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Title:

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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Running title page

Running title: A novel SNRI with 5-HT_{2A} antagonist activity for treatment of depression

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Abbreviations: ADs: antidepressants; SSRIs: selective serotonin reuptake inhibitors; NRIs: norepinephrine reuptake inhibitors; SNRIs: serotonin and norepinephrine reuptake inhibitors; MAOIs: monoamine oxidase inhibitors; TCAs: tricyclic antidepressants; SERT: serotonin transporter; NET: norepinephrine transporter; DAT: dopamine transporter; H05:(3-(benzo[d] [1, 3] dioxol-4-yloxy)-3- (4-fluorophenyl)-N, N-dimethylpropan-1-amine; FST: forced swimming test; TST: tail suspension test; MED: minimal effective doses; CUMS: chronic unpredictable mild stress; PK: pharmacokinetic; ACTH: adrenocorticotropic hormone; CORT: corticosterone; DOI: R(-)-2,5-Dimethoxy-4-iodoamphetamine; HPA: hypothalamic–pituitary-adrenal;

FLIPR: Fluorescent Image Plate Reader.

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Abstract

Multi-target antidepressants selectively inhibiting monoaminergic transporters and 5-HT_{2A} receptor have demonstrated higher efficacy and fewer side effects than selective serotonin reuptake inhibitors (SSRIs). 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 (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 duloxetine, and showed no stimulatory effect on locomotor activity. The administration of **H05** (5, 10 and 20 mg/kg, p.o.) significantly shortened the immobility time of ACTH-treated rats that serve as a model of treatment-resistant depression, while imipramine (30 mg/kg, p.o.) and duloxetine (30 mg/kg, p.o.) showed no obvious effects. Chronic treatment with **H05** reversed the depressive-like behaviors in rat model of chronic unpredictable mild stress and mouse model of corticosterone-induced depression. Microdialysis analysis revealed that administration of **H05** at either 10 or 20 mg/kg increased the release of 5-HT and NE from the frontal cortex. The pharmacokinetic 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 norepinephrine 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 is the highest burden to patients and society, which can lead to disability and suicide (Whiteford et al., 2013).

Over the past 60 years, several different types of antidepressants (ADs), such as tricyclic antidepressants (TCAs), monoamine oxidase inhibitors (MAOIs), selective serotonin reuptake inhibitors (SSRIs), norepinephrine reuptake inhibitors (NRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs), have been developed. TCAs and MAOIs 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 in 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 signficantly 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 the first line ADs treatment (Trivedi et al., 2006; Warden et al., 2007; Mathew, 2008). Such treatment-resistant depression suggests substantial unmet

medical needs (Little, 2009). Therefore, more effective ADs with less adverse reactions, especailly for treatment-resistant depression therapy, are highly desired.

Accumulative evidence indicates that multi-target drugs selectively targeting monoaminergic transporters and the subtypes of 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 multi-action antidepressant with affinity for α2, 5-HT_{2A} (Ki = 10 nM), 5-HT_{2C}, and 5-HT_3 receptor, 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 antidepressant, acts at 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 antidepressants in the improvement of cognitive symptoms of depression (Pehrson et al., 2015). Trazodone and nefazodone, two antidepressants, have both high affinity at

rat 5-HT_{2A} receptor (K_i is 20 nM and 7.1 nM, respectively) and relatively low affinity at human SERT and NET (K_i >500 nM) (Owens et al., 1997). These two drugs, due to their 5-HT_{2A} receptor antagonist activity, can produce less sleep disorders and sexual dysfunction than SSRIs in 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 FDA as an adjunctive antidepressant treatment for major depressive disorder (Mathew, 2008). All these studies suggest that ADs with both monoaminergic reuptake inhibition effect and 5-HT_{2A} antagonism property represent an attractive strategy for depression treatment, especially for treatment-resistant depression therapy.

In the present work, we screened and identified some compounds that are capable of selectively inhibiting serotonin and norepinephrine 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 the (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 (see supplemental materials for details). **H05** exhibited robust antidepressant effects *in vitro* and *in vivo*. The pharmacokinetics and blood-brain penetration analysis suggest that **H05** possesses druggable properties with potential application in depression therapy.

Materials and Methods

Animals

Male healthy ICR mice of 18-22 g or male Sprague-Dawley (SD) rats of 180-350g were purchased from Vital River Laboratory Animal Technology Co., Ltd (Beijing, China). Animals were housed in groups under standard conditions at room temperature of 22 ± 2 °C, humidity 50 % ± 10 % and a 12 h 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 consistent with the guidelines of the Bylaw of Experiments on Animals.

Chemicals

Compound **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 via p.o. in a volume of 10 ml/kg unless specified.

Measurement of binding affinity of H05 to monoamine transporters

The competitive binding assays for H05 and reference antidepressant agents against rat serotonin transporter (SERT), norepinephrine transporter (NET) and

dopamine transporter (DAT) were performed as 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, NET) or striatum (for DAT). Competitive binding assays were performed in reaction buffer containing 50 μg membrane proteins, [^{3}H]-paroxetine (0.5 nM), [^{3}H]-nisoxetine (0.5 nM) or [^{3}H]-WIN-35428 (0.5 nM) and various concentrations of compounds (10^{-1}-10^4 nM) at 23°C for 60 min (for SERT)，or 25°C for 30 min (for NET) or 4°C for 120 min (for DAT). Non-specific binding to SERT, NET and DAT was determined using 10 μM paroxetine, 10 μM desipramine and 10 μM nomifensine, respectively. Experimental conditions for use of radioligands such as [^{3}H] paroxetine, [^{3}H] nisoxetine and [^{3}H] WIN-35428 and non-specific ligands such as paroxetine, nomifensine, and desipramine were summarized in Table 1. 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 Krebs bicarbonate solution 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 min. The non-specific 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 triplicates 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's Psychoactive Drug Screening Program (NIMH-PDSP) (Roth, 2008). The conditions and standard procedures were 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 membrane proteins, [^{3}H]-ketanserin (0.6 nM) and various concentrations of **H05** (10^{-1}-10^4 nM) at 37°C for 25 min. Non-specific binding to 5-HT$_{2A}$ was determined using 10 μM methysergide. Inhibition rate was calculated by 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, CHO-K1 cells stably expressing human 5-HT$_{2A}$ receptors (Accession number NM_000621) were lightly trypsinized and seeded in 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 test.

For agonist test, 20 μl calcium assay loading buffer (20 mM HEPES/HBSS with 2.5 mM probenecid and 4 μM Fluo-4, pH7.4) was added to each well after removal of culture medium. The cell plate was first placed into a 37°C incubator for 60 min, followed by 15 min at room temperature before transfer to reading position of Fluorescent Image Plate Reader (FLIPR) (Molecular Devices, Sunnyvale, CA, USA) for fluorescence signals for 20 s. Addition of 5 μl in 5 × final concentration of compounds, such as 5-HT (as positive control) or 0.25% DMSO (as negative control) to the reading plate at 20 s was made and the change in cell fluorescence signal was measured for an additional 100 s (21 s to 120 s).

For antagonist test, the medium was removed and replaced with 20 μl calcium assay loading buffer (20 mM HEPES/HBSS with 2.5 mM probenecid and 4 μM Fluo-4, pH7.4) and 5 μl of compound or ketanserin (as positive control) in 5-time concentration or 0.25% DMSO (as negative control). The cell plate was first incubated at 37°C for 60 min, followed by 15 min at room temperature before transfer to the reading position of FLIPR for fluorescence signals for 20 s. Adding 5 μl of control agonist 5-HT in 6-time concentration to the reading plate at 20 s was made and the change in fluorescence signals was measured for an additional 100 s (21 s to 120 s).

For both agonist and antagonist tests, the average value of 20 s (1 s to 20 s) in reading as the baseline was calculated, and the relative fluorescent unit (∆RFU)

intensity was calculated as the maximal fluorescent units (21 s to 120 s) after subtraction of the average value of baseline readings. The percentage effect of compound was calculated using the following equation:

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

Dose response curves for agonist/antagonist were fitted with four-parameter logistic equation using the software GraphPad Prism 5.0.

Forced swim test (FST) in mice

The 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 eleven 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 min of total 6 min was recorded, and animals were judged to be immobile when they floated motionless, making only necessary movements to keep their heads above the water.

Tail suspension test (TST) in mice

The TST procedure in mice was adapted from descriptions by Steru et al (Steru et al., 1985). 110 mice were randomly assigned to eleven 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 of 10 mice. After oral administration of compounds for 60 min, each mouse was suspended on the top of apparatus using adhesive tape placed approximately 1 cm from the tail tip. The immobility duration of the last 4 min of total 6 min period was recorded. Mice were considered to be immobile when hung passively without moving.

Locomotor activity measurement

To assess the effect of H05 on locomotion, forty naive mice were randomly divided into four groups with 10 mice in each group. Animals were transferred into the testing room at least 2 h 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 (L × W × H: 25 × 25 × 30 cm) for a 5 min acclimation period. Sixty minutes after drug administration, spontaneous locomotor activity was recorded for 30 min using a tracking and computerized analysis system (Clever Sys Inc., Leesburg, VA, USA). The area was wiped and cleaned with 75% alcohol solution and dried before each test.

Rat model of chronic unpredictable mild stress (CUMS)

To further evaluate the antidepressant-like effects of H05, rat model of 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 h before one of the two bottles of sucrose solution was replaced with water for 24 h. Prior to the sucrose baseline test, rats ware food and water deprived for 20 h. In the sucrose baseline test, each rat was presented simultaneously with 2 bottles, one containing

sucrose (0.8%), and the other containing water. The baseline for sucrose intake was calculated and animals were divided into 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 control group, were subjected to 4-week stress. The stressors contain: water and food deprivation (24 h), overnight illumination, stroboscope (120 flashes/min), intermittent illumination (lights on and off in every 2 hours), white noise (110 dB), soiled cage (200 ml water in sawdust bedding), forced swimming (5 min at 4 °C), tail pinch (1 min), cage tilting (45°), electrical shock (0.8 mA, 10 s duration) and restraint (2 h). 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 non-stressed rats as control, except for the deprivation period of 20 h prior to each sucrose test. Details of stressors and schedule for model of CUMS in rats were listed in Table 2.

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

Mouse model of corticosterone (CORT) -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 min prior to the CORT (40 mg/kg, s.c.) injection for 21 days. The sucrose preference test was employed 24 h after the last administration of drug. 72 h before the test, mice were individually trained to adapt to sucrose solution (0.8%, w/v) by placing two bottles of sucrose solution in each cage for 24 h before one of the two bottles was replaced with water for 24 h. Mice were deprived of water and food for 24 h 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). Sucrose preference test was conducted at 9:00 a.m. After 24 h, the amount of consumed sucrose solution and water were recorded and the sucrose preference was calculated. The protocol schematic of CORT-induced depression in mice was shown in Figure 6b.

DOI-induced head twitch in mice

The R(-)-2,5-Dimethoxy-4-iodoamphetamine (DOI) induced head twitch test in mice was performed as previously described (Fantegrossi et al., 2010). Briefly, a total of 48 naive mice were randomly assigned to six groups with 8 mice in each group: 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 min 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 min by observers.

Rat model of ACTH-induced treatment-resistant depression

The adreno-cortico-tropic-hormone (ACTH) induced rat model of treatment-resistant depression (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 into 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); 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 two sessions: the 15 min pre-swim session, and a 6-min swimming test session followed 24 h later. The last injection of ACTH was given immediately following the pre-swim test. Imipramine or duloxetine was injected intraperitoneally, H05 was orally administrated 0.5 h, 5 h and 23 h before the swimming test session. The total period of immobility during the 6-min period of testing was recorded. The schematic protocol of ACTH-induced treatment-resistant depression in rats was shown in Figure 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 to 350g were anaesthetized with 10% chloral hydrate and placed in a stereotaxic apparatus (RWD Life Science Co., Ltd., Shenzhen, Guangdong Province, China).

After exposure of skull, a hole for probe was drilled. The CXG-2 guide cannula (Eicom Co., Kyoto, Japan) was implanted into the medial prefrontal cortex (mPFC) (AP + 3.2mm, L + 0.8 mm and DV -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, dialysis probe (Eicom; 0.22 mm o.d., 4 mm length, cut-off 50 kDa) 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 $CaCl_2$, 1mM $MgCl_2$ and 1.4mM Na_2HPO_4, PH 7.4) at a flow rate of 1 μl/min. After an initial 2 h equilibration period, samples of dialysate were collected every 30 min. Two samples were collected for analysis of basal levels of 5-HT, DA and NE, before the animals were administrated of compound H05 (10, 20 mg/kg, p.o.). The dialysate samples were collected for an additional period of 240 min after dosing. 1 μl 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 UPLC-MS/MS method as previously described (Song et al., 2012). Briefly, 10 μl dialysate samples were 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 benzoyl chloride-(phenyl-$^{13}C_6$) ($^{13}C_6$BZ) was added to improve quantitation. A Waters Acquity UPLC (Waters, Milford, MA) system was used with automatic injection of 10 μl sample into a Waters UPLC BEH C_{18} column (100 mm × 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^{TM} 5500 tandem mass spectrometer (AB Sciex, Framingham, MA).

Pharmacokinetic (PK) assay and brain penetration analysis

PK study

12 male SD rats were randomly divided into two groups with 6 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 h and had free access to water before dosing. Serial blood samples (approximately 0.3 ml) were collected from tail veins at time point 0 min, 5 min, 15 min, 30 min, 1.0 h, 2.0 h, 3.0 h, 7.0 h, 9.0 h, 11.0 h, 24 h, 30 h, 48 h and 54 h after H05 administration. The plasma was separated by centrifugation at 3000 g for 10 min, and stored at -70 °C until analysis. The plasma samples were analyzed for test compounds/drugs and internal standard using Agilent G6120B LC-MS (Agilent Co. Ltd, USA).

Blood-brain barrier penetration test

Six male SD rats were used for brain penetration study of the compound. Animals were fasted 8 hours before compound administration. The H05 was administered orally (30 mg/kg), and two hours (approximately the T_{max} of H05) later blood samples and whole brain from each rat were collected. Blood samples were collected in heparinized tubes and immediately centrifuged at 3000 g for 10 min. 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 G6120B LC-MS (Agilent Co. Ltd, USA).

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/uptake assays, the transporter binding and monoamine uptake results were analyzed using one-site nonlinear regression of concentration-effect curve. The K_i values were calculated using the Cheng-Prusoff equation (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-measure ANOVA, followed by Tukey's test, was used to compare the percentage increase from the baseline among different groups for microdialysis experiments. 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 of 2.30 ± 0.06 nM, 2.11 ± 0.17 nM, and 439.00 ± 35.11 nM, respectively (Table 3, Figure 2a-c). The K_i of **H05** against SERT and NET was determined to be 3.54 ± 0.25 nM and 2.05 ± 0.07 nM, respectively, suggesting its potent binding affinities (Table 3, Figure 2a-b). However, **H05** exhibited a weak binding affinity to DAT with K_i >1000 nM (Table 3 and Figure 2c).

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} of 4.81 ± 0.31 nM and 6.72 ± 1.02 nM, respectively (Table 3, Figure 3a-b). Unlike duloxetine that 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 with a negligible potency to inhibit [^{3}H] DA uptake (IC_{50}>1000 nM) (Table 3, Figure 3a-c).

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 (Figure 4a and 4b). DA

levels were also elevated by **H05**, but not at the statistically significant level (Figure 4c).

Efficacious antidepressant activity of H05 in multiple animal models of depression

The antidepressant 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 Figure 5a and 5b, the oral administration of duloxetine (10, 20 and 40 mg/kg) decreased the duration of immobility in a dose-dependent manner with the minimal effective doses (MED) 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 lower MED of 2.5 mg/kg in both tests, suggesting 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 min period, indicating that the **H05**-induced decrease in the immobility time in FST and TST did not result from psychostimulant effect, but rather antidepressant activity (Figure 5c).

The antidepressant effects of **H05** were further evaluated in the rat model of CUMS and 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 four-week period of CUMS caused significant decreases with an average value of 27% ($p<0.05$, n=10) in the sucrose preference of stress group,

compared with the non-stressed 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 stressed (Figure 6c). Compared with the vehicle control, chronic subcutaneous injection of CORT (40 mg/kg) resulted in an obvious decline in sucrose consumption in mice (Figure 6d), which was successfully reversed by oral administration of **H05** (5, 10 and 20 mg/kg), suggesting the antidepressant-like effects of **H05** on the CORT-induced depression in mice (Figure 6d).

5-HT2A receptor antagonist activity and antidepressant-like effects of H05 in the ACTH-induced rat model of treatment–resistant depression

A moderate binding affinity of **H05** to 5-HT_{2A} receptor was found with IC_{50} of 71.05 ± 6.58 nM by the binding test (Supplemental Table 3, Table 3 and Figure 7a). As agonist or antagonist might exert different biological activities, the intrinsic activity of **H05** at 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 (Table 3, Figure 7b-c), without agonistic effect on 5-HT_{2A} receptor, suggesting that **H05** is a 5-HT_{2A} receptor antagonist.

R(-)-2,5-Dimethoxy-4-iodoamphetamine (DOI) is a 5-$HT_{2A/2C}$ agonist that can elicit the head twitch behavior mediated by the activation of 5-HT_{2A} receptor in mice. To further evaluate the inhibitory effect **H05** on 5-HT_{2A} receptor *in vivo*, the DOI-induced head twitch in mice was treated with **H05**. As shown in Figure 7d, DOI caused head twitch responses in mice, which were suppressed by pretreatment with

H05 in a dose dependent manner.

The effect of **H05** treatment on treatment-resistant depression 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/day, s.c.) for 14 days, indicating that neither of them were effective antidepressants for treatment-resistant depression induced by ACTH (Figure 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 antidepressant effect of **H05** on treatment-resistant depression (Figure 7f).

Favorable PK properties and good blood-brain penetration ability for H05

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 the oral and intravenous administrations in rats. As shown in Table 3, the oral administration of **H05** to rats (30 mg/kg, n = 6) resulted in $t_{1/2}$ of 3.87 h, T_{max} of 2 h, and C_{max} of 165.20 ng/ml. The $AUC_{(0-\infty)}$ was measured to be 912.02 ng·h/ml for oral administration (30 mg/kg) and 868.88 ng·h/ml for intravenous administration (5 mg/kg) with the 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 two 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 lead compound with inhibition effects on both monoaminergic transport and 5-HT_{2A} receptor (Supplemental Table 2-3).

H05 exhibits strong binding affinities to SERT and NET and 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 SERT and NET, respectively, suggesting that it is an SNRI. In addition, **H05** shows more balanced affinities to NET and SERT with the IC_{50} ratio of 1.40 than conventional SNRIs. For example, the K_i ratios of NET/SERT reuptake 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 profile, as Dreyfus *et al.* indicated that SNRIs with comparable potency can lead to a drug with more safety profile and superior efficacy(Dreyfus et al., 2013). Unlike duloxetine (K_i>700 nM), venlafaxine (K_i>100000 nM), paroxetine (K_i=6320 nM) and fluoxetine (K_i=141 nM) that

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 IC_{50} of 60.37 nM. The 5-HT_{2A} receptor antagonistic property may render **H05** more potency and 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 antidepressant effects of **H05** are partly resulted from its ability to inhibit NE and 5-HT uptake.

Behavioral TST and FST are widely used to predict the antidepressant activity of 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 much a lower MED of 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 antidepressant activity. Locomotor activity analyses indicate that **H05** does not significantly alter total travel distance at the doses of 10, 20, and 40 mg/kg, which can likely exclude false positive antidepressant effects.

In addition to the acute behavioral despair model, two chronic stress models,

mouse model of CORT-induced depression and rat model of CUMS, were also used to further evaluate the antidepressant effects of H**05**. 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 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** can be potentially developed as an antidepressant.

Up to date, the psychopathological and neurobiological mechanisms of treatment-resistant depression still remain unclear. Some studies suggest that the hyperactivity of hypothalamic–pituitary-adrenal (HPA) axis is correlated with treatment-resistant depression (Murphy et al., 1991; Wolkowitz et al., 1993). Kitamura *et al.* showed that the chronic administration of adrenocorticotropic hormone ($ACTH_{1\text{-}24}$) could effectively inhibit the antidepressant effects of various antidepressants including imipramine, desipramine and milnacipran in rat (Kitamura et al., 2002a; Kitamura and Gomita, 2008). Similarly, Srikumar *et al.* found that chronic ACTH administration did not exhibit depression-like phenotype in mice, but could diminish the antidepressant-like effects of duloxetine, imipramine, fluoxetine and bupropion in FST (Srikumar et al., 2017). In addition, effective clinical therapies for treatment-resistant depression, such as the co-administration of lithium or carbamazepine with imipramine and repeated electroconvulsive stimulation, can

decrease the immobility duration in ACTH-treated rats (Kitamura et al., 2002a; Li et al., 2006; Kitamura et al., 2008). These findings suggest that ACTH-treated rat is a useful animal model of treatment-resistant depression (TRD). Consistent with these findings, we find that chronic administration of ACTH (100 μg/d, 14d, s.c.) attenuates antidepressant-like effects of imipramine (30 mg/kg, i.p.) or duloxetine (30 mg/kg, i.p.) in FST, while acute administration of **H05** (5, 10 and 20 mg/kg, p.o.) significantly shortens the immobility time of ACTH treated rats, suggesting that **H05** might be a promising AD for the treatment of refractory depression. However, we used only one dose of duloxetine or imipramine under acute administration procedure, the both were ineffective in this TRD model, and multiple doses and chronic administration of duloxetine or imipramine might yield efficacy. Takao *et al.* showed that the activation of HPA axis could regulate the function of 5-HT_{2A} receptor (Takao et al., 1997). Kuroda and Mikuni reported that the ACTH induced activation of HPA axis could increase the 5-HT_{2A} receptor expression level in frontal cortex and the number of DOI, a 5-HT_{2A} receptor agonist, induced wet-dog shakes (Kuroda et al., 1992). Other studies demonstrate that the antidepressant-like effects on ACTH-treated rats caused by co-administration 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 treatment-resistant depression 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 CNS penetrant with the penetration ratio (brain concentration:plasma concentration) of 3.94, much higher than that (human cerebrospinal fluid concentration:plasma concentration=0.023) of duloxetine (Paulzen et al., 2016). The better CNS penetrant ability of **H05** may explain why it has similar binding potencies towards SERT and NET *in vitro* as duloxetine, but lower MED in TST and FST. These results also indicate that **H05** may have less 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% remained in 2 hours. In contrast, **H05** is highly stable under the acidic conditions with >99% remained in 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 indentified as a lead compound with developmental potential for an antidepressant with superior efficacy. **H05** is a potent SNRI with 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 antidepressant activities in a variety of behavioral tests, with much lower MED 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 drugable. 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 treatment-resistant depression.

Authorship Contributions

Participated in research design: Xiangqing Xu, Qiang Guo, Guisen Zhang, KeWei Wang

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Footnotes

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Competing financial interest

All authors declare that there is no any competing financial interest in this submitted work.

Figure legends

Figure 1. Chemical structure of the lead compound **H05**.

Figure 2. Binding affinities of **H05** to SERT **(a)**, NET **(b)**, and DAT**(c)** determined by radioligand receptor binding assay. Data are presented as the means ± S.E.M. of three independent tests that were conducted in triplicates.

Figure 3. Inhibition effects of **H05** on the [^{3}H]-5-HT **(a)**, [^{3}H]-NE **(b)** and [^{3}H]-DA **(c)** uptakes into rat brain synaptosomes. Data are presented as the means ± S.E.M. of three independent tests that were conducted in triplicates.

Figure 4. The extracellular expression levels of 5-HT **(a)**, NE **(b)** and DA **(c)** in the frontal cortex of 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 means ± S.E.M., relative to basal pretreatment value that 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.

Figure 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;

Figure 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 two-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. Following the initial 2 weeks of CUMS, animals received daily intraperitoneal injections of vehicle or drugs 30min before another 2 weeks CUMS test. $^{*}p<0.05$ vs. CON, $^{\#}p<0.05$ vs. STR. CON, control; STR, stress; DLX, duloxetine (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, p.o.) was administrated 60 min 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 means ± S.E.M.

Figure 7. Inhibition effects of **H05** on 5-HT_{2A} receptor and its antidepressant-like effects in ACTH-induced rat model of treatment–resistant depression. **(a)** Binding affinities of **H05** to 5-HT_{2A} determined by radioligand receptor binding assays. Data are presented as mean ± S.E.M. from three independent tests, and each test was performed in triplicates; **(b-c)** Dose response curves and IC_{50} in antagonist mode (b) and EC_{50} in agonist mode (c) in 5-HT_{2A} calcium assay of **H05**. 5-HT, ketanserin and **H05** were dissolved in DMSO at 30 mM, and diluted with assay buffer in 11 serial concentrations. Each compound was tested in three duplicates. The fluorescent signals were conversed to % 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 DOI-induced head twitch response in mice. Mice were administered with **H05** (0.3, 1, 3 and 10 mg/kg, p.o.) 60 min prior to DOI injection (1 mg/kg, i.p.). The number of head twitches was counted for a total of 20 min.**p<0.01 vs vehicle, $^{\#\#}p$<0.01 vs the DOI group (n=8). Data are expressed as the means ± S.E.M. **(e)** Schematic protocol for ACTH-induced treatment-resistant depression test. **(f)** Effects of imipramine (IMI), duloxetine (DLX) and **H05** on the immobility in ACTH treated rats. ACTH (100 μg/day, s.c.) was administered to rats once every day for 14 days. The immobility time was measured the day following the final ACTH treatment. Imipramine (30 mg/kg, i.p.), duloxetine (30 mg/kg, i.p.) and **H05** (5, 10 and 20 mg/kg, p.o.) were administrated three times (0.5, 5 and 23 h) before the forced swim test. **P<0.01 vs. vehicle (n=12). Data are expressed as the means ± S.E.M.

Tables

Table 1. Conditions for binding and uptake assays.

Assays	Species	Tissue	[^{3}H]Ligand(nM)	Non-specific ligand(μM)	Reaction conditions
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	WIN35,428 (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

Table 2. Chronic unpredictable mild stress regime

Day	Weeks			
	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: 5min	Restraint stress:2h	Unpredictable shocks 30 min
Thursday	Social defeat: 1h; Water deprivation: 24 h	Social defeat: 1 h; Water deprivation: 24 h	Social defeat: 1h; 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

Table 3. Summary of pharmacologic and pharmacokinetic properties of H05 and duloxetine

Assays	H05	Duloxetine
SERT binding, [^{3}H]-Paroxetine, Ki (nM)	3.54 ± 0.25	2.30 ± 0.06
NET binding, [^{3}H]-Nisoxetine, Ki (nM)	2.05 ± 0.07	2.11 ± 0.17
DAT binding, [^{3}H]-WIN35,428, Ki (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, PO, $T_{1/2}$(h)	3.87	ND
Rat PK, 30 mg/kg, PO, T_{max}(h)	2	ND
Rat PK, 30 mg/kg, PO, Bioavailability	17.49%	ND
Blood-brain barrier penetration, C_{brain}/C_{plasm}	3.94	0.023[b]

Data are presented as the means ± S.E.M (n=3) from three independent experiments, and each experiment was performed in triplicates; ND, not determined. [a]*(Wong et al., 1993; Karpa et al., 2002)* ; [b]*(Paulzen et al., 2016).*

Figures

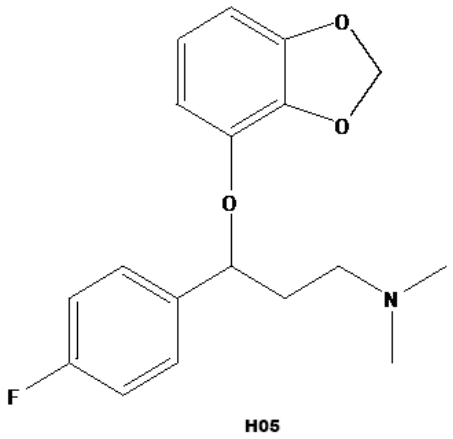

Figure 1

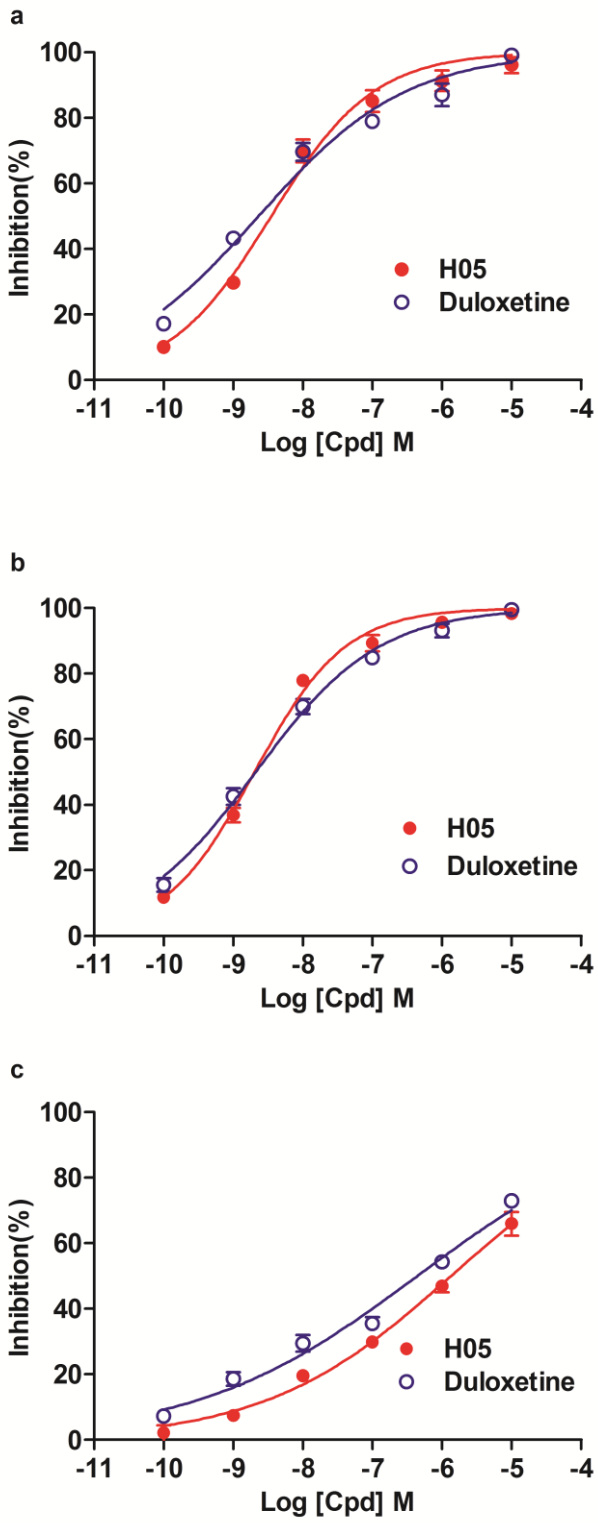
a
Inhibition(%)
100
80
60
40
20
0
-11
-10
-9
-8
-7
-6
-5
-4
Log [Cpd] M
H05
Duloxetine
b
Inhibition(%)
Log [Cpd] M
H05
Duloxetine
c
Inhibition(%)
Log [Cpd] M
H05
Duloxetine

Figure 2

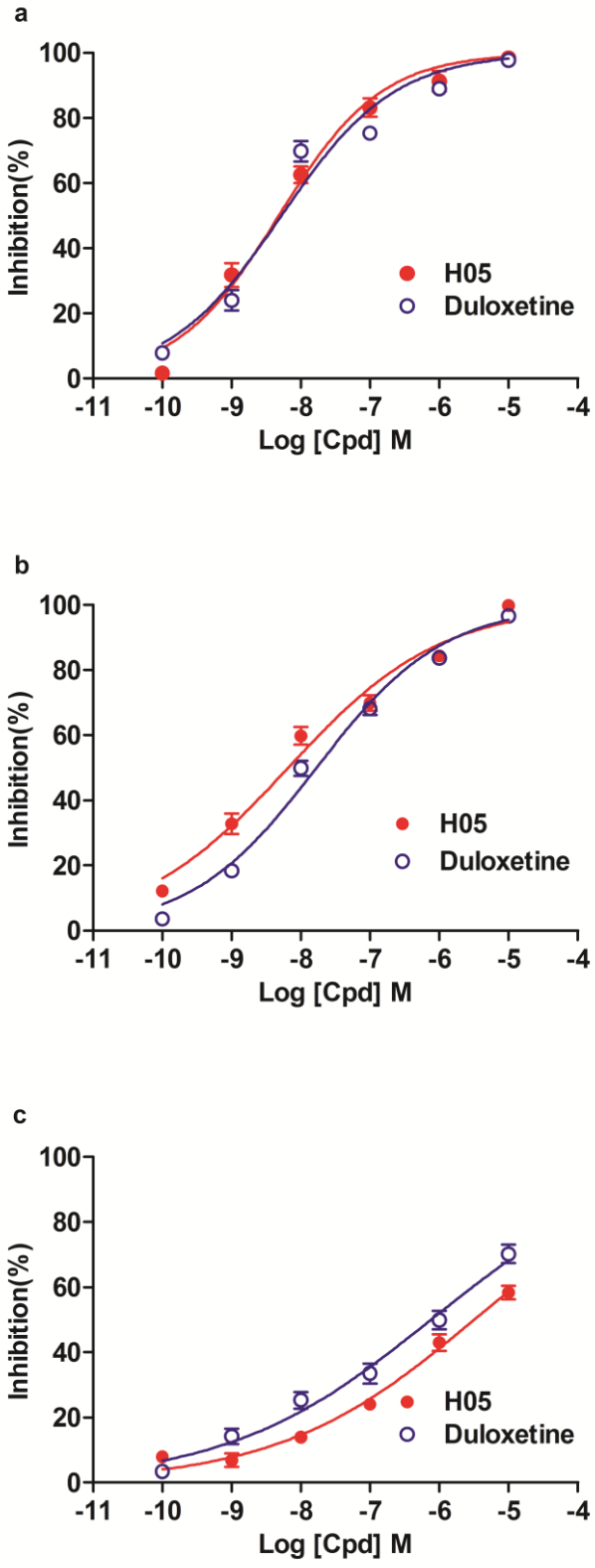
a
100
80
60
40
20
0
Inhibition(%)
-11
-10
-9
-8
-7
-6
-5
-4
Log [Cpd] M
H05
Duloxetine
b
100
80
60
40
20
0
Inhibition(%)
-11
-10
-9
-8
-7
-6
-5
-4
Log [Cpd] M
H05
Duloxetine
c
100
80
60
40
20
0
Inhibition(%)
-11
-10
-9
-8
-7
-6
-5
-4
Log [Cpd] M
H05
Duloxetine

Figure 3

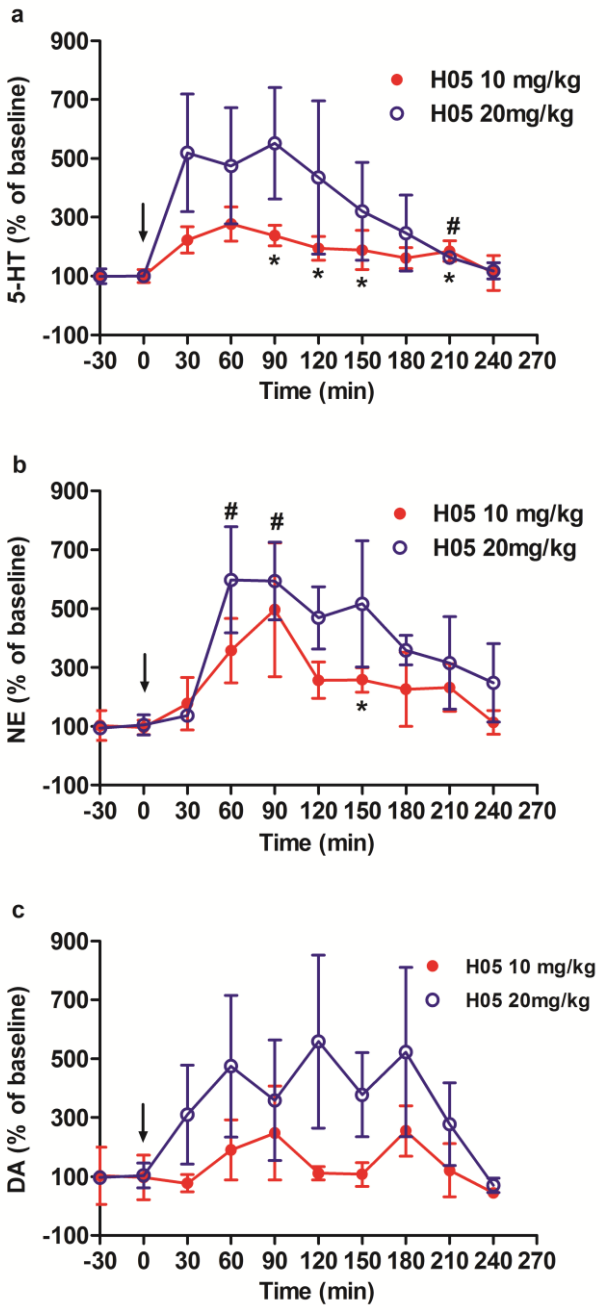
a
900
700
500
300
100
-100
5-HT (% of baseline)
H05 10 mg/kg
H05 20mg/kg
#
*
*
*
*
-30 0 30 60 90 120 150 180 210 240 270
Time (min)
b
900
700
500
300
100
-100
NE (% of baseline)
H05 10 mg/kg
H05 20mg/kg
#
#
*
-30 0 30 60 90 120 150 180 210 240 270
Time (min)
c
900
700
500
300
100
-100
DA (% of baseline)
H05 10 mg/kg
H05 20mg/kg
-30 0 30 60 90 120 150 180 210 240 270
Time (min)

Figure 4

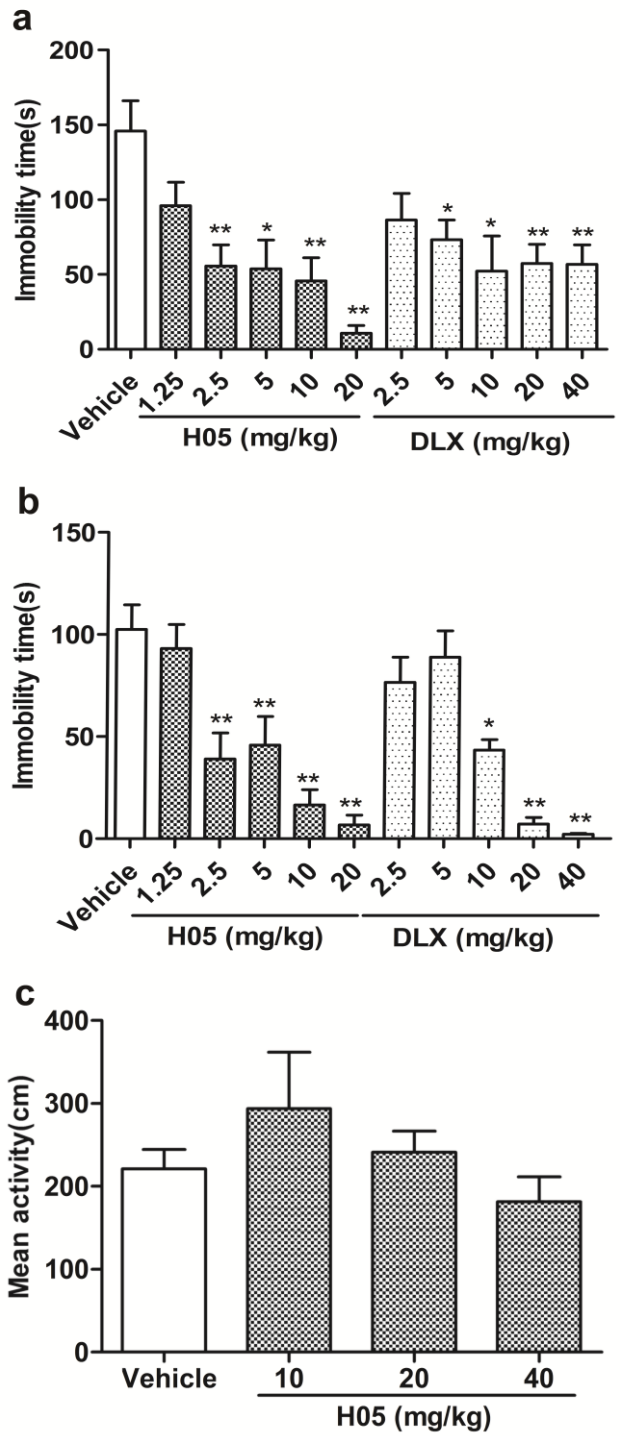
a
Immobility time(s)
200
150
100
50
0
Vehicle
1.25
2.5
5
10
20
2.5
5
10
20
40
H05 (mg/kg)
DLX (mg/kg)
b
Immobility time(s)
150
100
50
0
Vehicle
1.25
2.5
5
10
20
2.5
5
10
20
40
H05 (mg/kg)
DLX (mg/kg)
c
Mean activity(cm)
400
300
200
100
0
Vehicle
10
20
40
H05 (mg/kg)

Figure 5

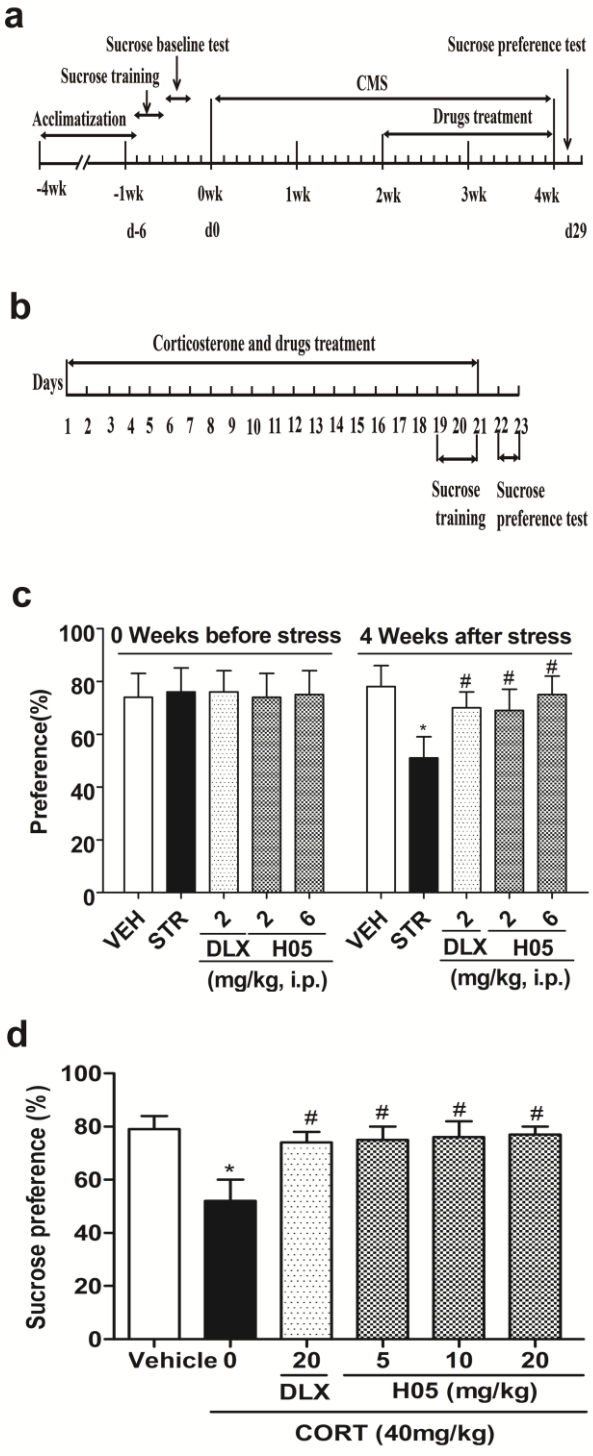
a
Sucrose baseline test
Sucrose preference test
Sucrose training
CMS
Acclimatization
Drugs treatment
-4wk
-1wk
0wk
1wk
2wk
3wk
4wk
d-6
d0
d29
b
Corticosterone and drugs treatment
Days
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Sucrose training
Sucrose preference test
c
0 Weeks before stress
4 Weeks after stress
Preference(%)
100
80
60
40
20
0
VEH
STR
2
2
6
DLX
H05
(mg/kg, i.p.)
d
Sucrose preference (%)
Vehicle 0
20
5
10
20
DLX
H05 (mg/kg)
CORT (40mg/kg)

Figure 6

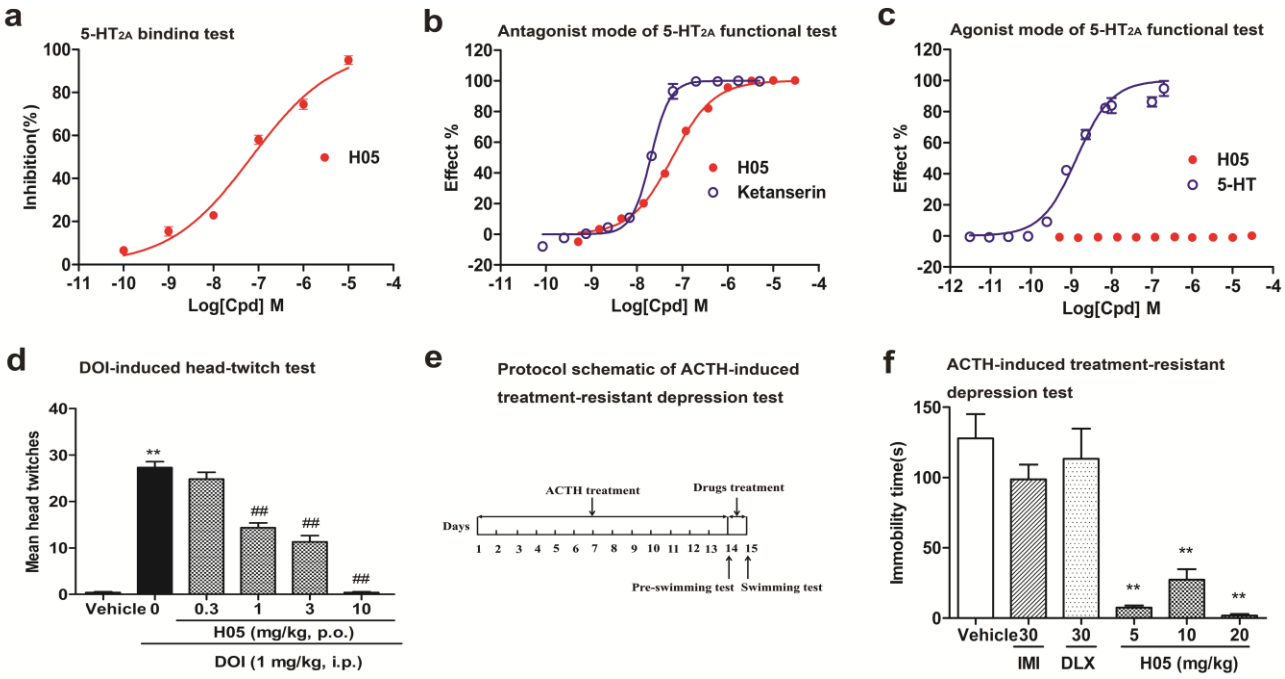
a
5-HT2A binding test
Inhibition(%)
H05
Log[Cpd] M
b
Antagonist mode of 5-HT2A functional test
Effect %
H05
Ketanserin
Log[Cpd] M
c
Agonist mode of 5-HT2A functional test
Effect %
H05
5-HT
Log[Cpd] M
d
DOI-induced head-twitch test
Mean head twitches
Vehicle 0
0.3
1
3
10
H05 (mg/kg, p.o.)
DOI (1 mg/kg, i.p.)
e
Protocol schematic of ACTH-induced treatment-resistant depression test
ACTH treatment
Drugs treatment
Days
Pre-swimming test
Swimming test
f
ACTH-induced treatment-resistant depression test
Immobility time(s)
Vehicle
IMI
DLX
H05 (mg/kg)

Figure 7