Designing Selective, High Affinity Ligands of 5-HT_{1D} Receptor by Covalent Dimerization of 5-HT_{1F} Ligands Derived From 4-Fluoro-*N*-[3-(1-methyl-4-piperidinyl)-1*H*-indol-5-yl]benzamide

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We demonstrate here that covalent dimerization of 5-HT_1 ligands is an effective design strategy to modulate affinity and selectivity of 5-HT_1 ligands. This approach was applied to LY-334370, a selective agonist of 5-HT_{1F} receptor, to generate structurally well-defined divalent molecules. Radioligand binding assays to three cloned 5-HT_1 receptor subtypes (5-HT_{1B}, 5-HT_{1D}, 5-HT_{1F}) demonstrated that the affinity of a series of homologous dimers varied significantly upon exploration of three structural variables (linker length, attachment position, functionality). In particular, the series of C_3-to-C_3 linked dimers derived from a monomer (**3**) showed high binding affinity to 5-HT_{1D} (for example, $K_i \approx 0.3$ nM for dimer **8**) but did not bind to 5-HT_{1F} ($K_i > 0.01$ mM), providing >10000-fold subtype selectivity. Results from a functional assay (rabbit saphenous vein contraction) demonstrate that certain dimers are 5-HT_1 receptor agonists.

Introduction

Migraine is a common disorder that affects about 10−15% of the general population, with particularly high prevalence in women.[1] Migraine headaches are of neurovascular origin and believed to be caused by local intracranial vasodilatation, dysfunctional nociceptive transmission, and neurogenic inflammation.[2–4] Clinical and experimental observations have demonstrated a role of the serotonergic system in migraine attacks,[5] and sumatriptan (3-[2-(dimethylamino)ethyl]-*N*-methyl-1*H*-indole-5-methanesulfonamide), a 5-HT receptor agonist selective for the 5-HT_1 subtypes, has been shown to be effective in treating migraine pain and associated symptoms.[4] Sumatriptan was the first of a new class of drugs termed "triptans", which include zolmitriptan ((4*S*)-4-[[3-[2-(dimethylamino)ethyl]-1*H*-indol-5-yl]methyl]- 2-oxazolidinone), rizatriptan (*N*,*N*-dimethyl-5-(1*H*-1,2,4-triazol-1-ylmethyl)-1*H*-indole-3-ethanamine), naratriptan (*N*-methyl-3-(1-methyl-4-piperidinyl)-1*H*-indole-5-ethanesulfonamide), almotriptan (*N*,*N*-dimethyl-5-[(1-pyrrolidinylsulfonyl)methyl]-1*H*-indole-3-ethanamine), eletriptan (3-[[(2*R*)-1-methyl-2-pyrrolidinyl]methyl]-5-[2-(phenylsulfonyl)ethyl]-1*H*-indole), and frovatriptan ((3*R*)-2,3,4,9-tetrahydro-3-(methylamino)-1*H*-carbazole-6-carboxamide). Selected examples are shown in Figure 1.[6–10] The antimigraine effect of all the triptans are mediated by both 5-HT_{1B} and 5-HT_{1D} receptors, and speculation remains as to whether or not a selective 5-HT_{1D} agonist would be as or more efficacious than current triptans with the benefit of improved tolerability and safety.[11]

Most of the triptans exhibit considerable potency at the 5-HT_{1F} subtype, in addition to their activity at 5-HT_{1B} and 5-HT_{1D} receptors.[7,8,10,11] It is therefore difficult to dissect the relative contribution of the different 5-HT_1 receptor subtypes to the mediation of adverse effects and beneficial effects of the triptans in migraine treatment. Consequently, the design of subtype-selective ligands is highly desirable and should provide tools for elucidating the role of individual 5-HT_1 receptor subtypes. It could also lead to a new class of agonists devoid of adverse effects on the cardiovascular and pulmonary systems.

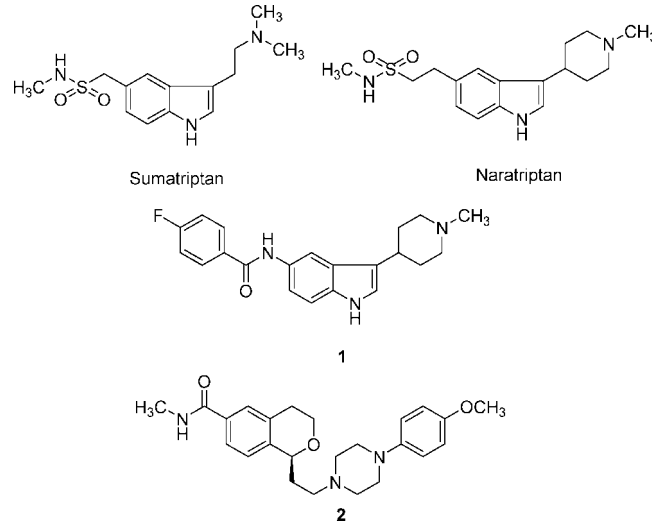

Figure 1. Structures of selected agonists to 5-HT_1 subtypes of human serotonergic receptor.

In order to achieve higher receptor subtype selectivity, we planned to exploit the concept of multivalency as a design principle for receptor ligands. Multivalent molecules interact with their biological target through the simultaneous binding of adjacent sites. These multivalent interactions may lead to a significant increase in avidity and specificity of the interaction, ultimately resulting in improved potencies, selectivities, and possibly duration of action of compounds.[12,13]

The 5-HT_1 class of serotonin receptors belongs to the superfamily of G-protein-coupled receptors (GPCRs). As single-chain proteins with seven membrane-spanning helices, GPCRs represent a target family that is particularly amenable to multivalent drug design. A growing list of GPCRs have been shown to dimerize in vivo, and both homo- and heterodimers have been reported.[14–16] While the structures of these GPCR oligomers are yet to be further characterized and the distance between adjacent binding sites in such oligomers is little studied, it is attractive to consider a homodivalent ligand model in which

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the ligand spans the receptor dimer and binds to both sites. For instance the atomic force microscopic images of rhodopsin dimers in native membranes suggest the possibility of designing such divalent ligands tethered at ~40−60Å in distance.[17]

In addition to the "primary" binding site for the endogeneous ligand, many GPCRs exhibit additional, "secondary" binding sites that, when occupied by small molecules, peptides, or proteins, allow allosteric modulation of receptor function.[18,19] Divalent ligands might allow for simultaneous interaction of the ligand with both primary and secondary binding sites. There are numerous examples of divalent ligands for GPCRs that have better pharmacological profiles than their respective monovalent counterparts,[13,20,21] with the most extensively studied examples being divalent ligands for opioid receptors,[22,23] 5-HT_4 serotonin receptors,[20] and 5-HT_1 serotonin receptors.[21,24]

This report describes our efforts toward identification of novel ligands with high affinity and selectivity for the 5-HT_1 class of serotonin receptors.[25,26] All the designed ligands are dimeric in nature; thus, each of the molecules represents two 5-HT ligands tethered via a linker. We identified a series of compounds for which dimerization of an initially 5-HT_{1F}-selective ligands led to a switch in subtype selectivity because of significantly increased binding affinity of the dimer for the 5-HT_{1D} receptor: ~260-fold and $>10^4$-fold selectivity over 5-HT_{1B} and 5-HT_{1F}, respectively. We observed a strong dependence of 5-HT_{1D} receptor binding affinity on linker length. Because of their high selectivity and binding affinity, these ligands are expected to serve as useful pharmacological tools to dissect the role(s) of the 5-HT_{1D} receptor subtype in the pathophysiology of migraine.

Chemistry

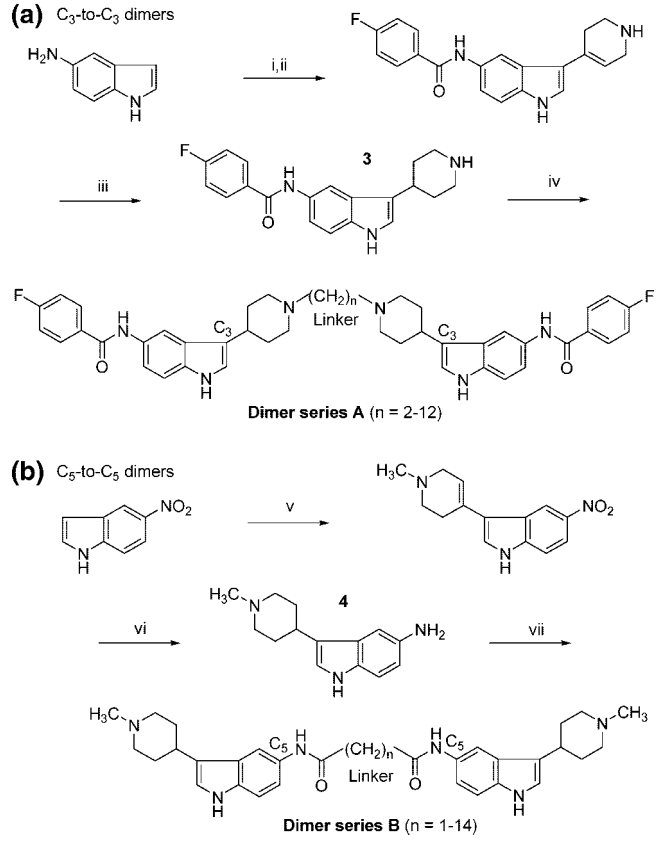
We considered three parameters in designing divalent molecules (ligand, nature of linker, and point of attachment) by which the molecular diversity of the library was generated. In evaluating 5-HT ligands as monovalent synthons, we surveyed various analogues and substructures of known 5-HT_1 agonists such as the triptans and **1** (LY-334370) (Figure 1). We chose two monomeric synthons, 5-(4-fluorobenzoyl)amino-3-(piperidin-4-yl)-1*H*-indole (**3**) and 5-amino-3-(*N*-methylpiperidin-4-yl)-1*H*-indole (**4**), for this study (Scheme 1).

Since length and functionality of the spacer are the important molecular parameters that define the linker diversity of the dimer library, we selected the linkers to incorporate such features. Therefore, the library included a range of linker length linkers (2 to >12 atoms) comprising a variety of functionality including alkane, (hetero)aromatics, (thio)ether, H-bond donor/acceptor group (amide, ester, alcohol).

The point of attachment used for the indole was either C_3 or C_5. Two representative classes of divalent indoles are shown in Scheme 1: (a) C_3-to-C_3 dimers and (b) C_5-to-C_5 dimers. Each is classified according to the location of the covalent bond on the indole ring that is used to cross-link two monomeric indole units. In the C_3-to-C_3 homodimer class, the functionality of the linker at the C_3-position of the indole remains as a tertiary amine. Such functionality is commonly observed in conventional 5-HT_1 agonists (Figure 1). In the C_5-to-C_5 class, an amide bond serves as the linking group to the indole core.

Synthesis of the C_3-to-C_3 dimers was based on the monomeric synthon **3**. The monomer was synthesized similarly according to the literature procedures for its *N*-Me piperidine analogue (**1**).[27−29] The synthesis of **3** began with 4-fluorobenzoylation of 5-aminoindole and was followed by the condensation of the amide with 4-piperidone in the presence of alcoholic KOH and

Scheme 1. (a) Synthetic Strategy for C_3-to-C_3 Dimer Series A Based on Bis-N-alkylation and (b) Strategy for C_5-to-C_5 Dimer Series B Based on Bis-amide Formation[a]



[a] Reagents and conditions: (i) 4-fluorobenzoyl chloride, Et_3N, CH_2Cl_2; (ii) 4-piperidone, KOH, MeOH; (iii) H_2 (1 atm), 10% Pd/C, EtOH; (iv) bis-halide (0.5 equiv), DMF, 70 °C, 2 days; (v) 1-methyl-4-piperidone, KOH, MeOH; (vi) H_2, 10% Pd/C, EtOH; (vii) bis-acid (0.5 equiv), *i*-Pr_2NEt, HATU, HOAt, DMF, room temp, 1 day.

subsequent reduction to **3** under standard hydrogenation conditions (H_2, 10% Pd/C) (for details, see Supporting Information).

The synthetic strategy for the preparation of the dimer series A (C_3-to-C_3) employed an N-alkylation reaction as described in Scheme 1. The monomeric ligand **3** was covalently homodimerized in a reaction with commercially available α,ω-bis-halides as linker precursors. Each of the dimers in this series (A) was prepared by heating a homogeneous solution of monomer **3** (0.1 mmol), bis-halide (0.5 equiv), and *i*-Pr_2NEt (1 equiv) in DMF in a sealed vial for 48 h, followed by purification of the reaction products by preparative reversed-phase high performance liquid chromatography (HPLC, purity of dimers greater than 98%). The identity of the dimers was confirmed by NMR spectroscopy and the combination technique of liquid chromatography and electrospray ionization mass spectrometry (LC−MS). Isolated yields of the bis-N-alkylation dimers were in the range of 10−30% depending on the nature of bis-halide component used.

Synthesis of the C_5-to-C_5 dimers linked through the bis-carboxamide (series B) is also described in Scheme 1. The synthon monomer **4** (3-(*N*-methyl-4-piperidinyl)-5-amino-1*H*-indole) was synthesized from 5-nitro-1*H*-indole using similar procedures as described previously.[27−29] By use of a standard coupling protocol (HATU, HOAt) commonly utilized for amide formation, a synthon **4** (0.2 mmol) reacted with a series of bis-

acids (0.5 equiv) to create dimer library B. The linker of these dimers (B) comprised of a linear aliphatic chain with $n = 1-14$.

Biological Results

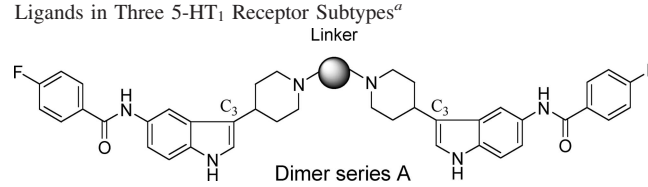
The in vitro binding affinity of the synthetic dimers for the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors, along with monomer controls, was measured by displacement of a bound radioligand [^{3}H]-5HT from the cloned human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors expressed in a CHO-K1 cell line. The binding affinity at the 5-HT$_{1F}$ subtype was measured similarly using a cloned 5-HT$_{1F}$ receptor from a rhesus monkey.[30,31]

Binding affinities of the dimers from series A, standards, and monomer-linker controls are given in Table 1. Sumatriptan showed potent binding at the 5-HT$_{1D}$ receptor, ~7-fold and ~195-fold higher than at the 5-HT$_{1B}$ receptor and the 5-HT$_{1F}$ receptor, respectively. Another monomeric standard **1** bound tightly and selectively to the 5-HT$_{1F}$ subtype with a value of K_i = 3.0 nM, though it showed reduced affinity to 5-HT$_{1B}$ and 5-HT$_{1D}$ subtypes (~27-fold and ~70-fold lower, respectively). The binding affinities of **1** are comparable to the values reported in literature:[32] K_i of 1.6 nM (human 5-HT$_{1F}$), 136 nM (human 5-HT$_{1B}$), 137 nM (human 5-HT$_{1D}$).[33]

To first study the influence of linker length on binding affinity, we chose aliphatic chains of length ($(CH_2)_2$ through $(CH_2)_{12}$) as the linker for the preparation of the C_3-C_3 dimers derived from **3**. This series of dimers led to exceptional activity at the 5-HT$_{1D}$ receptor where several dimers showed nanomolar affinity (Table 1). The optimum activity (K_i = 0.30 nM) was obtained for $(CH_2)_7$ (**8**), shorter or longer linkers having decreased affinity.[34] This trend appears clearly in a plot of linker length (n = number of atoms in linker) against pK_i of the dimers from series A at the three 5-HT$_1$ subtypes as shown in Figure 2a. A bell-shaped curve of affinity against linker distance was observed at 5-HT$_{1D}$ receptor. All of the series A dimers had no binding activity for the 5-HT$_{1F}$ receptor at 10 μM concentration. This observation was unexpected and interesting considering the strong affinity of the comparable monomer controls (**20**, **23**) at this subtype. For the 5-HT$_{1B}$ subtype, the binding activities of the dimers were lower than, or comparable to, that of **1** over the range of linker length (Figure 2a). Their affinities at 5-HT$_{1B}$ receptor varied with linker length with optimal activities for $(CH_2)_{10}$ (**10**) and $(CH_2)_7$ (**8**).

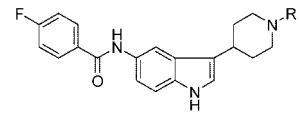
In extending the structure−activity relationship (SAR) of the linker in this C_3-C_3 dimer series A, we varied the functionality of the linker from simple alkyl chains to a selected set of functional groups such as ether, (hetero)aromatic, amide, alcohol, and ester within a comparable range of linker length (less than 11 atoms). For example, in an oligoether-linked dimer **13** with eight-atom length ($[CH_2CH_2O]_2CH_2CH_2$), the K_i value at the 5-HT$_{1D}$ subtype was 1.5 nM, about 12- to 18-fold lower than at 5-HT$_{1B}$ and 5-HT$_{1F}$, respectively. In contrast to **8** with a linker of $(CH_2)_7$, this dimer showed 5-HT$_{1F}$ activity with a K_i value, 9-fold higher than that of **1**. At a linker distance of 11 atoms, the activities of another dimer **14** (linker = $[CH_2CH_2O]_3CH_2CH_2$) were slightly lowered (~2-fold) or similar compared to those of the previous shorter ether analogue **13**. These examples in the aliphatic and ether linker series demonstrate that the composition of the atoms in the linker is an influential factor for binding activity. The dimers with relatively short linkers and a rigid conformation such as *trans*-2-butene (**15**), and *m*-xylyl (**16**) spacer showed activities at 5-HT$_{1D}$ that appear to reflect the linker-activity trend from the aliphatic series. At a short distance (two to three atoms), incorporation of CF_3 (**17**), $CONH_2$ (**18**), or $CO_2{}^t$Bu group into the alkane linker structure improved the affinity at 5-HT$_{1F}$ relative to the parent analogue **6**.

Table 1. Binding Affinities of Series A Dimers and Monovalent Ligands in Three 5-HT$_1$ Receptor Subtypes[a]



Dimer series A

Divalent Molecule	Linker =	K_i (nM) [h5-HT$_{1B}$]	K_i (nM) [h5-HT$_{1D}$]	K_i (nM) [rh5-HT$_{1F}$]
5	$(CH_2)_2$	>10000[b]	219 (±30)	>10000
6	$(CH_2)_3$	>10000	79 (±15)	>10000
7	$(CH_2)_6$	100 (±27)	0.60 (±0.20)	>10000
8	$(CH_2)_7$	79 (±33)	0.30 (±0.10)	>10000
9	$(CH_2)_9$	126 (±74)	1.0 (±0.4)	>10000
10	$(CH_2)_{10}$	38 (±8)	13 (±3)	>10000
11	$(CH_2)_{11}$	126 (±73)	13 (±4)	>10000
12	$(CH_2)_{12}$	>10000	13 (±4)	>10000
13	$[CH_2CH_2O]_2CH_2CH_2$	18	1.5	27
14	$[CH_2CH_2O]_3CH_2CH_2$	32	3.9	28
15	$CH_2CH{=}CHCH_2$ (E)	103	24	>1000
16	*m*-$CH_2C_6H_4CH_2$	453	151	>1000
17	$CH_2CH(CF_3)$	>1000	>1000	24
18	$CH_2CH(CONH_2)$	>1000	>1000	48



Monomer series

Monovalent Control	R =	K_i (nM) [h5-HT$_{1B}$]	K_i (nM) [h5-HT$_{1D}$]	K_i (nM) [rh5-HT$_{1F}$]
19	$(CH_2)_7CH_3$	>1000	>1000	>1000
20	$CH_2C_6H_5$	>1000	>1000	32
21	$(CH_2)_2OH$	>1000	>1000	9
22	$(CH_2)_2$-*N*-piperidine	>1000	>1000	>1000
23	$(CH_2)_3N(CH_3)_2$	>1000	>1000	123.5
24	$(CH_2)_9CO_2H$	>1000	>1000	>1000
1	CH_3	83 (±27)	210 (±120)	3.0 (±0.6)
Sumatriptan	-	9.1 (±2.2)	1.2 (±0.4)	234 (±115)
5-HT	-	1.1 (±0.3)	0.50 (±0.30)	35 (±9)

[a] The values of K_i represent the mean of more than two independent measurements. Each of the measurements was performed in duplicate or triplicate where individual values were observed within the range of ±60% of the mean value. Numbers within parentheses refer to the value of standard error of the mean (SEM). [b] No binding activity observed up to at 10 000 nM.

The monomers in Table 1 were tested to determine whether monomer-linker was attributable to the activity of a comparable dimer. Such monomers were designed to serve as representative examples where the structures of N-substituents range from a long aliphatic chain $(CH_2)_7CH_3$, benzyl group, alkyl chain

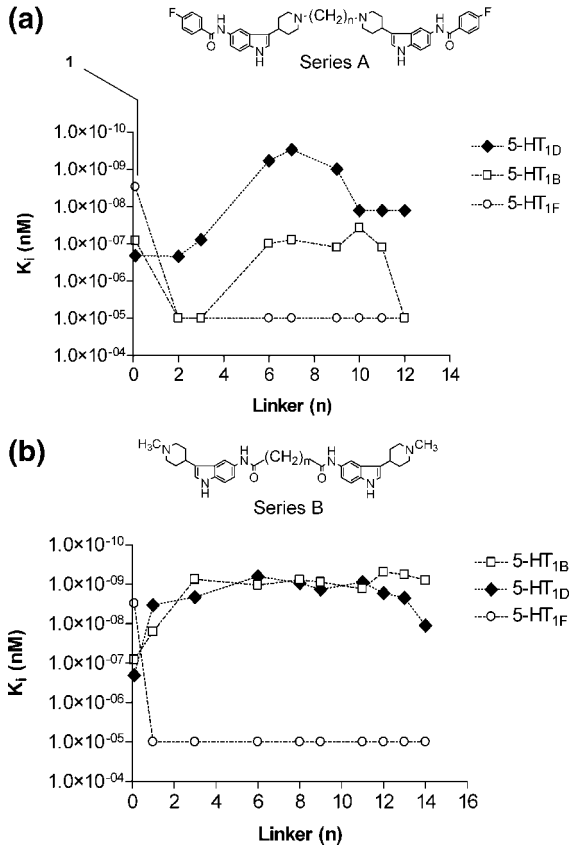


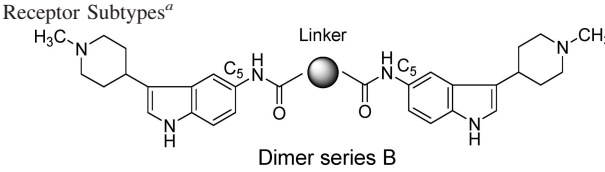
Figure 2. (a) Plot of K_i values (5-HT$_{1D}$, 5-HT$_{1B}$, 5-HT$_{1F}$) against linker length (number of atoms) of C_3-to-C_3 dimer series A. (b) Plot of K_i values against linker length of C_5-to-C_5 dimer series B.

containing hydroxyl, tertiary amine to a quaternary ammonium group. All of these monomers did not show any binding activity at 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors at >1000 nM. However, several monomer controls (e.g., N-substituent = CH_2CH_2OH, $CH_2C_6H_5$, *m*-CH_2pyridine) retained affinity at the 5-HT$_{1F}$ receptor, though these affinities were ~3- to 40-fold less than that of **1**.

As one of the major considerations in the design of divalent compounds, the point of attachment can be adjusted to modulate affinity and selectivity. The dimer series B, which represents an alternative to the C_3-to-C_3 indole system of series A, was constructed of alkyl-linked C_5-to-C_5 homodimers of 3-(*N*-methylpiperidin-4-yl)-1*H*-indole. The K_i values for series B dimers at 5-HT$_{1B}$, 5-HT$_{1D}$, and 5-HT$_{1F}$ (Table 2) are plotted vs linker length in Figure 2b. The binding activities of series B dimers at 5-HT$_{1F}$ were very low with K_i >1000 nM over almost an entire range of linker length examined. However, in comparison to the activity of the monomer **1** at the 5-HT$_{1B}$ subtype, the activities of the dimers ($n = 1-14$) were improved from ~5-fold ($n = 1$) up to 100-fold ($n = 8$). The affinity was correlated positively with linker length but relatively insensitive to an increase in length for $n > 2$. A similar trend was observed from binding of the dimers to 5-HT$_{1D}$. The combined results in Figure 2b show that C_5-to-C_5 dimers in general demonstrated a complete reversal of activity profile relative to monomer **1**, losing most activity at 5HT$_{1F}$ and gaining significant nonselective activity at the 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors.

Finally, our divalent design strategy led to identification of many 5-HT$_{1D}$-selective dimers particularly from the C_3-to-C_3 class. The results are highly encouraging because there are only a few reported ligands with ability to distinguish between 5-HT$_{1B}$ and 5-HT$_{1D}$ receptors such as **2** (PNU-109291, Figure 1) (K_i = 0.9 nM at 5-HT$_{1D}$; >5000-fold selectivity over 5-HT$_{1B}$)[35] and 3-[3-[4-[2-(3-fluorophenyl)ethyl]-1-piperazinyl] propyl]-5-(4*H*-1,2,4-triazol-4-yl)-1*H*-indole (L-775606) (IC_{50} at 5-HT$_{1D}$ = 0.6 nM; 125-fold selectivity over 5-HT$_{1B}$).[36]

Table 2. Binding Affinities of Series B Dimers in Three 5-HT$_1$ Receptor Subtypes[a]



divalent molecule	linker	K_i (nM) [h5-HT$_{1B}$]	[h5-HT$_{1D}$]	[rh5-HT$_{1F}$]
25	$(CH_2)_1$	16	3.4	>10000[b]
26	$(CH_2)_3$	0.76	0.95	>10000
27	$(CH_2)_6$	1.3	0.9	>10000
28	$(CH_2)_8$	0.79	0.95	>10000
29	$(CH_2)_9$	1.3	0.9	>10000
30	$(CH_2)_{11}$	0.1	1.4	>10000
31	$(CH_2)_{12}$	0.5	1.7	>10000
32	$(CH_2)_{13}$	0.58	2.3	>10000
33	$(CH_2)_{14}$	0.81	11.3	>10000
4	monomer	353	39.6	>10000

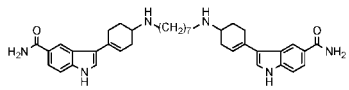
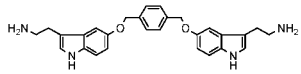
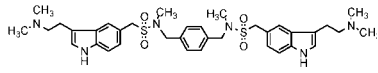
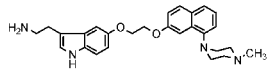
[a] The values of K_i represent the mean of more than two independent measurements. Each of the measurements was performed in duplicate or triplicate where individual values were observed within the range of ±60% of the mean value. Numbers within parentheses refer to the value of standard error of the mean (SEM). [b] No binding activity observed up to at 10 000 nM.

Preliminary studies on the pharmacological activity of dimers were performed in an isolated tissue contraction assay with rabbit saphenous vein, where activation of 5-HT$_{1B}$ receptors by either 5-HT or other 5-HT$_{1B}$ agonists is reported to cause contraction.[37,38] Three representative dimers **8**, **12**, and **31**, which were selected on the basis of their affinity at the 5-HT$_{1B}$ receptor, were tested at a single concentration (30 μM). Dimer **31** (K_i = 0.5 nM at 5-HT$_{1B}$, 1.7 nM at 5-HT$_{1D}$) elicited a 24% contractile response relative to sumatriptan when tested alone and inhibited the response to sumatriptan by 58% after preincubation of tissue, suggesting that **31** is a partial agonist with lower intrinsic activity relative to sumatriptan. A second dimer **8** (K_i = 79 nM at 5-HT$_{1B}$, 0.3 nM at 5-HT$_{1D}$) did not elicit tissue contractility when tested alone but showed weak antagonism (18%) of sumatriptan-induced contractility. A 5-HT$_{1D}$-selective dimer **12** with only low binding affinity for the 5-HT$_{1B}$ receptor (K_i > 10 000 nM at 5-HT$_{1B}$, 13 nM at 5-HT$_{1D}$) demonstrated neither agonist nor antagonist effects in the assay, confirming a predominant role of the 5-HT$_{1B}$ receptor in mediating rabbit saphenous vein contractions.

Discussion

In our understanding of a divalent recognition model, we propose the existence of a secondary site proximal to a primary recognition site of a ligand such that the combined nature of the two sites could be unique to a specific receptor subtype in their location, shape, and intersite distance within the receptor.[12,23,39] Targeting such proximal binding sites simultaneously with a divalent molecule could be an effective way to identify high affinity, subtype-selective ligands. The utility of this concept for GPCRs has been documented most thoroughly by Portoghese and colleagues for the opioid receptor,[23] such as their use of β-naltrexamine in the modulation of affinity and selectivity at opioid receptor subtypes (μ, κ, δ).[40] Others have applied various forms of divalent ligand design to GPCRs (adrenergic,[41–43] dopaminergic,[44] muscarinic,[45] serotonergic[20,21,24,46,47]), as well as membrane-bound transporter proteins.[48]

Table 3. Relative Affinities of Reported Divalent 5-HT$_1$ Ligands to Monovalent Controls

Divalent Molecules of 5-HT$_1$ receptor	Linkage Class	5-HT$_1$ Affinity (nM)	Enhancement Ratio[a]
Carboxamidoindole homodimer		IC_{50} ($_{1A}$) = 1.83	~1
	C_3-to-C_3	IC_{50} ($_{1D}$) = 0.05	40
		IC_{50} ($_{1E}$) = 99	-
Serotonin homodimer		K_i ($_{1A}$) = 1.6	2
	C_5-to-C_5	K_i ($_{1B}$) = 0.10	68
		K_i ($_{1D}$) = 0.11	46
Sumatriptan homodimer		K_i ($_{1A}$) = 17.7	23
	C_5-to-C_5	K_i ($_{1B}$) = 0.64	30
		K_i ($_{1D}$) = 0.89	10
Serotonin-antagonist heterodimer		K_i ($_{1A}$) = 0.96	~3
	C_5	K_i ($_{1B}$) = 0.76	9
		K_i ($_{1D}$) = 0.40	13

[a] Enhancement ratio refers to the increase in the binding affinity of a divalent ligand relative to a monovalent ligand: ratio = [IC_{50}(monomer)/IC_{50}(dimer)] or [K_i(monomer)/K_i(dimer)].

Prior to our investigation of divalent ligands for the 5-HT$_1$ receptors, several groups reported encouraging results, as summarized in Table 3. Examples include 5-carboxamidoindole dimers (LeBoulluec et al.),[49] serotonin dimers,[21] sumatriptan dimers,[24] and a serotonin heterodimer (Halazy et al.).[50] These previous examples demonstrated that connecting two 5-HT ligands through a linker is an effective strategy to enhance binding activity of a dimer over a corresponding monovalent counterpart (range of enhancement ratio of up to 68-fold in Table 3). In the present study, we have expanded this divalent design approach by varying multiple divalent parameters (5-HT ligands, linker composition, and point of attachment) in a systematic fashion aimed at enhancing our understanding of the importance of such parameters to binding affinity/selectivity of the resulting divalent 5-HT$_1$ ligands.

The primary role of the linker in the design of divalent molecules is to connect two primary binding groups at neighboring sites. The linker must be therefore sufficiently long and flexible to span the two sites. The linker may also provide a means to estimate the distance between the two sites.[51] In the C_3-to-C_3 dimer series A (Scheme 1), the monomer **3** was initially dimerized using linear aliphatic chains providing conformational flexibility with a simple extension of length. Within this series the maximal length of the constructs is estimated to be less than 12Å, allowing the molecules to target proximal sites located within a single receptor (intrareceptor) rather than multiple receptors (inter-receptor) on the surface of membrane. We observed a correlation between spacer distance and affinity at the 5-HT$_{1D}$ and 5-HT$_{1B}$ receptor subtypes with the largest enhancement in activity obtained with a linker $(CH_2)_7$ at 5-HT$_{1D}$. This observation at the two receptor subtypes is in contrast to the inactivity of the C_3-linked series A dimers at 5-HT$_{1F}$ receptor and was surprising in light of the activity of the monomeric component at the 5-HT$_{1F}$ subtype. Our observation is similar to that by Tamiz et al. in their design of monoamine transporter inhibitors, in which covalent dimerization using alkyl chains of a weak inhibitor of norepinephrine transporter (3,4-disubstituted piperidine-based) led to potent, selective inhibitors of the serotonin reuptake transporter rather than the norepinephrine transporters.[52]

Our results suggest a certain level of distinction between the 5-HT$_{1B}$ and 5-HT$_{1D}$ subtypes versus the 5-HT$_{1F}$ subtype for binding larger divalent constructs. For both the C_3-to-C_3 dimers (A) and the C_5-to-C_5 dimer series (B), binding activities at 5-HT$_{1F}$ were very low (K_i > 1000 nM). The results might be understood on the basis of the structural differences between the 5-HT$_1$ receptor subtypes. The 5-HT$_{1D}$ and 5-HT$_{1B}$ subtypes are related together closely and diverged from 5-HT$_{1F}$ subtype on the basis of the degree of homology in the sequence alignment.[53] Such primary distinction might be associated with the higher level of binding activities observed at either the 5-HT$_{1B}$ or the 5-HT$_{1D}$ subtype than at the 5-HT$_{1F}$ subtype.

The classes of attachment point used in designing the two dimer series led to interesting profiles regarding 5-HT$_{1B}$ vs 5-HT$_{1D}$ selectivity. The C_3-to-C_3 dimers were more selective for 5-HT$_{1D}$ with an optimal linker length reaching a maximum (up to 250-fold over 5-HT$_{1B}$) at $n = 7$, whereas most of the C_5-to-C_5 dimers showed enhanced but virtually nonselective activities with less variation to linker length. This linkage selectivity is primarily attributable to the structural differences in the ligand recognition domain of the 5-HT$_1$ receptor. According to a model proposed by Sternfeld et al.,[54] the human 5-HT$_{1D}$ subtype readily tolerates bulky and extended substituents at the 3-position of indole (5-HT) while the 5-HT$_{1B}$ subtype prefers smaller substitutions instead.

Another factor to be considered in the interpretation of the binding affinity of a dimer is the potential contribution from interaction of the linker itself with a receptor. For example, a hydrophobic alkane linker may interact favorably with hydrophobic residues around proximal sites on the receptor. This issue

is addressed by selected examples of monovalent controls bearing an alkane linker terminated with simple functionality such as methyl, phenyl, hydroxyl, acidic, and basic groups (Table 1). Considering their inactivity in 5-HT_{1B}, and 5-HT_{1D} receptors (**19**−**24**, all >1000 nM), we believe that the potential binding contribution from a hydrophobic linker of the dimer class A might be insignificant compared to that expected from a tight divalent interaction provided by the additional second ligand.

Linker functionality in general can be considered to influence the molecular shape and conformation of a dimer and to provide an additional binding contribution from a specific linker−receptor interaction. In dimer series A, we varied the linker structure from linear alkanes to those that select functionality (ether, rigid, aromatic, H-bond donor/acceptor). For example, dimer **13**, which is linked with tri(ethylene glycol) of eight-atom length, is a 5-HT_{1D}-selective ligand, though its potency for 5-HT_{1D} (or 5-HT_{1B}) receptor is several-fold lower than that of an equivalent alkane dimer (**8** or **9**) of comparable linker length. However, its affinity for 5-HT_{1F} receptor is much improved compared to the alkane dimers. This example demonstrates that linker composition is influential for binding activity dependent on the physicochemical nature of functionality and the specific receptor.

Given the discovery of 5-HT_{1D}-selective dimers, it would be interesting to evaluate their functional activity at the 5-HT_{1D} receptor. In the absence of a suitable functional assay, we assessed functional activity of a selected set of compounds in the rabbit saphenous vein isolated tissue contraction assay, which represents an in vitro model for 5-HT_{1B} receptor-mediated functional activity.[37,38] Our results demonstrate that **31** is an agonist at the rabbit saphenous vein preparation. Since **31** can partially inhibit the contractile response to sumatriptan and since the 5-HT_{1D}-selective dimer **12** demonstrated neither agonist nor antagonist activity in the preparation, we conclude that **31** is a partial agonist at the 5-HT_{1B} receptor with a lower intrinsic activity relative to sumatriptan.

In summary, we observed structure-defined trends in affinity and selectivity between two linkage classes of dimers. The patterns of activity variation dependent on attachment sites are uniquely interesting. However, because of lack of structural information regarding the dimer−receptor interaction, it is difficult to interpret the observed results clearly with supporting evidence. Therefore, their modes of binding at the receptors, functional activities, and pharmacokinetic properties will be the subject of future investigations.

Conclusion

We demonstrated the practical utility of covalent dimerization strategy in our search for potent selective ligands at the 5-HT_{1D} receptor. We designed indole-based divalent ligands that are composed of homodimers (C_3-to-C_3, C_5-to-C_5). The structure−activity correlation derived from the binding activity of those dimers at 5-$HT_{1B,}$ 5-HT_{1D}, and 5-HT_{1F} receptors suggests that affinity and selectivity of the dimers depended significantly upon a combination of three main variables involved in dimer design (linker distance, point of attachment, ligand fragment). The series of C_3-to-C_3 linked dimers derived from a monomer (**3**) led to potent 5-HT_{1D}-selective ligands with lack of activity at 5-HT_{1F}. The results of this study demonstrate that the covalent dimerization of a monovalent ligand can be tailored to afford selectivity to either 5-HT_{1D} alone or 5-HT_{1B}/5-HT_{1D}.

Experimental Section

Chemistry. Unless noted otherwise, starting material (including bis-amine, bis-acid, bis-halide) and solvents were purchased from commercial suppliers and used without further purification. Reactions were run under nitrogen atmosphere, unless noted otherwise. Progress of reaction mixtures was monitored by analytical high performance liquid chromatography (HPLC) and mass spectrometry, the details of which are given below and separately in specific examples of reactions. Reaction mixtures were worked up as described specifically in each reaction and routinely purified by preparative HPLC; a general protocol is described below. Test compounds with >98% purity, assessed by analytical HPLC (Bonus-RP, 2.1 mm × 50 mm, particle size of 5 μm, flow rate of 0.5 mL/min, linear gradient method A (10% MeCN/H_2O/0.1% TFA to 40% MeCN/H_2O/0.1% TFA over 6 min), linear gradient method B (10% MeCN/H_2O/0.1% TFA to 70% MeCN/H_2O/0.1% TFA over 5 min), UV detection at 214, and 254 nm), were submitted for biological evaluation. Characterization of reaction products was performed by ^{1}H and ^{13}C NMR spectrometry in deuterated solvent (CD_3OD, DMSO-d_6) acquired under standard parameters using a Varian Gemini 2000 instrument (400 or 300 MHz). Mass spectrometric identification of compounds was performed using standard electrospray ionization methods (ESMS) with a Perkin-Elmer SCIEX API 150 EX.

Preparation of Monomers (Scheme 1). Monomer **3** [5-(4-fluorobenzoyl)amino-3-(piperidin-4-yl)-1*H*-indole] was synthesized from 5-aminoindole and *N*-(4-fluoro)benzoic acid (also using other requisite starting materials) according to a literature protocol (see Supporting Information).[29,55] Purity 99% (analytical HPLC: method A; t_R = 3.17 min). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 10.91 (d, J = 2.40 Hz, 1H), 10.15 (s, 1H), 8.89 (br s, 1H), 8.10−8.05 (m, 2H), 8.03 (s, 1H), 7.39−7.34 (t, 2H), 7.33 (d, J = 0.80 Hz, 2H), 7.13 (d, J = 2.00 Hz, 1H), 3.37−3.34 (d, 2H), 3.10−3.03 (dt, 2H), 2.13−2.10 (d, J = 11.99 Hz, 2H), 1.93−1.82 (dq, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 165.1, 163.9, 162.6, 133.6, 131.7, 131.6, 130.3, 130.2, 130.1, 125.6, 121.5, 118.1, 116.6, 115.3, 115.1, 111.3, 111.3, 43.5, 30.8, 29.2. ESMS ($C_{20}H_{20}FN_3O$): calcd 337.4, found 338.1 $[M + H]^+$, 675.2 $[2M + H]^+$. HRMS (Bruker TOF) calcd for $C_{20}H_{20}FN_3O$ 338.1663, found 338.1669.

Monomer **4** [5-amino-3-(1-methylpiperidin-4-yl)-1*H*-indole] was synthesized from 5-nitroindole according to a literature protocol[56] (see Supporting Information). Purity 95% (analytical HPLC: method B; t_R = 0.8 min). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 10.30 (br s, 1H), 7.03−7.00 (d, J = 8.80 Hz, 1H), 6.87 (s, 1H), 6.70 (d, J = 1.60 Hz, 1H), 6.46−6.44 (dd, J = 8.40, 1.99 Hz, 1H), 2.86−2.83 (m, 2H), 2.55 (m, 1H), 2.19 (s, 3H), 2.01−1.92 (m, 2H), 1.88−1.85 (d, 2H), 1.71−1.58 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 140.4, 130.3, 127.1, 120.1, 118.0, 111.6, 111.4, 102.0, 56.0, 46.3, 32.8, 32.5. ESMS ($C_{14}H_{19}N_3$): calcd 229.3, found 230.1 $[M + H]^+$. HRMS (Bruker TOF) calcd for $C_{14}H_{19}N_3$ 230.1658, found 230.1627.

Preparation of C_3-to-C_3 Dimer Series A (Scheme 1). A Representative Procedure, 5 (n = 2). A solution of DMF (1 mL) containing monomer **3** (67.4 mg, 0.2 mmol) and 1, 2-dibromoethane (18.79 mg, 0.1 mmol) in a sealed vial was heated at 70 °C for 48 h with shaking. The reaction mixture was added to ethyl ether (45 mL) contained in a plastic bottle, and the whole mixture was shaken to homogeneity. After a few minutes a pale-brown oily residue precipitated. It was collected by centrifugation at 3500 rpm for 20 min followed by decanting of the supernatant solution. The solid residue was rinsed with ether (50 mL) and dried in air. The crude product was dissolved in 2 mL of 50% MeCN/H_2O (0.1% TFA) and purified by reversed phase HPLC (YMC Pack-Pro C18 column, 50 mm × 20 mm and particle size of 5 μm; linear gradient from 2% to 40% MeCN/H_2O (0.1% TFA) over 15 min, flow rate of 40 mL/min; detection at 214 nm) to afford **5**. Purity of >99% (analytical HPLC: method B; t_R = 2.50 min). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 10.4 (br s, 2H), 10.10 (s, 2H), 8.10−8.0 (m, 6H), 7.35−7.25 (m, 8H), 7.14 (br s, 2H), 3.7−3.0 (m, 12H), 2.20 (br d, 4H), 1.90 (br, 4H). ESMS ($C_{42}H_{42}F_2N_6O_2$): calcd 700.83, found 701.3 $[M + H]^+$.

Other C_3-to-C_3 dimers shown in Table 1 were prepared in an analogous manner from monomer **3** and a requisite bis-halide (0.5 equiv) (see Supporting Information).

Preparation of C_5-to-C_5 Dimer Series B (Scheme 1). A Representative Procedure 26 ($n = 3$). To a solution of 5-amino-3-(1-methylpiperidin-4-yl)-1*H*-indole **4** (46 mg, 0.2 mmol) with diisopropylethylamine (54 μL, 0.3 mmol) in anhydrous DMF (200 μL) was added a solution of glutaric acid (0.1 mmol), HOAt (34 mg, 0.25 mmol), and HATU (95 mg, 0.25 mmol) in anhydrous DMF (400 μL). The reaction mixture was shaken overnight at ambient temperature and then concentrated in vacuo. This mixture was dissolved in 2 mL of 50% aqueous acetonitrile with 0.1% trifluoroacetic acid and purified by preparative HPLC to afford **26**. Purity of 98% (analytical HPLC: method B; $t_R = 1.8$ min). ^{1}H NMR (400 MHz, MeOH-d_4): δ (ppm) 7.92 (s, 2H), 7.36−7.30 (d, $J = 8.40$ Hz, 2H), 7.08−7.03 (m, 4H), 3.60−3.53 (m, 4H), 3.20−2.99 (m, 4H), 2.89 (s, 6H), 2.52−2.49 (t, $J = 7.19$ Hz, 2H), 2.45−2.31 (m, 4H), 2.25−2.10 (m, 4H), 1.99−1.83 (m, 4H). ESMS ($C_{33}H_{42}N_6O_2$); calcd 554.74, found 555.4 $[M + H]^+$.

Other C_5-to-C_5 dimers were prepared in an analogous manner from monomer **4** and a requisite bis-acid (0.5 equiv) (see Supporting Information).

Radioligand Binding Assays. Membranes from CHO-K1 cell lines expressing the cloned human 5-HT$_{1D}$ and 5-HT$_{1B}$ receptors were purchased from Euroscreen (Brussels, Belgium). The 5-HT$_{1F}$ receptor was cloned from rhesus monkey genomic DNA by polymerase chain reaction.[30,31] The 5-HT$_{1F}$ receptor coding sequence was subcloned into the expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA). COS-7 cells (American Type Culture Collection, Manassas, VA) were transiently transfected with the 5-HT$_{1F}$ expression plasmid, and membranes were prepared by homogenization in 50 mM Tris-HCl, 5 mM EDTA, pH 7.4, and centrifugation.

The binding affinities of compounds for the 5-HT$_{1B}$, 5-HT$_{1D}$, and 5-HT$_{1F}$ receptors were determined in radioligand displacement assays using membrane preparations. Assays were carried out at a constant concentration of the radioligand [^{3}H]5-hydroxytryptamine (Amersham Biosciences, Piscataway, NJ) with increasing concentrations (10^{-12} to 10^{-6} M) of test compounds. Binding reactions were incubated for 1 h at room temperature in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 12.5 mM $MgCl_2$, 0.1% ascorbic acid and were stopped by rapid filtration over GF/B glass fiber filter plates using a cell harvester (PerkinElmer, Boston, MA). Filter-bound radioactivity was determined by liquid scintillation counting. Inhibition constants K_i were calculated from IC_{50} values, and the dissociation constants (K_d) of the radioligand were calculated according to the Cheng−Prusoff equation.[57]

Isolated Tissue Contraction Assay. The functional activity of selected compounds was evaluated at isolated rabbit saphenous vein preparations in vitro at MDS Pharma Services (Taipei, Taiwan), according to published procedures.[37,38] To assess serotonin receptor agonism, the contractile response to 30 μM of test compounds was measured and expressed relative to the response to 30 μM sumatriptan after 5 min of incubation at 37 °C. To assess antagonism, tissue was incubated with 30 μM test compound for 5 min at 37 °C, followed by a challenge with 30 μM sumatriptan. The antagonistic effect was expressed as percent inhibition of the sumatriptan response.

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Supporting Information Available: Synthetic details and characterization data for monomers (**3**, **4**), C_3-to-C_3 dimers (**5**−**14**), and C_5-to-C_5 dimers (**25**−**31**). This material is available free of charge via the Internet at http://pubs.acs.org.

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