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Pharmacological Characterization of H05, a Novel Serotonin and Noradrenaline Reuptake Inhibitor with Moderate 5-HT_{2A} Antagonist Activity for the Treatment of Depression[S]

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ABSTRACT

Multitarget antidepressants selectively inhibiting monoaminergic transporters and 5-hydroxytryptamine (5-HT) 2A receptor have demonstrated higher efficacy and fewer side effects than selective serotonin reuptake inhibitors. In the present study, we synthesized a series of novel 3-(benzo[d][1,3]dioxol-4-yloxy)-3-arylpropyl amine derivatives, among which compound H05 was identified as a lead, exhibiting potent inhibitory effects on both serotonin (K_i = 4.81 nM) and norepinephrine (NE) (K_i = 6.72 nM) transporters and moderate 5-HT_{2A} antagonist activity (IC_{50} = 60.37 nM). H05 was able to dose-dependently reduce the immobility duration in mouse forced swimming test and tail suspension test, with the minimal effective doses lower than those of duloxetine, and showed no stimulatory effect on locomotor activity. The administration of H05 (5, 10, and 20 mg/kg, by mouth) significantly shortened the immobility time of adrenocorticotropin-treated rats that serve as a model of treatment-resistant depression, whereas imipramine (30 mg/kg, by mouth) and duloxetine (30 mg/kg, by mouth) showed no obvious effects. Chronic treatment with H05 reversed the depressive-like behaviors in a rat model of chronic unpredictable mild stress and a mouse model of corticosterone-induced depression. Microdialysis analysis revealed that the administration of H05 at either 10 or 20 mg/kg increased the release of 5-HT and NE from the frontal cortex. The pharmacokinetic (PK) and brain penetration analyses suggest that H05 has favorable PK properties with good blood-brain penetration ability. Therefore, it can be concluded that H05, a novel serotonin and NE reuptake inhibitor with 5-HT_{2A} antagonist activity, possesses efficacious activity in the preclinical models of depression and treatment-resistant depression, and it may warrant further evaluation for clinical development.

Introduction

Depression, a serious worldwide health problem with a lifetime prevalence of 21%, is a common mood disorder characterized by a persistent feeling of sadness and loss of interest (McKenna et al., 2005). Among all mental diseases, depression carries the highest burden for patients and society, which can lead to disability and suicide (Whiteford et al., 2013).

Over the past 60 years, several different types of antidepressant (AD) agents, such as tricyclic ADs (TCAs), monoamine oxidase inhibitors, selective serotonin reuptake inhibitors (SSRIs), norepinephrine (NE) reuptake inhibitors, and serotonin and NE reuptake inhibitors (SNRIs), have been developed. TCAs and monoamine oxidase inhibitors are the first-generation ADs with unfavorable side effects due to their high affinities to muscarinic, adrenergic, and histaminergic receptors. The second-generation ADs, such as SSRIs and SNRIs, are the most commonly used in the clinic treatment currently because of their ease of use, relative safety regarding

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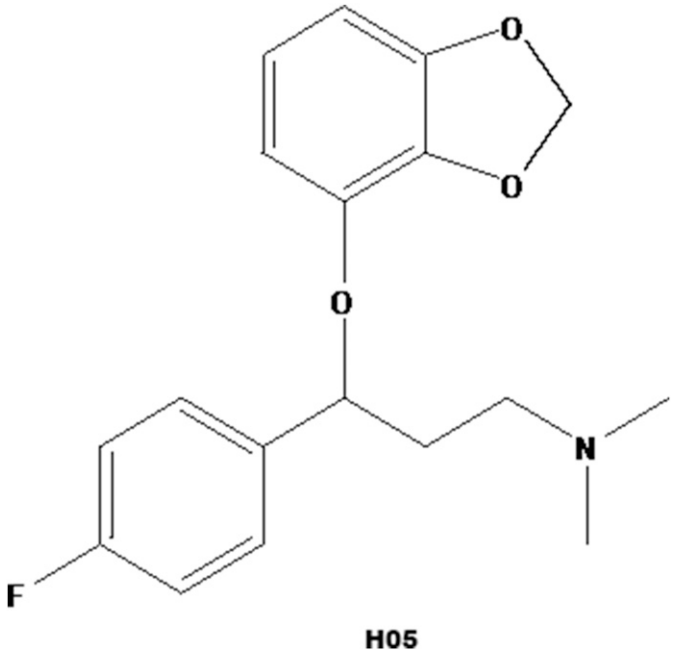
ABBREVIATIONS: ACTH, adrenocorticotropin; AD, antidepressant; ANOVA, analysis of variance; BZ, benzoyl chloride; CORT, corticosterone; CUMS, chronic unpredictable mild stress; DA, dopamine; DAT, dopamine transporter; DMSO, dimethylsulfoxide; DOI, *R*(−)-2,5-dimethoxy-4-iodoamphetamine; FLIPR, Fluorescent Image Plate Reader; FST, forced swimming test; H05, 3-(benzo[d][1,3]dioxol-4-yloxy)-3-(4-fluorophenyl)-*N*,*N*-dimethylpropan-1-amine; HPA, hypothalamic-pituitary-adrenal; 5-HT, 5-hydroxytryptamine; K_i, inhibition constant; MED, minimal effective dose; mPFC, medial prefrontal cortex; NE, norepinephrine; NET, norepinephrine transporter; PK, pharmacokinetic; ΔRFU, change in relative fluorescent unit; SD, Sprague-Dawley; SERT, serotonin transporter; SNRI, serotonin and norepinephrine reuptake inhibitor; SSRI, selective serotonin reuptake inhibitor; TCA, tricyclic antidepressant; T_{max}, time to maximal concentration; TRD, treatment-resistant depression; TST, tail suspension test; WIN-35428, ((−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane); UPLC, ultra-performance liquid chromatography.

overdose, and high tolerability (Goldstein and Goodnick, 1998; Millan, 2009). Although SSRIs and SNRIs are safer than TCAs, they still can cause some side effects, such as cardiac toxicity, sexual dysfunction, and sleep disturbance (Goldstein and Goodnick, 1998) with no significantly improved efficacy over the first-generation ADs (Goodnick and Goldstein, 1998; Anderson, 2000; Montgomery et al., 2007; Souery et al., 2011; Magni et al., 2013). There are about one-third to one-half of patients who fail to respond adequately to treatment with first-line ADs (Trivedi et al., 2006; Warden et al., 2007; Mathew, 2008). Such treatment-resistant depression (TRD) suggests substantial unmet medical needs (Little, 2009). Therefore, more effective ADs with fewer adverse reactions, especially for TRD therapy, are highly desired.

Accumulative evidence indicates that multitarget drugs selectively targeting monoaminergic transporters and the subtypes of 5-hydroxytryptamine (5-HT) receptor may be more effective and better tolerated than SSRIs (Rajkumar and Mahesh, 2010; Reinhold et al., 2012; Celada et al., 2013; Katona and Katona, 2014). 5-HT$_{2A}$ receptor, a subtype of 5-HT$_2$ receptor belonging to the serotonin receptor family, is a G protein–coupled receptor involved in the pathology of depression (Rosel et al., 2000; Bhagwagar et al., 2006; Aznar and Klein, 2013; Muguruza et al., 2014), and it can regulate the monoaminergic function in a direct or indirect manner (Di Giovanni, 2013). Drugs with 5-HT$_{2A}$ receptor antagonistic activities can either augment the clinical response to SSRIs in treatment-resistant patients (Carpenter et al., 1999; Ostroff and Nelson, 1999; Shelton et al., 2001; Carvalho et al., 2009) or cause fewer side effects, such as sexual dysfunction and sleep disorders (Feiger et al., 1996; Davis et al., 1997; Rush et al., 1998). Several clinical investigations have shown that mirtazapine, a multiaction AD with affinity for α2, 5-HT$_{2A}$ (K_i = 10 nM), 5-HT$_{2C}$, and 5-HT$_3$ receptors, can augment the clinical response to SSRIs in refractory depression patients (Carpenter et al., 2002; Wan et al., 2003; Blier et al., 2009). Vortioxetine, another multimodal AD, acts at the serotonin transporter (SERT) and several 5-HT receptor subtypes (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5-HT$_{3A}$, and 5-HT$_7$), showing some advantages over current ADs in the improvement of cognitive symptoms of depression (Pehrson et al., 2015). Trazodone and nefazodone, two ADs, have both high affinity at rat 5-HT$_{2A}$ receptor (K_i = 20 and 7.1 nM, respectively) and relatively low affinity at human SERT and norepinephrine transporter (NET) (K_i > 500 nM) (Owens et al., 1997). These two drugs, because of their 5-HT$_{2A}$ receptor antagonist activity, can produce fewer sleep disorders and less sexual dysfunction than SSRIs in the treatment of major depression (Davis et al., 1997; Khazaie et al., 2015). The atypical antipsychotic aripiprazole, a potent 5-HT$_{2A}$ receptor antagonist, was approved by the Food and Drug Administration as an adjunctive AD treatment of major depressive disorder (Mathew, 2008). All of these studies suggest that ADs with both monoaminergic reuptake inhibition effect and 5-HT$_{2A}$ antagonism properties represent an attractive strategy for depression treatment, especially for TRD therapy.

In the present work, we screened and identified some compounds that are capable of selectively inhibiting serotonin and NE transporters, as well as possessing 5-HT$_{2A}$ receptor antagonist properties. A series of 3-(benzo[d][1,3]dioxol-4-yloxy)-3- arylpropyl amine derivatives were synthesized, and 3-(benzo[d][1,3]dioxol-4-yloxy)-3-(4-fluorophenyl)-*N*,*N*-dimethylpropan-1-amine (H05) was characterized as a lead compound worthy of further studies in preclinical assays (for details, see Supplemental Materials). H05 exhibited robust AD effects in vitro and in vivo. The pharmacokinetic (PK) and blood-brain penetration analysis suggest that H05 possesses druggable properties with potential application in depression therapy.



H05

Fig. 1. Chemical structure of the lead compound H05.

Materials and Methods

Animals

Healthy male ICR mice (weight, 18–22 g) or male Sprague-Dawley (SD) rats (weight, 180–350 g) were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, People's Republic of China). Animals were housed in groups under standard conditions at a mean room temperature of 22 ± 2°C, humidity of 50% ± 10%, and a 12-hour light/dark cycle (lights on at 8:00 AM). Water and food were provided ad libitum except during the tests. Animals were adapted to laboratory conditions for about 1 week prior to testing. Animal experimental protocols were approved by the Animal Ethics Committee of Peking University Health Science Center and were performed in a manner consistent with the guidelines of the Bylaw of Experiments on Animals.

Chemicals

Compound H05 was synthesized by Jiangsu Nhwa Pharmaceutical Co., Ltd (Jiangsu, People's Republic of China). Its structure is depicted in Fig. 1. Chemicals such as duloxetine, fluoxetine, paroxetine, nomifensine, desipramine, 1-[2-[bis (4-fluorophenyl) methoxy] ethyl]-4-(3-phenylpropyl) piperazine dihydrochloride (GBR12909), adrenocorticotropin (ACTH; 1-24), *R*(−)-2,5-dimethoxy-4-iodoamphetamine (DOI), 5-HT, NE, and dopamine (DA) were purchased from Sigma-Aldrich (St. Louis, MO). Radioligands were purchased from PerkinElmer (Boston, MA). All compounds were dissolved in distilled water and administered by mouth in a concentration of 10 ml/kg, unless otherwise specified.

Measurement of Binding Affinity of H05 to Monoamine Transporters

The competitive binding assays for H05 and reference AD agents against rat SERT, NET, and DA transporter (DAT) were performed as

TABLE 1
Conditions for binding and uptake assays

Assays	Species	Tissue	[^{3}H]Ligand	Nonspecific Ligand	Reaction Conditions
			(nM)	(μM)	
SERT binding	Rat	Frontal cortex	Paroxetine (0.5)	Paroxetine (10)	23°C, 60 min
NET binding	Rat	Frontal cortex	Nisoxetine (0.5)	Desipramine (10)	25°C, 30 min
DAT binding	Rat	Striatum	WIN35428 (0.5)	Nomifensine (10)	4°C, 120 min
5-HT uptake	Rat	Frontal cortex	5-HT (20)	Paroxetine (10)	37°C, 10 min
NE uptake	Rat	Frontal cortex	NE (20)	Desipramine (10)	37°C, 10 min
DA uptake	Rat	Striatum	DA (20)	Nomifensine (10)	37°C, 10 min

previously described with minor modifications (Orjales et al., 2003; Wang et al., 2014). Briefly, rats were sacrificed by guillotine apparatus, and membrane proteins were prepared from rat frontal cortex (for SERT and NET) or striatum (for DAT). Competitive binding assays were performed in reaction buffer containing 50 μg of membrane proteins, [^{3}H]paroxetine (0.5 nM), [^{3}H]nisoxetine (0.5 nM), or [^{3}H] WIN-35428 [((−)-2-β-carbomethoxy-3-β-(4-fluorophenyl)tropane)] (0.5 nM) and various concentrations of compounds (10^{-1}–10^4 nM) at 23°C for 60 minutes (for SERT), at 25°C for 30 minutes (for NET), or at 4°C for 120 minutes (for DAT). Nonspecific binding to SERT, NET, and DAT was determined using 10 μM paroxetine, 10 μM desipramine, and 10 μM nomifensine, respectively. Experimental conditions for the use of radioligands such as [^{3}H]paroxetine, [^{3}H]nisoxetine, and [^{3}H] WIN-35428 and nonspecific ligands such as paroxetine, nomifensine, and desipramine are summarized in Table 1. The inhibition rate was calculated by the following formula: inhibition rate = (total binding − drug binding) × 100%/(total binding − nonspecific binding). All data are expressed as the mean values of three independent experiments.

Monoamine Uptake Transporter Assay

The reuptake assays were performed using crude synaptosomes freshly prepared from rat brains, as previously described with minor modifications (Artaiz et al., 2005; do Rego et al., 2007). Briefly, the uptake of [^{3}H]5-HT by SERT or [^{3}H]NE by NET was carried out using membrane preparations of rat cerebral cortex, and the uptake of [^{3}H] DA by DAT was determined using rat striatum preparations (Table 1). Crude synaptosomes were incubated in a solution of Krebs bicarbonate buffer that contains [^{3}H]5-HT (20 nM), [^{3}H]NE (20 nM), or [^{3}H]DA (20 nM) and various concentrations of test compounds (10^{-1}–10^4 nM) at 37°C for 10 minutes. The nonspecific uptake to SERT, NET, and DAT was determined with 10 μM paroxetine, 10 μM desipramine, and 10 μM nomifensine, respectively. All experiments were performed in triplicate from three independent tests.

Measurement of Binding Affinity of H05 to 5-HT$_{2A}$ Receptor

H05 binding for H05 to 5-HT$_{2A}$ receptor was carried out under the National Institute of Mental Health Psychoactive Drug Screening Program (Roth, 2008). The conditions and standard procedures are summarized in Supplemental Table 1. Briefly, rats were sacrificed by guillotine apparatus, and membrane proteins were prepared from rat frontal cortex. Competitive binding assays were performed in reaction buffer containing 50 μg of membrane proteins, [^{3}H] ketanserin (0.6 nM), and various concentrations of H05 (10^{-1}–10^4 nM) at 37°C for 25 minutes. Nonspecific binding to 5-HT$_{2A}$ was determined using 10 μM methysergide. The inhibition rate was calculated using the following formula: inhibition rate = (total binding − drug binding) × 100%/(total binding − nonspecific binding). All data are presented as the mean values of three independent experiments.

Determination of 5-HT$_{2A}$ Receptor Intrinsic Activity

The calcium flux assay for agonist or antagonist activity of 5-HT$_{2A}$ receptor was previously described with minor modifications (Chen et al., 2013). Briefly, Chinese hamster ovary-K1 cells stably expressing human 5-HT$_{2A}$ receptors (accession number NM_000621) were lightly trypsinized, seeded in a 384-well plate at a density of 2×10^4 cells/well in 25 μl of cell culture medium, and maintained in 5% CO_2 at 37°C for 24 hours before the test.

For the agonist test, 20 μl of calcium assay loading buffer (20 mM HEPES/Hanks' balanced salt solution with 2.5 mM probenecid and 4 μM Fluo-4, pH 7.4) was added to each well after the removal of culture medium. The cell plate was first placed into a 37°C incubator for 60 minutes, followed by 15 minutes at room temperature before transfer to reading position of the Fluorescent Image Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA) for fluorescence signals for 20 seconds. An addition of 5 μl in 5× final concentration of compounds, such as 5-HT (as positive control) or 0.25% dimethylsulfoxide (DMSO) (as negative control), to the reading plate at 20 seconds was made, and the change in the cell fluorescence signal was measured for an additional 100 seconds (range, 21–120 seconds).

For the antagonist test, the medium was removed and replaced with 20 μl of calcium assay loading buffer (20 mM HEPES/Hanks' balanced salt solution with 2.5 mM probenecid and 4 μM Fluo-4, pH 7.4) and 5 μl of compound or ketanserin (as positive control) in 5× concentration or 0.25% DMSO (as negative control). The cell plate was first incubated at 37°C for 60 minutes, followed by 15 minutes at room temperature before transfer to the reading position of the FLIPR for fluorescence signals for 20 seconds. Adding 5 μl of the control agonist 5-HT in 6× concentration to the reading plate at 20 seconds was carried out, and the change in fluorescence signals was measured for an additional 100 seconds (range, 21–120 seconds).

For both agonist and antagonist tests, the average value of 20 seconds (range, 1–20 seconds) in reading as the baseline was calculated, and the change in relative fluorescent unit (△RFU) intensity was calculated as the maximal fluorescent units (range, 21–120 seconds) after subtraction of the average value of baseline readings. The percentage effect of compound was calculated using the following equation:

$$\%Effect = (\Delta RFU_{Compound} - \Delta RFU_{negative\ control}) \Big/ (\Delta RFU_{positive\ control} - \Delta RFU_{negative\ control}) \times 100$$

Dose-response curves for agonist/antagonist were fitted with four-parameter logistic equation using the software GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

Forced Swimming Test in Mice

The forced swimming test (FST) was performed in mice according to the procedures as previously described (Porsolt et al., 1977). Briefly, a total of 110 naive mice were randomly assigned to 11 groups consisting of vehicle control, duloxetine (2.5, 5, 10, 20, and 40 mg/kg), and H05 (1.25, 2.5, 5, 10, and 20 mg/kg) with each group containing 10 mice. One hour after oral administration of test compounds, each mouse was forced to swim in an open cylindrical container (diameter of 10 cm, height of 25 cm, containing 15 cm of water with temperature maintained at 24 ± 1°C). The duration of immobility during the last 4 minutes of a total time of 6 minutes was recorded, and animals were judged to be immobile when they floated motionless, making only necessary movements to keep their heads above the water.

TABLE 2
CUMS regimen

Day	Week 1	Week 2	Week 3	Week 4
Monday	Social defeat: 1 h; empty water bottles: 2 h	Social defeat: 1 h; restricted food: 2 h	Social defeat: 1 h; tail pinch: 1 min	Social defeat: 1 h; stroboscope: 7 h
Tuesday	Water/food deprivation: 24 h; soiled cage: 17 h	Water/food deprivation: 24 h; tilted cage: 17 h	Water/food deprivation: 24 h; water in cage: 17 h	Water/food deprivation: 24 h; empty cage: 17 h
Wednesday	Stroboscope: 7 h	4°C swimming: 5 min	Restraint stress: 2 h	Unpredictable shocks: 30 min
Thursday	Social defeat: 1 h; water deprivation: 24 h	Social defeat: 1 h; water deprivation: 24 h	Social defeat: 1 h; water deprivation: 24 h	Social defeat: 1 h; water deprivation: 24 h
Friday	Glucocorticoid injection; overnight illumination	Glucocorticoid injection; white noise: 7 h	Glucocorticoid injection; intermittent illumination: 8 h	Glucocorticoid injection; stroboscope: 7 h
Saturday	Food deprivation: 24 h	Water deprivation: 24 h	Water deprivation: 24 h	Soiled cage: 17 h
Sunday	Water deprivation: 24 h	Food deprivation: 24 h	Food deprivation: 24 h	Water/food deprivation: 20 h

Tail Suspension Test in Mice

The tail suspension test (TST) procedure in mice was adapted from descriptions by Steru et al. (1985). One hundred ten mice were randomly assigned to 11 groups consisting of vehicle control, duloxetine (2.5, 5, 10, 20, and 40 mg/kg), and H05 (1.25, 2.5, 5, 10, and 20 mg/kg) groups, with each group consisting of 10 mice. After oral administration of compounds for 60 minutes, each mouse was suspended on the top of the apparatus using adhesive tape placed approximately 1 cm from the tip of the tail. The immobility duration of the last 4 minutes of a total time period of 6 minutes was recorded. Mice were considered to be immobile when hung passively without moving.

Locomotor Activity Measurement

To assess the effect of H05 on locomotion, 40 naive mice were randomly divided into four groups with 10 mice in each group. Animals were transferred into the testing room at least 2 hours prior to drug administration. Fifty-five minutes after oral administration of different doses of H05 (10, 20, and 40 mg/kg) or vehicle, mice were individually placed into the corner of each test chamber (length × width × height: 25 × 25 × 30 cm) for a 5-minute acclimation period. Sixty minutes after drug administration, spontaneous locomotor activity was recorded for 30 minutes using a tracking and computerized analysis system (Clever Sys Inc., Leesburg, VA). The area was wiped and cleaned with a 75% alcohol solution and dried before each test.

Rat Model of Chronic Unpredictable Mild Stress

To further evaluate the AD-like effects of H05, a rat model of chronic unpredictable mild stress (CUMS) was adopted according to previous descriptions (Willner et al., 1987). Briefly, rats were first trained to consume two bottles of 0.8% sucrose solution for 24 hours before one of the two bottles of sucrose solution was replaced with water for 24 hours. Prior to the sucrose baseline test, rats were food and water deprived for 20 hours. In the sucrose baseline test, each rat was presented simultaneously with two bottles, one containing sucrose (0.8%), and the other containing water. The baseline for sucrose intake was calculated, and animals were divided into the following five matched groups: control, CUMS + vehicle, CUMS + duloxetine (2 mg/kg), CUMS + H05 (2 mg/kg), and CUMS + H05 (6 mg/kg).

All animals, except for those in the control group, were subjected to 4 weeks of stress. The stressors contain the following: water and food deprivation (24 hours), overnight illumination, stroboscope (120 flashes/min), intermittent illumination (lights on and off every 2 hours), white noise (110 dB), soiled cage (200 ml of water in sawdust bedding), forced swimming (5 minutes at 4°C), tail pinch (1 minute), cage tilting (45°), electrical shock (0.8 mA, 10 seconds duration), and restraint (2 hours). All stressors were applied continuously and randomly. Control rats were housed in a separate room, and they had no contact with the stressed animals. Water and food were freely available to the nonstressed rats as a control, except for the deprivation period of 20 hours prior to each sucrose test. Details of stressors and the schedule for the model of CUMS in rats are listed in Table 2.

After the initial 2 weeks of stress, animals received once a day intraperitoneal injection of vehicle or drugs 0.5 hour before the stress procedure for another 2 weeks. After a total of 4 weeks of stress, the sucrose preference test (on day 29) was carried out for 24 hours in the absence of acute drug treatment after rats received vehicle or drugs for 14 days. The scheme for chronic unpredictable stress and behavioral tests is shown in Fig. 6A.

Mouse Model of Corticosterone-Induced Depression

The effect of H05 on corticosterone (CORT)-induced depression–like behaviors was evaluated in mice. The test was performed as described previously (Ali et al., 2015). Briefly, mice were divided into six groups with 12 mice in each group. H05 and duloxetine were administered orally 60 minutes prior to the CORT (40 mg/kg, s.c.) injection for 21 days. The sucrose preference test was used 24 hours after the last administration of drug. Seventy-two hours before the test, mice were individually trained to adapt to a sucrose solution (0.8%, w/v) by placing two bottles of sucrose solution in each cage for 24 hours before one of the two bottles was replaced with water for 24 hours. Mice were deprived of water and food for 24 hours after the adaptation. Mice were given free access to the two bottles containing 100 ml of water or 100 ml of sucrose solution (0.8%, w/v). A sucrose preference test was conducted at 9:00 AM After 24 hours, the amount of consumed sucrose solution and water was recorded, and the sucrose preference was calculated. The protocol schematic of CORT-induced depression in mice is shown in Fig. 6B.

DOI-Induced Head Twitch in Mice

The DOI-induced head twitch test in mice was performed as previously described (Fantegrossi et al., 2010). Briefly, a total of 48 naive mice was randomly assigned to six groups, with eight mice in each of the following groups: vehicle control, DOI + vehicle, DOI + H05 (0.3 mg/kg), DOI + H05 (1 mg/kg), DOI + H05 (3 mg/kg), and DOI + H05 (10 mg/kg). Various doses of H05 were administered orally 60 minutes prior to the DOI (1 mg/kg, i.p.) injection. Immediately after the DOI injection, mice were individually placed into a Plexiglas box. The number of head twitches was counted for a total of 20 minutes by observers.

Rat Model of Adrenocorticotropin-Induced TRD

The ACTH-induced rat model of TRD was generated according to previous descriptions (Kitamura et al., 2002a; Kawaura et al., 2016). Briefly, rats received chronic treatment with ACTH (100 μg/rat, s.c.) once a day for a period of 14 days before they were randomly assigned to the following six treatment groups: ACTH + saline, ACTH + imipramine (30 mg/kg), ACTH + duloxetine (30 mg/kg), ACTH + H05 (5 mg/kg), ACTH + H05 (10 mg/kg), and ACTH + H05 (20 mg/kg).

The FST was performed as previously described at day 14 (Porsolt et al., 1978). Each rat was placed in an opaque cylinder (height, 40 cm; diameter, 20 cm) containing 30 cm of water at 25°C. The test contained the following two sessions: the 15-minute preswim session, and a 6-minute swimming test session followed 24 hours later. The last injection of ACTH was given immediately after the preswim test. Imipramine or duloxetine was injected intraperitoneally, H05 was orally administrated 0.5, 5, and 23 hours before the swimming test session. The total period of immobility during the 6-minute period of testing was recorded. The schematic protocol of ACTH-induced TRD in rats was shown in Fig. 7E.

Microdialysis

The microdialysis tests were carried out on freely moving rats according to the protocol previously described (Si et al., 2010). Adult male SD rats weighing 250–350 g were anesthetized with 10% chloral hydrate and placed in a stereotaxic apparatus (RWD Life Science Co., Ltd., Shenzhen, Guangdong Province, People's Republic of China). After exposure of the skull, a hole for the probe was drilled. A CXG-2 guide cannula (Eicom Co., Kyoto, Japan) was implanted into the medial prefrontal cortex (mPFC) (anteroposterior +3.2 mm, lateral + 0.8 mm, and dorsoventral −2 mm relative to the bregma and dura) and fixed firmly to the skull surface using dental cement. Rats were housed individually after operation. Three days after surgery, a dialysis probe (0.22 mm o.d., 4 mm length, cutoff of 50 kDa; Eicom Co.) was inserted into the guide cannula to replace the dummy cannula and was perfused with artificial cerebrospinal fluid (145 mM NaCl, 3.0 mM KCl, 1.26 mM $CaCl_2$, 1 mM $MgCl_2$, and 1.4 mM Na_2HPO_4, pH 7.4) at a flow rate of 1 μl/min. After an initial 2-hour equilibration period, samples of dialysate were collected every 30 minutes. Two samples were collected for analysis of basal levels of 5-HT, DA, and NE, before the animals were administered compound H05 (10 and 20 mg/kg, by mouth). The dialysate samples were collected for an additional period of 240 minutes after dosing. One microliter of antioxidant, which contained 0.1 M acetic acid, 3.3 mM L-cysteine, and 0.5 mM ascorbic acid was added to each vial before sample collection. The concentrations of DA, NE, and 5-HT in collected microdialysis samples were determined using a benzoylation derivatization ultra-performance liquid chromatography (UPLC)-tandem mass spectrometry method, as previously described (Song et al., 2012). Briefly, 10 μl of dialysate samples was derivatized by adding 5 μl of 100 mM sodium carbonate, 5 μl of 2% benzoyl chloride (BZ) in acetonitrile before 5 μl internal standard mixture of BZ-(phenyl-$^{13}C_6$) ($^{13}C_6$BZ) was added to improve quantitation. An Acquity UPLC system (Waters, Milford, MA) was used with automatic injection of a 10-μl sample into a Waters UPLC BEH C_{18} column (100 × 2.1 mm, 1.7 μm) at a flow rate of 0.4 ml/min. The mobile phase consisted of 10 mM ammonium formate with 0.15% formic acid in water (A) and acetonitrile (B). Analytes were detected by a Triple Quad 5500 Tandem Mass Spectrometer (AB Sciex, Framingham, MA).

PK Assay and Brain Penetration Analysis

PK Study. Twelve male SD rats were randomly divided into two groups with six rats in each group for PK studies: oral administration (30 mg/kg) and intravenous administration (5 mg/kg) of H05. Animals were fasted for 12 hours and had free access to water before dosing. Serial blood samples (approximately 0.3 ml) were collected from tail veins at time points of 0, 5, 15, and 30 minutes, and 1.0, 2.0, 3.0, 7.0, 9.0, 11.0, 24, 30, 48, and 54 hours after H05 administration. The plasma was separated by centrifugation at 3000*g* for 10 minutes, and stored at −70°C until analysis. The plasma samples were analyzed for test compounds/drugs and internal standard using the G6120B liquid chromatography-mass spectrometry system (Agilent Technologies, Santa Clara, CA).

Blood-Brain Barrier Penetration Test. Six male SD rats were used for a brain penetration study of the compound. Animals were fasted for 8 hours before compound administration. The H05 was administered orally (30 mg/kg), and 2 hours (approximately the T_{max} of H05) later blood samples and the whole brain from each rat were

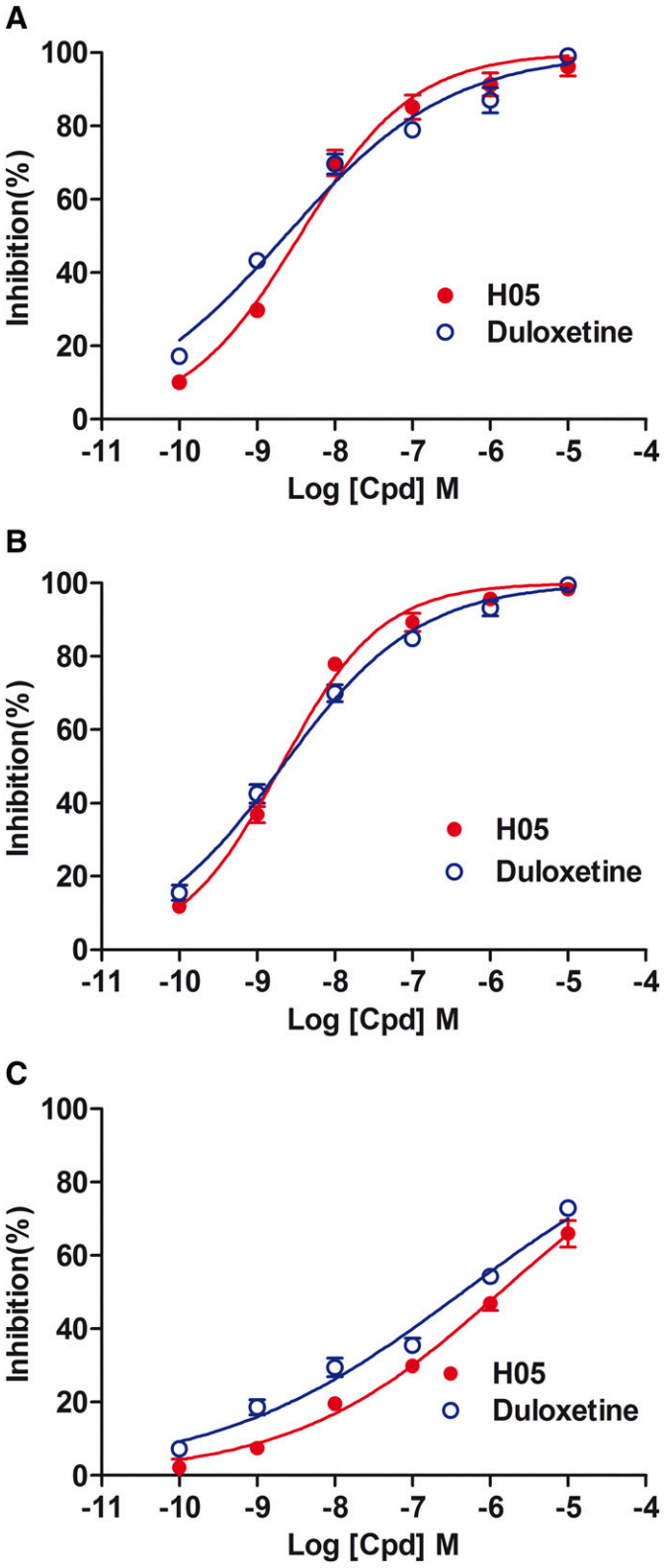


Fig. 2. Binding affinities of H05 to SERT (A), NET (B), and DAT (C) determined by radioligand receptor binding assay. Data are presented as the mean ± S.E.M. of three independent tests, which were conducted in triplicate.

TABLE 3
Summary of pharmacologic and PK properties of H05 and duloxetine
Data are presented as the mean ± S.E.M (n = 3) from three independent experiments, and each experiment was performed in triplicate.

Assays	H05	Duloxetine
SERT binding, [^{3}H]Paroxetine, K_i (nM)	3.54 ± 0.25	2.30 ± 0.06
NET binding, [^{3}H]Nisoxetine, K_i (nM)	2.05 ± 0.07	2.11 ± 0.17
DAT binding, [^{3}H]WIN35,428, K_i (nM)	>1000	439.00 ± 35.11
5-HT uptake, [^{3}H]5-HT, IC$_{50}$ (nM)	4.81 ± 0.31	5.26 ± 0.42
NE uptake, [^{3}H]NE, IC$_{50}$ (nM)	6.72 ± 1.02	17.02 ± 2.03
DA uptake, [^{3}H]DA, IC$_{50}$ (nM)	>1000	754.21 ± 227.42
5-HT$_{2A}$ binding, [^{3}H]Ketanserin, IC$_{50}$ (nM)	71.05 ± 6.58	K_i > 700 nM[a]
5-HT$_{2A}$ inhibition, calcium influx, IC$_{50}$ (nM)	60.37	ND
Rat PK, 30 mg/kg, by mouth, $t_{1/2}$ (h)	3.87	ND
Rat PK, 30 mg/kg, by mouth, T$_{max}$ (h)	2	ND
Rat PK, 30 mg/kg, by mouth, Bioavailability	17.49%	ND
Blood-brain barrier penetration, C_{brain}/C_{plasma}	3.94	0.023[b]

ND, not determined; $t_{1/2}$, half-life. C_{brain}/C_{plasma}: the concentration ratio of brain to plasma.
[a]Wong et al. (1993) and Karpa et al. (2002).
[b]Paulzen et al. (2016).

collected. Blood samples were collected in heparinized tubes and immediately centrifuged at 3000g for 10 minutes. Harvested plasma samples were stored at −20°C until analysis. The whole brain was removed after rats were euthanized, and the brain was stored at −70°C after an iced saline wash to remove residual blood. The concentration of H05 in plasma and the brain was analyzed using the Agilent Technologies G6120B liquid chromatography-mass spectrometry system.

Statistical Analysis

All data are presented as the mean ± S.E.M. Statistical analyses were conducted using GraphPad Prism version 5.0 (GraphPad Software).

For binding/uptake assays, the transporter binding and monoamine uptake results were analyzed using one-site nonlinear regression of the concentration-effect curve. The K_i values were calculated using the equation of Cheng and Prusoff (1973).

For data from behavioral tests, the analysis was made using one-way analysis of variance (ANOVA) followed by Dunnett's test.

A two-way repeated-measures ANOVA, followed by Tukey's test, was used to compare the percentage increase from the baseline among different groups for microdialysis experiments. A difference with $P < 0.05$ was considered to be statistically significant.

Results

Effects of H05 on Monoamine Uptake In Vitro and Monoamine Level in the Frontal Cortex of Conscious Rat. The binding affinities of H05 to monoamine transporters. including SERT, NET, and DAT, were determined by the radioligand receptor binding assay using duloxetine, an SNRI, as a positive control. Duloxetine exhibited potent binding affinities to SERT, NET, and DAT with K_i values of 2.30 ± 0.06, 2.11 ± 0.17, and 439.00 ± 35.11 nM, respectively (Fig. 2, A–C; Table 3). The K_i of H05 against SERT and NET was determined to be 3.54 ± 0.25 and 2.05 ± 0.07 nM, respectively, suggesting its potent binding affinities (Fig. 2, A and B; Table 3). However, H05 exhibited a weak binding affinity to DAT with K_i >1000 nM (Fig. 2C; Table 3).

Consistent with its strong binding affinities to SERT and NET, H05 was able to dramatically inhibit the uptake of both [^{3}H]5-HT and [^{3}H]NE in the cerebral synaptosomes of rat with IC$_{50}$ values of 4.81 ± 0.31 and 6.72 ± 1.02 nM, respectively (Fig. 3, A and B; Table 3). Unlike duloxetine, which potently inhibited the uptake of [^{3}H]5-HT (IC$_{50}$ = 5.26 ± 0.42 nM), [^{3}H]NE (IC$_{50}$ = 17.02 ± 2.03 nM), and [^{3}H]DA (IC$_{50}$ = 754.21 ± 227.42 nM) into synaptosomes, H05 was found to have a negligible potency to inhibit [^{3}H]DA uptake (IC$_{50}$ > 1000 nM) (Fig. 3, A–C; Table 3).

Microdialysis was conducted to determine the effects of H05 on the release of 5-HT, NE, and DA in vivo. The results suggest that systemic administration of H05 (10 and 20 mg/kg) elevated the 5-HT and NE levels up to 551% and 598%, respectively, in mPFC in a dose-dependent and time-dependent manner (Fig. 4, A and B). DA levels were also elevated by H05, but not at the statistically significant level (Fig. 4C).

Efficacious AD Activity of H05 in Multiple Animal Models of Depression. The AD effects of H05 were evaluated by TST and FST in a mouse model of acute depression using duloxetine as the positive control. As shown in Fig. 5, A and B, the oral administration of duloxetine (10, 20, and 40 mg/kg) decreased the duration of immobility in a dose-dependent manner with minimal effective doses (MEDs) of 10 mg/kg in TST and 5 mg/kg in FST. H05 also caused a dose-dependent reduction in immobility time (Dunnett's test, $P < 0.01$ vs. vehicle), but with a lower MED of 2.5 mg/kg in both tests, suggesting that it is more potent than duloxetine in these models.

The spontaneous locomotion test indicated that oral administrations of H05 at doses of 10, 20, and 40 mg/kg were not able to significantly alter the total distance traveled during the 30-minute period, indicating that the H05-induced decrease in the immobility time in FST and TST did not result from a psychostimulant effect, but rather from AD activity (Fig. 5C).

The AD effects of H05 were further evaluated in the rat model of CUMS and the mouse model of CORT-induced depression. For the CUMS model, the baselines of sucrose preference of all test groups were in the range from 74% to 76% at week 0 before stress. A 4-week period of CUMS caused significant decreases with an average value of 27% ($P < 0.05$, $n = 10$) in the sucrose preference of the stress group, compared with the nonstressed control group. Both 2-week treatments with H05 (2 or 6 mg/kg, i.p.) and duloxetine (2 mg/kg, i.p.) significantly increased the sucrose preference to the level close to the baseline of rats before they were stressed (Fig. 6C). Compared with the vehicle control, chronic subcutaneous

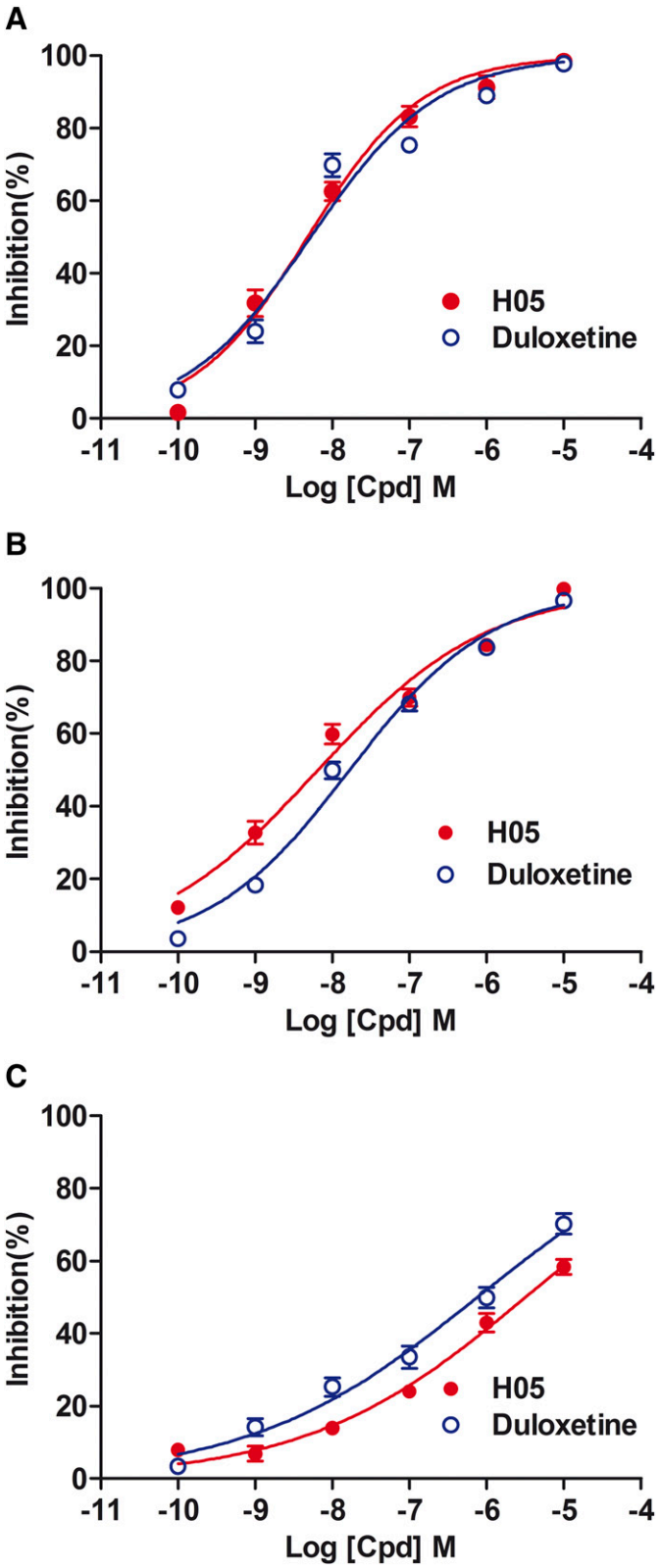


Fig. 3. Inhibition effects of H05 on the uptakes of [^{3}H]5-HT (A), [^{3}H]NE (B), and [^{3}H]DA (C) into rat brain synaptosomes. Data are presented as the mean ± S.E.M. of three independent tests, which were conducted in triplicate.

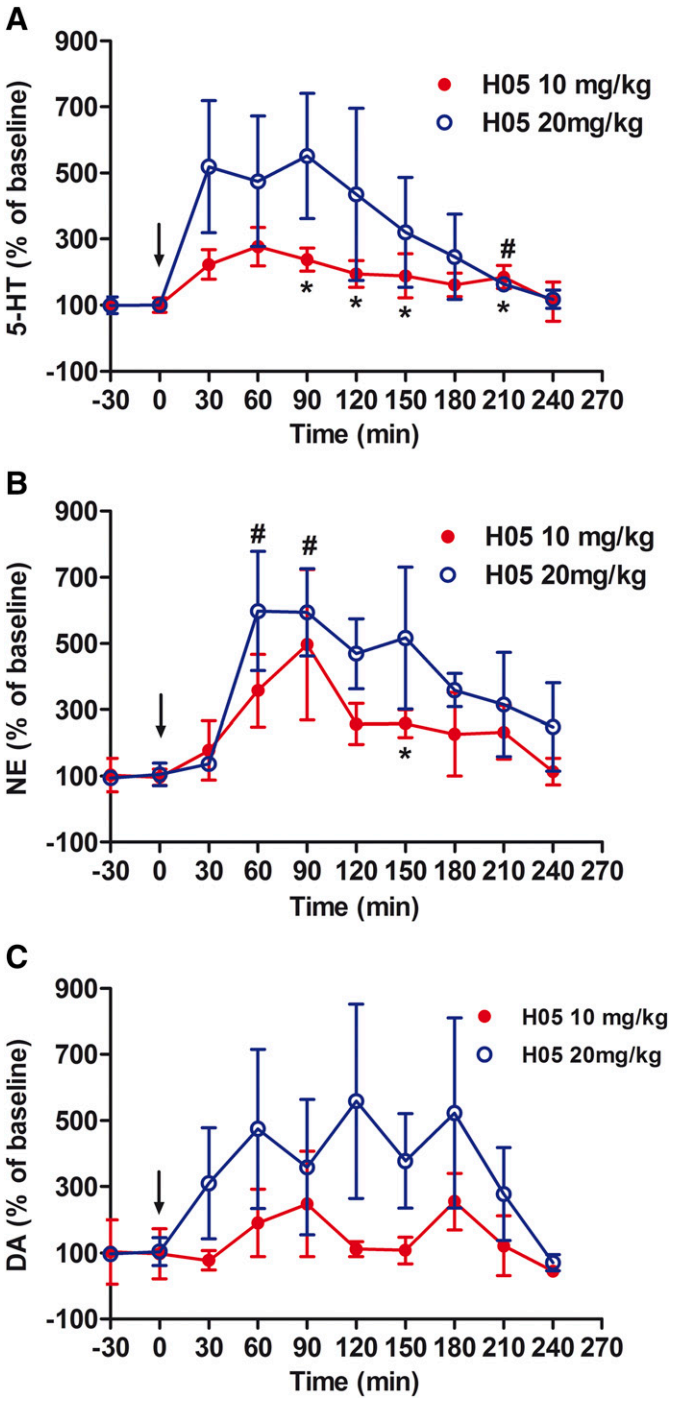


Fig. 4. The extracellular expression levels of 5-HT (A), NE (B), and DA (C) in the frontal cortex of the rat were increased by oral administration of H05 in a dose-dependent manner. Monoamines were measured by microdialysis (n = 3). Arrows indicate the administration time. Data are expressed as the mean ± S.E.M., relative to the basal pretreatment value, which is defined as 100%. *$P < 0.05$, 10 mg/kg H05 group vs. basal levels; #$P < 0.05$: 20 mg/kg H05 group vs. basal levels.

injection of CORT (40 mg/kg) resulted in an obvious decline in sucrose consumption in mice (Fig. 6D), which was successfully reversed by oral administration of H05 (5, 10, and 20 mg/kg), suggesting the AD-like effects of H05 on the CORT-induced depression in mice (Fig. 6D).

5-HT$_{2A}$ Receptor Antagonist Activity and AD-Like Effects of H05 in the ACTH-Induced Rat Model of TRD. A moderate binding affinity of H05 to 5-HT$_{2A}$ receptor was found with an IC$_{50}$ value of 71.05 ± 6.58 nM by the binding test (Fig. 7A; Supplemental Table 3; Table 3). Because

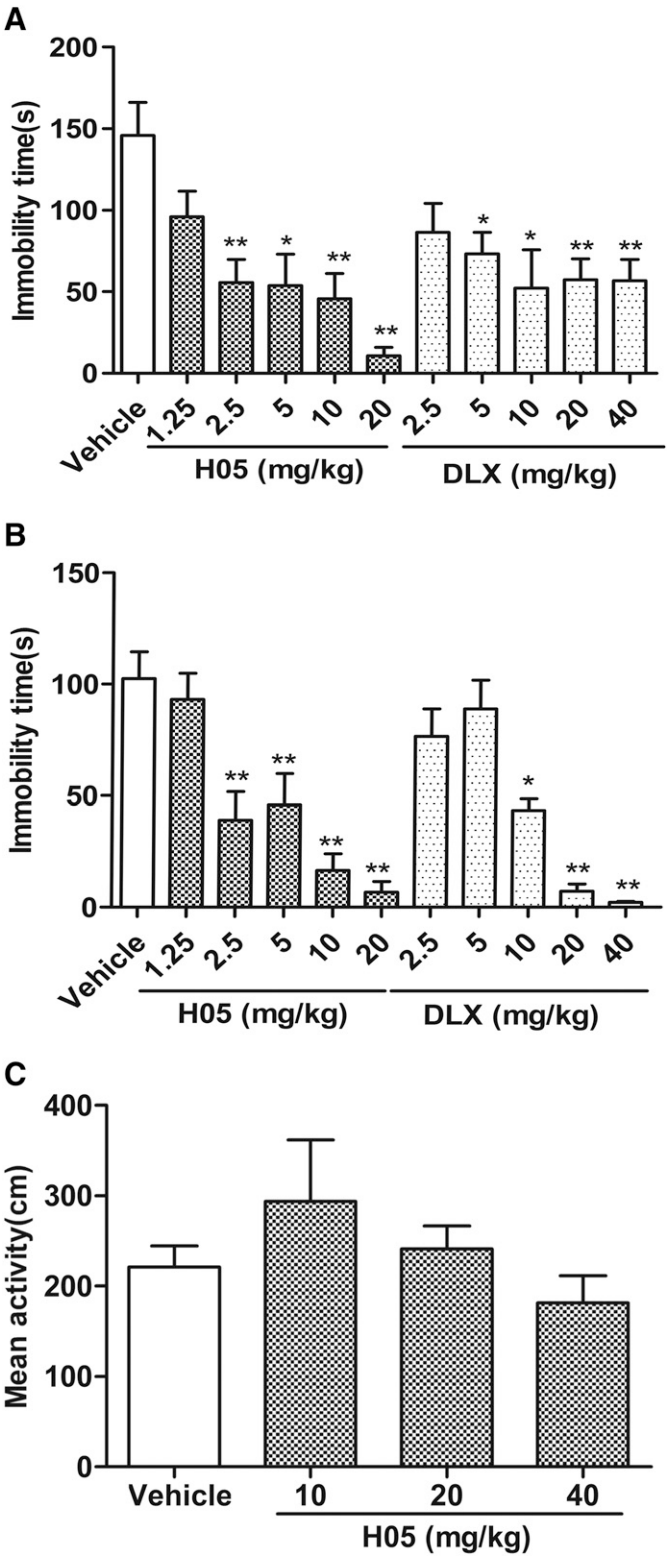


Fig. 5. Effects of different doses of H05 on acute behavioral despair models in mice. (A) FST. (B) TST. (C) Spontaneous locomotor test. One-way ANOVA followed by Dunnett's test: $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the vehicle control group (n = 10). DLX, duloxetine.

an agonist or antagonist might exert different biologic activities, the intrinsic activity of H05 at the 5-HT$_{2A}$ receptor was measured by the calcium influx FLIPR assay. The results confirmed the highly potent antagonistic activity of H05 with IC$_{50}$ = 60.37 nM (Fig. 7, B and C; Table 3), without an agonistic effect on 5-HT$_{2A}$ receptor, suggesting that H05 is a 5-HT$_{2A}$ receptor antagonist.

DOI is a 5-HT$_{2A/2C}$ agonist that can elicit the head twitch behavior mediated by activation of the 5-HT$_{2A}$ receptor in mice. To further evaluate the inhibitory effect of H05 on the 5-HT$_{2A}$ receptor in vivo, the DOI-induced head twitch in mice was treated with H05. As shown in Fig. 7D, DOI caused head twitch responses in mice, which were suppressed by pre-treatment with H05 in a dose-dependent manner.

The effect of H05 treatment on TRD was investigated in a rat model of ACTH-induced depression. The administrations of imipramine (30 mg/kg, i.p.) and duloxetine (30 mg/kg, i.p.) showed no obvious effects on the immobility time in FST in rats treated with ACTH (100 μg/d, s.c.) for 14 days, indicating that neither of them were effective ADs for TRD induced by ACTH (Fig. 7F). In contrast, the oral administration of H05 (5, 10, and 20 mg/kg) was able to dramatically decrease the immobility time in ACTH-treated rats, suggesting the AD effect of H05 on TRD (Fig. 7F).

Favorable PK Properties and Good Blood-Brain Penetration Ability for H05. The PK property is a critical parameter for drug discovery and development. About 10% of all drugs in clinical trial fail due to poor PK profiles (Kola and Landis, 2004). The PK properties of H05 were examined by oral and intravenous administration in rats. As shown in Table 3, the oral administration of H05 to rats (30 mg/kg, n = 6) resulted in a half-life of 3.87 hours, a T$_{max}$ of 2 hours, and a C_{max} of 165.20 ng/ml. The area under the curve for time 0 to infinity was measured to be 912.02 ng/h per milliliter for oral administration (30 mg/kg) and 868.88 ng/h per milliliter for intravenous administration (5 mg/kg) with oral bioavailability of ~17.49%. The concentrations of H05 in brain and plasma were determined to be 30.03 ng/g and 7.63 ng/ml, respectively, by the blood-brain penetration analysis 2 hours after oral administration when T$_{max}$ was reached. The concentration ratio of brain to plasma reached 3.94 (Table 3), demonstrating the excellent brain penetration properties of H05 for further development.

Discussion

Although SSRIs and SNRIs have been widely used as the first-line ADs in the clinic, their efficacy, safety, and side effects still cannot meet the need of depression treatment (Goldstein and Goodnick, 1998; Trivedi et al., 2006; Warden et al., 2007; Mathew, 2008). One effective strategy to improve the depression therapy is to inhibit monoaminergic reuptake and antagonize 5-HT$_{2A}$ receptor simultaneously (Carpenter et al., 1999; Ostroff and Nelson, 1999; Shelton et al., 2001; Carvalho et al., 2009). Among the 3-(benzo[d] [1, 3] dioxol-4-yloxy)-3-arylpropyl amine derivatives synthesized in the present work, H05 was identified as the lead compound, with inhibition effects on both monoaminergic transport and 5-HT$_{2A}$ receptor (Supplemental Tables 2 and 3).

H05 exhibits strong binding affinities to SERT and NET, and a potent inhibition effect on 5-HT and NE reuptakes in vitro, yet a weak affinity to DAT, at least 282-fold and 488-fold lower than those for SERT and NET, respectively, suggesting that it is an SNRI. In addition, H05 shows more balanced affinities to NET and SERT, with an IC$_{50}$ ratio of 1.40, than conventional SNRIs. For example, the K_i ratios of

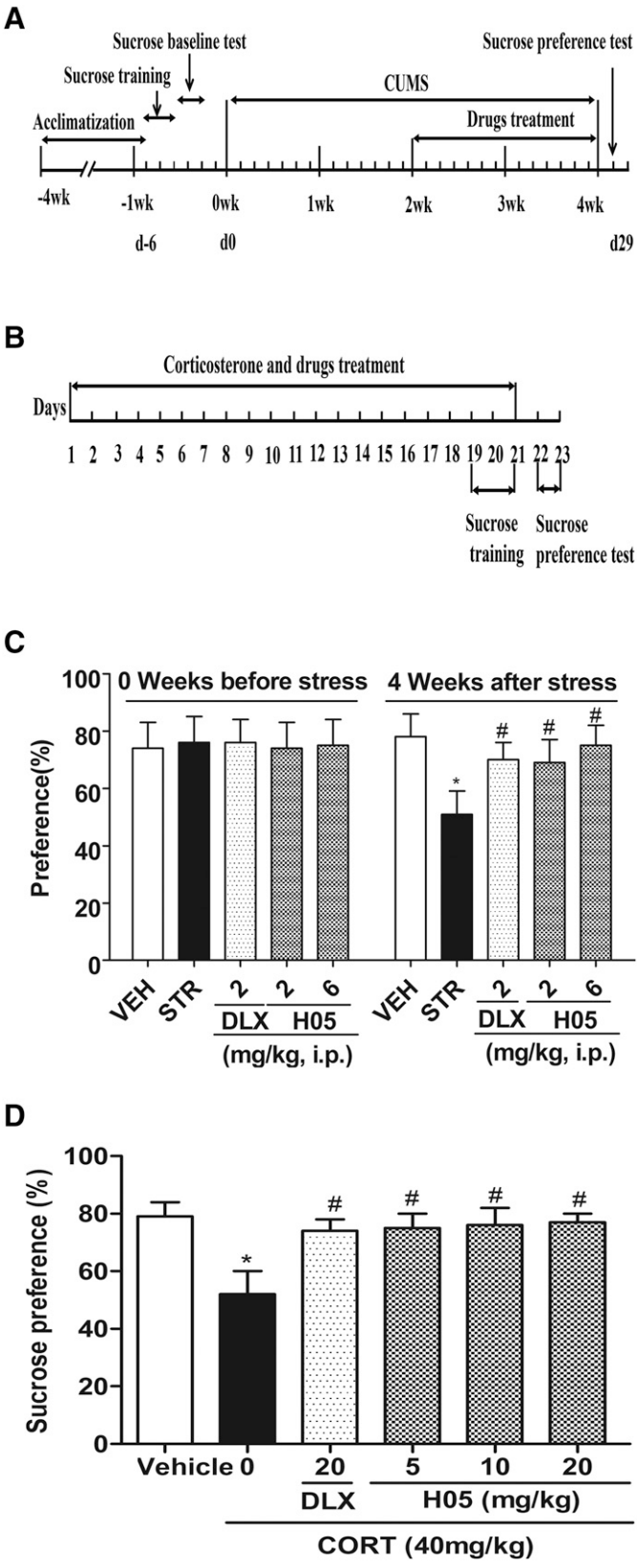


Fig. 6. Effects of different doses of H05 on chronic stress models. (A) Schematic outline of the protocol for CUMS test. Wk: week; d: day. (B) Schematic outline of the protocol for CORT-induced depression test. (C) Effects of 2-week intraperitoneal administration of H05 (2 and 6 mg/kg) and duloxetine (2 mg/kg) on the sucrose preference in rats subjected to 4-week CUMS. After the initial 2 weeks of CUMS, animals received daily intraperitoneal injections of vehicle or drugs 30 minutes before another 2-week CUMS test. *$P < 0.05$ vs. CON, #$P < 0.05$ vs. STR. CON, control; DLX, duloxetine; STR, stress. ($n = 10$). (d) Effects of H05 on the sucrose preference in CORT-treated mice. Mice were administered with CORT (40 mg/kg, s.c.) once a day for 21 days, and H05 (5, 10 and 20 mg/kg, by mouth) was administrated 60 minutes prior to CORT injection for 21 days. *$P < 0.05$ vs. vehicle, #$P < 0.05$ vs. CORT. DLX, duloxetine. ($n = 12$). Data are expressed as the mean ± S.E.M.

NET/SERT reuptake values of duloxetine and venlafaxine are 3.24 and 6.99, respectively (Bymaster et al., 2001). This characteristic feature of H05 makes it a better SNRI than duloxetine and venlafaxine in safety and efficacy profiles, as Dreyfus et al. (2013) indicated that SNRIs with comparable potency can lead to a drug with more safety profile and superior efficacy. Unlike duloxetine ($K_i > 700$ nM), venlafaxine ($K_i > 100{,}000$ nM), paroxetine ($K_i = 6320$ nM), and fluoxetine ($K_i = 141$ nM), which exhibited no significant effects on 5-HT_{2A} receptor (Wong et al., 1993; Owens et al., 1997; Karpa et al., 2002), H05 exerts moderate inhibition against 5-HT_{2A} receptor with an IC_{50} of 60.37 nM. The 5-HT_{2A} receptor antagonistic property may render H05 more potent and with fewer side effects than duloxetine.

The serotonergic and noradrenergic properties of H05 were also confirmed by in vivo microdialysis. Consistent with its potent inhibitory effects on 5-HT and NE reuptake, H05 increases extracellular 5-HT and NE levels in the mPFC in a dose-dependent manner, up to 551% and 598%, respectively, above the basal level at 20 mg/kg, supporting the conclusion drawn from the behavioral analyses. These results indicate that the AD effects of H05 are partly a result of its ability to inhibit NE and 5-HT uptake.

Behavioral TST and FST are widely used to predict the AD activity of a compound at the early stage of drug discovery (Porsolt et al., 1977; Steru et al., 1985; Cryan et al., 2002). The oral administration of low doses of H05 is able to dramatically reduce the immobility time in both TST and FST with a much lower MED (2.5 mg/kg) than those of duloxetine (10 and 5 mg/kg), fluoxetine (30 and 16 mg/kg) (Da-Rocha et al., 1997; Cryan et al., 2005), and imipramine (60 and 30 mg/kg) (Porsolt et al., 1977; Cryan et al., 2005), suggesting its more potent AD activity. Locomotor activity analyses indicate that H05 does not significantly alter the total travel distance at doses of 10, 20, and 40 mg/kg, which can likely exclude false-positive AD effects.

In addition to the acute behavioral despair model, two chronic stress models, a mouse model of CORT-induced depression and a rat model of CUMS, were also used to further evaluate the AD effects of H05. Studies have shown that chronic stress can induce significant declines in sucrose preference in animals (Willner et al., 1987; Ali et al., 2015), and such a behavioral deficit can manifest anhedonia in human beings, one of the core symptoms of depression. Therefore, these two models have better face and construct validity than TST and FST. Our results indicate that H05 can dose-dependently increase the percentage of sucrose consumption in both chronic unexpected mild stress and repeated CORT treatment models. Therefore, H05 potentially can be developed as an AD.

To date, the psychopathological and neurobiological mechanisms of TRD still remain unclear. Some studies suggest that the hyperactivity of the hypothalamic-pituitary-adrenal

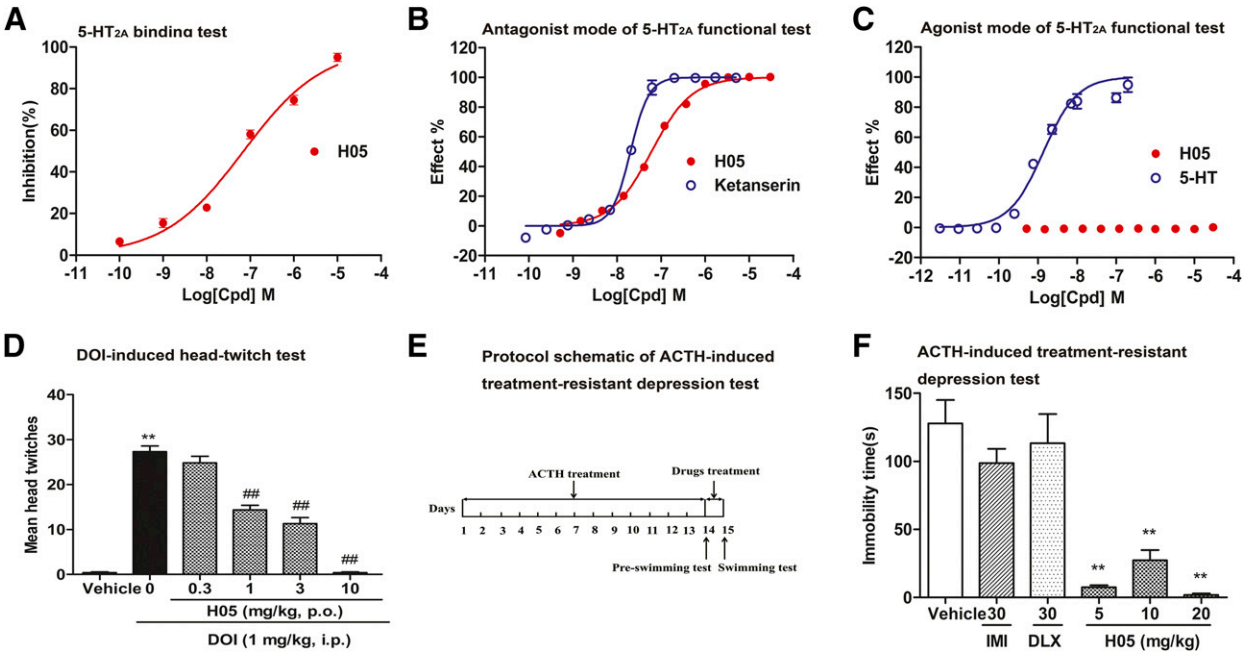


Fig. 7. Inhibition effects of H05 on 5-HT$_{2A}$ receptor and its AD-like effects in an ACTH-induced rat model of TRD. (A) Binding affinities of H05 to 5-HT$_{2A}$ determined by radioligand receptor binding assays. Data are presented as the mean ± S.E.M. from three independent tests, and each test was performed in triplicate. Dose-response curves and IC_{50} values in antagonist mode (B) and EC_{50} in agonist mode (C) in a 5-HT$_{2A}$ calcium assay of H05. 5-HT, ketanserin, and H05 were dissolved in DMSO at a concentration of 30 mM, and diluted with assay buffer in 11 serial concentrations. Each compound was tested three times in duplicate. The fluorescent signals were converted to the percentage of effect (Effect %) by the equation: Effect % = ($\triangle RFU_{Compound} - \triangle RFU_{negative\ control}$)/($\triangle RFU_{positive\ control} - \triangle RFU_{negative\ control}$) × 100. (D) Effects of H05 on the DOI-induced head twitch response in mice. Mice were administered H05 (0.3, 1, 3, and 10 mg/kg, by mouth) 60 minutes prior to DOI injection (1 mg/kg, i.p.). The number of head twitches was counted for a total of 20 minutes. $^{**}P < 0.01$ vs. vehicle; $^{\#\#}P < 0.01$ vs. the DOI group ($n = 8$). Data are expressed as the mean ± S.E.M. (E) Schematic protocol for the ACTH-induced TRD test. (F) Effects of imipramine (IMI), duloxetine (DLX), and H05 on immobility in ACTH-treated rats. ACTH (100 μg/d, s.c.) was administered to rats once every day for 14 days. The immobility time was measured the day after the final ACTH treatment. Imipramine (30 mg/kg, i.p.), duloxetine (30 mg/kg, i.p.), and H05 (5, 10, and 20 mg/kg, by mouth) were administrated three times (at 0.5, 5, and 23 hours) before the FST. $^{**}P < 0.01$ vs. vehicle ($n = 12$). Data are expressed as the mean ± S.E.M.

(HPA) axis is correlated with TRD (Murphy et al., 1991; Wolkowitz et al., 1993). Kitamura et al. (Kitamura et al., 2002a; Kitamura and Gomita, 2008) showed that the chronic administration of $ACTH_{1\text{-}24}$ could effectively inhibit the AD effects of various ADs, including imipramine, desipramine, and milnacipran, in rat. Similarly, Srikumar et al. (2017) found that chronic ACTH administration did not exhibit depression-like phenotype in mice, but could diminish the AD-like effects of duloxetine, imipramine, fluoxetine, and bupropion in FST. In addition, effective clinical therapies for TRD, such as the coadministration of lithium or carbamazepine with imipramine and repeated electroconvulsive stimulation, can decrease the immobility duration in ACTH-treated rats (Kitamura et al., 2002a, 2008; Li et al., 2006). These findings suggest that ACTH-treated rat is a useful animal model of TRD. Consistent with these findings, we find that chronic administration of ACTH (100 μg/d, s.c., for 14 days) attenuates AD-like effects of imipramine (30 mg/kg, i.p.) or duloxetine (30 mg/kg, i.p.) in FST, whereas acute administration of H05 (5, 10, and 20 mg/kg, by mouth) significantly shortens the immobility time of ACTH-treated rats, suggesting that H05 might be a promising AD agent for the treatment of refractory depression. However, we used only one dose of duloxetine or imipramine under the acute administration procedure, and both were ineffective in this TRD model; multiple doses and chronic administration of duloxetine or imipramine might yield efficacy. Takao et al. (1997) showed that the activation of the HPA axis could regulate the function of the 5-HT$_{2A}$ receptor. Kuroda et al. (1992) reported that the ACTH-induced activation of the HPA axis could increase the 5-HT$_{2A}$ receptor expression level in frontal cortex and the number of wet-dog shakes induced by DOI, a 5-HT$_{2A}$ receptor agonist. Other studies have demonstrated that the AD-like effects on ACTH-treated rats caused by the coadministration of lithium and imipramine are mediated by inhibiting hyperfunctional 5-HT$_{2A}$ receptors (Kitamura et al., 2002b). Based on the antagonistic effects of H05 on 5-HT$_{2A}$ receptors observed in vitro (antagonism of 5-HT$_{2A}$ receptor in functional test) and in vivo (suppressing DOI-induced head twitch behavior in mice), we speculate that the therapeutic potential of H05 in TRD largely relies on its 5-HT$_{2A}$ receptor antagonistic activity.

The PK profile of a compound can also be used to predict its druggability. In the present work, the main PK parameters and in vivo brain penetration of H05 in rats suggest that it is highly central nervous system penetrant, with a penetration ratio (brain concentration/plasma concentration) of 3.94, much higher than that (human cerebrospinal fluid concentration/plasma concentration ratio of 0.023) of duloxetine (Paulzen et al., 2016). The better ability of H05 to penetrate the central nervous system may explain why it has similar binding potencies toward SERT and NET in vitro as duloxetine, but lower MEDs in TST and FST. These results also indicate that H05 may have fewer peripheral adverse effects, such as cardiovascular and gastrointestinal effects, than duloxetine. In addition, we show that duloxetine is prone to hydrolysis under acidic conditions (pH 1), with only ~18%

remaining after 2 hours. In contrast, H05 is highly stable under the acidic conditions with >99% remaining after 2 hours (data not shown), which makes it favorable for a variety of dosage forms.

In conclusion, a 3-(benzo[d][1,3]dioxol-4-yloxy)-3-arylpropyl amine derivative, H05, was identified as a lead compound with developmental potential for an AD agent with superior efficacy. H05 is a potent SNRI with a moderate 5-HT_{2A} antagonist activity that contributes to its better efficacy. Compared with other SNRIs, such as duloxetine and venlafaxine, H05 exhibits more balanced affinities to NET and SERT, and displays robust AD activities in a variety of behavioral tests, with much lower MEDs in TST and FST than those of duloxetine, fluoxetine, and imipramine. In addition, H05 is efficacious in the rat model of ACTH-induced depression that is resistant to current ADs, suggesting the potential of H05 in the refractory depression treatment. The high stability in acidic solution, favorable PK properties, and excellent blood-brain penetration ability of H05 make it druggable. In all, H05, a novel potent serotonin and noradrenaline reuptake inhibitor with moderate 5-HT_{2A} antagonist activity, possesses developmental and therapeutic potential for the treatment of depression or TRD.

Authorship Contributions

Participated in research design: Xu, Guo, Zhang, and Wang.

Conducted experiments: Xu, Wei, Guo, Zhao, Z. Liu, Xiao, Y. Liu, Qiu, and Hou.

Contributed new reagents or analytic tools: Guo and Zhang.

Preformed data analysis: Xu, Zhao, Z. Liu, Qiu, and Hou.

Wrote or contributed to the writing of the manuscript: Xu, Xiao, and Wang.

References

Ali SH, Madhana RM, K v A, Kasala ER, Bodduluru LN, Pitta S, Mahareddy JR, and Lahkar M (2015) Resveratrol ameliorates depressive-like behavior in repeated corticosterone-induced depression in mice. *Steroids* **101**:37–42.

Anderson IM (2000) Selective serotonin reuptake inhibitors versus tricyclic antidepressants: a meta-analysis of efficacy and tolerability. *J Affect Disord* **58**:19–36.

Artaiz I, Zazpe A, Innerárity A, Del Olmo E, Díaz A, Ruiz-Ortega JA, Castro E, Pena R, Labeaga L, Pazos A, et al. (2005) Preclinical pharmacology of F-98214-TA, a novel potent serotonin and norepinephrine uptake inhibitor with antidepressant and anxiolytic properties. *Psychopharmacology (Berl)* **182**:400–413.

Aznar S and Klein AB (2013) Regulating prefrontal cortex activation: an emerging role for the 5-HT_2A serotonin receptor in the modulation of emotion-based actions? *Mol Neurobiol* **48**:841–853.

Bhagwagar Z, Hinz R, Taylor M, Fancy S, Cowen P, and Grasby P (2006) Increased 5-HT(2A) receptor binding in euthymic, medication-free patients recovered from depression: a positron emission study with [(11)C]MDL 100,907. *Am J Psychiatry* **163**:1580–1587.

Blier P, Gobbi G, Turcotte JE, de Montigny C, Boucher N, Hébert C, and Debonnel G (2009) Mirtazapine and paroxetine in major depression: a comparison of monotherapy versus their combination from treatment initiation. *Eur Neuropsychopharmacol* **19**:457–465.

Bymaster FP, Dreshfield-Ahmad LJ, Threlkeld PG, Shaw JL, Thompson L, Nelson DL, Hemrick-Luecke SK, and Wong DT (2001) Comparative affinity of duloxetine and venlafaxine for serotonin and norepinephrine transporters in vitro and in vivo, human serotonin receptor subtypes, and other neuronal receptors. *Neuropsychopharmacology* **25**:871–880.

Carpenter LL, Jocic Z, Hall JM, Rasmussen SA, and Price LH (1999) Mirtazapine augmentation in the treatment of refractory depression. *J Clin Psychiatry* **60**:45–49.

Carpenter LL, Yasmin S, and Price LH (2002) A double-blind, placebo-controlled study of antidepressant augmentation with mirtazapine. *Biol Psychiatry* **51**: 183–188.

Carvalho AF, Machado JR, and Cavalcante JL (2009) Augmentation strategies for treatment-resistant depression. *Curr Opin Psychiatry* **22**:7–12.

Celada P, Bortolozzi A, and Artigas F (2013) Serotonin 5-HT1A receptors as targets for agents to treat psychiatric disorders: rationale and current status of research. *CNS Drugs* **27**:703–716.

Chen Y, Wang S, Xu X, Liu X, Yu M, Zhao S, Liu S, Qiu Y, Zhang T, Liu BF, et al. (2013) Synthesis and biological investigation of coumarin piperazine (piperidine) derivatives as potential multireceptor atypical antipsychotics. *J Med Chem* **56**: 4671–4690.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099–3108.

Cryan JF, Markou A, and Lucki I (2002) Assessing antidepressant activity in rodents: recent developments and future needs. *Trends Pharmacol Sci* **23**:238–245.

Cryan JF, Mombereau C, and Vassout A (2005) The tail suspension test as a model for assessing antidepressant activity: review of pharmacological and genetic studies in mice. *Neurosci Biobehav Rev* **29**:571–625.

Da-Rocha MA, Jr, Puech AJ, and Thiébot M-H (1997) Influence of anxiolytic drugs on the effects of specific serotonin reuptake inhibitors in the forced swimming test in mice. *J Psychopharmacol* **11**:211–218.

Davis R, Whittington R, and Bryson HM (1997) Nefazodone. A review of its pharmacology and clinical efficacy in the management of major depression. *Drugs* **53**: 608–636.

Di Giovanni G (2013) Serotonin in the pathophysiology and treatment of CNS disorders. *Exp Brain Res* **230**:371–373.

do Rego JC, Benkiki N, Chosson E, Kabouche Z, Seguin E, and Costentin J (2007) Antidepressant-like effect of hyperfoliatin, a polyisoprenylated phloroglucinol derivative from Hypericum perfoliatum (Clusiaceae) is associated with an inhibition of neuronal monoamines uptake. *Eur J Pharmacol* **569**:197–203.

Dreyfus N, Myers JK, Badescu VO, de Frutos O, de la Puente ML, Ding C, Filla SA, Fynboe K, Gernert DL, Heinz BA, et al. (2013) Discovery of a potent, dual serotonin and norepinephrine reuptake inhibitor. *ACS Med Chem Lett* **4**:560–564.

Fantegrossi WE, Simoneau J, Cohen MS, Zimmerman SM, Henson CM, Rice KC, and Woods JH (2010) Interaction of 5-HT2A and 5-HT2C receptors in R(-)-2,5-dimethoxy-4-iodoamphetamine-elicited head twitch behavior in mice. *J Pharmacol Exp Ther* **335**:728–734.

Feiger A, Kiev A, Shrivastava RK, Wisselink PG, and Wilcox CS (1996) Nefazodone versus sertraline in outpatients with major depression: focus on efficacy, tolerability, and effects on sexual function and satisfaction. *J Clin Psychiatry* **57** (Suppl 2):53–62.

Goldstein BJ and Goodnick PJ (1998) Selective serotonin reuptake inhibitors in the treatment of affective disorders—III. Tolerability, safety and pharmacoeconomics. *J Psychopharmacol* **12** (3 Suppl B):S55–S87.

Goodnick PJ and Goldstein BJ (1998) Selective serotonin reuptake inhibitors in affective disorders—II. Efficacy and quality of life. *J Psychopharmacol* **12** (3 Suppl B):S21–S54.

Karpa KD, Cavanaugh JE, and Lakoski JM (2002) Duloxetine pharmacology: profile of a dual monoamine modulator. *CNS Drug Rev* **8**:361–376.

Katona CL and Katona CP (2014) New generation multi-modal antidepressants: focus on vortioxetine for major depressive disorder. *Neuropsychiatr Dis Treat* **10**:349–354.

Kawaura K, Ogata Y, Honda S, Soeda F, Shirasaki T, and Takahama K (2016) Tipepidine, a non-narcotic antitussive, exerts an antidepressant-like effect in the forced swimming test in adrenocorticotropic hormone-treated rats. *Behav Brain Res* **302**:269–278.

Khazaie H, Rezaie L, Rezaei Payam N, and Najafi F (2015) Antidepressant-induced sexual dysfunction during treatment with fluoxetine, sertraline and trazodone; a randomized controlled trial. *Gen Hosp Psychiatry* **37**:40–45.

Kitamura Y, Akiyama K, Kitagawa K, Shibata K, Kawasaki H, Suemaru K, Araki H, Sendo T, and Gomita Y (2008) Chronic coadministration of carbamazepine together with imipramine produces antidepressant-like effects in an ACTH-induced animal model of treatment-resistant depression: involvement of 5-HT(2A) receptors? *Pharmacol Biochem Behav* **89**:235–240.

Kitamura Y, Araki H, and Gomita Y (2002a) Influence of ACTH on the effects of imipramine, desipramine and lithium on duration of immobility of rats in the forced swim test. *Pharmacol Biochem Behav* **71**:63–69.

Kitamura Y, Araki H, Suemaru K, and Gomita Y (2002b) Effects of imipramine and lithium on wet-dog shakes mediated by the 5-HT2A receptor in ACTH-treated rats. *Pharmacol Biochem Behav* **72**:397–402.

Kitamura Y and Gomita Y (2008) [Development of animal models of treatment-resistant depression in rats]. *Nihon Shinkei Seishin Yakurigaku Zasshi* **28**:93–100.

Kola I and Landis J (2004) Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov* **3**:711–715.

Kuroda Y, Mikuni M, Ogawa T, and Takahashi K (1992) Effect of ACTH, adrenalectomy and the combination treatment on the density of 5-HT2 receptor binding sites in neocortex of rat forebrain and 5-HT2 receptor-mediated wet-dog shake behaviors. *Psychopharmacology (Berl)* **108**:27–32.

Li B, Suemaru K, Cui R, Kitamura Y, Gomita Y, and Araki H (2006) Repeated electroconvulsive stimuli increase brain-derived neurotrophic factor in ACTH-treated rats. *Eur J Pharmacol* **529**:114–121.

Little A (2009) Treatment-resistant depression. *Am Fam Physician* **80**:167–172.

Magni LR, Purgato M, Gastaldon C, Papola D, Furukawa TA, Cipriani A, and Barbui C (2013) Fluoxetine versus other types of pharmacotherapy for depression. *Cochrane Database Syst Rev* (7):CD004185.

Mathew SJ (2008) Treatment-resistant depression: recent developments and future directions. *Depress Anxiety* **25**:989–992.

McKenna MT, Michaud CM, Murray CJ, and Marks JS (2005) Assessing the burden of disease in the United States using disability-adjusted life years. *Am J Prev Med* **28**:415–423.

Millan MJ (2009) Dual- and triple-acting agents for treating core and co-morbid symptoms of major depression: novel concepts, new drugs. *Neurotherapeutics* **6**: 53–77.

Montgomery SA, Baldwin DS, Blier P, Fineberg NA, Kasper S, Lader M, Lam RW, Lépine JP, Möller HJ, Nutt DJ, et al. (2007) Which antidepressants have demonstrated superior efficacy? A review of the evidence. *Int Clin Psychopharmacol* **22**: 323–329.

Muguruza C, Miranda-Azpiazu P, Díez-Alarcia R, Morentin B, González-Maeso J, Callado LF, and Meana JJ (2014) Evaluation of 5-HT2A and mGlu2/3 receptors in postmortem prefrontal cortex of subjects with major depressive disorder: effect of antidepressant treatment. *Neuropharmacology* **86**:311–318.

Murphy BE, Dhar V, Ghadirian AM, Chouinard G, and Keller R (1991) Response to steroid suppression in major depression resistant to antidepressant therapy. *J Clin Psychopharmacol* **11**:121–126.

Orjales A, Mosquera R, Toledo A, Pumar MC, García N, Cortizo L, Labeaga L, and Innerárity A (2003) Syntheses and binding studies of new [(aryl)(aryloxy)-methyl]piperidine derivatives and related compounds as potential antidepressant drugs with high affinity for serotonin (5-HT) and norepinephrine (NE) transporters. *J Med Chem* **46**:5512–5532.

Ostroff RB and Nelson JC (1999) Risperidone augmentation of selective serotonin reuptake inhibitors in major depression. *J Clin Psychiatry* **60**:256–259.

Owens MJ, Morgan WN, Plott SJ, and Nemeroff CB (1997) Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J Pharmacol Exp Ther* **283**:1305–1322.

Paulzen M, Gründer G, Veselinovic T, Wolf B, Hiemke C, and Lammertz SE (2016) Duloxetine enters the brain - but why is it not found in the cerebrospinal fluid. *J Affect Disord* **189**:159–163.

Pehrson AL, Leiser SC, Gulinello M, Dale E, Li Y, Waller JA, and Sanchez C (2015) Treatment of cognitive dysfunction in major depressive disorder—a review of the preclinical evidence for efficacy of selective serotonin reuptake inhibitors, serotonin-norepinephrine reuptake inhibitors and the multimodal-acting antidepressant vortioxetine. *Eur J Pharmacol* **753**:19–31.

Porsolt RD, Anton G, Blavet N, and Jalfre M (1978) Behavioural despair in rats: a new model sensitive to antidepressant treatments. *Eur J Pharmacol* **47**:379–391.

Porsolt RD, Bertin A, and Jalfre M (1977) Behavioral despair in mice: a primary screening test for antidepressants. *Arch Int Pharmacodyn Ther* **229**:327–336.

Rajkumar R and Mahesh R (2010) The auspicious role of the 5-HT3 receptor in depression: a probable neuronal target? *J Psychopharmacol* **24**:455–469.

Reinhold JA, Mandos LA, Lohoff FW, and Rickels K (2012) Evidence for the use of vilazodone in the treatment of major depressive disorder. *Expert Opin Pharmacother* **13**:2215–2224.

Rosel P, Arranz B, San L, Vallejo J, Crespo JM, Urretavizcaya M, and Navarro MA (2000) Altered 5-HT(2A) binding sites and second messenger inositol trisphosphate (IP(3)) levels in hippocampus but not in frontal cortex from depressed suicide victims. *Psychiatry Res* **99**:173–181.

Roth BL (2008) *Assay Protocol Book. National Institute of Mental Health Psychoactive Drug Screening Program*, University of North Carolina, Chapel Hill, NC.

Rush AJ, Armitage R, Gillin JC, Yonkers KA, Winokur A, Moldofsky H, Vogel GW, Kaplita SB, Fleming JB, Montplaisir J, et al. (1998) Comparative effects of nefazodone and fluoxetine on sleep in outpatients with major depressive disorder. *Biol Psychiatry* **44**:3–14.

Shelton RC, Tollefson GD, Tohen M, Stahl S, Gannon KS, Jacobs TG, Buras WR, Bymaster FP, Zhang W, Spencer KA, et al. (2001) A novel augmentation strategy for treating resistant major depression. *Am J Psychiatry* **158**:131–134.

Si W, Aluisio L, Okamura N, Clark SD, Fraser I, Sutton SW, Bonaventure P, and Reinscheid RK (2010) Neuropeptide S stimulates dopaminergic neurotransmission in the medial prefrontal cortex. *J Neurochem* **115**:475–482.

Song P, Mabrouk OS, Hershey ND, and Kennedy RT (2012) In vivo neurochemical monitoring using benzoyl chloride derivatization and liquid chromatography-mass spectrometry. *Anal Chem* **84**:412–419.

Souery D, Serretti A, Calati R, Oswald P, Massat I, Konstantinidis A, Linotte S, Kasper S, Montgomery S, Zohar J, et al. (2011) Citalopram versus desipramine in treatment resistant depression: effect of continuation or switching strategies: a randomized open study. *World J Biol Psychiatry* **12**:364–375.

Srikumar BN, Paschapur M, Kalidindi N, Adepu B, Das ML, Sreedhara MV, Kuchibhotla VK, Pieschl RL, Li YW, Ega DSP, et al. (2017) Characterization of the adrenocorticotrophic hormone-induced mouse model of resistance to antidepressant drug treatment. *Pharmacol Biochem Behav* **161**:53–61.

Steru L, Chermat R, Thierry B, and Simon P (1985) The tail suspension test: a new method for screening antidepressants in mice. *Psychopharmacology (Berl)* **85**: 367–370.

Takao K, Nagatani T, Kitamura Y, and Yamawaki S (1997) Effects of corticosterone on 5-HT1A and 5-HT2 receptor binding and on the receptor-mediated behavioral responses of rats. *Eur J Pharmacol* **333**:123–128.

Trivedi MH, Rush AJ, Wisniewski SR, Nierenberg AA, Warden D, Ritz L, Norquist G, Howland RH, Lebowitz B, McGrath PJ, et al.; STAR*D Study Team (2006) Evaluation of outcomes with citalopram for depression using measurement-based care in STAR*D: implications for clinical practice. *Am J Psychiatry* **163**:28–40.

Wan DD, Kundhur D, Solomons K, Yatham LN, and Lam RW (2003) Mirtazapine for treatment-resistant depression: a preliminary report. *J Psychiatry Neurosci* **28**: 55–59.

Wang S, Chen Y, Liu X, Xu X, Liu X, Liu BF, and Zhang G (2014) Synthesis and evaluation of novel 2,3-dihydrobenzo[b][1,4]dioxin- and indolealkylamine derivatives as potential antidepressants. *Arch Pharm (Weinheim)* **347**:32–41.

Warden D, Rush AJ, Trivedi MH, Fava M, and Wisniewski SR (2007) The STAR*D Project results: a comprehensive review of findings. *Curr Psychiatry Rep* **9**: 449–459.

Whiteford HA, Degenhardt L, Rehm J, Baxter AJ, Ferrari AJ, Erskine HE, Charlson FJ, Norman RE, Flaxman AD, Johns N, et al. (2013) Global burden of disease attributable to mental and substance use disorders: findings from the Global Burden of Disease Study 2010. *Lancet* **382**:1575–1586.

Willner P, Towell A, Sampson D, Sophokleous S, and Muscat R (1987) Reduction of sucrose preference by chronic unpredictable mild stress, and its restoration by a tricyclic antidepressant. *Psychopharmacology (Berl)* **93**:358–364.

Wolkowitz OM, Reus VI, Manfredi F, Ingbar J, Brizendine L, and Weingartner H (1993) Ketoconazole administration in hypercortisolemic depression. *Am J Psychiatry* **150**:810–812.

Wong DT, Bymaster FP, Mayle DA, Reid LR, Krushinski JH, and Robertson DW (1993) LY248686, a new inhibitor of serotonin and norepinephrine uptake. *Neuropsychopharmacology* **8**:23–33.

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Pharmacological characterization of (3-(benzo[d][1,3] dioxol-4-yloxy) -3-(4-fluorophenyl)-N, N-dimethylpropan-1-amine (H05), a novel serotonin and noradrenaline reuptake inhibitor with moderate 5-HT_{2A} antagonist activity for the treatment of depression

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Supplementation Experimental Procedure

Chemicals

H05 was synthesized by Jiangsu Nhwa Pharmaceutical Co., Ltd (Jiangsu, China). Its structure is depicted in Figure 1. Chemicals such as duloxetine, fluoxetine, paroxetine, nomifensine, desipramine, GBR12909, adrenocorticotropic hormone (ACTH 1-24), DOI, 5-HT, NE and DA were purchased from Sigma Co., Ltd (St. Louis, MO, USA). Radioligands were purchased from PerkinElmer (Boston, MA, USA). All compounds were dissolved in distilled water and administered p.o. in a volume of 10 ml/kg unless specified.

Determination of selectivity of H05 over other CNS receptors

H05 binding was screened and evaluated against the members of 5-HT subfamilies and other CNS receptors using standard receptor binding procedures carried out under the National Institute of Mental Health's Psychoactive Drug Screening Program (NIMH-PDSP) (Roth, 2008). The tested receptors and standard procedures were summarized in Supplemental Table 1. The default concentration of H05 for primary binding was 10 μM. When the inhibition was >50% at 10 μM in primary test, compound **H05** was further tested in dose-dependent fashion to calculate IC_{50} value.

Statistical analysis

All data are presented as the means ± standard error of the mean (S.E.M). Statistical analyses were conducted using GraphPad Prism 5.0 (GraphPad Software,

San Diego, CA).

For binding assays, the receptors binding were analyzed using one-site nonlinear regression of concentration-effect curve. The IC_{50} values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).

As for data from behavioral tests, the data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test.

Results

Identification of the lead compound

SNRIs are the first line ADs used in the clinic with higher efficacies and better safety profiles than TCAs and SSRIs (Machado et al., 2006). Duloxetine is the most commonly used SNRI antidepressant. Aiming at discovering novel ADs with better efficacies and fewer side effects than duloxetine, we designed and synthesized a series of novel 3-(benzo[d][1, 3] dioxol-4-yloxy)-3-arylpropyl amine derivatives. Their binding affinities to SERT, NET and DAT were determined by the radioligand receptor binding assay *in vitro*. As shown in Table 4, five of the derivatives including **H01**, **H03**, **H04**, **H05**, and **H06** exhibited remarkable binding affinities to SERT and NET with the inhibition efficiencies over 95% at 10 μM. Further screening i*n vivo* revealed that **H01**, **H03**, **H04**, **H05**, **H06**, **H07** and **H08** were able to significantly reduce the immobility time in TST and FST at the dose of 10 mg/kg, and **H05** and **H06** exhibited evident antidepressant effects at a lower dose of 5 mg/kg (Supplemental Table 2). The inhibition efficiencies of **H05** in TST and FST were determined to be 63.0%-87.4%, higher than those of **H06** (34.8%-70%). Based on its dual action on SERT and NET *in vitro* and strong antidepressant activity *in vivo* (Supplemental Table 2), **H05** was identified as the lead compound for further evaluation.

Selectivity of H05 to CNS receptors

To evaluate the selectivity of H05, its binding affinities to a variety of CNS receptors were measured. As shown in Supplemental Table 3, **H05** displayed low

affinities to most tested receptors with the binding inhibition in the range of 0% - 60% at 10 μM, except for 5-HT_{2A} and 5-HT_{2C} that were inhibited 104.1% and 102.1%, respectively. Further tests with different doses of **H05** revealed that it inhibited [^{3}H] ketanserin and [^{3}H] mesulergine binding with IC_{50} of 71.05 ± 6.58 nM (n=3) and 482.4 ± 32.17 nM (n=3), respectively (Table 5, Table 6 and Figure 7a). These results indicate that, in addition to the strong binding affinities to both SERT and NET, **H05** also possesses moderate binding affinity to 5-HT_{2A} receptor.

References

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.

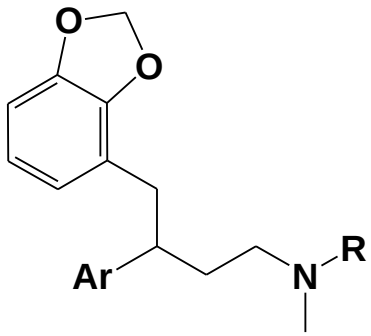
Machado M, Iskedjian M and Einarson TR (2006) Pmh4 Meta-Analysis of SNRIS, SSRIS, and TCAS in the Treatment of Major Depressive Disorder Using Remission as the Clinical Outcome. *Value Health* **9**:A65.

Roth BL (2008) Assay protocol book. National Institute of Mental Health - Psychoactive Drug Screening Program in *Chapel Hill: University of North Carolina* pp 3-66.

Supplemental Table 1. Conditions for receptor-binding selectivity assays

Target receptor	[^{3}H]Ligand (nM)	Non-specific ligand (μM)	Reaction conditions
5-HT_{1A}	[^{3}H]-8-OH-DPAT	5-HT(10)	25 °C, 60 min
5-HT_{1B}	[^{3}H]-GR-125743	5-HT(10)	25 °C, 60 min
5-HT_{2A}	[^{3}H]-Ketanserin	Methysergide (10)	37 °C, 25 min
5-HT_{2C}	[^{3}H]-Mesulergine	Ketanserin (10)	37 °C, 25 min
D_1	[^{3}H]-SCH23390	Butaclamol (10)	37 °C, 15 min
D_2	[^{3}H]-Spiperone	Haloperidol (10)	37 °C, 25 min
D_3	[^{3}H]-Spiperone	Haloperidol (10)	27 °C, 30 min
α_1	[^{3}H]-Prazosin	Prazosin (10)	25 °C, 60 min
α_2	[^{3}H]-Rauwolscine	Rauwolscine (10)	25 °C, 60 min
M_1	[^{3}H]-QNB	Atropine (10)	37 °C, 60 min
H_1	[^{3}H]-Pyrilamine	Promethazine (10)	30 °C, 60 min
H_3	[^{3}H]-Methylhistamine	Thioperamide (10)	30 °C, 40 min
Sigma-1	[^{3}H]-Pentazocine	Haloperidol (10)	25 °C, 135 min
Sigma-2	[^{3}H]-DTG	DTG(5)	25 °C, 135 min
NMDA	[^{3}H]-MK801	MK-801 (10)	25 °C, 120 min
$GABA_A$	[^{3}H]-Flunitrazepam	Clonazepam (10)	4 °C, 60 min

Supplemental Table 2. Effects of compounds on binding affinities in radioligand receptor binding tests and immobility time in FST and TST in mice



Compounds	Ar	R	In vitro test (n=3)				In vivo test (n=10)				
			Concentration	% Inhibition [a]			Dose	TST		FST	
			(μM)	SERT	NET	DAT	(mg/kg)	Immobility time(s) [b]	Inhibition rate (%) [c]	Immobility time(s) [b]	Inhibition rate (%) [c]
Vehicle	--	--	--	--	--	--	--	73.2 ± 23.4	--	113.7± 13.4	-
Duloxetine	--	--	10^{-5}	99.23	102.19	40.09	10	34.7±13.4	52.6	76.0±18.2	33.2

							20	17.8±5.8**	75.7	41.2±10.5**	63.8
H01	2-thiophene	CH_3	10^{-5}	97.54	98.23	50.62	5	41.0±8.3	44.0	80.1±11.4	29.6
							10	17.3±8.1**	76.3	46.5±13.1**	59.1
H02	3-OCH_3 benzene	CH_3	10^{-5}	71.24	60.35	36.08	5	53.1±15.4	27.5	81.6±20	28.2
							10	67.8±19.2	7.4	82.8±24.8	27.2
H03	benzene	CH_3	10^{-5}	98.14	99.52	18.33	5	42.9±18.0	32.8	70.1±17.4*	38.3
							10	12.5±9.1**	82.9	59.8±20.2*	47.4
H04	benzene	H	10^{-5}	97.58	101.38	50.60	5	44.3±10.9	39.5	38.9±10.9**	65.7
							10	8.2±4.3**	88.8	28.6±6.1**	74.8
H05	4-F benzene	CH_3	10^{-5}	99.98	107.53	74.40	5	22.0±8.92**	69.9	42.0±17.8**	63.0
							10	9.2±4.2**	87.4	31.0±17.2**	72.7
H06	4-F benzene	H	10^{-5}	99.05	96.18	61.31	5	21.4±4.05**	70.0	74.1±11.6*	34.8
							10	29.6±11.1*	59.6	60.1±13.4**	47.2

H07	3-Cl benzene	CH_3	10^{-5}	95.31	74.54	55.60	5	35.9±12.9	50.9	75.6±18.5	33.5
							10	16.3±2.5**	77.7	51.6±10.4**	54.6
H08	3-Cl benzene	H	10^{-5}	98.27	81.97	54.31	5	54.8±10.7	25.2	87.2±17.7	23.3
							10	34.0±9.6*	53.5	63.7±14.5*	40.8
H09	4-Cl benzene	CH_3	10^{-5}	90.58	61.07	10.34	5	64.4±11.6	12.0	89.9±13.4	20.9
							10	31.1±7.7*	57.5	81.6±15.2	28.2
H10	4-Cl benzene	H	10^{-5}	88.54	65.48	29.68	5	85.4±14.8	-16.8	101.9±16.6	10.3
							10	70.7±20.4	3.4	103.7±20.3	8.8

[a] Compound exhibiting >95% inhibition was considered as a bioactive compound in vitro; [b] *p<0.05, **p<0.01 compared with the vehicle control group;

[c] Inhibition rate (%) = (vehicle group immobility time – compound group immobility time) × 100 / vehicle group immobility time.

Supplemental Table 3. Binding affinities of H05 for brain receptors

Target receptor	Radioligand	% Inhibition of binding at 10 μM H05	IC_{50} for H05 (nM) [a]
5-HT_{1A}	[^{3}H]-8-OH-DPAT	32.4	
5-HT_{1B}	[^{3}H]-GR-125743	21.2	
5-HT_{2A}	[^{3}H]-Ketanserin	104.06	71.05 ± 6.58
5-HT_{2C}	[^{3}H]-Mesulergine	102.14	482.4 ± 32.17
D_1	[^{3}H]-SCH23390	40.32	
D_2	[^{3}H]-Spiperone	18.23	
D_3	[^{3}H]-Spiperone	44.27	
α_1	[^{3}H]-Prazosin	56.58	> 10,000
α_2	[^{3}H]-Rauwolscine	-2.15	
M_1	[^{3}H]-QNB	9.85	
H_1	[^{3}H]-Pyrilamine	35.80	
H3	[^{3}H]-Methylhistamine	-1.91	
Sigma-1	[^{3}H]-Pentazocine	38.98	
Sigma-2	[^{3}H]-DTG	61.81	> 10,000
NMDA	[^{3}H]-MK801	17.43	
$GABA_A$	[^{3}H]-Flunitrazepam	29.29	

The default concentration of H05 for primary binding test was 10 μM, and % inhibition was obtained from at least three independent experiments, and each experiment was performed in triplicates.

[a]Compound exhibiting >50% inhibition at any target receptor was further evaluated in dose-dependent effect in binding assays for calculation of IC_{50} values (means ± S.E.M). The data were from three independent experiments, and each experiment was performed in triplicates