Brominated Cyclodipeptides from the Marine Sponge Geodia barretti as Selective 5-HT Ligands

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Brominated Cyclodipeptides from the Marine Sponge *Geodia barretti* as Selective 5-HT Ligands

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The brominated cyclodipeptides barettin (cyclo[(6-bromo-8-entryptophan)arginine]) and 8,9-dihydrobarettin (cyclo[(6-bromotryptophan)arginine]) isolated from the marine sponge *Geodia barretti* have previously been shown to inhibit settlement of barnacle larvae in a dose-dependent manner in concentrations ranging from 0.5 to 25 μM. To further establish the molecular target and mode of action of these compounds, we investigated their affinity to human serotonin receptors. The tryptophan residue in the barettins resembles that of endogenous serotonin [5-hydroxytryptamine]. A selection of human serotonin receptors, including representatives from all subfamilies (1−7), were transfected into HEK-293 cells. Barettin selectively interacted with the serotonin receptors 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 at concentrations close to that of endogenous serotonin, with the corresponding K_i values being 1.93, 0.34, and 1.91 μM, respectively. 8,9-Dihydrobarettin interacted exclusively with the 5-HT_{2C} receptor with a K_i value of 4.63 μM; it failed to show affinity to 5-HT_{2A} and 5-HT_4, indicating that the double bond between the tryptophan and arginine residue plays an important role in the interaction with the receptor proteins.

Interactions among organisms have led to the evolution of bioactive chemical compounds selected for diverse purposes such as deterring fouling and feeding, capturing prey, and combating invasive pests and pathogens. The production of bioactive compounds by plants, animals, and microorganisms has long been exploited in the search for drug candidates or for compounds to serve as leads in drug development.[1,2] Traditionally, such bioprospecting for drug candidates has focused on terrestrial microorganisms and plants; the equivalent research in marine systems is in its infancy, but the much larger diversity of major lineages in the sea promises a wealth of new molecular structures with as yet unknown functions.

In the ocean, sessile sponges have proved to be a rich source of bioactive compounds, many of which are believed to constitute a chemical defense against predators or foulers aimed at protecting the body surface.[3] We have previously reported on the production of two brominated cyclodipeptides in the marine sponge *Geodia barretti* Bowerbank (family Geodiidae, class Demospongiae, order Astrophorida), compounds that strongly inhibit the settlement of the barnacle larva of *Balanus improvisus* and the blue mussel *Mytilus edulis*.[4,5] These two cyclodipeptides, barettin (87/13 mixture of *Z*/*E* isomers) and 8,9-dihydrobarettin, have both been isolated, elucidated, and synthesized by our group[5,6] and, in the case of barettin, by others.[7] Barettin and 8,9-dihydrobarettin both inhibit settlement of barnacle larvae of *B. improvisus* in a dose-dependent manner in concentrations ranging from 0.5 to 25 μM. Their respective EC_{50} values are 0.9 (barettin) and 7.9 μM (8,9-dihydrobarettin).[5] The dose-dependence of settlement inhibition is indicative of a specific molecular target in the barnacle larva. In order to further establish the mode of action of the barettins, we investigated their affinity to a wide range of human serotonin receptors from all subfamilies (1−7). Serotonin receptors were chosen on the basis of the chemical structures of the barettins; like serotonin, the barettins carry an indole nucleus by way of their tryptophan residue. However, serotonin is hydroxylated in the 5-position, whereas barettin and 8,9-dihydrobarettin are substituted with a bromine atom in the 6-position. This brominated tryptophan residue can also be found as a 6-Br-Trp moiety in the 41-amino-acid peptide σ-conotoxin, a polypeptide that selectively inhibits the 5-HT_3 receptor through competitive antagonism.[8] The structural similarities of barettin and 8,9-dihydrobarettin to ligands with high affinity for 5-HT receptors, such as the drug tegaserod, which was recently approved for treatment of irritable bowel syndrome (IBS),[9] also prompted us to investigate a possible interaction with the serotonergic system. Here, we describe the affinity and selectivity of the marine cyclodipeptides barettin and 8,9-dihydrobarettin to serotonin receptors.

Results and Discussion

Of the panel of receptors tested, covering all the major 5-HT subfamilies, the HEK-293 cell membranes expressing 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors showed receptor−ligand affinity with barettin below a concentration of 10 μM (Table 1). Barettin displaced the radioligands ([*N*-methyl-^{3}H]LSD for 5-$HT_{2A,\ 2C}$; [*N*-methyl-^{3}H]GR113808 for 5-HT_4) with dose-responsive kinetics. The affinity constant K_i for barettin at the 5-HT_{2A} receptor was determined to be 1.93 μM (Figure 2). Barettin does not have the high affinity to 5-HT_{2A} as the more selective ligand methysergide displays, but is selective enough to bind nearly as good as endogenous 5-HT (0.69 μM). In the case of 5-HT_{2C}, both barettin and 8,9-dihydrobarettin were able to displace [*N*-methyl-^{3}H]LSD, producing K_i values of 0.34 and 4.63 μM, respectively (Figure 3). Once again methysergide showed a better affinity (K_i = 2.5 nM) and the displacement with 5-HT gained a 10-fold lower K_i than for barettin. Competing with [*N*-methyl-^{3}H]GR113808 for binding to the 5-HT_4 receptor, barettin showed a K_i of 1.91 μM, where 5-HT had a K_i of 0.50 μM. 8,9-Dihydrobarettin was not able to displace the radioligand at the 5-HT_4 receptor. Tegaserod was introduced as a selective ligand and produced a K_i of 0.031 μM, approximately 10 times better than 5-HT, 100 times better than barettin.

Taken together, barettin clearly has its advantages at the 5-HT_{2C} receptor, producing a selectivity ratio of 5.68 (5-HT_{2A}/5-HT_{2C}) between the two 5-HT_2 receptor subtypes included in the study. The small differences between barettin and 8,9-dihydrobarettin

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Table 1. Affinities of Barettin, 8,9-Dihydrobarettin, 5-Hydroxytryptamine, Methysergide (5-$HT_{2A,2C}$), and Tegaserod (5-HT_4) to the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 Receptors Expressed in HEK-293 Cell Membranes (data presented as mean ± SEM with three independent experiments performed in triplicate)

	K_i [μM]			
receptor[a]	barettin	8,9-dihydrobarettin	5-hydroxytryptamine	selective ligand[b]
5-HT_{1A}	>10	>10		
5-HT_{1D}	>10	>10		
5-HT_{2A}	1.93 ± 0.59	>10	0.69 ± 0.1	0.01 ± 0.003
5-HT_{2C}	0.34 ± 0.14	4.63 ± 0.13	0.020 ± 0.004	0.0025 ± 0.001
5-HT_{3A}	>10	>10		
5-HT_4	1.91 ± 0.12	>10	0.50 ± 0.07	0.031 ± 0.01
5-HT_{5A}	>10	>10		
5-HT_6	>10	>10		
5-HT_{7A}	>10	>10		

[a] [1,2-^{3}H]5-Carboxamidotryptamine used for 5-$HT_{1A,1D,5A,7A}$; [*N*-methyl-^{3}H]LSD used for 5-$HT_{2A,2C,6}$; [9-methyl-^{3}H]BRL-43694 used for 5-HT_{3A}; [*N*-methyl-^{3}H]GR113808 used for 5-HT_4. [b] Methysergide (5-$HT_{2A,2C}$); tegaserod (5-HT_4).

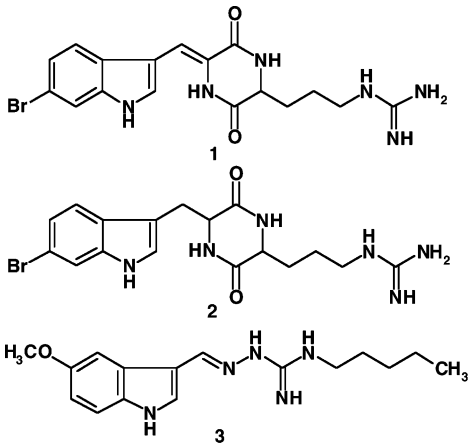

Figure 1. Chemical structures of barettin (**1**), 8,9-dihydrobarettin (**2**), and tegaserod (**3**).

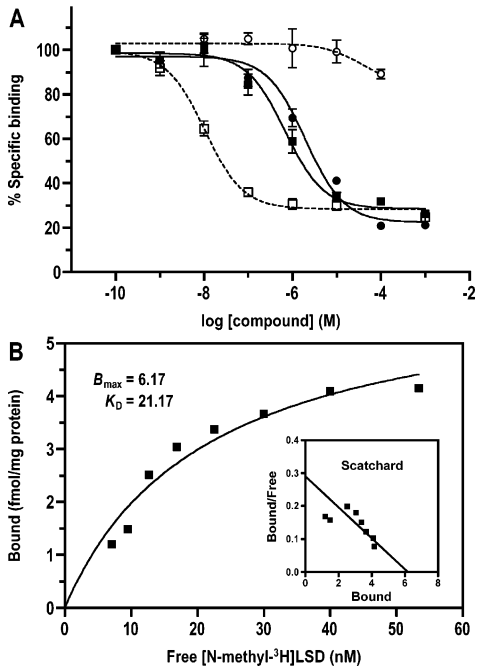

Figure 2. (A) Displacement of [*N*-methyl-^{3}H]LSD on the serotonin subreceptor 5-HT_{2A} by (●) barettin, (○) 8,9-dihydrobarettin, (■) 5-hydroxytryptamine, and (□) methysergide. (B) Saturation analysis of [*N*-methyl-^{3}H]LSD binding to HEK-293 cell membranes expressing the human 5-HT_{2A} receptor. Data points are triplicate values.

greatly affected the affinity. It has proven difficult to develop selective ligands within the 5-HT_2 receptor subfamily. Barettin, showing a clear selectivity for the 5-HT_{2C} receptor over the 5-HT_{2A} receptor, could therefore be of interest for further investigation.

The cell lines were subjected to saturation analysis with a high-affinity radioligand to determine the receptor expression levels. Saturation of transfected HEK-293 cell membranes is due to receptors being occupied with the radioligand. When saturation occurs, all the receptors are occupied and no further specific binding site is available. The saturation experiments were performed in order to confirm that we have a robust cellular system that expresses the desired receptor and that this receptor binds the radioligand used in the competition-binding assays. The straight line in the three Scatchard analyses indicates that the radioligands [*N*-methyl-^{3}H]-LSD and [*N*-methyl-^{3}H]GR113808 bind to a single site on the transfected HEK-293 cell membrane as well as that the binding occurs with a single affinity to the receptor (Figures 2−4). B_{max} expresses the number of available receptors/mg protein, which is illustrated by the Scatchard curve's intersection of the *x*-axis. K_D is the value of free radioligand at half B_{max} and expresses the affinity of the radioligand to the cell receptor: [*N*-methyl-^{3}H]LSD bound to 5-HT_{2A} receptors (B_{max} = 6.17 fmol mg protein^{-1} and K_D = 21.17 nM) (Figure 2), [*N*-methyl-^{3}H]LSD bound to 5-HT_{2C} receptors (B_{max} = 4.50 fmol mg protein^{-1} and K_D = 52.84 nM) (Figure 3), [*N*-methyl-^{3}H]GR113808 bound to 5-HT_4 receptors (B_{max} = 8.04 fmol mg protein^{-1} and K_D = 15.81 nM) (Figure 4). No specific radioligand binding was detected in the parental cell lines (data not shown).

We have thus shown that the marine natural product barettin specifically interacts with 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors, while 8,9-dihydrobarettin interacts with the 5-HT_{2C} receptor (Figures 2−4; Table 1). Among the selected 5-HT receptor subtypes, 8,9-dihydrobarettin displayed affinity only for 5-HT_{2C} receptors (Figure 2; Table 1). and the difference in affinity to the 5-HT_{2C} receptor between barettin and 8,9-dihydrobarettin was approximately 10-fold (Table 1). We suggest that the small difference in chemical backbone between the two substances, a double bond in the tryptophan residue, may explain the considerable differences between them in affinity to the serotonin 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors; the double bond in barettin causes a more rigid steric orientation of the bromotryptophan residue, resulting in a better fit of barettin in the receptor-binding pocket.

The bromine substituent is another chemical constituent that may play a profound role in the interaction between the cyclodipeptides and the serotonin receptor. The importance of halogenated substituents for serotonin analogues has recently been highlighted in structure−activity studies. Halogenation not only accounted for drastic improvements in affinity but also produced changes in selectivity between serotonin subtypes.[10,11] However, further conformational and structure−activity studies of barettin and 8,9-dihydrobarettin are needed to elucidate how the double bond and the bromination influence the interactions with the 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4 receptors.

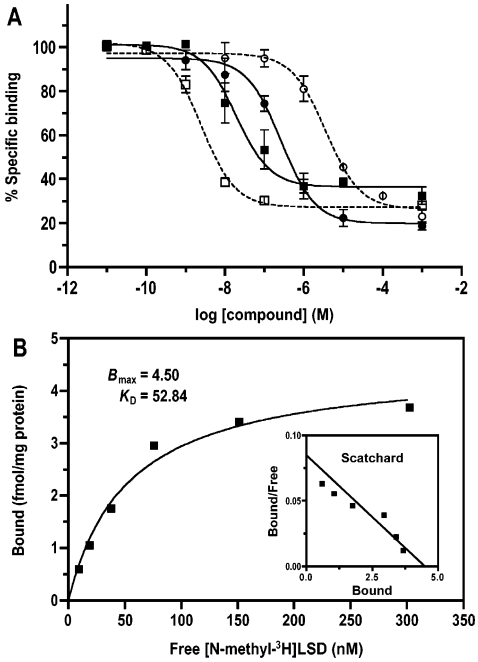

Figure 3. (A) Displacement [*N*-methyl-^{3}H]LSD on the serotonin subreceptor 5-HT_{2C} by (●) barettin, (○) 8,9-dihydrobarettin, (■) 5-hydroxytryptamine, and (□) methysergide. (B) Saturation analysis of [*N*-methyl-^{3}H]LSD binding to HEK-293 cell membranes expressing the human 5-HT_{2C} receptor. Data points are triplicate values.

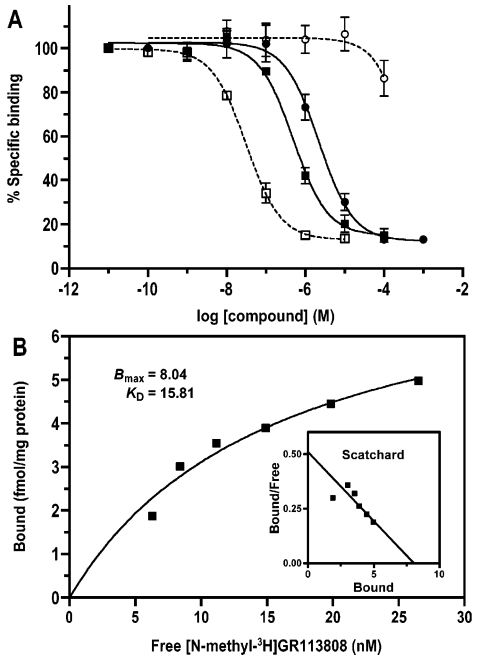

Figure 4. (A) Displacement of [*N*-methyl-^{3}H]GR113808 on the serotonin subreceptor 5-HT_4 by (●) barettin, (○) 8,9-dihydrobarettin, (■) 5-hydroxytryptamine, and (□) tegaserod. (B) Saturation analysis of [*N*-methyl-^{3}H]GR113808 binding to HEK-293 cell membranes expressing the human 5-HT_4 receptor. Data points are triplicate values.

The structure−activity relationship of barettin and 8,9-dihydrobarettin in the receptor−ligand binding assay, i.e., the affinities of the compounds for the 5-HT_{2C} receptor, corresponds closely to the difference in activity found in the in vivo assay of settling larvae of *B. improvisus*.[5] Several serotonin receptors have been cloned and functionally characterized in invertebrates. Receptors related in DNA sequence, which activate intracellular signal pathways identical to mammalian 5-HT_1 and 5-HT_2 receptors, have been found in *Drosophila melanogaster*, *Caenorhabditis elegans*, and the pond snail *Lymnaea stagnalis*.[12] In *D. melanogaster*, a receptor corresponding to the mammalian 5-HT_7 receptor has also been found.[13,14] The functional roles of 5-HT_2 receptors in invertebrates such as arthropods need to be further defined. It has been suggested that 5-HT_2 receptors are of particular importance in *D. melanogaster* embryogenesis.[13] Cypris larvae of the barnacle, *B. amphitrite*, show DNA sequences homologous to a human 5-HT_{1A} receptor.[15] However, this barnacle serotonergic G protein-coupled receptor has not been functionally cloned, nor has its pharmacology been fully established. However, a 5-HT_2 receptor has recently been cloned and functionally characterized from another crustacean, the spiny lobster, *Panulirus interruptus*. This receptor is widely expressed in the gastrointestinal nervous system, where it is involved in the neurohormonal modulation of the gastrointestinal ganglion.[16] We suggest that the primary molecular target of the barettins in cyprids of *B. improvisus* is the 5-HT_2 receptor, but further studies are needed to confirm the presence of this receptor in barnacles. It is plausible that the marine sponge *G. barretti* has evolved the ability to produce defense compounds that deter foulers and predators by interacting with serotonergic functions in these organisms; the sponge lacks a nervous system and so may not itself be affected by the release of these compounds.

In addition to interactions with 5-HT_{2A} and 5-HT_{2C} receptors, barettin also displayed significant affinity for the 5-HT_4 receptor, with a K_i value of 1.91 μM (Figure 4; Table 1). 8,9-Dihydrobarettin on the other hand lacked affinity for the 5-HT_4 receptor. No invertebrate 5-HT_4 receptor has yet been cloned and functionally characterized, and so the importance of barettin's affinity for 5-HT_4 receptors in the cyprid settlement assay remains to be elucidated.

Tegaserod (**3**) is a drug that has recently been approved for treatment of irritable bowel syndrome (IBS).[17] We selected it as a control due to a certain degree of similarity in chemical structure to barettin and 8,9-dihydrobarettin; in particular, the position of the double bond relative to the tryptophan ring in tegaserod is similiar to that in barettin. Tegaserod has a high affinity to 5-HT_4 as well as affinity to all the 5-HT_2 receptors.[9,18]

Neither barettin nor 8,9-dihydrobarettin displayed any significant interaction with the 5-HT_3 receptor. This was somewhat unexpected since England et al. (1998) have previously reported that σ-conotoxin from the cone snail *Conus geographus*, a peptide bearing a 6-bromotryptophan residue like the barettins, caused an inactivation of 5-HT_3 receptors transfected in HEK-293 cells. Additionally, σ-conotoxin did not interact with the serotonin receptors 5-HT_{2A}, 5-HT_{2C}, and 5-HT_4.[8] The similarities of the 6-bromo-substituted tryptophan residue in σ-conotoxin with barettin and 8,9-dihydrobarettin is not reflected in affinity for the 5-HT_3 receptor.

In conclusion, we have identified the marine cyclodipeptides barettin and 8,9-dihydrobarettin as selective serotonin receptor ligands. This may prove useful in further defining the functional roles of 5-HT receptors in invertebrates. Moreover, the small difference in chemical backbone between barettin and 8,9-dihydrobarettin results in striking differences in affinity to the human 5-HT receptors. This suggests a possible function for these compounds as templates to provide clues in drug discovery research aimed at disease states associated with the serotonergic system.

Experimental Section

Isolation of Barettin and 8,9-Dihydrobarettin. The cyclodipeptides barettin (cyclo[(6-bromo-8-entryptophan)arginine]) and 8,9-dihydrobarettin (cyclo[(6-bromotryptophan)arginine]) were isolated from a specimen of *Geodia barretti* collected in the Swedish Koster Fjord, 1 nautical mile from Tjärnö Marine Biological Laboratory (58°53′ N, 11°8′ E). The isolation and structure elucidation of the compounds were conducted as outlined in our previous publication.[5]

Chemicals. [*N*-methyl-^{3}H]LSD, [9-methyl-^{3}H]BRL-43694, and [1,2-^{3}H]5-carboxamidotryptamine were obtained from Perkin-Elmer (Boston,

MA). [*N*-methyl-^{3}H]GR113808 was obtained from Amersham Bioscience (Buckinghamshire, UK). Tegaserod (**3**) was obtained from APIN Chemical Ltd. (Oxon, UK). All other chemicals used were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Membrane Preparation. Membranes were prepared from human embryonic kidney-293 cells (HEK-293) transfected with human serotonin receptors. The following serotonin receptor subtypes were prepared from a glycerol stock (Guthrie, Sayre, PA): 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_4, 5-HT_{5A}, 5-HT_6, and 5-HT_{7A}. Plasmids were purified according to QIAGEN Highspeed Plasmid Purification Maxi Kit protocol. HEK-293 cells were maintained in a 5% CO_2 humidified incubator (37 °C) in 10 cm Petri dishes with a growth medium consisting of Dulbecco's modified Eagles medium (Gibco, Paisley, UK) (DMEM) supplemented with d-glucose (4500 mg L^{-1}), 10% FBS (Gibco, Paisley, UK), 100 μg/mL pencillin G (Gibco, Paisley, UK), and 100 μg/mL streptomycin (Gibco, Paisley, UK). The growth medium was replaced with 2 × 3 mL of OptiMEM (Gibco, Paisley, UK) prior to transfection. Six micrograms of receptor plasmid was transfected to each Petri dish, using Plus reagent (Gibco, Paisley, UK) and Lipofectamine reagent (Gibco, Paisley, UK). The transfection procedure was carried out according to the instructions given by the manufacturer. The HEK-293 cells and the transfection mixture were incubated for 4 h at 37 °C, 5% CO_2. The cells were washed, cultured in growth medium, and incubated for 24 h, followed by a reculture of growth medium and then incubated for an additional 24 h. Medium was gently removed by suction, and cold phosphate buffer was added. The resuspended DNA was centrifuged at 4 °C (1000*g* for 5 min). The pellet was resuspended in 5 mL of cold 50 mM TRIS buffer (supplemented with 1 mM EDTA, pH 7.4). The suspension was mixed with a polytron and centrifuged at 4 °C (15000*g* for 20 min). The pellet was resuspended in 10 mM TRIS buffer (supplemented with 10% sucrose, 0.2 mM EDTA, pH 7.4) and mixed. The transfected HEK-293 cell membranes were stored at −80 °C. Protein determination was conducted according to Lowry et al. (1951).

Radioligand Binding Assay. Barettin and 8,9-dihydrobarettin were evaluated in an in vitro radioligand binding assay based on the displacement of radioligands from human 5-HT receptors expressed in HEK-293 cell membranes. [*N*-methyl-^{3}H]LSD (86.8 Ci/mmol), [9-methyl-^{3}H]BRL-43694 (69.50 Ci/mmol), [*N*-methyl-^{3}H]GR113808 (81.0 Ci/mmol), and [1,2-^{3}H]5-carboxamidotryptamine (29.70 Ci/mmol) were used as radioligands. Nonspecific radioligand binding was determined in the presence of 10 μM 5-hydroxytryptamine. Cell membrane suspension expressing the 5-HT receptor subtype (∼10 μg protein) was suspended in a 50 mM TRIS buffer (supplemented with 3 mM $MgCl_2$, pH 7.4) and incubated (60 min at 25 °C; total volume = 500 μL) with radioligand (0.8 mM [*N*-methyl-^{3}H]LSD; 0.2 mM [9-methyl-^{3}H]BRL-43694; 0.8 mM [1,2-^{3}H]5-carboxamidotryptamine; or 0.05 mM [*N*-methyl-^{3}H]GR113808) and concentrations ranging from 0.01 nM to 1 μM of the compound tested. The incubation was terminated by rapid filtration under vacuum through Whatman GF/B glass fiber filter sheets presoaked in washing buffer (50 mM TRIS, 1 mM EDTA, pH 7.4) using a Brandel cell harvester. The filters were washed with 3 × 2 mL of the same buffer, dissolved in 4 mL of EcoScint A (National Diagnostics, Atlanta, GA), and incubated overnight. The residual radioligand bound to the filter was determined by liquid scintillation counting (LS6000IC, Beckman, Fullerton, CA).

Saturation Analysis. Saturation studies were conducted on transfected HEK-293 cell membranes expressing 5-HT_{2A}, 5-HT_{2C}, or 5-HT_4 receptors. The radioligand [*N*-methyl-^{3}H]LSD (86.8 Ci/mmol) was used for 5-HT_{2A} and 5-HT_{2C} and [*N*-methyl-^{3}H]GR113808 (81.0 Ci/mmol) was used for 5-HT_4. Total binding for each radioligand was determined with 10−12 concentrations of radioligand ranging from 6 to 300 nM. Nonspecific binding for each radioligand was determined in the same manner as total binding but with the addition of 400 μM 5-hydroxytryptamine to each concentration point (Figures 2, 3, 4). Specific binding was determined by substracting nonspecific binding from total binding. Scatchard analysis was calculated by dividing bound radioligand by free radioligand. Approximately 7 μg of membrane protein was suspended together with radioligand in a 50 mM TRIS buffer (supplemented with 3 mM $MgCl_2$, pH 7.4) and incubated (60 min at 25 °C; total volume = 100 μL). Scintillation was determined as described in the radioligand binding assay.

Statistical Analysis. Serotonin binding data were analyzed with nonlinear regression analysis using GraphPad Prism 4 (GraphPad Software, San Diego, CA). K_i values were calculated based on K_D values for [*N*-methyl-^{3}H]LSD and [N-methyl-^{3}H]GR113808. IC_{50} values were converted to K_i using the Cheng and Prusoff method.[19] The B_{max} and K_D values were obtained from three independent saturation experiments.

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