ORIGINAL ARTICLE

Carbazole and tetrahydro-carboline derivatives as dopamine D₃ receptor antagonists with the multiple antipsychotic-like properties

Zhongtang Li[a,†], Fan Fang[a,†], Yiyan Li[a,†], Xuehui Lv[a], Ruqiu Zheng[a], Peili Jiao[a], Yuxi Wang[a], Guiwang Zhu[a], Zefang Jin[a], Xiangqing Xu[b], Yinli Qiu[b], Guisen Zhang[c], Zhongjun Li[a], Zhenming Liu[a,*], Liangren Zhang[a,*]

[a]State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China
[b]Jiangsu Nhwa Pharmaceutical Co., Ltd., Xuzhou 221116, China
[c]Jiangsu Key Laboratory of Marine Biological Resources and Environment, Jiangsu Key Laboratory of Marine Pharmaceutical Compound Screening, School of Pharmacy, Jiangsu Ocean University, Lianyungang 222005, China

Abstract Dopamine D₃ receptor (D₃R) is implicated in multiple psychotic symptoms. Increasing the D₃R selectivity over dopamine D₂ receptor (D₂R) would facilitate the antipsychotic treatments. Herein, novel carbazole and tetrahydro-carboline derivatives were reported as D₃R selective ligands. Through a structure-based virtual screen, ZLG-25 (D₃R K_i = 685 nmol/L; D₂R K_i > 10,000 nmol/L) was identified as a novel D₃R selective bitopic ligand with a carbazole scaffold. Scaffolds hopping led to the discovery of novel D₃R-selective analogs with tetrahydro-β-carboline or tetrahydro-γ-carboline core. Further functional studies showed that most derivatives acted as hD₃R-selective antagonists. Several lead compounds could dose-dependently inhibit the MK-801-induced hyperactivity. Additional investigation revealed that **23j** and **36b** could decrease the apomorphine-induced climbing without cataleptic reaction. Furthermore, **36b** demonstrated unusual antidepressant-like activity in the forced swimming tests and the tail suspension tests, and alleviated the MK-801-induced disruption of novel object recognition in mice. Additionally, preliminary studies confirmed the favorable PK/PD profiles, no weight gain and limited serum

*Corresponding authors.

E-mail addresses: zmliu@bjmu.edu.cn (Zhenming Liu), liangren@bjmu.edu.cn (Liangren Zhang).

†These authors made equal contributions to this work.

prolactin levels in mice. These results revealed that **36b** provided potential opportunities to new antipsychotic drugs with the multiple antipsychotic-like properties.

1. Introduction

G protein-coupled receptors (GPCRs) are the most prominent family of membrane proteins and are comprised of more than 800 members, of which the majority are implicated in the pathophysiology of neurodegenerative disorders[1–3]. Dopamine receptors belong to the class A GPCR family and mediate the physiological functions of dopamine, a neurotransmitter and hormone with a catecholamine structure. Based on the coupling to either $G\alpha_{s/olf}$ proteins, or $G\alpha_{i/o}$ proteins to stimulate or inhibit the production of the second messenger cAMP, respectively, dopamine receptors are categorized into two subfamilies: D_1-like (D_1R and D_5R) and D_2-like (D_2R, D_3R, and D_4R)[4]. D_3R belongs to the same D_2-like receptor family as D_2R, sharing a high level of homology with them. Since it was cloned and characterized, the D_3R has been a target of therapeutic interest due to its relatively localized expression within mesolimbic neurocircuitry, including the nucleus accumbens, islands of Calleja, and ventral striatum[5–7]. The polymorphism of ser9gly in the first exon of D_3R gene is associated with schizophrenia[8]. The level of D_3R in the striatum increases in patients with schizophrenia and decreases with in those treated with antipsychotic drugs[9].

A number of studies employed a strategy for schizophrenia drug development that avoided EPS elicited with many antipsychotics is targeting D_3R and avoiding the D_2R effects[10]. As shown in Supporting Information Table S1, the diverse bitopic ligands with highly D_3R-selectivity had been explored to develop novel D_3R compounds with clinical ambitions, exploiting unique interactions at the D_3R binding pocket[11–22]. Novel therapeutic applications of these known molecules with D_3R-selectivity have been disclosed, which encouraged the continuous discovery of novel series of D_3R selective ligands[23]. D_3R-selective ligands in clinic and clinical trials were shown in Fig. 1A. S33138 was a preferential dopamine D_3 versus D_2 receptor antagonists identified preserving, enhancing cognitive function and increasing front cortical cholinergic transmission[24,25]. ABT-925 is a selective D_3R antagonist with an approximately 100-fold higher in vitro affinity for dopamine D_3 versus D_2 receptors, which had been examined in a phase I trial for acute exacerbation of schizophrenia but ABT-925 failed in a phase II. The reason of the failure might be the insufficient occupancy to D_3 receptors at the doses used in this trail[26,27]. GSK598809, a D_3R antagonist with >100-fold selectivity for $D_3 > D_2$ receptors, belonged to a novel series of 1,2,4-triazol-3-yl-azabicyclo[3.1.0]hexanes with high affinity and selectivity for the D_3 receptor and excellent pharmacokinetic profiles[28,29]. The treatment of substance addiction by GSK598809 had been examined in obese individuals and severe chronic smokers (i.e., NCT1039454 and NCT01188967 on Clinicaltrial.gov). However, the potential cardiovascular liability

of GSK598809 at high doses increased the hurdle of clinical trials[30]. Buspirone was developed as a D_3-preferring $5HT_{1A}$ antagonist studied in alcoholics and found to reduce anxiety, in particular during withdrawal (i.e., NCT00360191 and NCT00875836 on clinicaltrial.gov). Interestingly, selective D_3R engagement of buspirone was only observed after oral dosing, while intramuscular administration was engaging also the D_2R, indicating buspirone metabolites generate by the first passage through the liver could be the actual active moiety[31]. RGH188 (cariprazine, CRP) was characterized as a D_3R preferential binding ligand with a 10-fold D_3R/D_2R selectivity. Notably, CRP exerted a cognitive-enhancing effect in the in vivo tests, which was thought to be related to its high affinity and preference for D_3 versus D_2 receptor[32]. F17464 was discovered as a unique >80-fold D_3 over D_2 receptor preferential antagonist, which has been demonstrated selective D_3 receptor occupancy in a human positron emission tomography (PET) imaging study[33]. In a phase II schizophrenia acute exacerbation study, clinical antipsychotic efficacy with no weight gain, no extrapyramidal disorder observed[34]. Furthermore, F17464 could rescue valproate induced impairment in a rat social interaction model of autism, probably by increasing dopamine release in the prefrontal cortex and lateral forebrain–dorsal striatum[35]. Although no highly selective D_3 ligands have been approved by US Food and Drug Administration (FDA), selective D_3 ligands have demonstrated many advantages in clinical trials for mood regulation, cognitive protection, and low side effects.

However, the development of selective D_3R ligands has been challenging due to the high sequence identity and homology. The D_2R and D_3R share 74% identity between their transmembrane domains (TMs) and 94% identity between their putative orthosteric binding sites (OBS), where the endogenous agonist dopamine binds[36]. To improve selectivity, most D_3R ligands that share several well-known scaffolds with a primary pharmacophore, bind to the OBS and a secondary pharmacophore binding to the second binding pocket (SBP) or an allosteric binding site (ABS)[37]. GPCR ligands with such a scaffold have been depicted as bitopic binders, which can benefit high subtype affinity and selectivity[38,39]. In this study, we report the discovery of a novel series of D_3R selective antagonists with desirable antipsychotic potency in vivo. From the initial hit compounds containing the carbazole scaffold, over 60 bitopic structural analogs were synthesized and evaluated for a comprehensive investigation of structure–activity relationships (SAR), which led to the identification of compound **36b** as a potent and blood brain barrier (BBB) penetrable D_3R selective antagonists suitable for in vivo characterization. Furthermore, compound **36b** exhibited potential antipsychotic activities in vivo with moderate safety profiles in psychotic side effects and hERG channel inhibition. In vivo,
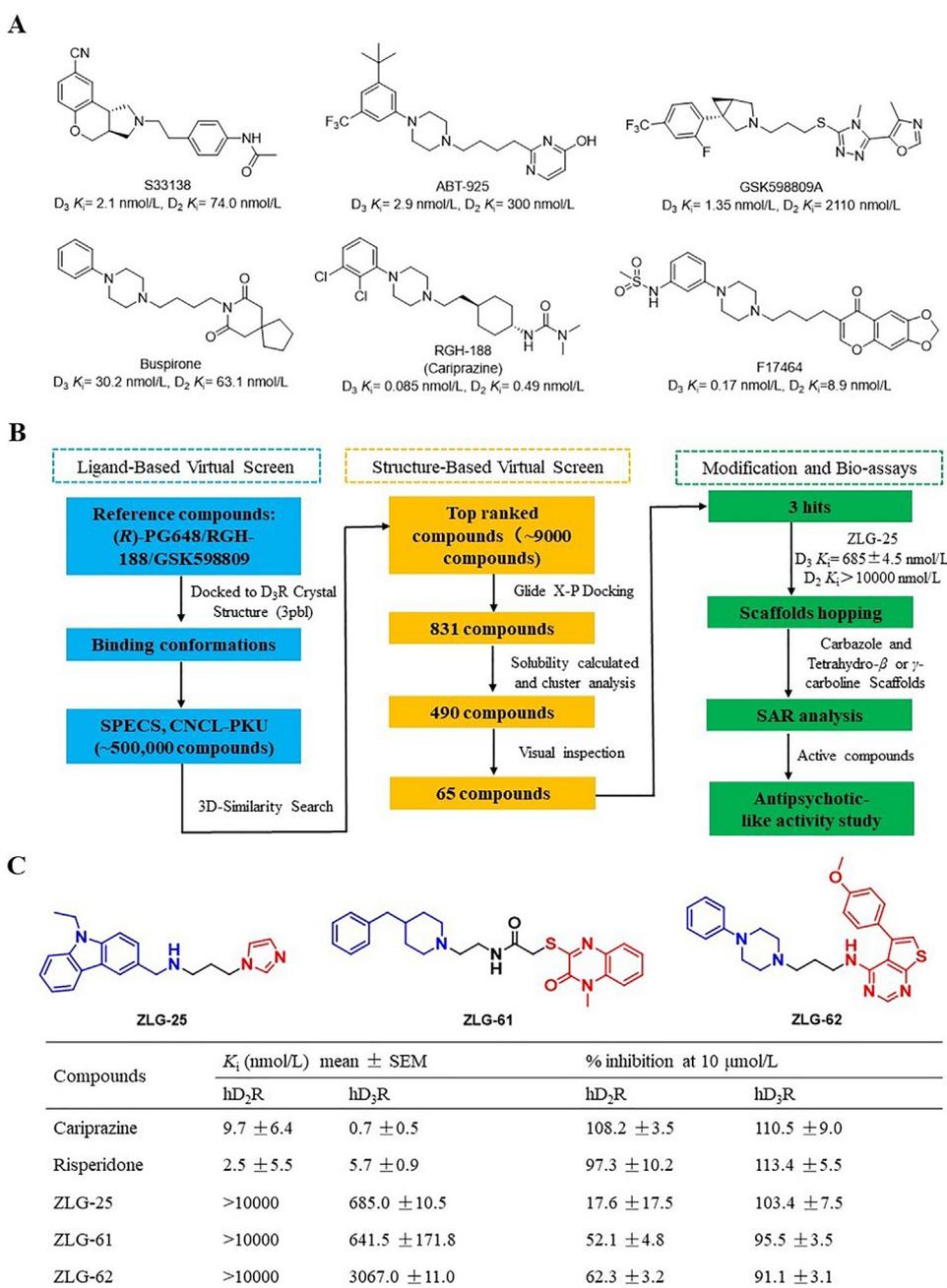
Figure 1 (A) D$_3$R-selective ligands in clinic and clinical trials. (B) General workflow of D$_3$R ligands identification. (C) Chemical structures of 3 identified hits and the binding affinities.

pharmacokinetics (PK) profiles and exquisite target selectivity of compound **36b** were also satisfactory. Taken together, compound **36b** was characterized as an excellent lead compound for further optimizations of the novel D$_3$R selective antagonists with distinct antipsychotic-like effects *in vivo*.

2. Results and discussion

2.1. Structure-based virtual screening

Our workflow of virtual screening is summarized in the protocol in Fig. 1B. A 3D shape-based similarity screening connected docking-based virtual screening approach was used in our

study[40−42]. Three compounds with high affinity and selectivity for the D$_3$R (*R*)-PG468, RGH-188, and GSK598809A were selected as the benchmark compounds for their representative secondary pharmacophore structures, which were to benefit high subtype selectivity. After preparing the co-crystal structure (PDB ID: 3pbl, Supporting Information Table S2) well, these three compounds were docked into the D$_3$R using the induced-docking module of Maestro obtaining three binding conformations for each compound (Supporting Information Fig. S1). Aiming to seek more potent ligands, 3D shape-based similarity virtual screening approaches were employed to search more than 600,000 compounds from the SPECS chemical library, the ChemDiv chemical library, and the Chinese National Compound Library of Peking University

(PKU-CNCL) databases by ROCS 3.1.2 (OpenEye Scientific Software, Inc.) program. As a result, the top 3000 compounds were retained based on the 'Shape Tanimoto' score. Next, the Glide molecular docking module was used to screen the selected compounds from the 3D-similarity search, which were sorted by docking score, and all compounds within the top one percent were obtained. Because the residue Asp110 was essential for the binding affinity, compounds forming the ionic interaction with Asp110 were isolated using the PoseFilter module in the Maestro platform. Based on compound docking results and physicochemical properties, 65 compounds were purchased for further biological activity tests (Supporting Information Fig. S2). All active compounds passed the pan assay interference compounds (PAINS) test by PAINS-remover[43].

For the primary screen, a single high concentration (10 μmol/L) of test compound was tested by radioligand binding assays on the CHO cells stably expressing hD$_2$R and hD$_3$R (Table S2), and those compounds that exhibited >75% inhibition were rescreened in full concentration−response format to estimate their affinity (K_i) values. Risperidone (RPD) and CRP were used as the positive drugs. From the 65 selected compounds, ZLG-25, ZLG-61, and ZLG-62 showed >90% inhibition for the D$_3$R at 10 μmol/L (Table S3). Further tests accessed the affinity values of ZLG-25 (K_i = 685 ± 4.5 nmol/L), ZLG-61 (K_i = 641.5 ± 171.8 nmol/L), and ZLG-62 (K_i = 3067.0 ± 11.0 nmol/L) for D$_3$R (Fig. 1C). Then, the D$_2$R K_i of these compounds were also tested and found to be 10−20 fold higher than that to D$_3$R. Moreover, representative molecular properties of all the tested compounds were predicted by the Maestro 11.5 *Qikprop* module. The CNS drug-like properties of the selected compounds were identified as favorable values defined as a log$P$ < 3, polar surface area (PSA) < 90 Å^2, and BBB permeability with the ADMET_BBB_Level< 3[44]. Among the selected compounds, ZLG-25 showed a superior selectivity, which provided a potential starting scaffold for structural optimization to improve biological activities and physicochemical properties (Supporting Information Table S4).

2.2. Molecular modeling of ZLG-25 and design of bitopic ligands

To explore the binding properties of ZLG-25 at D$_3$R, the induced-fit docking (Fig. 2A−B) and molecular dynamic (MD) simulation (Supporting Information Fig. S3) were performed in Schrodinger 2018.1. The results of MD were well consistent with the docking results, depicting a bitopic binding mode. The carbazole moiety of ZLG-25 was found located at the OBS forming π−π interactions with Phe345 and Phe346. The protonated nitrogen atom in the linker chain formed a salt bridge with the carboxylate of Asp110 offering a key and strong interaction. The imidazole moiety was oriented toward the SBP constructed by extracellular loop1, loop2, helices I, helices II and VII, forming the ionic interaction with Glu90. Based on the scaffold of ZLG-25, a series of more typical bitopic molecules were expected to be obtained as D$_3$R selective ligands by attaching a D$_3$R-preferring SBP binding motif to the amine group (Fig. 2C). The carboxamide replaced the SBP binding moiety-linked aromatic group, which was thought to occupy more space in the SBP and provide more interactions than the imidazole moiety. The scaffold hopping or structural simplification strategy was further used to improve the rigid carbazole structure by moving the nitrogen atom in the linker chain to the ring B of the carbazole moiety[45]. In 'hopping I', the secondary amine was converted to the tertiary amine at position 4, obtaining

the tetrahydro-β-carboline scaffold. Furthermore, 'hopping II' was processed by moving the tertiary amine to position 3 to obtain the tetrahydro-γ-carboline scaffold by (Fig. 2C). Therefore, started from ZLG-25, a series of carbazole and tetrahydrocarboline derivatives were designed to discover novel D$_3$R-selective ligands.

2.3. Chemistry

In Scheme 1A, carbazoles with different alkyl (methyl, ethyl, and isopropyl) groups were used as the starting materials. The formyl group at position 4 was incorporated in the solution of DMF and POCl$_3$ in 80%−95% yield. The subsequent incorporation of the linker moiety was achieved by the reductive amination reaction affording derivatives 3a−c, with adding NaBH(OAc)$_3$ for multiple times. However, benzaldehyde 7 was not obtained with the same procedures as 2a−c, but rather through Fisher indole synthesis reaction and oxidative dehydrogenation in one pot, obtaining intermediate 6 (Scheme 1B). It was found that if the reaction was carried out in the absence of acid environment in 100 °C, the phenyl hydrazine must be with hydrochloride for the formation of NH$_4$Cl. The aromatization by oxidation sequentially happened in one pot under O$_2$. Intermediates 6 were oxidized to an aldehyde group offering intermediates 7. Derivatives 8 was obtained by a similar fashion as 3a−c.

As shown in Scheme 1C, the cyclohexanone (4) and the methyl 4-hydrazineylbenzoate (9) was refluxed in AcOH to synthesize the intermediate 10 in high yield. Another two steps were proceeded for converting methyl formate intermediate to a formyl intermediate 12. A similar process was performed to obtain derivatives 13. To further understand the importance and effect of the SBP binding moiety; derivatives 17a−f were synthesized by replacement of the imidazole moiety with various aromatic rings (Scheme 1D). Compound 2b was reacted with the 3-aminopropanol or 4-amino-1-butanol and reduced to obtain intermediates 14a/b. The secondary amines were sequentially protected with the Boc group, and sulfonated with benzenesulfonyl group affording intermediates 16a/b. Subsequent substitution reactions and *N*-Boc deprotection resulted into the desired products 17a−f.

The derivatives 22a−c were accessed through a three-step synthesis outlined in Scheme 2A. The *tert*-butyl (3-aminopropyl) carbamate (18) was used to prepare intermediates 20a in the cooperation with dimethylcarbamic chloride and dimethylsulfamoyl chloride. Compounds 20b/c were obtained through the amide coupling in the presence of EDCI and DMAP, followed by the reductive amination to furnish the final products 22b/c. Additionally, a longer chain linker moiety was explored with the starting material 19 through the same three-step synthesis route to afford the desired final derivatives 23a−m (Scheme 2A). A cyclohexyl ring as linker was also investigated. As detailed in Scheme 2B, *tert*-butyl ((1*R*,4*R*)-4-aminocyclohexyl) carbamate (24) and 1*H*-indole-2-carboxylic acid (25) were utilized to prepare the intermediate 26 and resulted in the final compound 27 through a similar two-step, one-pot synthesis methods. Two intermediates, 28a/b, were obtained to synthesize the 29a/b with *tert*-butyl (4-bromobutyl) carbamate in potassium carbonate. Treatment of 29a/b with TFA afforded amine 30a/b. To furnish the desired targets 31a−e in high yield, the amide coupling catalyzed by EDCI and DMAP was used (Scheme 2C).

Tetrahydro-γ-carboline derivatives 36a−k were prepared as mentioned in Scheme 3A, from the starting materials
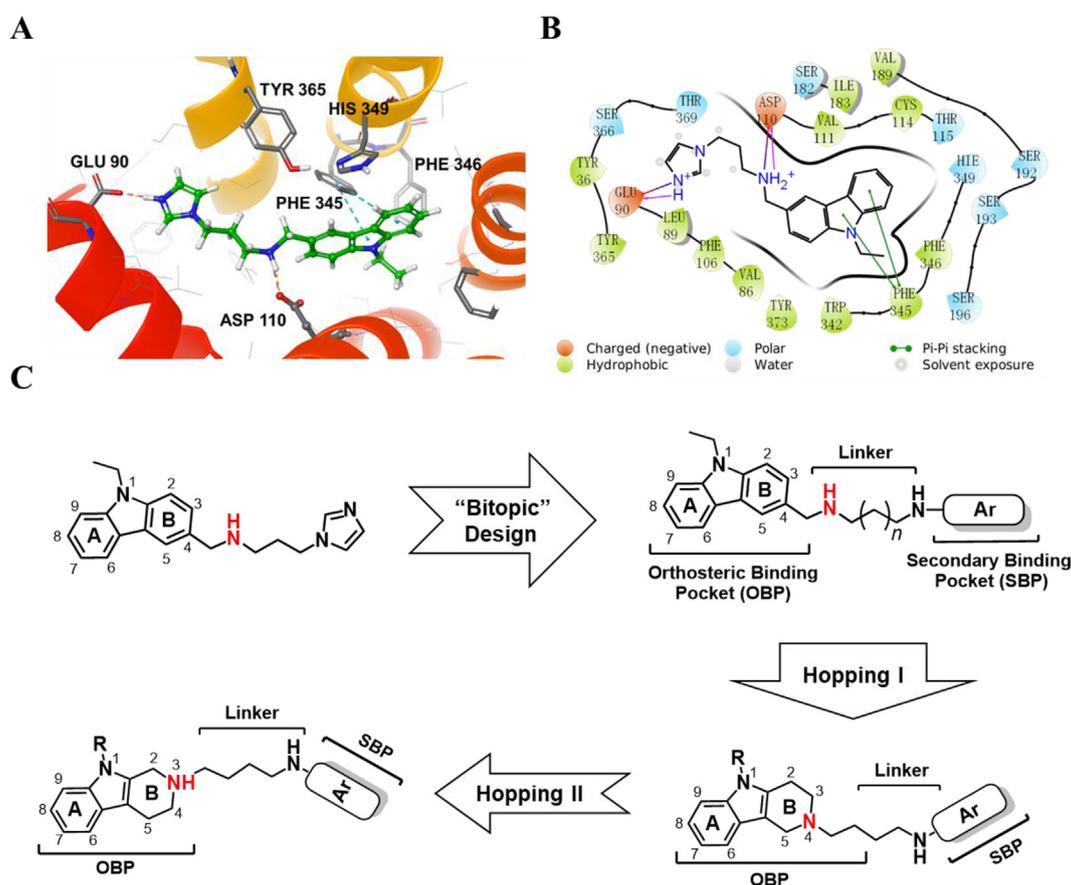
Figure 2   Protein−ligand contacts histogram ZLG-25 (A) and corresponding 2D diagram (B); The structure design strategy in this work (C).

phenylhydrazine **32a/b** and piperidine-4-one. Compounds **34a/b** served as the critical intermediates for synthesizing compounds, of which the synthetic protocol was the same as the intermediate **10**. Intermediates **35a/b** were prepared with commercially available *tert*-butyl (4-bromobutyl)carbamate. Then, compounds **35a/b** on treatment with CF₃COOH in CH₂Cl₂ afforded the primary amine intermediates to obtain the target compounds **36a–k** by the same amide coupling reaction. Tetrahydro-β-carboline derivatives **42a–f** were synthesized from the starting materials 2,3,4,9-tetrahydro-1*H*-pyrido[3,4-*b*]indole (**37**) (Scheme 3B). The NH at piperidyl structure was selectively protected by the Boc group and then the NH at indole structure was alkylated by CH₃I or CH₃CH₂I to offer intermediates **39a/b**. After the secondary amine intermediates **40a/b** afforded on treatment with CF₃COOH in CH₂Cl₂, compounds **41a/b** was obtained by the reaction with *tert*-butyl (4-bromobutyl)carbamate. Final compounds **42a–f** were prepared by the same process as **36a–k**.

2.4.   Structure−activity relationships study

To increase the binding affinity of hit compound ZLG-25 at the D₃R, the SAR study and scaffold hopping of the carbazole scaffold were investigated. More than 60 derivatives were synthesized and the binding affinities and selectivity profiles at D₂R and D₃R were identified. To explore the key role of carbazole moiety in a high selectivity for D₃R over D₂R, different alkyl substitutions on the 1-position nitrogen of the carbazole moiety and the impact of aromaticity on the carbazole nucleus were first examined. The

linker and imidazole moiety were then replaced with other entities to increase the binding affinity at D₃R as described above.

Radioligand competition binding assays were performed by measuring the ligands' ability to compete with [³H] spiperone for the CHO cells stably expressing hD₂R and hD₃R[9,39,46−49]. The results are expressed as the inhibition values at 10 μmol/L for all the derivatives presented in Tables 1−5. The K_i values were tested for any compound with an inhibition above 75% for D₂R or D₃R. Following the discovery of novel structures with high selectivity for D₃R, a structural analysis to understand the basis for this was initiated from the modification of carbazole moiety. As shown in Table 1, the alkylation at the N position of carbazole was explored, and the comparison of affinities to D₃R showed that ethyl (**3b**) and isopropyl (**3c**) groups had improved potency compared to the methyl group (**3c**) and hydrogen (**8**). The binding mode of ZLG-25 (**3b**) displayed strong π−π interactions between the carbazole and the aromatic residues (Phe345/Phe346/His349). To verify the importance of aromaticity, 2,3,4,9-tetrahydro-1*H*-carbazole was introduced to the analogs (**13**), which suggested the decreased potency as expected. The linker chain composed of four carbon atoms (**17a**) showed comparative potency with ZLG-25 (Table 1). Subsequently, a survey of heterocyclic replacements for the imidazole moiety was performed, such as 1,2,3-triazole (**17b**), 1,3,4-triazole (**17c**), benzimidazole (**17d**), purine (**17e**) and 6-Cl-purine (**17f**), among which only **17b** exhibited an improved binding affinity compared to ZLG-25.

As shown in Table 2, when the linker length was kept at three-carbon units, the replacement of the imidazole group by
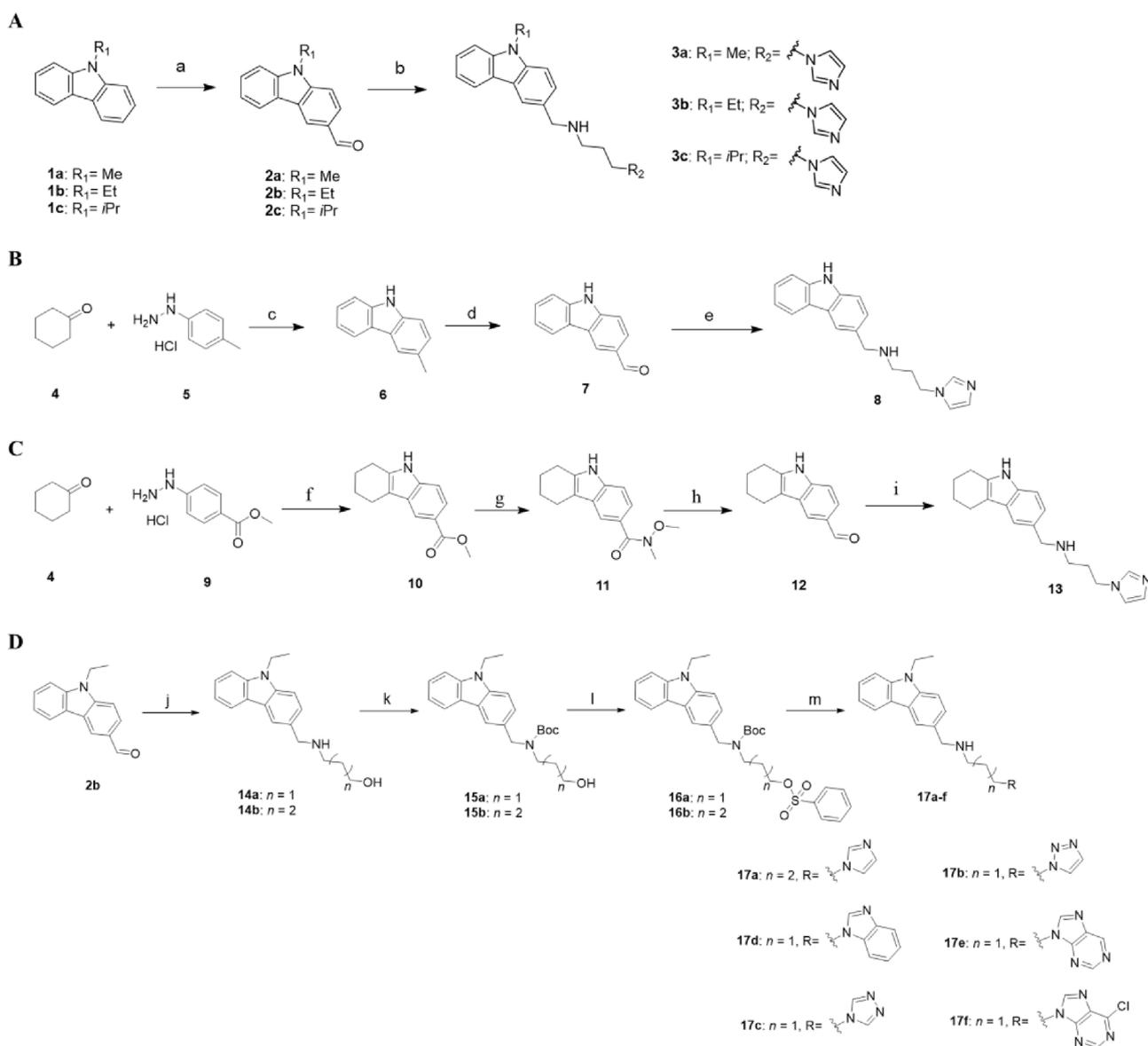
Scheme 1 Synthesis of 9-ethyl-9H-carbazole derivatives. Reagents and conditions: (a) DMF, POCl$_3$, 130 °C; (b) 3-(1H-imidazole-1-yl)propan-1-amine for **3a−c**, MeOH. (c) O$_2$ (1 atm), DMSO, 100 °C; (d) DDQ, MeOH/H$_2$O, r.t.; (e) 3-(1H-imidazole-1-yl)propan-1-amine, NaBH(OAc)$_3$, MeOH. (f) AcOH, TsOH, reflux; (g) 1) NaOH, MeOH/H$_2$O, r.t.; 2) N,O-Dimethylhydroxylamine hydrochloride, EDCI, DMAP, CH$_2$Cl$_2$; (h) LiAlH$_4$, THF, 0 °C; (i) 3-(1H-Imidazole-1-yl)propan-1-amine for **13**, NaBH(OAc)$_3$, MeOH. (j) 3-Aminopropanol or 4-amino-1-butanol, MeOH/NaBH(OAc)$_3$, r.t.; (k) (Boc)$_2$O, CH$_2$Cl$_2$, r.t.; (l) Benzenesulfonyl chloride, DMAP, 0 °C, 5 h; (m) 1) 1H−N-Heterocycle, NaH, TBAI, DMF, −5 °C; 2) CF$_3$COOH, CH$_2$Cl$_2$, r.t.

dimethylurea (**22a**) failed to give improved binding profiles. And, the aromatic carboxamide moieties (**22b/c**) were also explored but were found to be less potent versus ZLG-25. When the propyl linker was replaced with a butyl (**23a**) and cyclohexyl (**27**) linker, both compounds showed a significant improvement in D$_3$R binding affinity (K_i = 62.6 nmol/L for **23a** and K_i = 52.1 nmol/L for **27**). Intending to improve the D$_3$R binding affinity, we realized that the butyl linker would likely be suitable to explore the second binding region of bitopic compounds. Next, we investigated the replacement of the indole-2-carboxamide moiety of **23a** with different aromatic carboxamide moieties (**23b−m**) to fit the SBP. Comparing with compound **23a**, 1H-benzo[d]imidazole (**23b/23d**) exhibited decreased binding affinity at D$_3$R (K_i = 577.6 nmol/L/

K_i = 125.1 nmol/L). The pyrazolo[1,5-a]pyridine (**23c**) showed a comparable D$_3$R binding affinity (K_i = 45.7 nmol/L) to **23a**. Moreover, 1,3-dihydro-2H-benzo[d]imidazole-2-one (**23e**) and benzo[d]oxazol-2(3H)-one (**23f**) were both used as they have been reported in numerous D$_3$R selective compounds, showing the K_i values of 99.3 and 95.1 nmol/L, respectively. Further replacements with larger moieties were also explored such as the 1,1′-biphenyl-4-carboxamide moiety (**23g**) which led to a decreased D$_3$R affinity while 4′-acetyl-[1,1′-biphenyl]-4-carboxamide moiety (**23h**) and showed an increased D$_3$R affinity (K_i = 24.2 nmol/L). When the substituent groups on the *para*-position of benzamide were converted into pyridyl groups (**23i/j**), the binding affinity was found to have an approximately 10-fold improvement for D$_3$R *versus* the
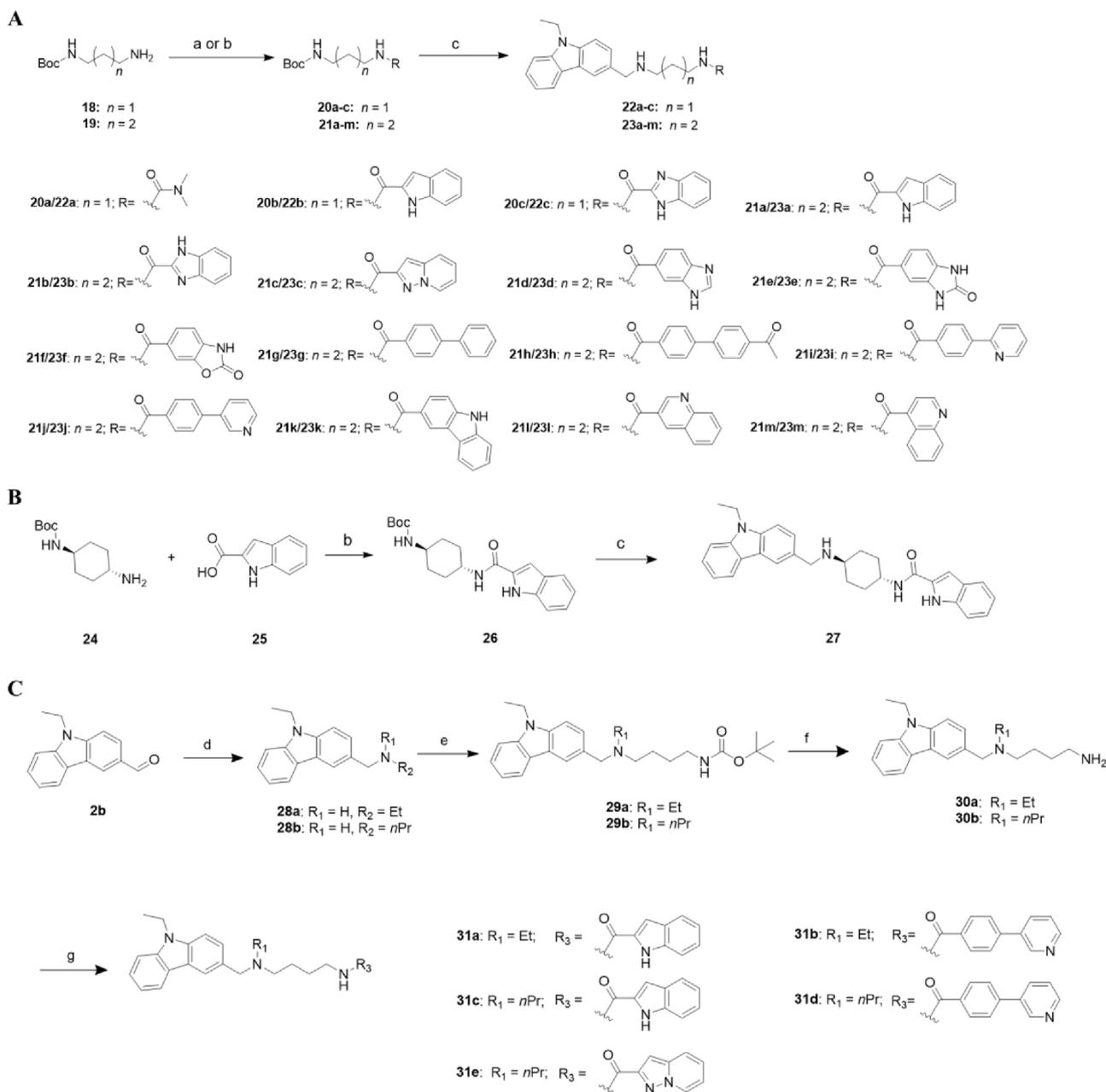
Scheme 2 Synthesis of carboline derivatives. Reagents and conditions: (a) for **20a**, dimethylcarbamic chloride, NaH, DMF; (b) for **20b/c** and **21a−m**, aryl formic acid, EDCI, DMAP, CH$_2$Cl$_2$, r.t.; (c) i) CF$_3$COOH, CH$_2$Cl$_2$, r.t.; ii) **2b**, NaBH(OAc)$_3$, MeOH, r.t. (d) MeOH, NaBH(OAc)$_3$, r.t.; (e) *tert*-Butyl (4-bromobutyl)carbamate, K$_2$CO$_3$, TBAI, DMF, 60 °C; (f) CF$_3$COOH, CH$_2$Cl$_2$; (g) EDCI, DMAP, CH$_2$Cl$_2$, r.t.

phenyl group (**23g**). Comparable receptor binding was observed with the fragments 9*H*-carbazole-3-carboxamide (**23k**, D$_3$R K_i = 144.7 nmol/L) and quinoline-3-carboxamide (**23l**, D$_3$R K_i = 85.7 nmol/L), but quinoline-4-carboxamide led to the reduced binding activity. As can be seen with compounds **23a–m**, all (9-ethyl-9*H*-carbazol-3-yl)methanamine analogs with the linker composed of four carbon atoms exhibited slight inhibition of D$_2$R at 10 μmol/L (<15%), showing excellent D$_3$R selectivity profiles over D$_2$R.

To investigate the potential interactions of N-4 as well as improved physicochemical properties, an ethyl or propyl group was introduced to the N atom on the linker of bitopic ligands. However, compared to the original compounds **23a/23c/23j**,

alkylated derivatives were found to have less potential in D$_3$R binding affinity. For **23a**, the alkylation of the benzyl N atom unexpectedly increased the D$_2$R binding affinity (D$_2$R K_i = 332.6 nmol/L for **31a** and D$_2$R K_i = 300.8 nmol/L for **31c**) (Table 3). It is speculated that the alkylation of the benzyl N atom of **23a** changed the bitopic binding mode by turning the 1*H*-indole-2-carboxamide moiety into an OBS binding part.

Binding studies continued to evaluate the tetrahydro-carboline core structure to determine if the scaffold hopping was engaging D$_3$R affinity and selectivity. Regarding superior binding structures for SBP, aromatic carboxamide moieties were also explored in the tetrahydro-carboline scaffold to obtain the bitopic ligands and binding affinities were shown in Tables 4 and 5. Replaced by 1*H*-
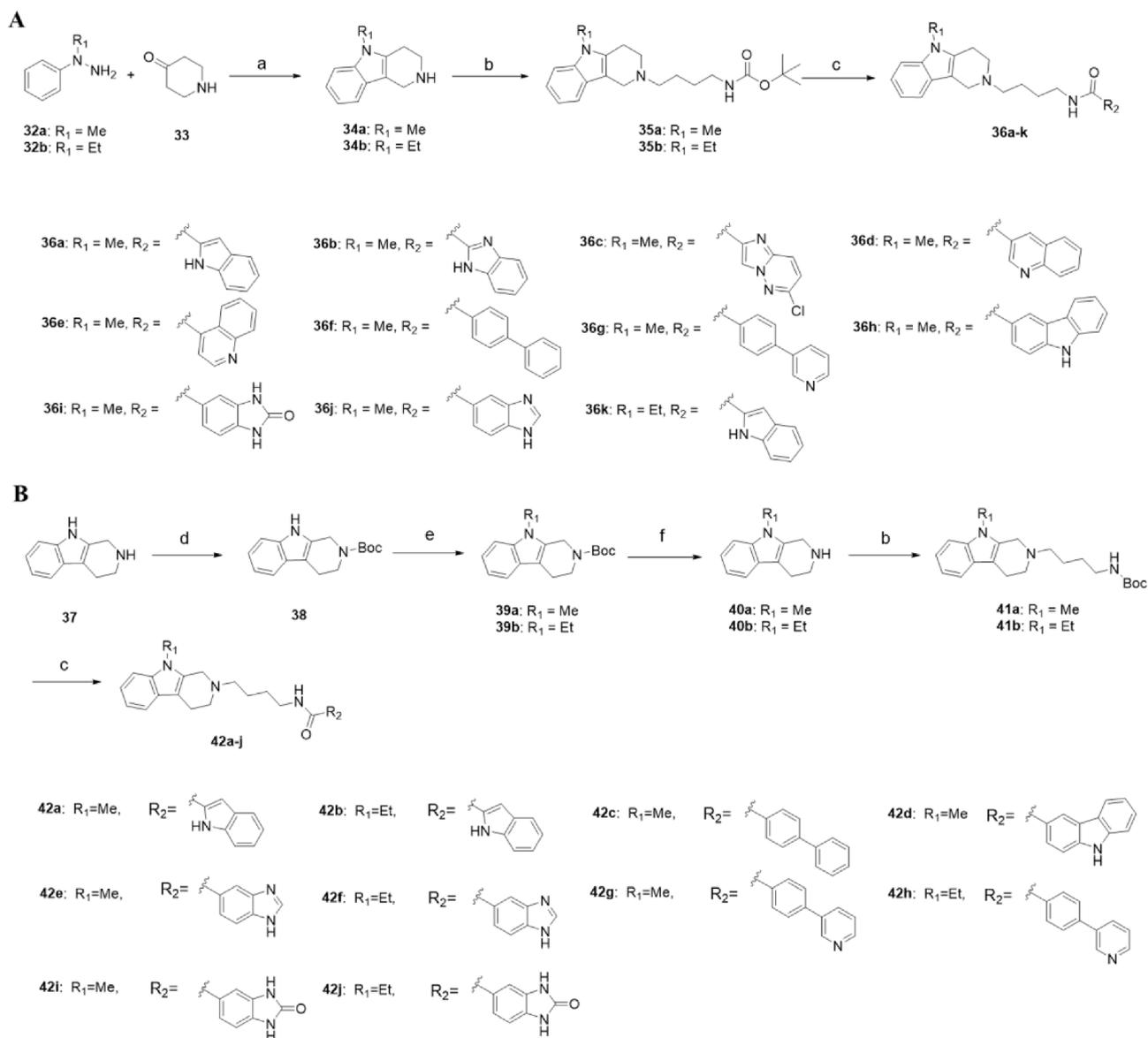
Scheme 3   Synthesis of carboline derivatives. Reagents and conditions: (a) AcOH, TsOH, reflux; (b) *tert*-butyl (4-bromobutyl)carbamate, K_2CO_3, TBAI, DMF; (c) i) CF_3COOH, CH_2Cl_2; ii) EDCI, DMAP, CH_2Cl_2; (d) $(Boc)_2O$, CH_2Cl_2; (e) MeI or CH_3CH_2I, NaH, THF, 0 °C; (f) CF_3COOH, CH_2Cl_2.

indole-2-carbamate, two tetrahydro-γ-carboline analogs (**36a/ 36k**) were found to have improved binding affinities (K_i = 10.6 and 15.5 nmol/L), and excellent D_3R selectivity profiles of which *N*-methyl or *N*-ethyl substituent at the tetrahydro-carboline showed no distinct difference. The same SAR was found for the tetrahydro-β-carboline analogs (**42a/b**). Ligand **36c** demonstrated a drastic decrease in D_3R binding affinity. Interestingly, due to the excellent D_3R binding properties observed with compounds **23l**, quinoline-3-carbamate (**36d**) was also explored, however it was found to dramatically reduced D_3R affinity. Almost all other aromatic carboxamide moieties replacements, as they are present in compound **36f–j**, led to the improved D_3R binding affinity, especially for **36f** and **36i** with an approximate K_i value of 4 nmol/L. Ligand **36g** (K_i = 29.0 nmol/L) was found to have a comparable binding affinity with **23j**. However, for the tetrahydro-β-carboline analogs (**42c/g/h**), D_3R affinities lost dramatically. Compared to the carbazole scaffold, tetrahydro-carboline

scaffold linked the 9*H*-carbazole-3-carbamate moiety (**36h**, K_i = 19.6 nmol/L and **42d**, K_i = 36.7 nmol/L) was more potential, affording an increase in D_3R binding. Compound **36j** (K_i = 14.2 nmol/L) with a tetrahydro-γ-carboline scaffold linked the 1*H*-benzo[*d*]imidazole moiety showed better binding affinity to D_3R than compounds with a tetrahydro-β-carboline scaffold (**42e**, K_i = 203.0 nmol/L and **42f**, K_i = 300.0 nmol/L). The same SAR was obtained from the tetrahydro-γ-carboline derivate **36i** (K_i = 3.7 nmol/L) and tetrahydro-γ-carboline derivates (**42i**, K_i = 112.0 nmol/L and **42j**, K_i = 149.0 nmol/L).

2.5. Functional study by measuring p-ERK1/2 mediated D_3 receptor signaling

To access the fundamental activities of these novel D_3R selective ligands, p-ERK1/2 as the key molecular in signal transduction was tested. To ensure the reliability of the ERK1/2

Table 1 Binding affinities of compounds **3a−c, 8, 13** and **17a−f** at hD$_2$ and hD$_3$ receptors.
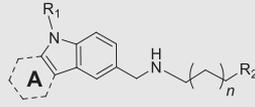
Compd.	A	R$_1$	R$_2$	% inhibition at 10 μmol/L		K_i (nmol/L)[a]	
				hD$_2$	hD$_3$	hD$_2$	hD$_3$
ZLG-25 (**3b**)		Et		17.6 ± 17.5	95.4 ± 3.0	>10,000[b]	685.0 ± 4.5
3a		Me		13.4 ± 23.8	63.8 ± 8.0	NA	NA
3c		iPr		1.7 ± 9.2	83.8 ± 10.2	>10,000[b]	665.4 ± 13.5
8		H		33.2 ± 17.5	54.2 ± 7.7	NA	NA
13		H		15.7 ± 1.7	36.9 ± 2.1	NA	NA
17a		Et		−23.6 ± 18.6	75.6 ± 3.5	>10,000[b]	994.1 ± 36.6
17b		Et		−24.6 ± 5.1	95.8 ± 6.9	>10,000[b]	96.7 ± 13.4
17c		Et		30.5 ± 4.0	63.0 ± 5.2	NA	NA
17d		Et		71.8 ± 3.6	75.0 ± 14.5	>10,000[b]	1104.5 ± 115.2
17e		Et		10.6 ± 7.4	−10.2 ± 13.3	NA	NA
17f		Et		12.8 ± 7.7	−7.9 ± 12	NA	NA

[a]K_i values are taken from three experiments, expressed as means ± SEM.
[b]The K_i values were not calculated because the inhibition percentages at 10 μmol/L were too low.

phosphorylation measurement assays to evaluate the functions to D$_{2/3}$R, several typical D$_{2/3}$R modulators were used as positive drugs. The results were shown in Supporting Information Fig. S4 that dopamine showed significantly increased level of pERK1/2 in CHO-hD$_2$R and CHO-hD$_3$R cells. Additionally, the full D$_{2/3}$R agonist, quinpirole at 20 μmol/L, induced a full ERK1/2 phosphorylation with the similar effects as dopamine. PD128907 was recognized as a D$_3$R-selective agonist with D$_3$R K_i of 1 nmol/L and D$_2$R K_i of 1183 nmol/L[50]. As a positive control, PD128907 robustly stimulated a full ERK1/2 phosphorylation in CHO-hD$_3$R cells but not CHO-hD$_2$R cells indicating the D$_3$R selective agonism. As an antagonist or weak partial agonist, cariprazine and haloperidol did not exhibit significant effects to the ERK1/2 phosphorylation. GSK598809 (a highly D$_3$R selective antagonist, D$_3$R K_i = 2.9 nmol/L, D$_2$R K_i = 2110 nmol/L) was measured the ERK1/2 phosphorylation to D$_2$R and D$_3$R and found no significant effects. As shown in Fig. 3A, dopamine significantly increased the level of pERK1/2 in hD$_3$R−CHO cells, but compounds **23j**, **36a**, **36b** and **36f** were found no influence in the ERK1/2 phosphorylation to hD$_3$R by the compounds alone. Competitive antagonism assays in the present of 20 μmol/L dopamine were also performed, which showed compounds **23j**, **36a**, **36b** and **36f** could dose-dependently inhibit the ERK1/2 phosphorylation mediated by D$_3$R (Fig. 3C). From the results (Fig. 3B), we found that compounds

23j, **36a**, and **36b** showed weak partial agonism to D$_2$R at a high level. These compounds could inhibit the ERK1/2 phosphorylation induced by dopamine but not eliminate (Fig. 3D). Compound **36f** showed no functional affects to D$_2$R. The selected compounds with excellent D$_3$R binding affinity failed to stimulate ERK1/2 phosphorylation in CHO-hD$_3$R cells, which corroborated that the carbazole or tetrahydro-carboline D$_3$R selective ligands possessed no intrinsic agonistic activity and acted as D$_3$R antagonists. All the results indicated **23j**, **36a**, **36b** and **36f** as the D$_3$R-sellective antagonists respectively with the IC$_{50}$ value of 17.7, 154.6, 594.4, and 673.9 nmol/L.

2.6. Binding modes analysis

To explain the SARs of novel bitopic scaffolds, the representative compounds **23j**, **36b**, **36f** and **42a**, were docked into the OBS of the D$_3$R (PDB ID: 3pbl). The OBS was a hydrophobic pocket surrounded by aromatic residues (Phe188, Phe345, Phe346, His349, and Tyr365), where the hydrophobic carbazole moiety of compound **23j** deeply buried in (Fig. 4A). The aromatic carbazole structure of compound **23j** formed $\pi-\pi$ interactions with Phe345, Phe346, and His349, and its hydrogen atom of NH$_2^+$ at the benzyl position of the carbazole group formed an ionic hydrogen bond with Asp110. In the comparison with the carbazole moiety, the carboline region was similarly located at the OBS region

Table 2 Binding affinities of compounds **22a–c** and **23a–m** at hD_2 and hD_3 receptors.
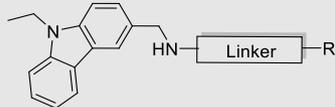
Compd.	Linker	R	% inhibition at 10 μmol/L		K_i (nmol/L)[a]	
			hD_2	hD_3	hD_2	hD_3
22a			-4.8 ± 27.2	42.1 ± 2.2	NA	NA
22b			8.1 ± 3.9	21.3 ± 8.5	NA	NA
22c			22.4 ± 5.7	32.7 ± 11.4	NA	NA
23a			30.0 ± 14.1	87.0 ± 2.5	>10,000[b]	62.6 ± 8.0
23b			-4.7 ± 14.2	86.7 ± 1.9	>10,000[b]	577.6 ± 85.0
23c			-17.6 ± 2.0	98.4 ± 2.0	>10,000[b]	45.7 ± 2.2
23d			2.7 ± 4.3	98.5 ± 2.3	>10,000[b]	125.1 ± 17.6
23e			1.9 ± 10.7	99.2 ± 0.5	>10,000[b]	99.3 ± 31.9
23f			5.5 ± 21.5	97.6 ± 0.3	>10,000[b]	95.1 ± 8.9
23g			-15.9 ± 21.9	102.3 ± 1.2	>10,000[b]	206.3 ± 2.9
23h			5.2 ± 7.6	105.0 ± 1.5	>10,000[b]	24.2 ± 4.4
23i			6.5 ± 2.3	101.0 ± 0.8	>10,000[b]	21.3 ± 4.3
23j			12.4 ± 5.8	107.3 ± 0.3	>10,000[b]	13.9 ± 3.4
23k			10.6 ± 3.3	98.1 ± 0.8	>10,000[b]	144.7 ± 11.9
23l			9.6 ± 0.1	100.0 ± 1.3	>10,000[b]	85.7 ± 2.9
23m			-19.1 ± 5.8	63.1 ± 0.2	NA	NA
27			15.6 ± 3.0	88.9 ± 0.9	>10,000[b]	52.1 ± 6.0

[a]K_i values are taken from three experiments, expressed as means $\pm$ SEM.
[b]The K_i values were not calculated because the inhibition percentages at 10 μmol/L were too low.

Table 3 Binding affinities of compounds **31a–e** at hD$_2$ and hD$_3$ receptors.

Compd.	R$_1$	R$_2$	% inhibition at 10 μmol/L		K_i (nmol/L)[a]	
			hD$_2$	hD$_3$	hD$_2$	hD$_3$
31a	Et	(2-indolyl)	88.1 ± 1.5	96.2 ± 0.2	332.6 ± 3.6	134.3 ± 9.6
31b	Et	(4-(pyridin-3-yl)phenyl)	36.9 ± 2.1	78.5 ± 0.3	>10,000[b]	421.8 ± 18.3
31c	nPr	(2-indolyl)	89.8 ± 2.9	98.6 ± 0.6	300.8 ± 11.9	448.2 ± 26.1
31d	nPr	(4-(pyridin-3-yl)phenyl)	26.8 ± 0.9	81.3 ± 0.6	>10,000[b]	994.4 ± 45.3
31e	nPr	(pyrazolo-pyridinyl)	18.6 ± 2.5	74.8 ± 1.5	>10,000[b]	478.2 ± 29.4
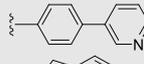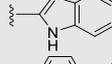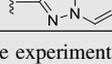
[a]K_i values are taken from three experiments, expressed as means ± SEM.
[b]The K_i values were not calculated because the inhibition percentages at 10 μmol/L were too low.

exhibiting fewer π-stacking interactions with Phe345, Phe346, and His349, which could be seen from **36b**, **36f,** and **42a** (Fig. 4B, Supporting Information Fig. S5A and S5B). The hydrogen atom of NH$^+$ at the 2-position of the carboline group formed an ionic hydrogen bond with Asp110 to stabilize the OBS binding conformation. For the SBP region, the 4-(pyridin-3-yl) benzamide moiety was predicted docked to the SBP region on the edge of the whole pocket, indicating the bitopic binding mode. The hydrogen atom of the amide structure formed a hydrogen bond with Glu90, but pyridin-3-yl group was almost exposed to water due to the long linear structure of **23j**. The similar binding mode with **23j** was observed in **36f**, which [1,1′-biphenyl]-4-carboxamide moiety extended into solvents. 1H-benzo[d]imidazole-2-carboxamide of **36b** and 1H-indole-2-carboxamide of **42a** both offered the hydrogen bond formed between NH and Glu90, suggesting that interactions with Glu90 played key roles in the bitopic binding mode.

To verify the binding modes in a dynamic condition, 300 ns MD simulations for compounds **23j**, **36b**, and **36f** and **42a** were performed with Desmond software. Compound **23j** exhibited unstable binding conformation with the fluctuant ligand RMSD (Supporting Information Fig. S6A) and therefore the entire MD of 300 ns was analyzed. The complexes of compound **36b** were stable after 30 ns from the beginning of simulations (Fig. S6B), and then the trajectories from 50 to 150 ns were extracted and analyzed. The MD analysis of **23j** were shown in Fig. 4C and E, indicating consistent results with docking. The stable interactions between Asp110 and NH$_2^+$ at the benzyl position of the carbazole group existed during 98% of the simulation time as the H-bond or ionic bond. The aromatic carbazole was predicted to form abundant hydrophobic interactions with Phe345, Phe346, and His349 in the 65%, 74%, and 85% stimulation time, respectively, indicating that the hydrophobic carbazole group could stabilize the OBS of hD$_3$R. Nevertheless, the 4-(pyridin-3-yl)benzamide moiety of **23j** seemed to broadly interact with the amino acids at the gorge entrance, such as Val86, Glu90, Tyr365, Ser366, Thr369, and Tyr373. The group embedded in the SBP span freely in the solvent in the absence of possible stabilizing interactions, which

might explain the fluctuant RMSD of the whole system. The MD analysis of **36b** showed (Fig. 4D and F), that the carboline region of **36b** was well embedded in the OBS forming stable interactions with Asp110 during 75% of the stimulation time as the H-bond, ionic bond, or water-bridge. Compared with **23j**, the ionic bond formed by **36b** with Asp110 seemed more prominent in all its interactions. However, fewer hydrophobic interactions by π–π stacking were predicted for the carboline group than for the carbazole group. The same situation appeared to the SBP binding moiety of **36b** viz., fewer interactions were observed during the extracted stimulation time. The SBP binding with 1H-benzo[d]imidazole-2-carboxamide significantly contributed to the stable conformation, especially the specific interactions with Glu90, which played a key role in about 60% time, such as H-bounds and water bridges. Unfortunately, the conformation of hD$_3$R with **36f** or **42a** was constantly perturbed during the 300 ns MD, and it was difficult to analyze a more stable conformation (Fig. S6C and D).

2.7. Binding selectivity profiles

The interactions between representative compounds and other receptors related to psychotic disorders were evaluated, and a selectivity profile was created using additional receptors (including hDR$_{1-5}$, h5-HT$_{1A}$, h5-HT$_{1B}$, h5-HT$_{2A}$, h5-HT$_{2C}$, h5-HT$_6$, hH1, hα1 receptors, Dopamine Transporter (hDAT), Norepinephrine Transporter (hNET), Serotonin Transporter (hSERT)) to investigate its potential off-target activity. As shown in Table 6, both selected compounds showed the highest affinity to the D$_3$R receptor (**23j** K_i = 16.8 nmol/L; **36b** K_i = 19.6 nmol/L) in the dopamine receptor families. Compound **23j** displayed a relatively higher affinity to the 5-HT$_{1B}$ receptor (K_i = 172.2 nmol/L). The other selected compound **36b** exhibited comparable selectivity to D$_3$R over 5-HT$_{1B}$ receptor (K_i = 138.0 nmol/L). Furthermore, **23j** and **36b** showed the comparatively potential binding activity to 5-HT$_{1A}$ receptor, and 5-HT$_{2A}$ receptor. To hD$_2$, h5-HT$_{2C}$ receptor, h5-HT$_6$ receptor, hNET, hDAT and hSERT, both **23j** and **36b** showed weak binding affinity (K_i > 2000 nmol/L). Overall, compounds **23j**

Table 4 Binding affinities of compounds **36a−k** at hD_2 and hD_3 Receptors.

Compd.	R_1	R_2	% inhibition at 10 μmol/L		K_i (nmol/L)[a]	
			hD_2	hD_3	hD_2	hD_3
36a	Me		20.9 ± 3.8	97.6 ± 2.0	>10,000[b]	10.6 ± 4.5
36b	Me		19.6 ± 5.7	97.9 ± 3.6	>10,000[b]	16.8 ± 1.8
36c	Me		21.1 ± 9.0	66.3 ± 9.1	NA	NA
36d	Me		15.6 ± 12.9	42.5 ± 6.8	NA	NA
36e	Me		−2.0 ± 17.4	68.1 ± 1.9	NA	NA
36f	Me		−20.7 ± 4.9	103.5 ± 1.4	>10,000[b]	4.1 ± 0.3
36g	Me		29.2 ± 5.8	83.8 ± 12.7	>10,000[b]	29.0 ± 3.3
36h	Me		74.4 ± 3.3	95.5 ± 0.7	NA	19.6 ± 7.3
36i	Me		38.9 ± 3.8	87.5 ± 6.1	>10,000[b]	3.7 ± 0.6
36j	Me		27.7 ± 1.4	74.4 ± 8.2	>10,000[b]	14.2 ± 0.7
36k	Et		13.2 ± 7.3	105.5 ± 2.2	>10,000[b]	15.5 ± 1.8

[a]K_i values are taken from three experiments, expressed as means ± SEM.
[b]The K_i values were not calculated because the inhibition percentages at 10 μmol/L were too low.

and **36b** showed excellent *in vitro* selective profiles with a preferred affinity for the D_3R.

2.8. Pharmacokinetic profiles and brain penetration properties study

Pharmacokinetics profiles of the selected compounds were extensively studied in ICR mice by i.v. administration at 3 mg/kg and *p.o.* administration at 10 mg/kg. As shown in Supporting Information Fig. S7, the plasma concentrations of each compound were monitored for 24 h. The related profiles were calculated and shown in Table 7. Both compounds showed a rapid distribution (T_{max} = 0.17 and 0.25 h for **23j** and **36b**) at a dose of 10 mg/kg (*p.o.*). Nevertheless, compound **36b** showed a much more effective blood plasma concentration (C_{max} = 505.2 ng/mL)

comparable to that of compound **23j** (C_{max} = 78.8 ng/mL) for the *p.o.* administration. Similarly, the C_{max} of the i.v. administration for compound **36b** was found to be more than one-fold higher than **23j**. Compounds **23j** and **36b** had the comparable half-life ($t_{1/2}$ = 1.1 h) for 10 mg/kg *p.o.* administration. Finally, the oral bioavailability of **23j** was rather low and did not reach a sustained pharmacologically relevant plasma concentration during an oral dosing period. Despite the high on-target potency and good selectivity for the carbazole scaffold, the results of **23j** indicated its poor PK profile (high i.v. clearance and no oral bioavailability). After hopping to the tetrahydro-carboline structure, the oral bioavailability seemed to be greatly improved, as can be seen from compound **36b** (F = 39.7%), by which the lower i.v. clearance was accompanied, perhaps due by its lower first-pass metabolism. Further tests for their brain penetration

Table 5 Binding affinities of compounds **42a−j** at hD$_2$ and hD$_3$ receptors.

Compd.	R$_1$	R$_2$	% inhibition at 10 µmol/L		K_i (nmol/L)[a]	
			hD$_2$	hD$_3$	hD$_2$	hD$_3$
42a	Me		9.1 ± 16.4	106.3 ± 1.3	>10,000[b]	6.8 ± 0.8
42b	Et		17.5 ± 1.5	105.5 ± 3.6	>10,000[b]	12.9 ± 2.1
42c	Et		−9.1 ± 20.7	63.9 ± 7.0	NA	NA
42d	Et		19.2 ± 8.3	97.1 ± 2.9	>10,000[b]	36.7 ± 1.5
42e	Me		49.7 ± 1.2	81.5 ± 0.6	NA	203.0
42f	Et		54.1 ± 0.1	80.6 ± 1.1	NA	300.0
42g	Me		37.8 ± 1.0	69.6 ± 0.8	NA	NA
42h	Et		28.9 ± 0.8	31.3 ± 0.6	NA	NA
42i	Me		37.6 ± 0.2	85.2 ± 0.5	NA	112.0
42j	Et		48.8 ± 0.7	85.9 ± 0.07	NA	149.0
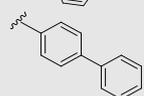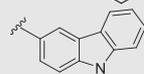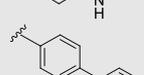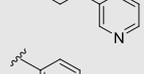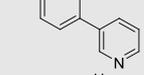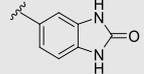
[a]K_i values are taken from three experiments, expressed as means ± SEM.
[b]The K_i values were not calculated because the inhibition percentages at 10 µmol/L were too low.

properties were performed at 0.5 and 2.0 h, and the brain/plasma ratios were calculated. The results showed that the brain/plasma ratio of **23j** was lower than one at 0.5 h after one single administration (3 mg/kg i.v.) and reached 1.5 at 2.0 h due to lower clearance in the brain. When analyzed after a single oral administration, the concentration of compound **23j** in the brain was too low to test. The BBB permeability of compound **36b** was found to be prominent after a single i.v. administration achieving a brain/plasma ratio high to 4.5 at 0.5 h, and this ratio was maintained at a high level above five until 2.0 h. Notably, the brain concentration of compound **36b** rapidly reached 1553 ng/mL, which was higher than the i.v. C_{max}. Considering the brain/plasma ratio after a single oral administration was calculated from 0.74 to 0.97, compound **36b** seemed to provide a moderate brain exposure (from 310 to 180 ng/mL). As can be seen from the pharmacokinetic profiles of **23j**, the carbazole derivatives may have a poor oral bioavailability. Then, in order to obtain candidate potential compounds in the next catalepsy tests and hyperactivity tests, i.p. administration was processed to evaluate the major therapeutic effects and side effects.

2.9. hERG channel blockade and cytotoxicity tests

Unwanted inhibition of the hERG channel can induce severe cardiac arrhythmias, such as long QT syndrome characterized by prolonged QT intervals and Torsades de pointes[51]. The inhibition of the hERG channel of the selected compounds was further tested by patch-clamp assays to predict cardiovascular toxicity (Supporting Information Fig. S8)[52]. As the results are shown in Table 8, RPD and compounds **23j**, **36a**, **36b**, **36f** were tested for their blockage to hERG showing the IC$_{50}$ as 96, 2620, 1410, 1750, and 46,950 nmol/L, respectively. Compound **36f** (hERG IC$_{50}$ > 40 µmol/L) exhibited good safety profile in terms of cardiotoxicity. Compound **23j** had a lower hERG inhibition than the others. Furthermore, **36a** showed potential selectivity to D$_3$R compared with hERG. Compound **36b** was found to have higher hERG inhibition but still had a comparative advantage *versus* RPD (hERG IC$_{50}$ = 96 nmol/L as tested at the same conditions). Next, further cytotoxicity tests to evaluate the safety of candidates were performed in HEK293T, BV2 and PC12 cells. The results were shown in Supporting Information Fig. S9 indicating that **36b** and
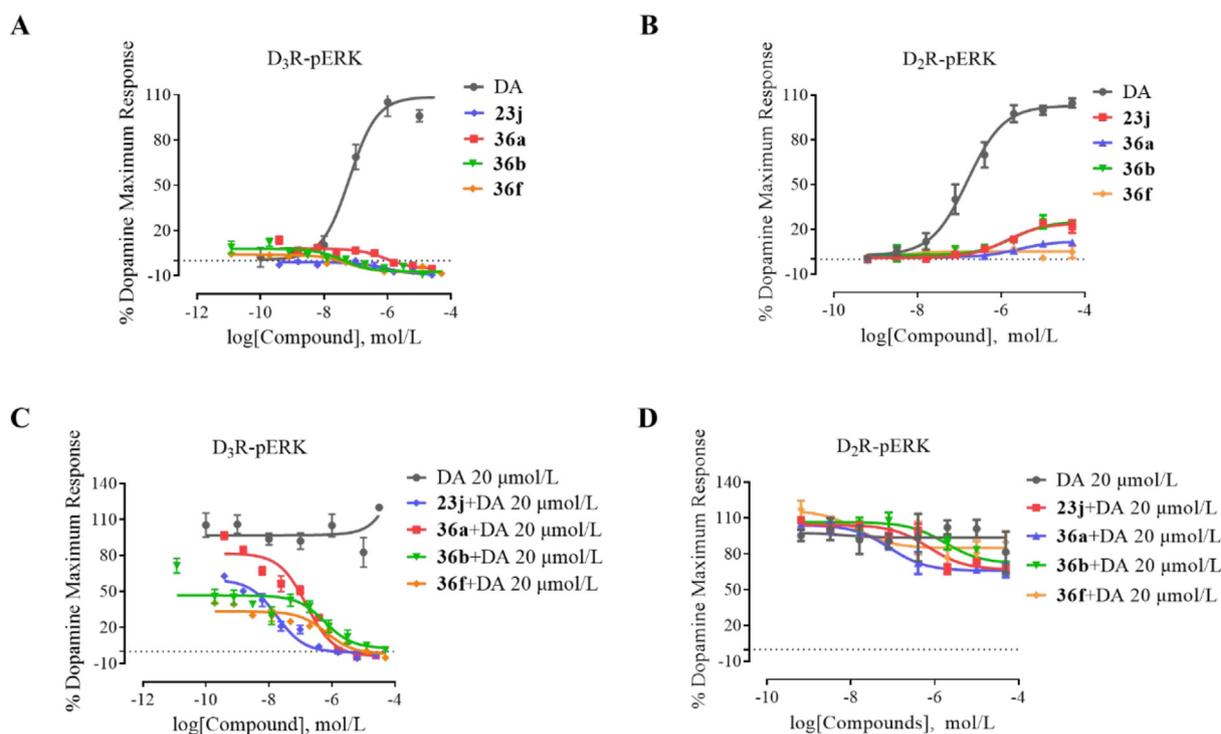
Figure 3 Functional characterization of lead compounds at D_3 dopamine receptors by ERK1/2 phosphorylation measurement. (A) Agonist dose−response curves for ERK1/2 phosphorylation mediated by hD_3R and (B) ERK1/2 phosphorylation mediated by hD_2R; (C) Competitive antagonist dose−response curves for ERK1/2 phosphorylation mediated by hD_3R and (D) ERK1/2 phosphorylation mediated by hD_2R in the present of 20 μmol/L dopamine.

36f exhibited less effects on the viability of HEK293T and BV2 than that of RPD. In PC12 cells, **36b** shown the best safety profiles. But compounds **23j** and **36a** unexpectedly exhibited significant cytotoxicity at high concentrations.

2.10. Acute toxicity assays and catalepsy tests in mice

One of the major obstacles to using antipsychotics is their propensity to produce extrapyramidal motor side effects. The catalepsy tests were used to detect the extrapyramidal side effects in mice during the antipsychotic drug discovery[53,54]. But before catalepsy tests, preliminary acute toxicity assays in mice were performed to observe the maximum tolerated dose for the tested compounds as before[55]. All mice were intraperitoneally injected with tested compounds and persistently observed for 24 h. The results were presented in Supporting Information Table S6, and compounds **36b** and **36f** were found well tolerant even at the highest concentration (600 mg/kg, i.p.). Compound **23j** showed a dose-dependent lethal toxicity from 240 to 600 mg/kg. For the dose of 240 mg/kg or above, the serious death often occurs after 12 h. As the example of **36f**, it took doses of 480 mg/kg or above to cause death in individual mice. Known the maximum tolerated dose for the tested compounds, catalepsy was then tested on each compound with increasing concentration gradients by i.p. administration[56]. The number of the mice with cataleptic reaction in each group was recorded. The ED_{50}, minimum effective doses (MED), and peak effects (percent of cataleptic animals) were accordingly calculated. As shown in Table 8, RPD was used as the positive control drug and induced a significant cataleptic effect from 2.4 mg/kg. It was found that almost all mice exhibited catalepsy at the high concentrations of RPD, while the ED_{50} was

approximately 1.51 mg/kg, which was related with its solid antagonistic effect on the D_2R. In contrast, compounds **23j**, **36a**, **36b** and **36f** did not elicit catalepsy under all tested doses (i.p.). The highest safe dose of **23j** was found between 240 and 480 mg/kg (i.p.) since 240 mg/kg (i.p.) induced no catalepsy but 480 mg/kg (i.p.) was lethal for mice. 480 mg/kg (i.p.) for **36a/b/f** was found to be safe, and no higher dosage was investigated, considering its effective dose of 10−30 mg/kg. These results showed that this series of D_3R-seletive ligands had a high threshold for catalepsy, which was associated with its low affinity to D_2R.

2.11. MK-801-induced hyperactivity tests and apomorphine-induced climbing tests of selected compounds

Schizophrenia is a heterogeneous disorder with positive symptoms (delusions, hallucinations, thought disorders), negative symptoms (anhedonia, avolition, social withdrawal, poverty of thought), and cognitive dysfunction[57,58]. But understanding of the biological origins of schizophrenia was still limited. Increased locomotor activity in response to psychotomimetic compounds such as apomorphine or noncompetitive N-methyl-D-aspartate (NMDA) glutamate receptor antagonists is commonly used as an indication of positive symptoms in schizophrenia[59]. It is well-known that, as an uncompetitive NMDA receptor antagonist, MK-801 could induce schizophrenic symptoms in healthy subjects and exacerbate existing psychoses in patients with schizophrenia[60]. So as the past research, MK-801-induced hyperactivity tests were used for relatively high-throughput evaluation of the candidate compounds in our study[47−49]. Selected compounds were tested in this model of ICR mice, before which the influences on the locomotion of selected compounds were firstly determined to avoid potential
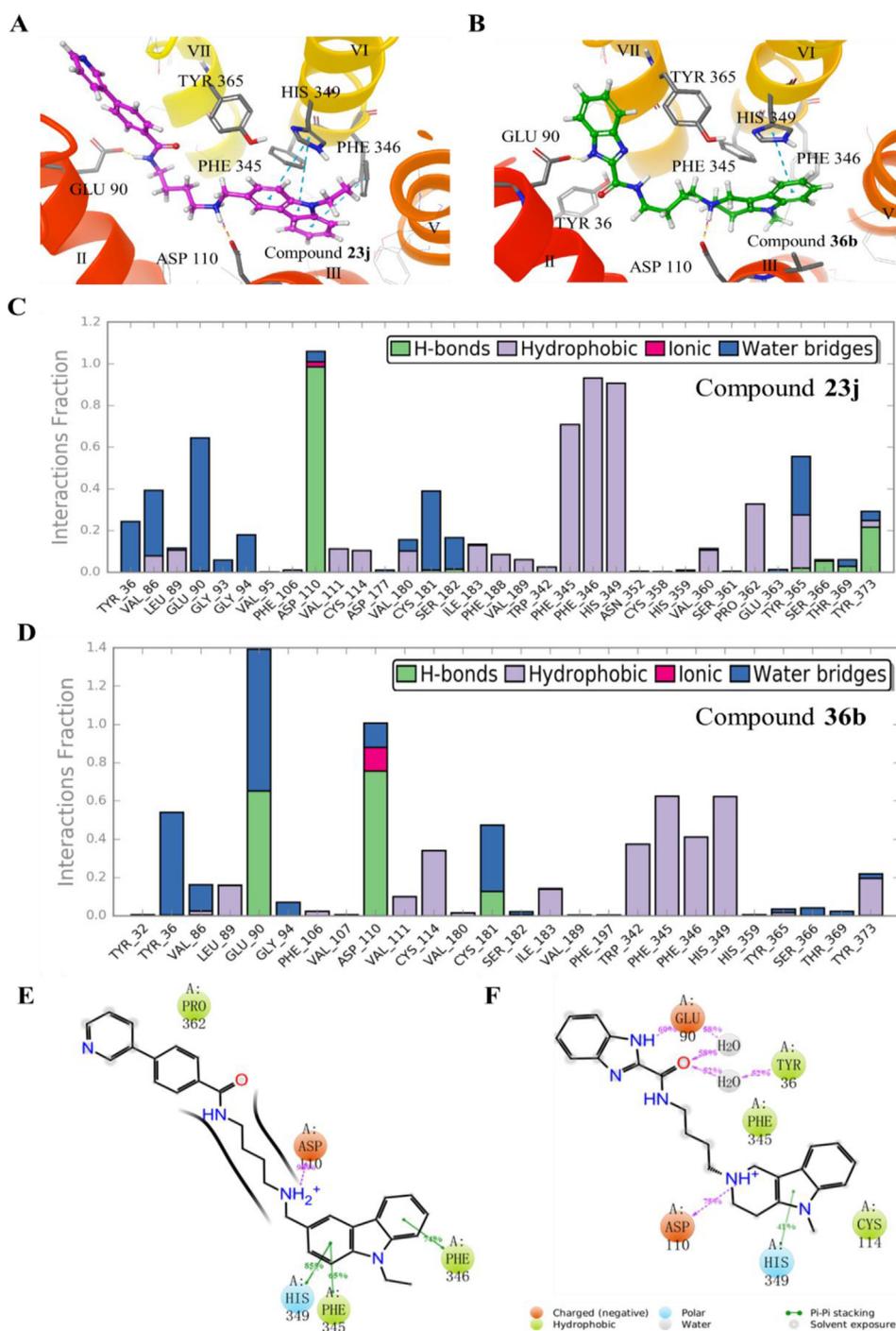
Figure 4 Binding modes analysis of **23j** and **36b**. (A) Predicted binding modes of compound **23j** with D$_3$R and (B) predicted binding modes of **36b** with D$_3$R. Images depicting the proposed binding modes were generated using Maestro software. Protein is shown as a cartoon, and small molecules are shown as sticks. Hydrogen bonds, $\pi-\pi$ stacking interactions, and electrostatic interaction are depicted by yellow, purple, and blue dashed lines, respectively. Residues of D$_3$R interacting with ligands are depicted by green sticks. (C) Protein–ligand contacts histograms of D$_3$R with **23j** and (D) protein–ligand contacts histograms of D$_3$R with **36b**. (E) Corresponding 2D interaction diagrams of **23j** and (F) corresponding 2D interaction diagrams of **36b** predicted through MD simulations; percentage suggests that for *X*% of the simulation time, the specific interaction is maintained.

psychotic side effects. Spontaneous locomotor results recorded for 15 min indicated that all tested compounds (**23j**, **36a**, **36b** and **36f**) showed no influences at 30 mg/kg i.p., and RPD (1 mg/kg) was tested as the positive drug showing a moderate inhibition (Fig. 5B). In the following tests, the process was shown in Fig. 5A

indicating that compounds and vehicle were administrated i.p. and followed by the s.c. administration of MK-801 (0.3 mg/kg) 10 min later. Then, the moving distance of mice in the next 60 min was recorded. As the results shown in Fig. 5C−H, MK-801 group was significantly longer compared with the control group. As the

Table 6 Binding affinities of compounds **23j** and **36b** for inhibiting radioligand binding to antischizophrenic drugs targets.

Targets	23j K_i (nmol/L)[a]	36b K_i (nmol/L)[a]	Targets	23j K_i (nmol/L)[a]	36b K_i (nmol/L)[a]
hD_1	> 10,000.0	> 10,000.0	$h5\text{-}HT_{2C}$	2230.0	5611.0
hD_2	> 10,000.0	> 10,000.0	$h5\text{-}HT_6$	8718.0	10,289.1
hD_3	16.8	19.6	hH1	5197.7	2314.5
hD_4	> 10,000.0	8813.0	$h\alpha1$	8353.1	2218.0
hD_5	> 10,000.0	> 10,000.0	hDAT	6097	1062.9
$h5\text{-}HT_{1A}$	578.7	274.5	hNET	> 10,000.0	> 10,000.0
$h5\text{-}HT_{1B}$	172.2	138.0	hSERT	5411.0	> 10,000.0
$h5\text{-}HT_{2A}$	214.4	398.2			

[a]K_i (nmol/L) values for the indicated compounds were determined as described in the Experimental Section.

Table 7 Pharmacokinetic and brain penetration properties of compound **23j** and **36b** in ICR mice ($n = 3$/group).

Parameters	Single-dose administration			
	3 mg/kg (i.v./**23j**)	10 mg/kg (*p.o.*/**23j**)	3 mg/kg (i.v./**36b**)	10 mg/kg (*p.o.*/**36b**)
C_{max} (ng/mL)	566.80	78.80	1226.50	505.20
T_{max} (h)	0.08	0.17	0.08	0.25
$t_{1/2}$ (h)	0.53	1.17	0.87.	1.14
AUC_{0-t} (h·ng/mL)	325.50	94.40	677.30	896.30
CL (mL/h/kg)	9208.50	—	4421.50	—
$MRT_{(0-t)}$ (h)	0.48	0.87	0.51	1.70
V_{dss} (mL/kg)	4439.60	—	2329.90	—
F	—	8.70	—	39.70
Brain concentration at 0.5 h (ng/mL)	98.04	—	1552.86	310.17
Plasma concentration at 0.5 h (ng/mL)	179.70	53.40	345.20	420.90
Brain/plasma ratio at 0.5 h	0.55	—	4.50	0.74
Brain concentration at 2.0 h (ng/mL)	11.48	—	138.55	181.65
Plasma concentration at 2.0 h (ng/mL)	7.17	2.89	24.87	188.03
Brain/plasma ratio at 2.0 h	1.60	—	5.57	0.97

positive drugs, RPD and CRP dose-dependently inhibited the MK-801-induced hyperactivity in our tests, and RPD showed more effective than CRP. Dose-dependent tests for **23j/36a/36b/36f** were performed at the doses of 3, 10, and 30 mg/kg. For all the tested carbazole derivatives, the dose of 30 mg/kg was found to be effective in the inhibition of MK-801-induced hyperactivity but not the spontaneous locomotion. Comparably, RPD by 1 mg/kg resulted in a significant inhibition of both spontaneous locomotion and MK-801-induced hyperactivity. Administration of 10 mg/kg was found effective ($P < 0.001$) for all derivatives, but the dose of 3 mg/kg did not show the inhibition activity at compounds **23j** and **36a**. Nevertheless, **36b** and **36f** administered i.p. at 3 mg/kg reduced the distance of mice movement compared with the MK-801 group ($P < 0.001$), suggesting the most potent inhibition of voluntary activity.

The antagonism of hyperlocomotion induced by dopamine receptor direct agonists (*e.g.*, apomorphine) is used to evaluate the antipsychotic efficacy[61]. Apomorphine-induced climbing tests could determine the attenuation of climbing behavior induced by apomorphine in mice for identifying potential antipsychotic activity. In this test, RPD and CRP exhibited dose-dependent inhibition to the apomorphine-induced climbing behavior (Fig. 5I and J). The concentration gradient as three times as RPD was set for the treatment with compounds **23j** and **36b**, showing that both compounds could dose-dependently inhibit the apomorphine-

induced hyperactivity (Fig. 5K and L). The results indicated that potent D_3R antagonism could significantly decrease the effects of apomorphine linked to behavioral agitation, one positive psychotic symptom.

2.12. *Antidepressant effects of compound 36b*

Regarding the exact nature relationship between depression and negative symptoms in a 'non-affective' psychotic illness such as schizophrenia[62], models of behavioral despair such as forced swimming test (FST) and tail suspension test (TST), were used to evaluate the negative symptom-like behavior in lots of reported works[63−65]. But due to limited antipsychotic drugs approved for depression, such as CRP, lurasidone, and quetiapine, there is a special significance to evaluating the antidepressant effects of antipsychotic compounds. In our study, considering more convincing safety, **36b** was further selected for more animal models of antipsychotic-drug-like activity and administrated by *p.o.* because of its satisfactory oral bioavailability. We continued investigating the antidepressant effects of **36b** using the FST and TST. The FST is widely used to study depressive-like behaviors in rodents; in this test, the rodent's immobility time and latency by first observed immobility reflect a measure of behavioral despair. TST is another measure of behavioral despair sensitive to a broad range of antidepressant drugs that makes it a suitable

Table 8 hERG channel inhibition and catalepsy induced by RPD and selected compounds[a].

Compd.	hERG inhibition IC$_{50}$ (nmol/L)[b]	Catalepsy		
		ED$_{50}$ i.p. (mg/kg)[b]	MED i.p. (mg/kg)[b]	Peak effect (%)[b]
RPD	96.0	1.5	2.4	100.0
23j	2460.0	NT[c]	≥240.0	0.0
36a	1315.0	NT	≥480.0	0.0
36b	1750.0	NT	≥480.0	0.0
36f	46,950.0	NT	≥480.0	0.0

[a]ED$_{50}$, Minimum effective doses (MED), and maximal effects (percent of cataleptic animals) are shown.
[b]All values were tested for three times.
[c]Not tested.

screening test. In our tests, duloxetine (DLX), considered one of the most effective antidepressant drugs in clinical practice, was used as the positive control. Administration of DLX caused a robust decrease in the immobility time in FST and TST, demonstrating its extraordinary antidepressant effect. Once

intraperitoneal treatment with compound **36b** dose-dependently decreased the immobility time in the TST compared with the control group ($P < 0.05$), but 3 mg/kg group did not show significant differences (Fig. 6A). For the latency by first observed TST immobility, only 30 mg/kg group was found to be prolonged

Figure 5 Evaluation of selected compounds in animal models of antipsychotic-drug-like activity. (A) The process of MK-801-induced hyperactivity tests and apomorphine-induced climbing tests. (B) Effects of RPD (1 mg/kg) and selected compounds (30 mg/kg) on spontaneous locomotor of mice. (C–H) Effects of RPD, CRP, and selected compounds in concentration gradients on MK-801-induced hyperlocomotion of mice. (I–L) Effects of RPD, CRP, and selected compounds in concentration gradients on apomorphine -induced hyperlocomotion of mice. Results are expressed as the means ± SEM of distance traveled ($n = 6-10$/group). Statistical evaluation was performed by one-way ANOVA followed by Dunnett's test for multiple comparisons. $^{\#\#}P < 0.005$ and $^{\#\#\#\#}P < 0.0001$ *versus* control group; $^{***}P < 0.001$ and $^{****}P < 0.0001$ *versus* vehicle group.

Figure 6 Evaluation of compound **36b** in animal models of behavioral despair and cognitive deficit. (A–B) Effects of compound **36b** (3, 10, 30 mg/kg) in the ICR mice TST ($n = 8-10$/group). (C–D) Effects of compound **36b** (3, 10, 30 mg/kg) in the ICR mice FST ($n = 8-10$/group). Effects of compound **36b** (3, 10, 30 mg/kg) on MK-801-induced object recognition disruption in mice ($n = 8-10$/group). (E) Exploration times in the training and (F) the recognition index in exploring the same objects were scored ($n = 8-10$/group). (G) Exploration times in the acquisition trial and (H) the novelty discrimination index (NDI) in exploring a familiar and novel object during acquisition trials (after 24 h training) were scored ($n = 8-10$/group). Results are expressed as the means ± SEM of distance traveled. Statistical evaluation was performed by one-way ANOVA followed by Dunnett's test for multiple comparisons. $^{\#\#}P < 0.005$ and $^{\#\#\#\#}P < 0.0001$ *versus* control group; $^{***}P < 0.001$ and $^{****}P < 0.0001$ *versus* vehicle group.

(Fig. 6B). So as immobility time of FST, 30 mg/kg group showed activities, but lower dose groups did not (Fig. 6C). DLX and compound **36b** failed to prolong the FST latency (Fig. 6D). The results indicated that compound **36b** exhibited potential antidepressant-like activity.

2.13. Effects of compound 36b on MK-801-induced novel object recognition (NOR) disruption in mice

Cognitive deficit is considered a core feature of schizophrenia, and it lacks responsiveness to many current antipsychotic drugs. NOR is a form of visual-recognition memory dependent on animals' innate preference to investigate novel objects, which is decreased in schizophrenia patients because of their visual-recognition memory impairments[66]. NOR models have been widely used to assess procognitive effects as quick, and straightforward preclinical models. The current study found that MK-801 could induce cognitive impairment in schizophrenia, consistent with previous reports disrupted NOR[67]. During this study, compound **36b** and RPD were administered intraperitoneally for four days before the acquisition trial. On the fifth day,

1 h after administration, the training trial was performed by recording the exploration time for the two identical objects A. Then, 1.5 h later, one of the objects A was replaced by a novel object B for the recognition trial. During the training trial, no difference was found among the vehicle, MK-801, RPD, or **36b** groups in the total object exploration time (Fig. 6E). Moreover, the recognition index, which was calculated as the percentage of novel object interaction time relative to total interaction time during the retention trial, was indiscriminate for all groups (Fig. 6F). In the testing session (Fig. 6G), total interaction time to objects A and B was increased significantly for the MK-801 group compared with control due to MK-801-induced hyperactivity. As shown in Fig. 6H, the control group of mice spent more time exploring the novel object than the familiar object. In contrast, throughout the experiment, MK-801 (0.2 mg/kg, i.p.)-treated mice spent less time exploring the novel object, indicating that MK-801 treatment resulted in a cognitive deficit. The deficit in NOR index induced by MK-801 was alleviated by **36b** (30 mg/kg, i.p.) but not RPD (1 mg/kg). These results suggested that compound **36b** improved cognitive ability during the NOR tasks in mice.

2.14. Weight gain and serum prolactin

The potential adverse effect profile was also assessed in terms of its ability to induce weight gain and high prolactin levels. Successive *p.o.* administrations of the compounds **23j** and **36b** at 30 mg/kg were performed for 30 days (one time/day). The positive group was administrated with 1 mg/kg RPD and control group was treated with vehicle in the same process. The Body weight for each mouse was recorded every day. No influence on body weight was observed for either compound compared with the control (Supporting Information Fig. S10A). Nevertheless, weight gain trends and the daily behavior of **23j** and **36b** administration groups were unaffected. The weight gain of RPD group grew obviously faster 15 days later from the beginning. Moreover, after 30 days of dosing, RPD raised the serum prolactin level but compounds **23j** and **36b** resulted in nonsignificant serum prolactin change (Fig. S10B). These results suggested that both compounds displayed a remarkable safety profile.

3. Conclusions

The D$_{2/3}$R binding affinities and functional selectivity have been studied for decades to explore the benefits for treating the schizophrenia. Highly D$_3$R selective antagonists exhibited some promising profiles in mood regulation, cognitive protection, and low side effects. We aimed to develop novel D$_3$R selective ligands with improved *in vivo* activity and drug properties for further development as clinical antipsychotics. Starting with virtual screens, a series of carbazole and tetrahydro-carboline derivatives were obtained through bitopic design and scaffold hopping strategy. A large proportion of compounds with the carbazole scaffold exhibited excellent binding affinities for the D$_3$R with good selectivity against D$_2$R. The tetrahydro-γ-carboline derivatives were found improved in D$_3$R binding affinities, but the tetrahydro-β-carboline showed less potential. In the functional studies, D$_3$R antagonism and weak D$_2$R partial agonism were identified for the novel ligands. Among the derivatives, compounds **23j** and **36b** were selected as our lead compounds, showing a moderate selectivity to D$_3$R over 5-HT$_{1A}$, 5-HT$_{1B}$, and 5-HT$_{2A}$ receptors and a weak affinity to the D$_2$, 5-HT$_{2C}$, and 5-HT$_6$ receptors. Binding mode analysis verified the scaffold hopping strategy's feasibility and understanding of the bitopic binding to D$_3$R. According to the data in MK-801-induced hyperactivity and catalepsy tests, these new D$_3$R selective antagonists were all effective in inhibiting the hyperactivity without cataleptic reactions. Further, compounds **23j** and **36b** dose-dependently reduced apomorphine-induced hyperactivity without cataleptic reaction. Compound **36b** also possessed unusual antidepressant-like activity in the FST and the TST. Moreover, compound **36b** alleviated the MK-801-induced disruption of novel object recognition in mice. Furthermore, the PK/PD studies of the compound **36b** in mice displayed superior profiles, including moderate drug exposure and excellent brain penetration properties. In addition, compound **36b** exhibited promising safety profiles in long-term drug administration without serum prolactin levels and weight gain change. Taken together, the results supported **36b** as a suitable lead compound, and additional investigation for developing **36b** into a promising pharmacotherapeutic for the treatment of psychoses is needed.

4. Experimental

4.1. Virtual screening and induced-docking

4.1.1. Ligand-based virtual screening
Pipeline Pilot 8.5 of Accelrys was used to perform all of the similarity searches[46]. The fingerprint ECFP_6 was generated for each structure of (R)-PG648, RGH-188, and GSK598809, then similarities were calculated using Tanimoto coefficient. Then, more than 9000 analogs were identified for further screening.

4.1.2. Structure-based virtual screening
Dopamine D$_3$ receptor crystal structure (PDB ID: 3pbl) was selected for the study in silico. Afterward, the D$_3$R structures was prepared by the Protein Preparation Wizard module in Schrodinger 10.2 software. The protein was assigned bond orders, added hydrogens, protonated, removed all crystallographic water molecules, and restrained minimization. Then, the eticlopride was docked into prepared model to check the reasonability for further docking screen. In next step, approximately more than 9000 compounds obtained from the similarity search were prepared to generate conformations by the LigPrep module of the Schrodinger suite (Schrodinger, NY, USA). Then, energy-minimized conformations were docked into the eticlopride binding site of the crystal structure. Then, three predicted binding poses were generated for each compound. According to the score obtained by the extra precision (XP) scoring function of the Glide module, 831 compounds (XP GScore < −9) were retained. The remaining 490 compounds were selected after structurally clustered into 26 clusters based on the Tanimoto coefficients computed using the ECFP_6 fingerprint. Lastly, 65 candidate compounds were purchased for further evaluation by radioligand competition binding assays.

4.1.3. Induced-docking
The prepared crystal structure (PDB ID: 3pbl) was used in the Induced Docking module with XP. For ligand preparation, conformations of ZLG-25, **23j** and **36b** were generated and energy-minimized by the LigPrep module. Images depicting the proposed binding modes were generated using Maestro 11.5.

4.2. Molecular dynamics simulations

Following molecular docking, 200 ns MD simulations for ZLG-25, 300 ns MD simulations for **23j** and **36b** were performed ligand−protein complex using the Desmond software[46]. According to the membrane position loading from the Orientations of Proteins in Membranes (OPM) database, D$_3$R crystal structure (PDB ID: 3pbl) was accurately set up to the phospholipid bilayer[68]. Na$^+$ and Cl$^−$ ions were added at the physiological concentration of 0.15 mol/L to ensure the overall neutrality of the systems. Simulations were conducted with an OPLS3 force field and a TIP3P explicit solvent model. We chose a 4.8 ps recording interval, and the NPT ensemble was employed with temperature fixed at 300 K and pressure at 1.01 bar. The integration time step was set at 2 fs. The model systems were relaxed using a six-step default protocol implemented in Desmond and utilized to prepare systems for production quality simulation. Default settings were used for all other parameters. The simulation interaction diagram analysis tool was used to monitor energetics, RMSD fluctuations,

hydrogen-bond distances, angles, and van der Waals interactions over the simulation trajectories.

4.3.   Chemical synthesis

All commercially available reagents and solvents were used without further purification. Reactions were monitored by thin-layer chromatography (TLC) on precoated glass silica gel plates (GF254, 0.25 mm, Yantai Xinde Chemical Co., Ltd.) using a CH_2Cl_2/MeOH/50% aq NH_4OH system or an EtOAc/petroleum ether system. Column chromatographic purification was carried out using silica gel. Melting points were determined using an X-4 micro melting point apparatus. ^1H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance III 400 spectrometer using chloroform-d or DMSO-d_6 as the solvent. J values were reported in hertz (Hz). Chemical shifts were given in δ values (ppm), using tetramethylsilane (TMS) as the internal standard. Signal multi-plicities are characterized as the following abbreviation: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), and br (broad signal). All final test compounds' purity is determined by highperformance liquid chromatography (HPLC), and all final test compounds display purity higher than 95%. HPLC methods used the following: Waters LC-20 AD spectrometer; Waters Xbridge BEH Amide column (4.6 mm × 250 mm, 5 μmol/L i.d.); mobile phase, H_2O aq/acetonitrile (30/70; Thermo Fisher Scientific Inc., USA) at a flow rate of 1 mL/min. The column temperature was 25 °C. The detection wavelength was 254 nm. The final formulations were characterized by elemental analysis recorded on a Vario EL III elementar, and results were shown in Supporting Information Table S4.

The synthesis and characterization of compounds are shown in the Supporting Information.

4.4.   In vitro studies

4.4.1.   Receptor binding studies[47−49]
The following specific radioligands and tissue sources were used: (a) the dopamine D_2 receptor [^3H] N-methylspiperone, from hD$_2$-CHO cells, (b) the dopaminergic D3 receptor [^3H] N-methyl-spiperone, from hD$_3$-CHO cells, (c) the serotonin 5-HT$_{1A}$ receptor [^3H] 8-OH-DPAT, from h5-HT$_{1A}$-CHO cells, (d) the serotonin 5-HT$_{1B}$ receptor [^3H] GR125743, from h5-HT$_{1A}$-CHO cells, (e) the serotonin 5-HT$_{2A}$ receptor [^3H] ketanserin in the presence of 4-piperidone hydrochloride hydrate (35 nmol/L), from h5-HT$_{2A}$-CHO cells, (f) the serotonin 5-HT$_{2C}$ receptor [^3H] mesulergine, in the presence of spiperone (40 nmol/L), from rat cerebral cortex, (g) the serotonin 5-HT$_6$ receptor [^3H] lysergic acid diethylamide, from h5-HT$_6$-CHO cells, (h) the dopamine transporter (DAT) [^3H] WIN35428, from rat striatum, (i) the noradrenaline transporter (NET) [^3H] nisoxetine, from hNET-CHO cells (j) the serotonin transporter (SERT) [^3H] paroxetine, from hSERT-CHO cells (k) the histamine H1 Receptor (H1) [^3H] mepyramine, from guinea pig cerebellum, and (l) the adrenergic α1 Receptor (α1) [^3H] prazosin, from rat cerebral cortex.

The radioligand competitive binding assay for each receptor was performed as follows. Compound **23j** was dissolved in 50% (v/v) DMSO, and the compound concentration was 2×10^{-3} mol/L; dilution to the initial concentration of the new compound, 2×10^{-4} mol/L, contained 5% DMSO. For one receptor binding assays, total binding (TB) was determined in the presence of the radioligand. Nonspecific binding (NB) was determined in the presence of the radioligand and competitive ligand for the related

receptor, whereas compound binding (CB) was determined in the presence of the radioligand and compound **23j**. Each specific binding (SB) was calculated as the total binding (TB) minus the nonspecific binding (NB) at a particular concentration of radio-ligand. Each percentage of inhibition (%) was calculated as Eq. (1):

Percentage of inhibition (%) = [(TB−CB)/(TB−NB)] × 100   (1)

Blank binding experiments contained 0.25% (v/v) DMSO were performed; DMSO had no effect. All compounds were tested at least three times over a 6-fold concentration range (10^{-5} to 10^{-10} mol/L). IC$_{50}$ values were determined by nonlinear regres-sion analysis with fitting to the Hill equation curve. K_i values were calculated using the Cheng and Prussoff equation as Eq. (2):

$$K_i = IC_{50}/(1 + C/K_d) \qquad (2)$$

where C represents the concentration of the hot ligand used and K_d is the receptor dissociation constant of each labeled ligand. The K_i value was derived from at least three independent experiments.

4.4.2.   Total ERK1/2 and pERK1/2 measurement[69,70]
Total ERK1/2 and pERK1/2 measurement was performed by using total ERK cellular kit (#64NRKPEG, Cisbio, Codolet, France) and advanced ERK phospho-T202/Y204 kit (#64AER-PEG, Cisbio, Codolet, France). All steps were carried out per manufacturer instructions. In brief, CHO-D$_2$R cells or CHO-D$_3$R cells were grown at 37 °C, 5% CO_2 in DMEM +10% FBS until approximately 80% confluent. Cells were seeded in polystyrene-treated 96-well cell culture plates at approximately 50,000 cells/well and cultured overnight in 50 μL serum-free growth media. After 18−24 h of overnight culture, tested compounds were pre-pared in DMEM+0.1% FBS, added to the cells and incubated at 37 °C, 5% CO_2, for 15 min. For the measure of the antagonism, dopamine was added to the wells in 20 μmol/L for p-ERK as-sessments and incubated at 37 °C, 5% CO_2 for 7−8 min after the incubation of tested compounds. For the measure of the agonism, DMEM+0.1% FBS was added instead. Then, the supernatant was removed, and cells were lysed with 1 × lysis buffer (prepared per manufacturer's protocol) for 30 min at room temperature (RT) while shaking. Next, 16 μL of cell lysates were transferred to an assay plate (384-OptiPlate, PerkinElmer). p-ERK antibodies were prepared and added per manufacturer's protocol: 2 μL of Eu3$^+$ Cryptate working antibody (donor) solution and 2 μL of d2 working antibody (acceptor) solution were added to each well and incubated overnight at RT. Fluorescence emission at 2 different wave-lengths (665 and 620 nm) were measured on an Envision plate reader (PerkinElmer, Waltham, Massachusetts), and the HTRF ratio of the acceptor and donor emission signals for each well was calculated as Eq. (3):

HTRF ratio = (665 nm/620 nm) × 10,000   (3)

All samples were tested in duplicates. The results were calculated as a percent of control after dividing the phospho-ERK1/2 by total-ERK1/2. Data for each group were averaged and presented as mean ± SEM. Data were assessed for normality (Shapiro−Wilk) and homoscedasticity (Brown-Forsythe). Statis-tical significance was determined by one-way analysis of variance

(ANOVA) with Tukey's multiple comparison post-test. Comparisons were considered statistically significant when $P < 0.05$.

4.4.3. hERG affinity

CHO cells were stably transfected with hERG cDNA and cultured in F12 medium (Gibco) supplemented with 10% (v/v) fetal bovine serum (FBS) and 0.5 mg/mL Geneticin (Invitrogen) at 37 °C in a humidified environment (5% CO$_2$/95% air). The cells were seeded out two days before reaching 70% confluency. Prior to use, the cells were washed in PBS and incubated with 5 mL Detachin (Genlantis) for 4−5 min at 37 °C to detach cells from the culture dish. The harvested cells were re-suspended in F12 medium at a density of 2 million cells/mL. The cells were transferred to a QPatch instrument (Sophion Bioscience, Denmark) and allowed to recover for 20 min in the Qstir cell preparation station on the Qpatch-8 before experiment. The tail currents of hERG channel were evaluated using the Q-patch automated patch clamp platform (Sophion Bioscience, Denmark). The following solutions were used during patch-clamp recording (compositions in mmol/L): internal solution: KCl 120, CaCl$_2$ 5.374, MgCl$_2$ 1.75, KOH 31.25, EGTA 10, HEPES 10, Na$_2$ATP 4, pH 7.2 (KOH); external solution: NaCl 145, KCl 4, MgCl$_2$ 1, CaCl$_2$ 2, HEPES 10, glucose 10, pH 7.4 (NaOH). All solutions were sterile filtered. Cells were clamped at −80 mV and hyperpolarized to −100 mV to monitor the change of series resistance. The voltage protocol for hERG ion channel started with a short (200 ms) −50 mV step to establish the baseline region. A depolarizing step was applied to the test potential of 20 mV for 2 s, and then the cell was depolarized to −50 mV to evoke outward tail currents. Currents were filtered using the internal Bessel filter in Qpatch. Recording started in external solution. After this control period, 5 increasing concentrations of the test compounds were applied, each for approximately 4 min to record a complete concentration−response curve. The last control period (saline) is used as baseline for data normalization. Cisapride (2 µmol/L) was applied as a reference inhibitor at the end of protocol. The sampling frequency is 2000 Hz. Data were acquired and analyzed using the PatchMaster software (HEKA). The compounds **23j**, **36a**, **36b** and **36f** were dissolved in extracellular solution to get different concentrations of solutions: 0.412, 1.23, 3.70, 11.1, 33.3, 100 µmol/L.

4.4.4. Cytotoxicity test

Cytotoxicity of selected compounds was determined using the cell counting kit-8 (CCK8) assay kit (Beyotime, Shanghai, China). Briefly, HEK293T, BV2, and PC12 were cultured in 96-well plates at a density of 8000 cells per well at 37 °C overnight. Then, different concentrations of selected compounds were added into cells and incubated for 24 h. According to the manufacture's instruction, 10 µL of CCK8 detection reagent was added into 100 µL of medium per well. After 1 h, absorbance was read at 450 nm for CCK8 using Spark® Multimode Microplate Reader. Cell inhibition was calculated as relative absorbance compared to a DMSO-only control. And dose−effect relationship curves were calculated for the IC$_{50}$.

4.5. In vivo efficacy studies

4.5.1. Animals and compounds

Male adult C57BL/6 mice (6−8 weeks old) and ICR mice (6−8 weeks old) were obtained from Beijing Vital River Lab Animal Technology Co., Ltd. All animals were housed in cages under artificial lighting from 7:00 AM to 7:00 PM, with free access to food and water. Animals were assigned to different experimental groups randomly, each kept in a separate cage. All experimental procedures were approved by the Peking University Institutional Animal Care and Use Committee. All compounds used for *in vivo* assays were produced as the formation of hydrochloride with the superior water solubility, and the final formulations were characterized by elemental analysis (Table S5).

4.5.2. Catalepsy test[32,49]

Mice were injected intraperitoneally with vehicle, RPD of 0.6, 1.2, 2.4, 4.8, 9.6 mg/kg, compound **23j** of 15, 30, 60, 120, 240 mg/kg, **36a/b/f** of 30, 60, 120, 240, 480 mg/kg. Catalepsy was tested individually 15, 30 and 45 min after injection. The test consisted in positioning the animal with its forepaws on the wood particle (3 cm in height) and recording how long it remained hanging onto the bar; the end point was 60 s and an all-or-none criterion was used. A mean immobility score of 30 s was used as the criterion for the presence of catalepsy. The number of the mice with positive reaction in each group was recorded. The ED$_{50}$, minimum effective doses (MED), and maximal effects (percent of cataleptic animals) were accordingly calculated.

4.5.3. Spontaneous locomotor test[47,49]

ICR mice (10 mice in each group) were dosed with the vehicle and selected compounds (30 mg/kg) by intraperitoneal injection. Animals were placed in Plexiglas cages for evaluating locomotor activity. After the environmental adaptation for 30 min, the total locomotor distance of each animal was recorded for 15 min and automatically measured by the spontaneous activity video analysis system. All cages were changed after every test.

4.5.4. MK-801-induced hyperactivity[47−49]

ICR mice (divided into several groups, 10 mice in each group) were intraperitoneally injected with vehicle (vehicle group and MK group) or increasing doses of selected compounds (3, 10, 30 mg/kg). RPD and CRP were used as the positive drug with the dose of 0.1, 0.3, 1, 3 mg/kg. After 10 min, were intraperitoneally injected with normal saline, while other groups of mice were challenged with 0.3 mg/kg of MK-801 s.c. After injection, mice were immediately placed in Plexiglas cages for evaluating locomotor activity for 60 min. The total locomotor distance of the mice movement during 60 min was automatically recorded and measured by the spontaneous activity video analysis system. All cages were changed after every test.

4.5.5. Apomorphine-induced climbing[47−49]

ICR mice were divided into several groups with 10 mice in each group. The vehicle group and APO group of mice were intraperitoneally injected with vehicle. The tested compounds groups were intraperitoneally injected with increasing doses of compound **23j** and **36b** (0.3, 1, 3, 10, 30 mg/kg). After 30 min, the model group and tested groups were challenged with 1.0 mg/kg of the apomorphine in 0.9% NaCl +0.1% ascorbic acid by subcutaneous injection. After the injection of apomorphine, the mice immediately were placed in cylindrical wire cages (12 cm in diameter, 14 cm in height), and observed for climbing behavior at 10, 20, 30 min post dose. The climbing behavior was scored as follows: 4 paws on the cage floor with normal activity = 0 score; 4 paws on the cage floor with an increase in activity or sniffing = 1; 2 paws on the reseau occasionally = 2; 4 paws on the reseau occasionally = 3; 4 paws on the reseau all

the time = 4. RPD was used as the positive drug with the dose of 0.1, 0.3, 1, 3, 10 mg/kg.

4.5.6. Tail suspension test[71]

ICR mice (10 mice in each group) were orally dosed with vehicle, duloxetine (15 mg/kg) or increasing doses of **36b** (3, 10, 30 mg/kg) for 3 days. After the last administration for 30 min, the mice were suspended individually by the tail using adhesive tape (attached 2 cm from the tip of the tail) to a hook attached to a strain gauge and the behavior changes in 6 min of the mice were recorded by a camera system. Then, the latency time (the immobility first observed) was calculated and duration of immobility in the last 4 min of the recorded time was measured.

4.5.7. Forced swimming test[71]

ICR mice (10 mice in each group) were orally dosed with vehicle, duloxetine (15 mg/kg) or increasing doses of **36b** (3, 10, 30 mg/kg) for 3 days. After the last administration for 30 min, each mouse was placed in a clear cylindrical tank (40 cm tall × 20 cm diameter), filled with water (30 cm tall) at 24 ± 2 °C. Mice were judged as immobile when they float motionlessly. Mice were forced to swim freely for 6 min with record by a camera system, and then the latency time (the immobility first observed) was calculated. Then, the duration of immobility in the last 4 min was measured when analyzing. Water was changed after every test.

4.5.8. Novel object recognition training and testing[49]

The first day of the experiment is the adaptation period. The mice were put into the empty box from the center of the side. The mice were allowed to adapt to the environment for 10 min freely. The second day is learning phase. Two object A were put into the box, 10 cm close to the same side of the box, and the distance between the two objects is 40 cm. Then the mice were put individually into the box and given 5 min explore the area and objects. Memory retention was tested 1.5 h after training. One of the two object A was replaced by an object B, then put the mice individually into the box and gave them 5 min to explore. The motion trails of the mice were recorded by the image processing system of computer. The NDI was calculated by Eq. (4):

$$NDI~(\%) = (\text{Novel object interaction time/Total interaction time}) \times 100 \qquad (4)$$

Mice were divided into 6 groups, 10 mice in each group. 5 groups of mice were orally dosed with vehicle, RPD (1 mg/kg) or increasing doses of compound **36b** (3, 10, 30 mg/kg) for 5 days, starting from 4 days before the adaption period. On the 5th day, the untreated group of mice and those dosed with RPD or compound **36b** were administrated i.p. with MK-801 (0.2 mg/kg) 30 min before the experiment.

4.5.9. Weight gain and serum prolactin

Mice (10 mice in each group) were orally dosed with vehicle, **23j** (30 mg/kg) or **36b** (30 mg/kg) for 28 days. The weight of each mouse was recorded before intragastric administration every day. The mice were killed by decapitation 180 min after the last treatment. Blood samples (2 mL) were collected and centrifuged (300×g for 30 min), and the serum prolactin of the resulting serum sample was determined by an ELISA kit from Elabscience cto.

4.6. In vivo metabolism studies

4.6.1. Pharmacokinetics study in mice[71]

The HPLC conditions were as follows: column, Diamonsil C18 (150 mm × 4.6 mm, 5 μmol/L, 120 A); mobile phase, 0.1% formic acid in water/acetonitrile (Merck Company, Germany) (v/v, 0−8.0 min, 40:60); flow rate, 0.2 mL/min; column temperature, 40 °C. UV detection, 254 nm. ICR mice (n = 3/group) were dosed with compounds **23j/36b** via the tail vein for i.v. administration (3 mg/kg) or p.o. administration (10 mg/kg). After the last administration, 80 μL of orbital blood was extracted at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8 and 24 h. After separated by centrifugation (rpm = 18,000, 10 min), the plasma sample (30 μL) was prepared for high-pressure liquid chromatography/tandem mass spectrometry (LC−MS/MS) analysis by protein precipitation with acetonitrile (100 μL). The plasma samples were analyzed for drug and internal standard via an API 4000 Q trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) coupled with a 1200 series HPLC system (Agilent, Santa Clara, CA, USA). Isocratic elution was used with 80% acetonitrile and 20% water with 0.1% formic acid to separate analytes. The total run time was 3 min, and the flow rate was 0.3 mL/min.

4.6.2. BBB penetration study in mice

At 0.5 and 2 h after the last administration, after euthanizing the mice using CO_2 gas, the blood was collected from the heart immediately and the plasma was treated the same way as above. The remaining blood was washed out from the circulation by performing cardiac perfusion with physiological saline containing 10 U/mL heparin. The brain was then removed from the skull and added to three volumes of PBS buffer per weight, homogenized, and stored at −20 °C. The compound concentrations in plasma and brain samples were determined via the LC−MS/MS protocol.

4.7. Statistical analysis

All values are presented as mean ± SEM or mean ± SD. For the calculation of EC_{50}, the variable slope model was used with Eq. (5):

$$Y = Y_{min} + (Y_{max} - Y_{min})/(1 + 10^{((\log~EC_{50} - X) \times HillSlope)}) \qquad (5)$$

Differences between groups were analyzed by two-tailed Student's t test. One-way ANOVA followed by post hoc Bonferroni's or Donnet's multiple comparisons was used to compare more than two groups. Two-way ANOVA followed by post hoc Bonferroni's multiple comparisons test was used for comparison of a series of data collection among groups.

Author contributions

Zhongtang Li devised the project and conceived the main conceptual ideas. Zhongtang Li and Fan Fang designed the compounds. Zhongtang Li and Fan Fang synthesized, purified, and characterized all compounds. Yiyan Li and Xuehui Lv performed receptors binding and functional study. Ruqiu Zheng and Peili Jiao characterized the selectivity study and hERG inhibiton. Guiwang Zhu and Zefang Jin carried out docking and modeling studies. Yuxi Wang, Xiangqing Xu and Yinli Qiu performed all *in vivo* experiments. The manuscript was written through contributions of all authors. Guisen Zhang, Zhongjun Li and Zhenming Liu revised and submitted the manuscript on behalf of other authors. Liangren Zhang provided the financial support for this project. All authors have given approval to the final version of the manuscript.

Conflicts of interest

The authors have no conflicts of interest to declare.

Appendix A.   Supporting information

Supporting data to this article can be found online at https://doi.org/10.1016/j.apsb.2023.07.024.

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