, 1H), 4.86 – 4.65 (m, 1H), 4.07 – 3.92 (m, 2H), 3.63 – 3.45 (m, 2H), 3.29 – 2.98 (m, 2H), 2.10 – 1.99 (m, 2H), 1.87 – 1.76 (m, 2H), 1.40 – 1.20 (m, 1H), 0.81 – 0.60 (m, 2H), 0.46 – 0.31 ppm (m, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) 199.33, 166.16-164.47 (d, J = 255.2 Hz), 164.59, 159.21, 140.74, 131.51, 130.42-130.36 (d, J = 9.1 Hz, 2C), 126.54, 125.91, 123.74, 115.56-115.42 (d, J = 21.1 Hz, 2C), 111.02, 73.94, 46.23, 42.82 (2C), 28.10 (2C), 9.40, 3.52 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{24}O_5N_2F$: 427.1669, found: 427.1665.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(2-(isopropylamino)-5-nitrophenyl)methanone (23g): **23g** was prepared from **13a** (0.1 g, 0.45 mmol) and **21d** (0.14 g, 0.54 mmol). Yellow solid (0.14 g, 73%). HPLC purity: 97.30%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.14 (dd, J = 9.3, 2.6 Hz, 1H), 8.03 (d, J = 2.7 Hz, 1H), 7.89 (td, J = 8.6, 6.5 Hz, 1H), 7.00 (ddd, J = 9.2, 7.6, 2.4 Hz, 1H), 6.90 (ddd, J = 11.1, 8.6, 2.4 Hz, 1H), 6.66 (d, J = 9.3 Hz, 1H), 6.08 (d, J = 7.3 Hz, 1H), 4.25 (s, 1H), 3.79 – 3.69 (m, J = 6.5 Hz, 1H), 3.47 – 3.37 (m, 1H), 3.18 (t, J = 12.9 Hz, 2H), 2.07 – 1.95 (m, 2H), 1.83 – 1.62 (m, 3H), 1.29 ppm (d, J = 6.4 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) 197.87-197.83 (d, J = 6.04 Hz), 167.80, 166.28-164.49 (dd, J = 12.08, 258.2 Hz), 162.40-160.63 (dd, J = 12.08, 255.2 Hz), 150.93, 135.29, 132.65 (m), 127.08, 124.77, 120.84-120.77 (d J = 10.57 Hz), 115.96, 112.25-112.09 (d, J = 21.14 Hz), 109.94 , 104.48-104.12 (t, J = 27.18 Hz), 47.13-47.08 (d, J = 7.55 Hz), 43.80 (3C), 27.74 (2C), 21.95 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{22}H_{24}O_4N_3F_2$: 432.1735, found: 432.1722.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(5-nitro-2-(piperidin-1-yl)phenyl)methanone (23h): **23h** was prepared from **13b** (0.1 g, 0.40 mmol) and **21d** (0.12 g, 0.48 mmol). Yellow solid (0.12 g, 66%). HPLC purity: 98.99%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.20 – 8.05 (m, 2H), 7.95 – 7.83 (m, 1H), 7.05 – 6.85 (m, 3H), 4.85 – 4.56 (m, 1H), 3.56 – 3.46 (m, 1H), 3.44 – 3.28 (m, 3H), 3.26 – 2.91 (m, 4H), 2.14 – 2.05 (m, 1H), 1.96 – 1.51 ppm (m,

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9H); ^{13}C NMR (151 MHz, $CDCl_3$) 198.22, 167.72, 166.23-164.61 (d, J = 244.6 Hz), 162.52-160.60 (d, J = 255.2 Hz), 154.24, 140.03, 132.78-132.52 (m), 127.54, 125.53, 125.01, 124.77 (m), 120.89, 116.58, 112.27-112.07 (d, J = 7.55 Hz), 104.58-104.17 (m), 51.85 (2C), 47.41, 46.03 (2C), 27.36 (2C), 25.27 (2C), 23.43. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{26}O_4N_3F_2$: 458.1891, found: 458.1881.

4-(Cyclopropylmethoxy)-3-(4-(2,4-difluorobenzoyl)piperidine-1-carbonyl)-N-methylbenzenesulfonamide (23i): **23i** was prepared from **10g** (0.1 g, 0.35 mmol) and **21.d** (0.11 g, 0.42 mmol). White solid (0.13 g, 80%). HPLC purity: 95.16%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.94 – 7.79 (m, 2H), 7.78 – 7.69 (m, 1H), 7.07 – 6.75 (m, 3H), 5.05 – 4.88 (m, 1H), 4.85 – 4.52 (m, 1H), 4.05 – 3.77 (m, 2H), 3.55 (d, J = 13.2 Hz, 1H), 3.37 (tt, J = 10.7, 3.8 Hz, 1H), 3.27 – 2.87 (m, 2H), 2.61 (d, J = 5.3 Hz, 3H), 2.06 (d, J = 13.5 Hz, 2H), 1.75 (h, J = 9.8, 8.6 Hz, 1H), 1.39 – 1.19 (m, 2H), 0.76 – 0.58 (m, 2H), 0.48 – 0.23 ppm (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.58, 167.12-164.44 (d, J = 257.5 Hz), 166.14, 163.20-160.70 (d, J = 252.5 Hz), 157.94, 133.09 (m), 130.96, 130.08, 127.37, 127.05, 121.44, 112.66-112.45 (d, J = 21.2 Hz), 111.67, 105.10-104.57 (t, J = 26.8 Hz), 73.86, 47.94, 46.90 (2C), 29.32 (2C), 28.21, 9.95, 3.14 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{27}O_5N_2F_2S$: 493.1609, found: 493.1595.

3-(4-(2,4-Difluorobenzoyl)piperidine-1-carbonyl)-N-methyl-4-(2,2,3,3,3-pentafluoropropoxy)benzenesulfonamide (23j): **23j** was prepared from **18c** (0.1 g, 0.27 mmol) and **21d** (0.086 g, 0.33 mmol). Off-white solid (0.13 g, 71%). HPLC purity: 95.93%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.95 – 7.74 (m, 3H), 7.11 – 7.03 (m, 1H), 7.03 – 6.96 (m, 1H), 6.95 – 6.83 (m, 1H), 5.17 – 5.00 (m, 1H), 4.74 – 4.47 (m, 3H), 3.48 (dt, J = 13.9, 4.1 Hz, 1H), 3.37 (dtd, J = 14.4, 10.5, 9.9, 4.7 Hz, 1H), 3.20 – 2.98 (m, 2H), 2.63 (dd, J = 5.4, 2.6 Hz, 3H), 2.07 (d, J = 13.7 Hz, 1H), 1.94 – 1.56 ppm (m, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) 197.89-197.76 (d, J = 19.6 Hz), 166.25-164.54 (d, J = 258.2 Hz), 164.46, 162.35-160.66 (d, J = 255.2 Hz), 155.35, 133.48, 132.67 (m), 129.68, 127.28, 127.00, 120.87, 116.98 (m), 112.20, 112.09 (m), 111.65, 104.43-104.07 (t, J = 27.2 Hz), 65.01-64.64 (m), 47.00, 45.60 (2C), 28.79 (2C), 27.35. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{22}O_5N_2F_7S$: 571.1138, found: 571.1121.

3-(4-(2,4-Difluorobenzoyl)piperidine-1-carbonyl)-4-(isopropylamino)-N-methylbenzenesulfonamide (23k): **23k** was prepared from **16c** (0.1 g, 0.37 mmol) and **21d** (0.11 g, 0.44 mmol). White solid (0.11 g, 63%). HPLC purity: 98.07%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.86 (td, J = 8.6, 6.5 Hz, 1H), 7.67 (dd, J = 8.8, 2.3 Hz, 1H), 7.55 (d, J = 2.3 Hz, 1H), 6.98 (ddd, J = 9.5, 7.5, 2.4 Hz, 1H), 6.88 (ddd, J = 11.1, 8.5, 2.4 Hz, 1H), 6.70 (d, J = 8.9 Hz, 1H), 5.49 (d, J = 7.3 Hz, 1H), 4.83 (q, J = 5.4 Hz, 1H), 3.68 (h, J = 6.4 Hz, 1H), 3.43 – 3.33 (m, 1H), 3.13 (t, J = 12.7 Hz, 2H), 2.57 (d, J = 5.4 Hz, 3H), 2.07 – 1.89 (m, 3H), 1.77 – 1.60 (m, 2H), 1.24 ppm (d, J = 6.4 Hz, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) 198.02-197.99 (d, J = 4.53 Hz), 168.32, 166.21-164.51 (d, J = 256.7 Hz), 162.37-160.67 (d, J = 256.7 Hz), 148.85, 132.64-132.55 (m), 129.96, 127.64, 123.00, 120.89, 117.13, 112.19-112.03 (d, J = 21.14 Hz), 110.58, 104.47-104.11 (t, J = 27.18 Hz), 47.11-47.06 (d, J = 7.55 Hz), 43.44 (2C), 38.10, 28.75 (2C), 27.71, 22.01 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{28}O_4N_3F_2S$: 480.1769, found: 480.1757.

3-(4-(2,4-Difluorobenzoyl)piperidine-1-carbonyl)-N-methyl-4-(piperidin-1-yl)benzenesulfonamide (23l): **23l** was prepared from **16d** (0.1 g, 0.34 mmol) and **21d** (0.10 g, 0.40 mmol). Off-white solid (0.12 g, 69%). HPLC purity: 97.67%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 – 7.82 (m, 1H), 7.81 – 7.63 (m, 2H), 7.09 – 6.96 (m, 2H), 6.89 (q, J = 9.6 Hz, 1H), 4.93 – 4.54 (m, 2H), 3.55 – 3.15 (m, 4H), 3.13 – 2.85 (m, 4H), 2.63 (d, J = 5.4 Hz, 3H), 2.18 – 1.65 (m, 8H), 1.29 ppm (d, J = 20.1 Hz, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.84, 168.81, 167.02-164.5 (d, J = 254.5 Hz), 153.51, 133.16-132.95 (m), 130.71, 130.07, 129.62, 129.43, 127.98, 121.36, 117.77, 112.69-112.52 (d, J = 17.2 Hz), 104.88-104.62 (m), 52.76 (2C), 47.82, 46.49 (2C), 29.68 (2C), 27.82, 26.35 (2C), 24.00. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{25}H_{30}O_4N_3F_2S$: 506.1925, found: 506.1911.

3-(4-(2,4-Difluorobenzoyl)piperidine-1-carbonyl)-N-methyl-4-morpholinobenzenesulfonamide (23m): **23m** was prepared from **16e** (0.1 g, 0.33 mmol) and **21d** (0.10 g, 0.40 mmol). White solid (0.13 g, 77%). HPLC purity: 98.45%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 – 7.85 (m, 1H), 7.82 (dd, J = 8.6, 2.3 Hz, 1H), 7.73 (dd, J = 21.7, 2.3 Hz, 1H), 7.11 – 6.97 (m, 2H), 6.95 – 6.85 (m, 1H), 4.88 (p, J = 5.4 Hz, 1H), 4.80 – 4.57 (m, 1H), 3.94 – 3.73 (m, 4H), 3.50 – 3.18 (m, 4H), 3.13 – 2.91 (m, 4H), 2.63 (d, J = 5.3 Hz, 3H), 2.16 – 1.90 (m, 2H), 1.80 – 1.48 ppm (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.60-198.20 (d, J = 40.4 Hz), 168.38, 167.26-164.6 (d, J = 268.6 Hz), 163.45-160.78 (d, J = 268.7 Hz), 152.42, 133.24-133.02 (m), 132.41, 130.95, 129.51, 127.70, 121.38-121.25 (d, J = 13.1 Hz), 117.98, 112.78-112.57 (d, J = 21.2 Hz), 104.88-104.60 (m), 66.70 (2C), 51.88 (2C), 47.70, 46.55 (2C), 29.35 (2C), 28.04. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{28}O_5N_3F_2S$: 508.1718, found: 508.1704.

3-(4-(2,4-Difluorobenzoyl)piperidine-1-carbonyl)-4-(isopropylthio)-N-methylbenzenesulfonamide (23n): **23n** was prepared from **18d** (0.1 g, 0.35 mmol) and **21d** (0.11 g, 0.42 mmol). Off-white solid (0.10 g, 58%). HPLC purity: 96.27%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.88 (q, J = 8.0 Hz, 1H), 7.81 – 7.74 (m, 1H), 7.66 (s, 1H), 7.47 (d, J = 8.4 Hz, 1H), 7.06 – 6.95 (m, 1H), 6.95 – 6.80 (m, 1H), 5.11 – 4.95 (m, 1H), 4.67 (s, 1H), 3.62 (hept, J = 6.8 Hz, 1H), 3.52 – 3.29 (m, 2H), 3.27 – 3.03 (m, 2H), 2.64 (d, J = 5.2 Hz, 3H), 2.16 – 1.57 (m, 4H), 1.37 ppm (d, J = 6.6 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.45, 167.16-164.61 (d, J = 257.5 Hz), 167.03, 163.18- 160.75 (d, J = 245.3 Hz), 140.10, 137.76, 136.10, 133.11, 128.85, 127.64, 125.26, 121.43-121.26 (d, J = 13.1 Hz), 112.72-112.51 (d, J = 21.2 Hz), 105.03-104.49 (t, J = 27.3 Hz), 47.63, 46.75 (2C), 37.24, 29.34 (2C), 27.62, 22.69 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}O_4N_2F_2S_2$: 497.1380, found: 497.1367.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(2-isopropoxy-5-(methylsulfonyl)phenyl)methanone (23o): **23o** was prepared from **10d** (0.1 g, 0.38 mmol) and **21d** (0.12 g, 0.46 mmol). White solid (0.14 g, 83%). HPLC purity: 98.06%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.91 – 7.82 (m, 2H), 7.82 – 7.71 (m, 1H), 7.02 (t, J = 8.7 Hz, 1H), 6.98 (t, J = 8.3 Hz, 1H), 6.92 – 6.83 (m, 1H), 4.75 – 4.57 (m, 2H), 3.52 – 3.43 (m, 1H), 3.42 – 3.31 (m, 1H), 3.18 – 3.07 (m, 1H), 3.04 (d, J = 3.5 Hz, 3H), 3.00 – 2.91 (m, 1H), 2.08 – 1.99 (m, 1H), 1.91 – 1.52 (m, 3H), 1.44 – 1.31 ppm (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) 198.21-197.97 (d, J = 36.2 Hz), 166.25-164.54 (d, J = 258.2 Hz), 165.57, 162.38- 160.77 (d, J = 243.1 Hz), 157.35, 132.61-132.41 (m), 131.79-131.60 (m),

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129.77, 129.61, 127.47, 120.88-120.79 (d, J = 13.60 Hz), 112.50, 112.23-112.01 (m), 104.53-104.18 (t, J = 26.43 Hz), 71.26, 47.11, 46.22, 44.17 (2C), 27.62 (2C), 21.11(2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{26}O_5NF_2S$: 466.1500, found: 466.1494.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(2-isobutoxy-5-(methylsulfonyl)phenyl)methanone (23p): **23p** was prepared from **10e** (0.1 g, 0.37 mmol) and **21d** (0.11 g, 0.44 mmol). White solid (0.13 g, 73%). HPLC purity: 98.82%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 – 7.74 (m, 3H), 7.09 – 6.96 (m, 2H), 6.95 – 6.82 (m, 1H), 4.78 – 4.62 (m, 1H), 4.02 – 3.73 (m, 2H), 3.57 – 3.28 (m, 2H), 3.22 – 3.08 (m, 1H), 3.04 (d, J = 2.8 Hz, 3H), 2.25 – 2.11 (m, 1H), 2.10 – 1.66 (m, 5H), 1.04 ppm (d, J = 6.6 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.52-198.27 (d, J = 25.3 Hz), 167.15-164.47 (d, J = 270.6 Hz), 165.71, 163.21-160.67 (d, J = 256.5 Hz), 159.15, 133.23-132.93 (m), 132.79, 130.35, 130.20, 127.27, 121.48-121.33(d, J = 15.1 Hz), 112.77-112.47 (m), 112.26, 105.09-104.56 (m), 75.52, 47.60 (m), 46.66, 44.76 (2C), 28.18 (2C), 27.71, 19.09 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{28}O_5NF_2S$: 480.1656, found: 480.1644.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(5-(methylsulfonyl)-2-(2,2,3,3,3-pentafluoropropoxy)phenyl)methanone (23q): **23q** was prepared from **18b** (0.2 g, 0.57 mmol) and **21d** (0.18 g, 0.69 mmol). White solid (0.26 g, 84%). HPLC purity: 98.78%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (dt, J = 8.7, 2.6 Hz, 1H), 7.93 – 7.83 (m, 2H), 7.09 (t, J = 9.0 Hz, 1H), 7.04 – 6.96 (m, 1H), 6.94 – 6.84 (m, 1H), 4.74 – 4.45 (m, 3H), 3.52 – 3.41 (m, 1H), 3.42 – 3.29 (m, 1H), 3.21 – 2.96 (m, 2H), 3.06 (d, J = 3.2 Hz, 3H), 2.12 – 2.00 (m, 1H), 1.90 – 1.80 (m, 1H), 1.79 – 1.62 ppm (m, 2H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.25, 167.10-164.65 (d, J = 247.5 Hz), 164.42, 163.27-160.75 (d, J = 254.5 Hz), 156.37, 135.49, 133.12 (m), 130.40, 128.23, 127.92, 121.42-121.29 (d, J = 13.1 Hz), 121.42 (m), 112.77(m), 112.99, 112.43, 104.71-104.46 (t, J = 12.6 Hz), 65.38 (m), 47.50 (m), 46.63, 44.65 (2C), 26.65 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{21}O_5NF_7S$: 556.1029, found: 556.1013.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(5-(methylsulfonyl)-2-((1,1,1-trifluoropropan-2-yl)oxy)phenyl)methanone (23r): **23r** was prepared from **18a** (0.10 g, 0.32 mmol) and **21d** (0.10 g, 0.38 mmol). White solid (0.13 g, 79%). HPLC purity: 98.73%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.99 – 7.94 (m, 1H), 7.94 – 7.83 (m, 2H), 7.18 – 7.07 (m, 1H), 7.00 (td, J = 9.2, 8.5, 2.5 Hz, 1H), 6.93 – 6.84 (m, 1H), 4.90 – 4.78 (m, 1H), 4.75 – 4.59 (m, 1H), 3.52 – 3.44 (m, 1H), 3.42 – 3.32 (m, 1H), 3.22 – 3.00 (m, 2H), 3.07 (d, J = 2.8 Hz, 3H), 2.07 (dd, J = 13.7, 3.8 Hz, 1H), 1.91 – 1.82 (m, 1H), 1.80 – 1.49 ppm (m, 5H); ^{13}C NMR (151 MHz, $CDCl_3$) 197.82, 166.10-164.19 (d, J = 286.5 Hz), 164.37, 162.23-160.54 (d, J = 255.2 Hz), 156.02-155.70 (m), 134.55, 132.71-132.61 (m), 129.73, 129.57, 127.35, 124.47 (m), 120.88-120.80 (d, J = 12.08 Hz), 114.21, 112.21-112.06 (d, J = 22.65 Hz), 104.42-104.06 (t, J = 27.18 Hz), 73.37-72.47 (m), 47.05, 46.31, 44.16 (2C), 27.31 (2C), 13.33-13.21 (m). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{23}O_5NF_5S$: 520.1217, found: 520.1205.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(2-(isopropylamino)-5-(methylsulfonyl)phenyl)methanone (23s): **23s** was prepared from **16a** (0.1 g, 0.39 mmol) and **21d** (0.12 g, 0.47 mmol). Off-white solid (0.11 g, 61%). HPLC purity: 98.63%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.93 – 7.82 (m, 1H), 7.74 (dd, J = 8.9, 2.1 Hz, 1H), 7.64 – 7.58 (m, 1H), 7.04 – 6.94 (m, 1H), 6.93 – 6.83 (m, 1H), 6.74 (d, J = 8.9 Hz, 1H), 5.65 (d, J = 7.3 Hz, 1H), 3.71 (h, J = 6.6 Hz, 1H), 3.46 – 3.34 (m, 1H), 3.15 (t, J = 12.6 Hz, 2H), 2.99 (d, J = 0.9 Hz, 3H), 2.00 (d, J = 13.9 Hz, 2H), 1.73 (q, J = 12.7, 12.1 Hz, 3H), 1.27 ppm (d, J = 6.3 Hz, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.34-198.30 (d, J = 4.04 Hz), 168.59, 167.16-164.47 (d, J = 258.5 Hz), 163.29-160.62 (d, J = 256.5 Hz), 150.11, 133.11-133.04 (m), 130.47, 128.17, 125.42, 121.37-121.26 (d, J = 11.1 Hz), 117.64, 112.70-112.49 (d, J = 21.2 Hz), 111.14, 105.01-104.48 (t, J = 26.7 Hz), 47.59-47.52 (d, J = 7.07 Hz), 45.00, 43.97 (2C), 38.56 , 28.19 (2C), 22.44 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}O_4N_2F_2S$: 465.1660, found: 465.1651.

(4-(2,4-Difluorobenzoyl)piperidin-1-yl)(5-(methylsulfonyl)-2-(piperidin-1-yl)phenyl)methanone (23t): **23t** was prepared from **16b** (0.1 g, 0.35 mmol) and **21d** (0.11 g, 0.42 mmol). Off-white solid (0.12 g, 72%). HPLC purity: 98.28%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.92 – 7.66 (m, 3H), 7.09 – 6.93 (m, 2H), 6.87 (t, J = 10.0 Hz, 1H), 4.62 (d, J = 12.6 Hz, 1H), 3.44 – 2.87 (m, 8H), 3.01 (s, 3H) 2.10 – 1.99 (m, 1H), 1.96 – 1.55 ppm (m, 9H); ^{13}C NMR (101 MHz, $CDCl_3$) 198.73, 168.48, 167.16-164.48 (d, J = 257.6 Hz), 163.15- 160.65 (d, J = 252.5 Hz), 154.10, 133.09, 132.11 (m), 129.54, 128.43, 121.43-121.27 (d, J = 13.1 Hz), 117.94 (2C), 112.73-112.52 (d, J = 21.2 Hz), 104.80 (m), 52.54 (2C), 47.81, 46.49 (2C), 38.61, 28.33 (2C), 26.21 (2C), 23.95. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{25}H_{29}O_4N_2F_2S$: 491.1816, found: 491.1806.

(4-(6-Fluorobenzo[*d*]isoxazol-3-yl)piperidin-1-yl)(5-(methylsulfonyl)-2-(2,2,3,3,3-pentafluoropropoxy)phenyl)methanone (24a): **24a** was prepared from **18b** (0.1 g; 0.29 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.09 g, 0.34 mmol, commercial available). White solid (0.11 g, 71%). HPLC purity: 96.45%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.03 – 7.86 (m, 2H), 7.70 – 7.61 (m, 1H), 7.27 (dq, J = 8.6, 2.1 Hz, 1H), 7.18 – 7.04 (m, 2H), 4.81 – 4.50 (m, 3H), 3.58 (td, J = 13.3, 3.7 Hz, 1H), 3.38 (td, J = 10.7, 5.0 Hz, 1H), 3.30 – 3.10 (m, 2H), 3.08 (dd, J = 4.5, 1.5 Hz, 3H), 2.29 – 1.97 ppm (m, 4H). ^{13}C NMR (151 MHz, $CDCl_3$) 164.55-162.89 (d, J = 250.6 Hz), 164.20, 163.46-163.37 (d, J = 13.6 Hz), 159.42 (m), 155.86, 134.82, 130.07, 127.82, 127.43, 121.73-121.67 (t, J = 4.53 Hz), 118.60 (m), 116.48, 112.60, 112.17 (m), 111.92, 97.15-96.98 (d, J = 25.67 Hz), 65.01 (m), 45.80 (2C), 44.07, 33.56, 29.81 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{21}O_5N_2F_6S$: 551.1075, found: 551.1060.

(4-(6-Fluorobenzo[*d*]isoxazol-3-yl)piperidin-1-yl)(2-isobutoxy-5-(methylsulfonyl)phenyl)methanone (24b): **24b** was prepared from **10f** (0.1 g, 0.37 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.11 g, 0.44 mmol). White solid (0.13 g, 76%). HPLC purity: 96.76%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.97 – 7.91 (m, 1H), 7.86 (dd, J = 32.0, 2.4 Hz, 1H), 7.70 – 7.59 (m, 1H), 7.28 – 7.24 (m, 1H), 7.13 – 7.02 (m, 2H), 4.80 (dd, J = 33.0, 13.5 Hz, 1H), 3.99 – 3.74 (m, 2H), 3.67 – 3.54 (m, 1H), 3.43 – 3.08 (m, 3H), 3.05 (d, J = 3.6 Hz, 3H), 2.32 – 1.94 (m, 5H), 1.11 – 0.96 ppm (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 165.82, 165.42-162.92 (d, J = 252.5 Hz), 163.88, 159.99, 159.13, 132.85, 130.40, 127.62, 127.32, 122.05 (m), 117.14, 112.60 (m), 112.15, 97.68-97.41 (d, J = 27.3 Hz), 75.54, 46.82 (2C), 44.75, 34.35, 30.08 (2C), 28.18, 19.09 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{28}O_5N_2FS$: 475.1703, found: 475.1690.

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(4-(6-Fluorobenzo[*d*]isoxazol-3-yl)piperidin-1-yl)(5-(methylsulfonyl)-2-((1,1,1-trifluoropropan-2-yl)oxy)phenyl)methanone (24c): **24c** was prepared from **18a** (0.1 g, 0.32 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.10 g, 0.38 mmol). White solid (0.13 g, 81%). HPLC purity: 98.52%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.98 (d, *J* = 8.4 Hz, 1H), 7.96 – 7.84 (m, 1H), 7.65 (td, *J* = 8.2, 4.7 Hz, 1H), 7.28 – 7.24 (m, 1H), 7.22 – 7.04 (m, 2H), 4.95 – 4.69 (m, 2H), 3.63 – 3.53 (m, 1H), 3.44 – 3.12 (m, 3H), 3.08 (s, 3H), 2.25 (dd, *J* = 13.6, 4.0 Hz, 1H), 2.14 – 1.89 (m, 3H), 1.66 – 1.52 ppm (m, 3H); ^{13}C NMR (101 MHz, $CDCl_3$) 165.53-163.03 (d, *J* = 252.5 Hz), 165.02, 164.08-163.94 (d, *J* = 14.1 Hz), 160.02, 156.59, 135.09, 130.43, 128.52, 127.95, 122.34 (m), 122.32-122.21 (d, *J* = 11.1 Hz), 117.08, 113.64, 112.82 (m), 97.82-97.55 (d, *J* = 27.3 Hz), 73.64 (m), 47.12 (2C), 44.76, 34.25, 30.17 (2C), 13.89. HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{23}O_5N_2F_4S$: 515.1264, found: 515.1251.

(2-(Cyclopentyloxy)-5-nitrophenyl)(4-(6-fluorobenzo[*d*]isoxazol-3-yl)piperidin-1-yl)methanone (24d): **24d** was prepared from **10b** (0.1 g, 0.40 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.12 g, 0.48 mmol). Light yellow solid (0.12 g, 65%). HPLC purity: 97.55%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.29 – 8.12 (m, 2H), 7.69 – 7.58 (m, 1H), 7.28 (d, *J* = 2.1 Hz, 1H), 7.09 (tt, *J* = 8.8, 2.1 Hz, 1H), 7.04 – 6.94 (m, 1H), 4.97 – 4.86 (m, 1H), 4.84 – 4.70 (m, 1H), 3.68 – 3.51 (m, 1H), 3.43 – 3.04 (m, 3H), 2.33 – 2.15 (m, 1H), 2.12 – 1.50 ppm (m, 11H); ^{13}C NMR (101 MHz, $CDCl_3$) 165.43-162.95 (d, *J* = 250.5 Hz), 165.26, 163.90, 160.02, 159.05, 141.03, 127.51, 126.18, 124.44, 124.01, 121.93 (m), 117.13, 112.80-112.49 (d, *J* = 31.3 Hz), 97.72-97.45 (d, *J* = 27.3 Hz), 81.64, 46.84 (2C), 33.97, 33.01 (2C), 30.29 (2C), 23.96 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{25}O_5N_3F$: 454.1778, found 454.1770.

(4-(6-Fluorobenzo[*d*]isoxazol-3-yl)piperidin-1-yl)(5-nitro-2-(piperidin-1-yl)phenyl)methanone (24e): **24e** was prepared from **13b** (0.1 g, 0.40 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.12 g, 0.48 mmol). Yellow solid (0.12 g, 67%). HPLC purity: 97.32%; ^{1}H NMR (600 MHz, $CDCl_3$): δ 8.24 – 8.08 (m, 2H), 7.70 – 7.60 (m, 1H), 7.31 – 7.24 (m, 1H), 7.10 (tdd, *J* = 8.8, 4.8, 2.1 Hz, 1H), 6.97 (dd, *J* = 11.2, 9.1 Hz, 1H), 4.95 – 4.59 (m, 1H), 3.67 – 3.53 (m, 1H), 3.43 – 3.25 (m, 4H), 3.23 – 3.02 (m, 3H), 2.35 – 2.21 (m, 1H), 2.19 – 1.82 (m, 3H), 1.80 – 1.59 ppm (m, 6H); ^{13}C NMR (151 MHz, $CDCl_3$) 167.81, 164.60-162.94 (d, *J* = 250.7 Hz), 163.54-163.34 (d, *J* = 30.2 Hz), 159.63, 154.25, 140.06, 127.44, 125.62, 125.08, 121.72-121.45 (d, *J* = 40.7 Hz), 116.64, 116.74, 112.33-112.16 (d, *J* = 25.6 Hz), 97.22-97.04 (d, *J* = 27.2 Hz), 51.86 (2C), 46.30 (2C), 33.98, 29.68 (2C), 25.71 (2C), 23.42. HRMS (ESI): *m/z* [M+H]+ calcd for $C_{24}H_{26}O_4N_4F$: 453.1938, found: 453.1927.

3-(4-(6-Fluorobenzo[*d*]isoxazol-3-yl)piperidine-1-carbonyl)-4-isopropoxy-N-methylbenzenesulfonamide (24f): **24f** was prepared from **10h** (0.1 g, 0.37 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.11 g, 0.44 mmol). Off-white solid (0.10 g, 60%). HPLC purity: 97.72%; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.85 – 7.68 (m, 2H), 7.68 – 7.59 (m, 1H), 7.25 – 7.18 (m, 1H), 7.06 (td, *J* = 8.8, 2.1 Hz, 1H), 6.98 (dd, *J* = 8.8, 2.9 Hz, 1H), 4.82 – 4.60 (m, 2H), 3.58 (d, *J* = 13.8 Hz, 1H), 3.42 – 3.01 (m, 3H), 2.58 (s, 3H), 2.27 – 1.82 (m, 5H), 1.42 – 1.31 ppm (m, 6H); ^{13}C NMR (101 MHz, $CDCl_3$) 166.07, 165.02-162.52 (d, *J* = 252.5 Hz), 163.40-163.27 (d, *J* = 13.1 Hz), 159.77, 156.41, 130.38, 129.83, 129.61,127.25, 126.96, 126.53, 121.96-121.68 (d, *J* = 28.3 Hz), 116.67-116.43 (d, *J* = 24.2 Hz), 112.19 (m), 71.11, 46.56 (2C), 33.77, 30.02, 28.78 (2C), 21.48 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{23}H_{27}O_5N_3FS$: 476.1655, found: 476.1645.

3-(4-(6-Fluorobenzo[*d*]isoxazol-3-yl)piperidine-1-carbonyl)-4-isobutoxy-N-methylbenzenesulfonamide (24g): **24g** was prepared from **10f** (0.1 g, 0.35 mmol) and 6-fluoro-3-(4-piperidinyl)-1,2-benzisoxazole hydrochloride (0.11 g, 0.42 mmol). White solid (0.13 g, 67%). HPLC purity: 96.83%; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.90 – 7.71 (m, 2H), 7.71 – 7.60 (m, 1H), 7.27 – 7.23 (m, 1H), 7.08 (tdd, *J* = 8.8, 3.9, 2.1 Hz, 1H), 7.01 (dd, *J* = 8.8, 5.2 Hz, 1H), 4.99 – 4.89 (m, 1H), 4.87 – 4.70 (m, 1H), 3.96 – 3.73 (m, 2H), 3.67 – 3.57 (m, 1H), 3.41 – 3.31 (m, 1H), 3.30 – 3.04 (m, 2H), 2.62 (d, *J* = 6.6 Hz, 3H), 2.31 – 1.88 (m, 5H), 1.07 – 1.00 ppm (m, 6H); ^{13}C NMR (126 MHz, $CDCl_3$) 166.25, 165.19-163.20 (d, *J* = 250.7 Hz), 163.87, 160.09, 158.27, 131.14, 130.35, 127.36, 126.92, 122.39-122.10 (d, *J* = 36.5 Hz), 117.18, 112.70 (m), 112.00-111.71 (d, *J* = 36.5 Hz), 97.68-97.46 (d, *J* = 27.7 Hz), 75.39, 46.90 (2C), 34.39, 30.36, 29.34 (2C), 28.25, 19.12 (2C). HRMS (ESI): *m/z* $[M+H]^+$ calcd for $C_{24}H_{29}O_5N_3FS$: 490.1812, found: 490.1798.

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

Rat glioma C6 cells that stably expressed GlyT1 were plated into 24-well culture plates (1×10^6 per well). After 18 h, the culture medium was discarded and HEPES buffer solution (160 μL) was added. Thereafter, tested compounds (20 μL) and ^{3}H-glycine (20 μL; final concentration ranges: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) were added to a total volume of 200 μL. In the total uptake wells, the tested compounds were replaced with Hank's balanced salt solution (HBSS; 20 μL). NFPS[24] (10 μM) was used to determine nonspecific uptake. After a 30 min incubation, HBSS was discarded and the plate was washed twice with phosphate-buffered saline (500 μL). Then, NaOH (100 μL, 2M) was added to lyse the cells. The lysate activity was examined, and the inhibiting ratio and IC_{50} values were calculated.

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

GlyT2 inhibiting activity was examined using cultured primary brain stem neurons. In brief, brain stems were removed from brains of rat pups. The tissues were minced with a sterile razor blade and digested with 0.05% trypsin (Sigma) and 0.01% Dnase I (Sigma) at 37 °C for 10 min. Then, the tissues were collected by centrifugation for 5 min. After centrifugation, complete culture media Dulbecco's modified Eagle's medium/F12 (3 mL; 1:1; Gibco BRL, Gaithersburg, MD, USA) was added, which contained 10% fetal bovine serum (Hyclone, Logan, UT, USA), penicillin (100 U/mL), and streptomycin. Thereafter, this tissue was mechanically dissociated into a single-cell suspension. The dissociated cells were plated into 24 plates pre-coated with polylysine (Sigma). At 48 h later, cytosine arabinoside (5 μM) was added to kill the glial cells. Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2. On the 10th day of culture, the inhibiting activity of GlyT2 was tested. Sarcosine (10^{-3} M) was added to all wells to block GlyT1 activity, and glycine (10^{-4} M) was used to determine nonspecific uptake action. The remaining procedures were identical to those in the GlyT1

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test.

Binding assay for dopamine and serotonin receptors

Dopamine receptors 1, 2, and 3 were obtained from HEK293 stable-transfecting cell lines. Binding assays were conducted as described before.[25, 26] ^{3}H-SCH23390 (10 nM) was used for the binding test of D_1 receptor, and ^{3}H-spiperone for the D_2 or D_3 receptor-binding assay. (+)-butaclamol (final concentration: 10 μM) was used to determine nonspecific binding activity. After incubating 1 h at 30 °C, Tris-HCl (pre-cooled, 3 ml) was added to stop the binding reaction. The receptor-bound isotope was collected with a Whatman GF/B glass fiber filter. Radioactivity on the fiber filter was examined, and the inhibiting ratio was calculated. In the binding test of 5-HT receptor, ^{3}H-8-OH-DPAT (10 nM) was used to test binding of 5-HT_{1A}, and nonspecific binding activity was determined with 5-HT (10 μM). ^{3}H-ketanserin was used for the 5-HT_{2A} test, and spiperone was used for nonspecific binding activity. The remaining procedures were identical to those in the dopamine receptor tests.

hERG channel assay

The K^+ current by the hERG was recorded using an Axopatch 200 A amplifier at 24–25 °C. HEK293 cells stably expressing hERG channels were held at a resting voltage of −80 mV. Voltage protocols were controlled by pClamp 9.0 software via the DigiData-1322A interface (Axon Instruments, USA). Electrodes (a tip resistance of 3–5 mΩ) were pulled from borosilicate grass pipettes (Sutter Instruments, USA) and filled with a pipette solution consisted of the following (in mM): 140 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 10 EGTA, pH 7.4 adjusted with KOH. The test compound was diluted (1 nM – 0.1 mM) in extracellular buffer (NaCl 150 mM, KCl 4 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1 mM, HEPES 10 mM, pH 7.4 with NaOH, 300–310 mOsm), and was added directly using a RSC-100 rapid solution changer with a 9-tube head (BioLogic Co, France). After the cells were stabilized and the currents steady, the amplitude and kinetics of I_{KhERG} were recorded. Offline analysis of the peak tail current was performed using pClamp software 9.0. The amplitude and kinetics of I_{KhERG} were recorded for each drug concentration. Concentration-response curves were fitted by nonlinear regression analysis and IC_{50} values were reported.

Pharmacokinetic study

Male ICR mice (body weight: 18-20 g) were purchased from the Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). Animals were fasted for 12h but free access to water before administration of the compound. All animals were fed in 3h after administration. Compound was formulated in 0.5% HMPC and administered orally by gavage at a dose of 10 mg/kg. Blood samples (0.1 mL) were drawn from retrobulbar venous plexus of mice at 0.5, 1，2，4，8 and 24h postdose (n = 3 mice/group) and stored in heparinized tubes. After centrifugation for 5 min, plasma (50 μL) was added MeOH/ACN (1:1, 200 μL) and centrifuged for 10 min. Supernatant fluid was stored at -20 °C for analysis. Brain tissues were collected at the same time and stored at -20 °C until analysis. Brain tissues were added 10 volumes of MeOH/ACN (1:1). After vortex and centrifugation, supernatant fluid was used to analysis. A LC-MS/MS method was used for the quantification of the tested compound. The PK parameters were calculated by non-compartmental model analysis on Phoenix WinNonlin 6.0 (Pharsight, Mountain View, CA).

Locomotion test

Male C57BL/6J mice were pre-treated with **23q** (20, 40 mg/kg, intragastric) 60 min before the injection of PCP (5 mg/kg, intraperitoneal). The mice were placed in a Plexiglas open field arena (40×40×45 cm, Jiliang Co. Ltd., Shanghai, China) connected with a video-based recording system. Automated activity was recorded, and the total travelling distance was calculated.

Social interaction test

In this test, C57BL/6J mice received daily injections of either saline or PCP (10 mg/kg, intraperitoneal) for 2 weeks prior to administration of vehicle control (saline) or compound **23q** (20, 40 mg/kg, intragastric) for an additional 2 weeks. After treatment, the mice were individually placed into an unfamiliar arena (40×40×45 cm, Jiliang Co., Ltd., Shanghai, China) simultaneously with a weight-matched male mouse. Their behavior was video-recorded for 10 min. The time spent in interaction was defined as sniffing at any part of the partner's body (mainly the anogenital area), grooming, following, crawling over/under, or boxing/wrestling. Interactions were manually scored.

Novel object recognition test

Animal treatment was identical to that described in the social interaction test. On the first day, mice were placed into an open field box (40×40×45 cm) for 5 min and then immediately returned to their home cages. This was repeated on the second day. On the third day, two identical objects were placed into the open-field box and animals were allowed to explore for 5 min. After this training, the box and objects were cleaned with 75% ethanol to avoid possible instinctive odorant cues. On the fourth day, mice were placed back into the same box, a novel object replaced one of the objects used during training, and mice were allowed to explore freely for 5 min. The familiar object and the novel one were different in shape and color, but similar in size. An exploratory preference index, or the ratio of time spent exploring novel object over total time spent exploring both objects, was used to score recognition memory.

Abbreviations

THF: tetrahydrofuran; DMF: N,N-dimethylformamide; DMA: N,N-dimethylacetamide; HATU: 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DIAD: diisopropyl azodicarboxylate; EWG: electron-withdrawing group; NFPS: N[3-(4'-fluorophenyl)-3-(4'-phenylphenoxy)propyl]sarcosine; PCP: phencyclidine; SAR: structure–activity relationship; HPLC: high performance liquid chromatography; EGTA: ethylene glycol tetraacetic acid; ACN: acetonitrile; HMPC: hydroxypropyl methyl cellulose; i.g.: intragastric; i.p.: intraperitoneal.

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Notes and references

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