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Novel Bivalent 5-HT_{2A} Receptor Antagonists Exhibit High Affinity and Potency *in Vitro* and Efficacy *in Vivo*

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Abstract

The 5-HT_{2A}R plays an important role in various neuropsychiatric disorders including cocaine use disorder and schizophrenia. Homodimerization of this receptor has been suggested but tools that allow direct assessment of 5-HT_{2A}R:5-HT_{2A}R homodimer relevance in these disorders are necessary. We chemically modified the selective 5-HT_{2A}R antagonist M100907 to synthesize a series of homobivalent ligands connected by ethylene glycol linkers of varying lengths that may be useful tools to probe 5-HT_{2A}R:5-HT_{2A}R homodimer function. We tested these molecules for 5-HT_{2A}R antagonist activity in a cell line stably expressing the functional 5-HT_{2A}R, and quantified a post-receptor signaling target, activation (phosphorylation) of extracellular regulated kinases 1/2 ($ERK_{1/2}$), in comparison to *in vivo* efficacy to alter spontaneous or cocaine-evoked locomotor activity in rats. All of the synthetic compounds inhibited 5-HT-mediated phosphorylation of $ERK_{1/2}$ in the cellular signaling assay; the potency of the bivalent ligands varied as a function of linker length with the intermediate linker lengths being the most potent. The K_i values for the binding of bivalent ligands to 5-HT_{2A}R were only slightly lower than the values for the parent (+)-M100907 compound, but retained significant selectivity for 5-HT_{2A}R over 5-HT_{2B}R or 5-HT_{2C}R binding. In addition, the 11-atom-linked bivalent 5-HT_{2A}R antagonist (2 mg/kg, i.p.) demonstrated efficacy on par with (+)-M100907 to inhibit cocaine-evoked hyperactivity. As we develop further strategies for ligand-evoked receptor assembly and analyses of diverse signaling and functional

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Author Contributions

C.A.S. performed the *in vitro* biological analyses, conducted pharmacological analyses and drafted the manuscript; M.J.S. and K.C.R. performed the chemical syntheses and analyses; R.G.F performed the *in vivo* biological analyses; C.S.W. and M.J.B. drafted the manuscript and provided biological interpretations; K.A.C., S.R.G., and N.C.A. conceptualized the project, oversaw experimental design/interpretation/analyses, and wrote/edited the manuscript.

Notes

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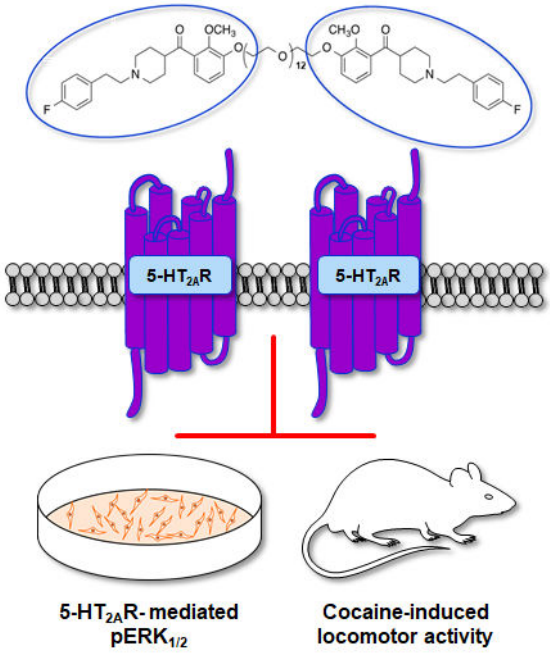
roles, these novel homobivalent 5-HT_{2A}R antagonist ligands will serve as useful *in vitro* and *in vivo* probes of 5-HT_{2A}R structure and function.

Graphical Abstract



Keywords

Serotonin; Serotonin 5-HT_{2A} receptor; bivalent ligand

Introduction

Serotonin (5-HT) neurotransmission is critically involved in the regulation of normal behavior (e.g., cognition, mood, satiety, sexual behavior, sleep) and pathological disorders (e.g., anxiety, depression, schizophrenia, substance use disorder) and is therefore, an important medications target. Actions of 5-HT are mediated by at least 14 subtypes of 5-HT receptors, 13 of which are G protein coupled receptors (GPCRs) and are presently grouped into seven families (5-HT_1R – 5-HT_7R) according to their structural and functional characteristics.[1] The metabotropic 5-HT_2R family (5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R) plays an important role in the regulation of CNS function and dysfunction. The receptors in the 5-HT_2 family couple predominantly to $G\alpha_{q/11}$ proteins to activate phospholipase C_β (PLCβ) resulting in downstream intracellular calcium (Ca_i^{++}) release and phosphorylation of $ERK_{1/2}$ ($pERK_{1/2}$).[2] Abnormalities of 5-HT_2R function have been implicated in several neuropsychological and neurological disorders[1, 3–6] and active initiatives are underway to develop novel 5-HT_2R ligands as therapies for such disorders.[7]

The 5-HT_{2A}R is of particular interest as a key target of atypical antipsychotics which are thought to improve symptoms and cognitive functioning in schizophrenia due to potent 5-

HT_{2A}R antagonist actions.[8–10] Other selective 5-HT_{2A}R antagonists show promise to improve symptomology in preclinical models of psychostimulant substance use disorder (i.e., cocaine, nicotine),[11–14] anxiety,[15] depression,[16–18] and sleep disorders.[19–21] Selective 5-HT_{2A}R antagonists have been in clinical trials for neurological and/or psychiatric disorders including volinanserin [MDL100907, M100907; (R)-(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl)ethyl]-4- piperidinemethanol; Aventis Pharmaceuticals; compound **1**; Fig. 1)[22, 23] and structurally similar 5-HT_{2A}R antagonists/inverse agonists for schizophrenia[24, 25] and sleep disorders.[26, 27] Of these, the 5-HT_{2A}R inverse agonist/antagonist pimavanserin (tradename NUPLAZID®; ACP-103; ACADIA Pharmaceuticals) has recently been approved by the FDA for Parkinson's Disease psychosis[4] and the 5-HT_{1A}R agonist/5-HT_{2A}R antagonist flibanserin (tradename Addyi®; BIMT 17; Sprout Pharmaceuticals) has been approved for the treatment of hypoactive sexual desire disorder in pre-menopausal women.[28]

Traditional drug discovery efforts for GPCRs like the 5-HT_{2A}R have been designed to target monomeric receptors and conceptualized as the pharmacophore interacting at one receptor binding site.[29] However, in recent years, the importance of GPCR dimerization and oligomerization on receptor signaling, trafficking and localization has been demonstrated for a variety of GPCRs, highlighting the need to investigate receptor-receptor interactions in disease pathology (for review, see[29]). Existence of homodimeric 5-HT_{2A}R:5-HT_{2A}R interactions is supported by co-immunoprecipitation and fluorescence resonance energy transfer (FRET) experiments[30] and 5-HT_{2A}R homoreceptor complexes have been proposed as the minimum functioning unit of the PLCβ- and PLA_2-mediated signaling pathways induced by 5-HT and synthetic 5-HT_{2A}R agonists.[31] Additionally, molecular dynamics modeling studies suggest that the putative 5-HT_{2A}R ligand binding sites displace differently in simulations of monomers versus homodimers, which suggests that receptor:receptor interactions may prefer different 5-HT_{2A}R ligands.[32] Therefore, tools to explore 5-HT_{2A}R:5-HT_{2A}R relevance in signal transduction as well as behavioral outcomes are necessary.

One proposed approach to probe receptor:receptor signaling and function is through the use of bivalent ligands. These ligands are comprised of two pharmacophores covalently tethered via a suitable spacer which are hypothesized to interact with a binding site on each receptor in the dimer pair.[33] Bivalent ligands have been synthesized to examine various neurotransmitter receptor systems,[34] including 5-HT receptors[35–37] such as the 5-HT_4R[38, 39] and 5-HT_{1B}R.[40–42] Here, we characterize homobivalent ligands with the pharmacophore of the piperidine M100907 which may serve as future tools to pharmacologically probe 5-HT_{2A}R:5-HT_{2A}R biology. The active (+)isomer of M100907 [(+)-M100907; compound **1**, Fig. 1] binds the 5-HT_{2A}R with high affinity and has >100 fold selectivity over the 5-HT_{2B}R and 5-HT_{2C}R.[43, 44] The pharmacology of M100907 has been demonstrated in a wide diversity of *in vitro*[45, 46] and *in vivo*[11, 13, 23, 47, 48] studies.

We have previously reported the synthesis and initial characterization of several bivalent analogues based on a modified structure of M100907 (compound **1**, Fig. 1).[45] To select the best location for the attachment of the tether used to link the molecules, derivatives with substitutions of the *p*-fluorine (compound **2**) or the methoxy group (compound **3**) were tested for their ability to inhibit 5-HT-stimulated intracellular calcium (Ca_i^{++}) release in a

Chinese hamster ovary (CHO) cell line stably expressing the human 5-HT_{2A}R (h5-HT_{2A}R). The replacement of the fluorine with a hydroxyl or ether moiety (compound **2**) resulted in significant loss of antagonist potency to inhibit 5-HT-induced Ca_i^{++} release.[45] Attachment of an ethylene glycol at the OH of the catechol (compound **3**) was found to retain significant activity.[45] Thus, this site was selected as the tether attachment site for synthesizing bivalent molecules. An active first pass metabolite of M100907 (des-3-methyl-ketone-M100907; compound **4**), which lacks a chiral center, also proved to be a potent (IC_{50} = 2.3 nM) 5-HT_{2A}R antagonist in the Ca_i^{++} release bioassays.[45] This molecule was selected as the starting material for the synthesis of tethered analogs to avoid generation of diasteromeric intermediates and eliminate the need for chiral resolution of the molecules. Two versions of compound **4** were synthesized with ethylene glycol groups (**5a–b**) and tested in the Ca_i^{++} bioassay to determine whether the polyether tether would have a deleterious effect on the activity of the ligand and found to retain nanomolar potency.[45] Finally, the desired bivalent molecules (**6a–g**) were synthesized and tested. The bivalent ligands exhibited sub-micromolar potency to inhibit 5-HT-induced Ca_i^{++} release demonstrating the retention of antagonist properties.[45] These studies suggested that intermediate tether lengths of 11–17 atoms in length are optimal for 5-HT_{2A}R:5-HT_{2A}R antagonism in the Ca_i^{++} assay.[45]

In the present report, we provide further functional characterization of these compounds. The compounds were additionally profiled through quantification of $ERK_{1/2}$ phosphorylation which represents a distal downstream signaling outcome of 5-HT_{2A}R activation. Phosphorylation of $ERK_{1/2}$ serves as an integration point of multiple upstream signaling pathways, including G protein dependent- and independent signal transduction.[46, 49, 50] In addition, affinity of these compounds for the 5-HT_{2A}R and selectivity over the highly homologous 5-HT_{2B}R or 5-HT_{2C}R was determined. We found that the bivalent ligands (Compound **6** series) retained activity and selectivity similar to that of (+)-M100907 (Compound **1**) and that the optimal tether length is between 8- and 17- atom linkers in the $ERK_{1/2}$ activation cellular assay. Thus, a homobivalent ligand with an intermediate tether length (compound **6c**) was selected for the first *in vivo* studies to evaluate the behavioral profile of a homobivalent 5-HT_{2A}R antagonist molecule. These studies open the door to the development of new bivalent molecules with the potential to elucidate the neurobiological role of 5-HT_{2A}R:5-HT_{2A}R homodimers in the CNS.

Results and Discussion

The inhibitory potency of M100907 analogs (Fig. 1) was evaluated in an $ERK_{1/2}$ activation assay in h5-HT_{2A}R-CHO cells (Fig. 2; Table 1). Activation of $ERK_{1/2}$ occurs by phosphorylation of the kinase which, in turn, phosphorylates other downstream targets to regulate gene expression and a variety of cellular processes (for review, see[51]). Serotonin induces a concentration-dependent increase in phosphorylated $ERK_{1/2}$ ($pERK_{1/2}$) with an EC_{50} of ~72 nM (Fig. 2A). Compound **1** and its analogs (**4–6g**; 10^{-10}–10^{-4} M) were examined for their ability to antagonize $ERK_{1/2}$ activation induced by a maximally effective concentration (1 μM) of 5-HT. The pIC_{50} and IC_{50} values are reported in Table 1. Compound **1**, (+)-M100907,[52] and compound **4**, the des-3-methyl-ketone derivative starting material for synthesis of the bivalent ligands, displayed low nanomolar potency in inhibiting 5-HT-evoked $ERK_{1/2}$ activation (Table 1, Fig. 2B).

The evaluation of inhibitory potency of compounds **5a** and **5b,** derivatives of compound **4** with 10- or 13-atom ethylene glycol linkers, revealed that the addition of the 10-atom linker (**5a**) retained comparable potency compared to the parent compounds, while addition of a 13-atom linker (**5b**) reduced the potency ~22-fold compared to the ketone starting material (**4**). This reduction in potency is not surprising since others have also noted that mere addition of the polyether tether diminishes the functional activity of the ligand.[33] Both **5a** (10-atom) and **5b** (13-atom) maintained sub-micromolar potency to inhibit 5-HT-mediated $ERK_{1/2}$ activation, thus, these data suggest that the 10-atom chain may be of optimal length. The potency of the homobivalent ligands (**6a–g**) to inhibit 5-HT-mediated $pERK_{1/2}$ was diminished compared to the parent compounds and varied modestly as a function of linker length (Table 1). Interestingly, the homobivalent with the shortest (5 atoms; **6a**) and the longest (20–23 atoms; **6f and 6g**) linkers had the lowest potency which suggests that there is an optimal linker length for maintenance of antagonist activity. Comparison of the inhibitory potency of the **6c**, the 11-atom linked bivalent ligand, and **5a**, its respective 10-atom monovalent counterpart, show that the potency of **6c** is decreased by ~22-fold versus **5a**, suggesting that addition of a second pharmacophore decreased the functional activity (Fig. 2B). To the contrary, comparison of the 14-atom linker bivalent ligand (**6d**) to its respective 13-atom monovalent counterpart (**5b**) resulted in a slight increase in potency (Table 1). These findings are consistent with previously published effects of these compounds in the Ca_i^{++} bioassay[45] and, together, may suggest that linker length impacts the potency of these ligands and should be considered for future experiments.

The affinity and selectivity of the synthesized M100907 derivatives for the 5-HT_{2A}R versus the highly homologous 5-HT_{2B}R and 5-HT_{2C}R, were assessed via radioligand binding assays at the Psychoactive Drug Screening Program (PSDP). The resultant K_i values for binding to 5-HT_{2A}R, 5-HT_{2B}R and 5-HT_{2C}R, as assessed by displacement of [^{3}H]-ketanserin (0.5 nM), [^{3}H]-LSD (1 nM) and [^{3}H]-mesulergine (0.5 nM), respectively, are shown in Table 2. All ligands displayed nanomolar affinity for the 5-HT_{2A}R, but K_i values were ~4–8 fold lower than that previously reported for the high affinity (+)-M100907 isomer (Compound **1**; K_i = 3 nM).[53] According to the PDSP, variations of this magnitude are expected in K_i assessments, thus the differences in K_i values observed at 5-HT_{2A}R for most of the M100907 analogs were modest versus compound **1** (Table 2). Only the bivalent ligand with the 20-atom linker (**6f**) exhibited a substantial decrease (~10 fold) in affinity for the (5-HT_{2A}R, K_i = 32 nM) which coincides with the decrease in potency observed for this molecule in the $ERK_{1/2}$ activation assay. In addition, the presence of the second pharmacophore in the 11- (**6c**) and 14-atom (**6d**) tethered bivalent ligands afforded no overt gain in affinity compared to their respective monovalent counterparts (**5a**, **5b**). These lack of differences may just be due to the inherent variability in K_i measurements, and/or the lack of measurement of actual association/dissociation constants of the ligands; the binding analysis was done at equilibrium, as opposed to during the dynamic state related to the functional endpoints.[54]

The inclusion of the second pharmacophore in the bivalent molecules did not afford any improvement in selectivity of the compounds for the 5-HT_{2A}R versus the 5-HT_{2B}R or 5-HT_{2C}R compared to the parent compounds. However, all the M100907 analogs maintained

~10-fold or greater affinity for the 5-HT_{2A}R over the 5-HT_{2C}R and >21-fold selectivity over the 5-HT_{2B}R. Due to the high degree of homology between these receptors, the selectivity of **6c** and **6d** bivalent compounds for the 5-HT_{2A}R was confirmed by assessment of these ligands in a functional assay of intracellular calcium (Ca_i^{++}) release, in 5-HT_{2C}R-expressing cells. Similar to compound **1**, compounds **6c** and **6d** had no effect upon 5-HT_{2C}R-mediated Ca_i^{++} release evoked by 1 μM 5-HT at concentrations <10 μM (data not shown).

The bivalent ligand with an intermediate (11-atom) linker (**6c**) was selected for initial *in vivo* efficacy studies based upon the *in vitro* results (above).[45] Previous studies have reported that (+)-M100907 (Compound **1**; 0.02–2 mg/kg) significantly suppresses cocaine-evoked, but not spontaneous, locomotor activity.[48, 55] Therefore, the effects of compound **6c** on spontaneous (saline) - versus cocaine-evoked locomotor activity was assessed in rats (Fig. 3). Locomotor activity was measured after (intraperitoneal) injection with vehicle (1% Tween 80 with 2% ethanol in 0.9% NaCl) or bivalent ligand **6c** (2 mg/kg) 30 minutes prior to saline (1 ml/kg) or cocaine (15 mg/kg) injection. Data are presented as mean total horizontal ambulations summed for the entire 90-minute session (Fig. 3A) and mean total vertical activity summed for the entire 90 min session (Fig. 3B). A main effect of treatment was observed for both mean total horizontal ambulations [$F_{(3,27)} = 9.71$, $p<0.05$; Fig. 3A] and mean total vertical activity [$F_{(3,27)} = 13.90$, $p<0.05$; Fig. 3B] summed across the 90-min session. *A priori* comparisons indicated that the bivalent ligand **6c** did not alter saline-evoked horizontal ambulation or vertical activity (n.s.; Fig. 3A and 3B). As expected, administration of vehicle plus cocaine (Veh + Coc) elevated total horizontal ambulation (Fig. 3A) and vertical activity (Fig. 3B) versus vehicle plus saline (Veh + Sal) administration for the entire 90-min session ($p<0.05$). *A priori* comparisons showed that the bivalent ligand **6c** significantly suppressed cocaine-evoked horizontal ambulation ($p<0.05$; Fig. 3A) and vertical activity ($p<0.05$; Fig. 3C). A three-way mixed model ANOVA indicated no pretreatment x treatment x time interaction ($F_{17,459)} = 1.12$, n.s.) for total horizontal ambulation (Fig. 3C). There was a pretreatment x treatment x time interaction ($F_{17,459)} = 1.74$, $p<0.05$) for vertical activity (Fig. 3D). Taken together and similar to the previously published (+)-M100907,[48] the bivalent ligand **6c** modestly suppressed spontaneous locomotor activity and displayed efficacy to suppress cocaine-evoked locomotor activity in a time-dependent manner. Of note, the calculated Central Nervous System Multiparameter Optimization (CNS MPO) value for **6c** was found to be 3. While this number is less than the optimal range of greater than 5, there are numerous CNS drugs with this value.[56] These data provide the first positive confirmation that a newly synthesized homobivalent ligand displays efficacy to produce behavioral effects similar to the parent 5-HT_{2A}R antagonist.

The present data indicate that the series of covalently-linked homobivalent 5-HT_{2A}R antagonist ligands based on the structure of the potent 5-HT_{2A}R antagonist (+)-M100907 (Compound **1**) retain potency and efficacy *in vitro* and *in vivo*. These ligands maintained nanomolar affinity for the 5-HT_{2A}R, selectivity over the 5-HT_{2B}R and 5-HT_{2C}R, and nanomolar potency as 5-HT_{2A}R antagonists in a cellular assay of $ERK_{1/2}$ activation. Furthermore, these results suggest that tether lengths of 8–17 atoms in length are optimal to suppress $ERK_{1/2}$ activation (present studies), which is consistent with our previous findings in the Ca_i^{++} assay.[45] Encouragingly, we also demonstrated that the covalently-linked

homobivalent ligand with optimal spacing suppresses cocaine-evoked behavior *in vivo*. Future studies are required to explore whether the addition of a second pharmacophore results in a second binding site which could represent the active site of a second interacting receptor, as might be expected given that the 5-HT_{2A}R has been demonstrated to exist as a receptor homodimer.[30–32, 57] Thus, these newly-designed bivalent ligands may provide useful tools to further explore 5-HT_{2A}R function and homodimerization.

Materials and Methods

Cell lines and cell culture

A CHO-K1 cell line stably transfected with the 5-HT_{2A}R (5-HT_{2A}R-CHO cells; FA4 line) was a generous gift of K. Berg and W. Clarke (University of Texas Health Science Center at San Antonio). This line expresses transfected h5-HT_{2A}R in the p198-DHFR-Hygro vector containing a hygromycin resistance gene.[58] Reverse transcription of RNA followed by a quantitative real time PCR assay confirmed that 5-HT_{2A}R-CHO cells expressed 5-HT_{2A}R mRNA (estimated to be approximately 3–4% of the mRNA level of the housekeeping gene cyclophilin; data not shown), but did not express either 5-HT_{2B}R or 5-HT_{2C}R mRNA;[59] the parental CHO-K1 cell line did not express detectable amounts of any 5-HT_2R mRNAs.[46] Cells were grown at 37°C, 5% CO_2 and 85% relative humidity in GlutaMax α-MEM (Invitrogen, Carlsbad CA), 5% fetal bovine serum (Atlanta Biologicals, Atlanta GA), 100 μg/ml hygromycin (Mediatech, Manassas VA) and were passaged when they reached 80% confluence.

Ligands

Serotonin (5-HT; Acros Organics, ThermoFisher Scientific, Pittsburgh, PA) was dissolved in 1X Hank's balanced salt solution (HBSS; Cellgro, Invitrogen) for *in vitro* studies. The (+)-M100907 (**1**) [R-(+)-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidine-methanol] was synthesized in the Drug Design and Synthesis Section, National Institute on Drug Abuse (National Institutes of Health) as described,[52] and was dissolved in 1X HBSS for *in vitro* studies. The (+)-M100907 (**1**), des-3-methyl-ketone-M100907 (**4**), and the monovalent and bivalent M100907 derivative compounds (Series **5** and **6**; see Fig. 1.) were synthesized as described previously[45] and dissolved in 1X HBSS for *in vitro* studies. Compound **6c** was dissolved in 1% Tween 80 + 2% ethanol in 0.9% NaCl for *in vivo* studies. (−)-Cocaine (National Institute on Drug Abuse, Research Triangle Park, NC, USA) was dissolved in 0.9% NaCl.

Plate immunoassay for $ERK_{1/2}$ activation

We adapted a previously developed plate immunoassay,[60] to measure levels of $pERK_{1/2}$ expression following ligand administration, with optimized fixation and antibody incubation conditions for use in this cell line.[46] Cells were plated in serum-replete medium at 16–20K cells in 150 μl in clear-sided, clear bottom 96-well tissue culture plates. Cells were grown for 24 hr in serum-replete medium, and then shifted overnight to serum-free medium. The day of the experiment, cells were fed with 80 μl of serum-free medium and returned to the incubator for 1–2 hrs, as adding medium alone caused a measurable $pERK_{1/2}$ signal that subsided by 1 hr (data not shown), as seen by others.[61]

Test compounds were added as 20 μl of a 5x stock concentration and plates were incubated at 37°C for 15 min. Cells were then stimulated with 1 μM of 5-HT (25 μl of a 5 μM stock) and incubated at 37°C for 5 min. Full concentration-response curves (10^{-11}-10^{-4} M) for 5-HT and each compound were performed in each experiment. Reactions were stopped by the addition of 2% paraformaldehyde (PFA) in phosphate buffered saline (PBS, pH 7.4). Cells were fixed for 45 min at room temperature (RT) then rinsed with PBS. Cells were then permeabilized with ice-cold methanol to ensure antibody access to intracellular antigens, washed with PBS and blocked for 45 min at RT with 0.1% fish gelatin (Sigma, St. Louis MO). Cells were then incubated with a 1:500 dilution of mouse monoclonal anti-p$ERK_{1/2}$ (p44/42; Cell Signaling, Danvers MA; #9106) overnight at 4°C with gentle shaking. Background was determined in wells incubated with no primary antibody. After washing with PBS, biotin-conjugated secondary antibody (Vector Labs, Burlingame CA; # BA-9200, 1:500 diluted in blocking solution) was added and incubated for 1 hr at RT. Following washing, alkaline phosphatase (AP) complexed with avidin (Vector Labs, #AK5000) was prepared according to the manufacturer's directions, added to the wells and incubated for 1 hr at RT. After washing, 50 μl of the AP substrate para-nitrophenyl-phosphate (pNpp; Vector Labs, #SK-5900) with levamisole (an inhibitor of endogenous phosphatases; Vector Labs, #SP-5000; two drops/10 ml), freshly prepared in 100 mM sodium bicarbonate was added, and the plate was incubated at 37°C for 30 min. The absorbance of the yellow product para-nitrophenol (pNp) was measured at 405 nm (A_{405}). Data were normalized to total cell mass in each well as measured by crystal violet staining (below) and expressed as A_{405}/A_{590}. The pIC_{50} values for $pERK_{1/2}$ expression were determined using 3-parameter nonlinear regression analysis (GraphPad Prism 7.02) and calculated from at least three independent experiments, each conducted in technical replicates of 3–8, and are presented as the mean ± SEM.

Crystal violet staining

Total cell mass in each well was measured by crystal violet staining, a value proportional to cell number used to estimate cell number in each well. Upon completion of an experiment, wells were rinsed with water, air dried and 50 μl of crystal violet solution (0.1% in water) was added for 30 min at RT, followed by one additional rinse. Cell-absorbed dye was extracted by the addition of 10% acetic acid (30 min, RT) and absorbance read at 590 nm (A_{590}).

Radioactive binding assays

K_i determinations were generously provided by the NIMH Psychoactive Drug Screening Program (PDSP; http://pdsp.med.unc.edu/). Briefly, binding assays were performed using crude membrane fractions from cell lines transiently or stably transfected with the appropriate receptor. The 5-HT_{2A}R binding was determined by displacement of [^{3}H] ketanserin (0.5 nM) relative to displacement by 10 μM clozapine. The 5-HT_{2B}R binding was determined by displacement of [^{3}H] LSD (1.0 nM) relative to displacement by 10 μM methysergide. The 5-HT_{2C}R binding was determined by displacement of [^{3}H] mesulergine (0.5 nM) relative to displacement by 10 μM chlorpromazine. An initial screen measured net displacement of bound ligand by 10 μM of each synthetic antagonist. K_i values were determined on all compounds that yielded > 50% displacement by performing competitive

binding curves using 11 concentrations spanning six orders of magnitude, with triplicate determinations for each concentration. Binding determinations were repeated for the racemic M100907 and for the bivalent molecules and the resultant K_i values are represented as mean ± SEM.

Animals

A total of 32 male Sprague-Dawley rats (Harlan, Inc., Indianapolis, IN, USA) weighing 225–325 g at the start of the experiments were used. Rats were allowed to acclimate for 5–7 days in a colony room at a constant temperature (21–23°C) and humidity (45–50%) on a 12 hr light-dark cycle (lights on 0700–1900 hr). Rats were housed two rats per cage and food and water was available *ad libitum*. All experiments were carried out in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (2011) and with the approval of the UTMB Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, when available.

In vivo assessment

Locomotor activity was monitored and quantified under low light conditions using a modified open field activity system (San Diego Instruments, San Diego, CA) according to previous publications with minor modifications.[48, 62–64] Clear Plexiglass chambers (40 × 40 × 40 cm) were surrounded by a 4 × 4 photobeam matrix positioned 4 cm from the chamber floor. Consecutive photobeam breaks within the 16 × 16 cm of the activity monitor were recorded as central ambulations. Peripheral ambulations were counted as consecutive beam breaks in the surrounding perimeter. Central and peripheral ambulations were summed to provide a measure of total horizontal ambulation. Vertical activity was quantified as the sum of the upper photobeam breaks that occurred within the activity monitor every 5 min. Rats were acclimated to the colony room and following 1 week of handling, rats were habituated to the activity monitors for 3 h/day for 2 days before the test day. Using a between-subjects design, rats (n=7–8/group) received vehicle (1% Tween 80 + 2% ethanol in 0.9% NaCl, 1 ml/kg, i.p.), or compound **6c** (2 mg/kg, i.p.), followed 30 minutes later by an injection of either saline (1 ml/kg, i.p.) or cocaine (15 mg/kg, i.p) and were immediately placed in activity monitors; locomotor activity was assessed for 90 min. Due to a misinjection, one rat was removed from vehicle plus cocaine group for a final n=7 in that group; all other groups were n=8.

Locomotor activity data are presented as mean total horizontal ambulation or vertical activity (± SEM) summed across the session the entire 90-min session or within 5 min time bins. The main effect of treatment on total horizontal ambulation and vertical activity were analyzed with a one-way ANOVA using the GLM procedure (SAS for Windows). Subsequent *a priori* comparisons between means for total horizontal ambulation and vertical activity were made using the Bonferroni correction. A three-way ANOVA was employed to analyze the between-subject factors of pretreatment (vehicle, **6c**) and treatment (saline, cocaine) and the within-subject factor of time (5-min bins across 90 min session) using a mixed general linear model (SPSS). All statistical analyses were conducted with an experimentwise error rate of α=0.05

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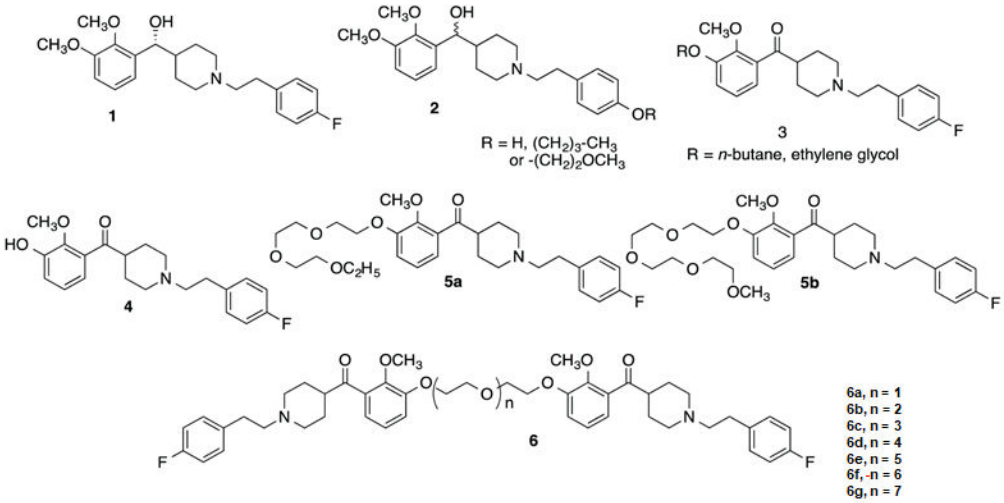

Figure 1.

Chemical structures of (+)-M100907 (**1**) and synthesized analogs. Modifications at the fluorine site (**2**), modifications at the 3-methoxy site (**3**), monomer of des-3-methyl ketone M100907 (**4**), monovalent ligands with 10-atom (**5a**) and 13-atom (**5b**) ethylene glycol tethers, and structure of bivalent ligands with tethers of varying lengths (**6**) are displayed.

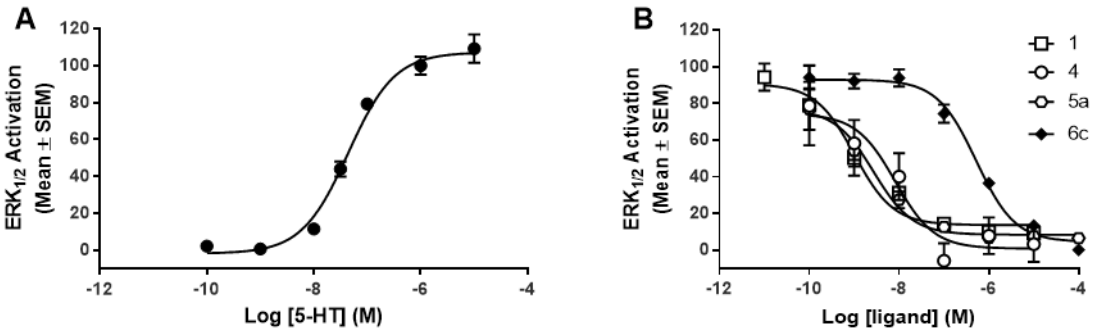

Figure 2.

Representative $ERK_{1/2}$ activation response in h5-HT_{2A}R-CHO cells. **[A]** 5-HT evokes a concentration-dependent elevation of $pERK_{1/2}$ expression (pEC_{50} = 7.14 ± 0.04; EC_{50} = 72.4 nM) and 1 μM 5-HT induces maximal $ERK_{1/2}$ activation. **[B]** M100907 derivatives induce a concentration-dependent inhibition of 1 μM 5-HT. pIC_{50} and IC_{50} values are listed in Table 1.

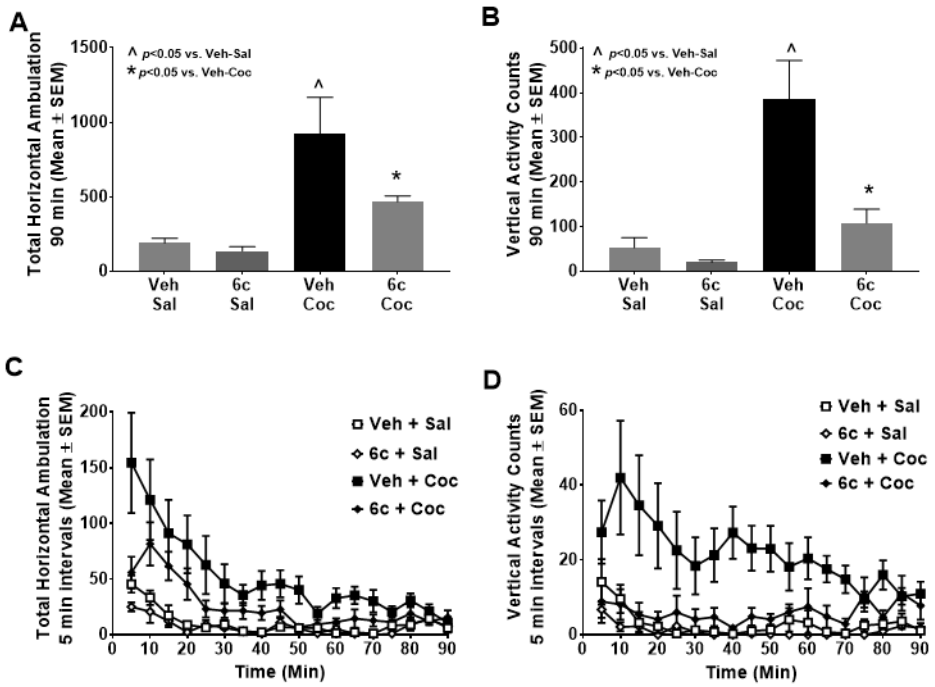

Figure 3.

Effects of pretreatment with the bivalent derivative **6c**. Rats (n=7–8/group) were treated (i.p.) with vehicle (Veh) or bivalent ligand **6c** (2 mg/kg) 30 min prior to injection with saline (Sal; 1 ml/kg) or cocaine (Coc; 15 mg/kg) immediately before the test session commenced. Data are presented as mean (± SEM) total horizontal ambulation **[A]** or vertical activity counts **[B]** summed across the entire 90 min session. Total horizontal ambulation **[C]** and vertical activity **[D]** in 5-min time bins are presented.

Table 1

Potency of M100907 derivatives on $ERK_{1/2}$ activation in h5-HT_{2A}R-CHO cells.[a]

ID	Linker #	pIC_{50} ± SEM	IC_{50} (nM)
1	—	8.29 ± 0.52	5.1
2	—	N.T.	N.T.
3	—	N.T.	N.T.
4	—	8.00 ± 0.05	10.1
5a	10	8.11 ± 0.51	7.9
5b	13	6.65 ± 0.04	225
6a	5	6.53 ± 0.24	298
6b	8	7.59 ± 0.53	25.6
6c	11	6.75 ± 0.16	178
6d	14	7.01 ± 0.13	98.2
6e	17	6.78 ± 0.39	164
6f	20	6.55 ± 0.12	284
6g	23	6.72 ± 0.14	192

[a] pIC_{50} is presented as mean ± SEM (n = 3–6). IC_{50} values were calculated from averaged pIC_{50} values.

"—" indicates that there is no atom linker on this compound. "NT" indicates compound has not been tested in this assay.

Table 2

Affinity profile of M100907 derivatives at the 5-HT_{2A}R, 5-HT_{2B}R, 5-HT_{2C}R.

ID	Linker #	5-HT_{2A}R		5-HT_{2B}R		5-HT_{2C}R	
		pK_i[1]	K_i (nM)	pK_i[2]	K_i (nM)	pK_i[3]	K_i (nM)
1	--	8.5[4]	3.0	6.2	612	7.4	41
2	--	N.T.		N.T.		N.T.	
3	--	N.T.		N.T.		N.T.	
4	--	8.8	1.6	6.6	261	7.8	15
5a	10	8.3	5.4	6.2	701	6.5	312
5b	13	8.2	6.9	N.D.[5]		6.3	472
6a	5	8.5[4]	3.1	6.7	202	7.6	27
6b	8	8.1[4]	7.2	6.3	476	7.2	64
6c	11	8.2[4]	5.8	6.9	126	7.1	79
6d	14	8.5[4]	3.4	6.8	157	7.1	84
6e	17	8.5[4]	3.0	6.6	267	7.3	50
6f	20	7.5[4]	32	5.9	1399	6.3	245
6g	23	8.4[4]	4.4	6.6	238	7.0	101

K_i determinations were generously provided by the NIMH Psychoactive Drug Screening Program.

[1]Determined by displacement of [^{3}H] ketanserin (0.5 nM) relative to displacement by 10 μM clozapine;

[2]Determined by displacement of [^{3}H] LSD (1.0 nM) relative to displacement by 10 μM methysergide;

[3]Determined by displacement of [^{3}H] mesulergine (0.5 nM) relative to displacement by 10 μM chlorpromazine.

[4]Determined in two independent assays; N.D. = Not determined; Displacement by 10 μM of methylsergide was < 50%. N.T.= Not tested in this assay