Published in final edited form as:

Bioorg Med Chem. 2015 March 15; 23(6): 1251–1259. doi:10.1016/j.bmc.2015.01.047.

Design, synthesis, and biological evaluation of a series of bifunctional ligands of Opioids/SSRIs

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Abstract

A series of opioid and serotonin re-uptake inhibitors (SSRIs) bifunctional ligands have been designed, synthesized, and tested for their activities and efficacies at μ -, δ - and κ opioid receptors and SSRIs receptors. Most of the compounds showed high affinities for μ - and δ -opioid receptors and lower affinities for SSRIs and κ opioid receptors. A docking study on the μ -opioid receptor binding pocket has been carried out for ligands **3-11**. The ligands **7** and **11** have displayed the highest binding profiles for the μ -opioid receptor binding site with G_{bind} (–12.14 kcal/mol) and K_i value (1.0 nM), and G_{bind} (–12.41 kcal/mol) and K_i value (0.4 nM), respectively. Ligand **3** was shown to have the potential of dual acting serotonin/norepinephrine re-uptake inhibitor (SNRI) antidepressant activity in addition to opioid activities, and thus could be used for the design of multifunctional ligands in the area of a novel approach for the treatment of pain and depression.

Keywords

Opioid; SSRIs; synergistic effect; bifunctional ligands; pain; depression

1. Introduction

Neuropathic pain is associated with disease or injury of the nervous system. In recent years, a number of drugs have been approved for neuropathic pain treatment, including some antidepressants. Antidepressants have been used for many years for the treatment of pain in

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Supporting Information

¹H NMR and ¹³C NMR spectra, and Mass spectrum of the ligands **1, 3-11** are provided.

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humans.¹ Selective serotonin re-uptake inhibitors (SSRIs) are commonly safer as compared to classical tricyclic antidepressants and are more often prescribed to treat chronic pain despite weaker antinociceptive effects.² The antinociceptive modes of action of antidepressants are not completely understood. Enhanced availability of norepinephrine (NE) and serotonin (5-HT) at neuronal terminals generated by antidepressant drugs^{3,4} may activate multiple monoamine receptors, *e.g.*, α_1 , α_{2A} , 5-HT₂ and 5-HT₃ receptors.^{1,5,6} The opioid system has been shown to be involved in the antinociceptive activity of antidepressants, including amitriptyline, paroxetine and sibutramine.⁷ In the same way activation of noradrenergic and serotonergic pathways occurs during opioid analgesia.⁸ On the basis of this fact, we have hypothesized that the development of novel ligands that possess properties of SSRIs at 5-HT receptors and of agonists at the opioid receptors, within the same molecule, may present a significant therapeutic advantage in the treatment of depression and pain states, and enhanced analgesic effects in chronic pain states *e.g.*, neuropathies and analgesia without the development of tolerance.

Among three (μ , δ and κ) opioid receptors, the activation of the μ -opioid receptors produces the highest antinociception. Most clinically used opioid drugs, including morphine, are μ opioid agonists. Previously, bifunctional compounds have shown concerted efficacy because of successful combination of the functions *e.g.*, chimera ESP6 (YPFFPLM-NH₂) containing overlapped pharmacophores of endomorphin-2 and substance-P.⁹⁻¹¹ Our design is based on the overlapping pharmacophore concept that has been developed for bifunctional ligands with opioid agonists and cholecystokinin (CCK), neurokinin-1 (NK-1) or melanocortin antagonist activities *e.g.*, opioid and melanocortin bifunctional ligands formed by overlapping of 2,6-dimethyltyrosine(Dmt)-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic) for δ -opioid receptor and Tic-D-Phe(*p*-Cl)-piperidin-4-yl-*N*-phenyl-propionamide for the melanocortin receptor *e.g.*.¹²⁻¹⁶ In our study, an enkephalin-based tetrapeptide, Tyr-*D*-Ala-Gly-Phe, and desmethylescitalopram (**1**) were used as an opioid agonist pharmacophore, and SSRIs pharmacophore, respectively.

2. Results and discussion

2.1. Design

The co-administration of opioid agonists and a type of antidepressants, SSRIs, is expected to give a synergistic effect^{17,18} and we herein set out to design an strategy to this co-administration, developing bifunctional ligands, an approach that has proven successful in the past for increasing potency and selectivity of many drugs. The drug design was based on bifunctional ligands interacting with two targets in a single molecule by combining two pharmacophores; opioid and SSRIs (Figure 1). The SSRIs pharmacophore desmethylescitalopram **1** was combined with an opioid pharmacophore so that the designed ligands maintain the topographical structures of both pharmacophores, bearing in mind the development of previously designed μ -opioid selective 4-anilidopiperidine, and *N*-phenyl-*N*-piperidin-4-yl-propionamide analogues.¹⁸ For the opioid agonist activity, enkephalin-like tetrapeptide structures (H-Tyr-*D*-Ala-Gly-Phe-) has been selected and modified. For the SSRIs activity, escitalopram, one of the most efficient SSRIs drug, has been selected and modified to get compound **1**. Butler *et al.* demonstrated that escitalopram has an *N,N*-

dimethyl propylamine group, a primary aromatic moiety with a electronegative but not hydrogen bond-donating cyano group, and a secondary aromatic ring with a uorine substituent.¹⁹ Structure-activity relationship (SAR) studies revealed that the aromatic CN substituent is crucial for high serotonin transporter inhibitory activity, despite the fact that the aromatic uorine atom and the amino substitution are less significant factors for serotonin transporter inhibition.²⁰ Further studies recognized that (S)-enantiomers of escitalopram analogues are also more selective and potent than (R)-enantiomers. In the meantime, escitalopram remains the most potent and selective compound among phenyl-substituted phthalanes as an inhibitor of serotonin transporter.^{20–23}

2.2. Chemistry

The designed ligands (**3–11**) (Table 1) were synthesized by stepwise solution phase peptide syntheses using *N*^α-Boc chemistry. During the peptide syntheses (chain elongation) the intermediates were isolated by precipitation from appropriate organic solvents, generally diethyl ether with high purity. The products were purified by preparative RP-HPLC to afford > 98 % pure compounds in 45–56 % yields (Scheme 1). The ligands (**3–11**) were analyzed by ¹H NMR, ¹³C NMR, Low Resolution and High Resolution Mass Spectroscopy and HPLC.

2.3. Biological Evaluation

2.3.1. Opioid receptors—Opioid binding were evaluated by radioligand competition analysis using [³H]DPDPE for the δ opioid receptor, [³H]DAMGO for the μ-opioid receptor and [³H]U69,593 for the κ opioid receptor in cell membrane preparations from transfected cells that stably express the respective receptor type. Ligand **3** in which a enkephalin-like tetrapeptide (H-Tyr-*D*-Ala-GlyPhe-) and desmethylescitalopram (**1**) are combined revealed nonselective moderate binding affinities at the three opioids receptors (*K*_i= 88 nM, 57 nM, 86 nM for μ, δ and κ-opioid receptors, respectively) (Table 2). Comparing to the binding affinities of tetrapeptide amide (H-Tyr-*D*-Ala-Gly-Phe-NH₂, *K*_{iδ}/*K*_{iμ} = 107), it is clear that the combination of the opioid pharmacophore with the SSRIs reduced μ receptor selectivity over δ receptor in all cases.

As discussed by the formerly established SAR, the replacement of *D*-Ala at position 2 with *D*-Nle and the halogenation at the para position of Phe aromatic ring at position 4 improved opioid activities at both μ- and δ-opioid receptors *in vitro*, that was in agreement with the other outcomes,^{24,25} in combination with increased lipophilicity (aLogP increase: 0.7 for *D*-Nle, 0.5 for Cl).²⁶ Herein, the modification in ligand **3** led to ligand **5**, which demonstrated augmented opioid binding affinity (MOR: *K*_i=16 nM, 5.5-fold increase; DOR: *K*_i=1.4 nM, 40-fold increase; KOR: *K*_i = 68 nM). The *D*-Nle and the F-substitutions increased the δ-opioid binding affinity more than the μ-opioid binding affinity. Likewise, Cl-replacement at the para position of Phe aromatic ring in ligand **3** directed to ligand **4**, which established augmented opioid binding affinities (MOR: *K*_i = 7.0 nM, 13-fold increase; DOR: *K*_i = 1.6 nM, 37- fold increase; KOR: *K*_i = 43 nM). It is evident from the literature that halogenation and methylation increase the lipophilicity and cell permeability.²⁷ It is also documented that substitution of a chlorine on the Phe residue of [D-Pen², D-Pen⁵]enkephalin (DPDPE) led to an important enhanced cell permeability in both *in vivo* and *in vitro* trials.²⁸ In our case, Cl-

substitution on the aromatic ring and Dmt-substitution in ligand **3** gave ligand **6**, which validated better opioid affinities [K_i = 12 nM (7-fold increase), K_i = 4.0 nM (14-fold increase) and K_i = 37 nM (2-fold increase) at the μ , δ and κ -opioid receptors, respectively] relative to **3**. Correspondingly replacement of *D*-Ala with *D*-Nle and F-substitution at the *para* position of Phe aromatic ring in ligand **3** led to ligand **7**, which displayed non-selective high opioid affinities [K_i = 1.0 nM (16-fold increase), K_i = 0.8 nM and K_i = 3.0 nM (23-fold increase) at the μ , δ and κ -opioid receptors, respectively].

Previously it has been shown that Dmt-Tic is a highly selective δ -opioid antagonist, and its modification lead to diverse changes in opioid activities and selectivities.²⁹ The Tic residue was changed to *D*-Tic in ligands **8-11**. These ligands retained similar biological activities with the other ligands, which suggested that the constrained and very hydrophobic *D*-Tic residue could be replaced for the flexible *D*-Ala and *D*-Nle residues in the enkephalin structure. The F-substitution in ligand **10** increased the opioid binding affinities [K_i = 7.0 nM (5-fold increase), 0.91 nM (22-fold increase), 9.0 nM (10-fold increase) for three the μ , δ and κ receptors, respectively] relative to **8**. However its δ receptor selectivity over μ and κ receptors was converted to μ receptor selectivity in ligand **11** by deleting the Gly at position 3.

With the aim of reducing the pharmacophore size but increasing the lipophilicity, the Gly³ residues in ligands **8** and **10** were deleted, achieving ligands **9** and **11**, respectively. It has been accepted that the comparative proximity of the Tyr aromatic ring and the Phe aromatic ring is critical for the opioid receptor selectivity.³⁰ In our findings, the deletion of Gly³ reduced the δ -opioid receptor activity while it increased the μ -opioid receptor affinity in one case (ligand **11**). The Gly³ deleted ligands exhibited increased δ -opioid receptor affinity over μ -opioid receptor affinity in the binding assays in our second case (ligand **9**). It is not clear how the F- and Cl-substitutions could be responsible for the contradictory effects on the receptor selectivities in ligands **9** and **11**.

2.3.2. Serotonin, adrenergic, dopamine, and sigma receptors and monoamine transporters—Ligands were subjected to the primary radioligand binding assays at serotonin receptors (5HT₁₋₇) using CHO cells or HEK cells.³¹ In the primary binding assays, all ligands did not show more than 50% inhibition at 10 μ M concentration, when the percent inhibition was calculated as follows: 100% - 0% radioactivity bound. In addition to the binding assays at opioid and serotonin receptors, assays were also examined against a panel of neurotransmitters transporters and brain receptors. While all serotonin receptors were devoid of sufficient inhibition, some of ligands exhibited affinities for the other receptors: adrenergic receptors, dopamine receptors, sigma receptors, and biogenic monoamine transporters (Table 3). Ligands **6** (K_i = 0.24 μ M) and **7** (K_i = 0.28 μ M) showed the submicromolar range of binding affinities at α_{2C} receptor. Ligand **5** exhibited selective binding affinity (K_i = 47 nM) at the sigma₁ receptor in the nanomolar range. However, binding at the rest of the tested receptors and transporters was not found to give significant affinity. Notably, ligand **3** was found to bind to NET (K_i = 0.75 μ M), SERT (K_i = 0.28 μ M), and σ_1 receptor (K_i = 0.41 μ M) in the submicromolar range. It is evident from the literature that newer dual acting antidepressants *e.g.*, SNRIs (*e.g.* venlafaxine and duloxetine)

possesses analgesic efficacy like TCAs, but have a more liable safety and tolerability profile and also have an efficacy advantage over SSRIs in treating the painful physical symptoms of depression and in achieving remission of all symptoms of depression.³² The ligand **3** emerged with the potential of a dual acting serotonin /norepinephrine re-uptake inhibitor (SNRI) in addition to excellent opioid activities. Therefore, the structure of ligand **3** can be considered as a good scaffold for designing a novel series of bifunctional ligands for the treatment of pain and depression.

2.3.3. Functional Assays at 5HT₂-family receptors—Functional assays of the ligands **6**, **7**, **10** and **11** were performed for 5HT₂ receptors. Ligands **6** and **7**, as we expected from the binding assay data, did not exhibit any significant functional activities of 5HT₂-family receptors (5HT_{2A}, 5HT_{2B}, 5HT_{2C}) (Table 4). Ligands **10** and **11**, which showed 94% and 99% of antagonist activities, respectively, at 10 μ M in the primary functional assays, were tested for secondary screening using concentration-response assays. From this assay, it was confirmed that both ligands do not possess any functional activity for the 5HT₂-family receptors.

Our aim was to develop bifunctional ligands of SSRIs and opioid in one structure using two known pharmacophores as a starting point. The bifunctional ligands showed excellent binding affinities at μ , δ and κ opioid receptors while these ligands did not show any significant interaction with 5-HT receptors. On the basis of the biological activity profile, these ligands are considered to show good analgesic effects rather than bifunctional opioid and SSRIs properties. It is likely that opioid decreases the release of the monoamines in the synaptic cleft while in case of SSRIs it inhibits the reuptake of serotonin neurotransmitter.^{33,34} The Selective inhibition was not possible and this may be the reason why ligands exhibited opioid but not SSRIs activities. However, this idea could be helpful for further design and compatibility of two different pharmacophores in the search of an approach for pain and depression management in a single molecule. In our other findings, during a multitargeted screen of opioids/SSRIs ligands **3** exhibited the potential of dual acting SNRIs antidepressant properties in addition to opioid activities.

2.3.4. Molecular Docking Studies—The 3D structure of Rattus norvegicus 5-HT_{2B} receptor (PDB ID 4nc3) was retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (PDB). The 3D structure of Rattus norvegicus μ -opioid receptor in active state was taken from Peptide Synthesis and Molecular Recognition Lab³⁵. Coordinate files were subjected to Discovery Studio Visualizer for pre-docking receptor preparation. The pre-docking ligand preparation was performed by *Energy Minimize* tool of MOE. It applied Merck Molecular Force Field 94x (MMFF94x) to obtain the lowest energy conformation of the ligands. The optimized coordinates of Ligands **3-11** were docked with the μ -opioid receptor and 5-HT_{2B} receptor separately by Molecular Operating Environment (MOE). The London dG and Affinity dG scoring functions were applied by MOE to estimate the free binding energy in Kcal/mol. MOE *Site Finder* tool estimated the potential binding sites for μ -opioid receptor and 5-HT_{2B} receptor. The selected binding pockets of both receptor proteins included the important amino acid residues as indicated in the respective crystal structures.^{35, 36}

To get qualitative assessment and to understand the molecular basis of the calculated biological activities (K_i) the docked complexes of ligands **3-11** were analyzed. The docked structures were evaluated by using the Ligand Interaction tool of MOE package. The binding affinity of the docked ligands was given as S-score (free binding energy or G_{bind} kcal/mol) calculation. Hydrogen bonding and hydrophobic interactions of each ligand were estimated within binding pocket of receptor protein. The conformation of each compound which exhibits the highest binding affinity has shown in Table 5 for the μ -opioid receptor and for the 5-HT_{2B} receptor (See Supporting Information: Table S9 and Figure S2). The results were used to suggest a preliminary structure-activity correlation, bearing in mind that the assays have been performed *in vitro*, enhancing the complexity of the biological response and, hence, of correlation to the theoretical data.

In μ -opioid binding pocket, ligand **11** exhibited the highest binding affinity as indicated by G_{bind} (−12.41 kcal/mol) which agrees with the experimentally determined binding affinity of this ligand (K_i = 0.4 nM) (Table 5, Figure 2). Visual inspection of this complex shows that the terminal amino group of the Dmt moiety potentially forms hydrogen bond with Asp²¹⁶ (1.78 Å), and the hydroxyl group of Dmt also forms two hydrogen bonds with Asn¹²⁷ with bond length of 2.74 Å and 2.45 Å. It is worth noting that these hydrogen bond contacts are playing an important role in giving ligand **11** superior binding affinity compared to other tested ligands. Also the free rotation of the heterocyclic rings around the axis of the carbon linker allows the best orientation inside the binding pocket, which is not allowed in the other ligands. All the benzo moieties of the tetrahydroisoquinoline, isobenzofuran, 4-fluorophenyl and 4-fluoro-phenylalanine were within Van der Waals contact distances with the hydrophobic side chains of the receptor amino acids. Such contacts involve the hydrophobic amino acids of active site such as Val²³⁶, Leu²³², Leu²¹⁹, Ile¹⁴⁴, Met¹⁵¹, Trp²⁹³, Val³⁰⁰ and Trp³¹⁸.

The binding affinity (K_i = 1.0 nM) of ligand **7** was correlated with G_{bind} (−12.14 kcal/mol) (Table 5, Figure 2). Visual inspection of this complex exhibits that the terminal amino group of the Dmt scaffold has a potential for hydrogen bond with Asp¹⁴⁷ amino acid with bond length of 1.55 Å. In this study, the salt bridge between Asp¹⁴⁷ residue in TMH3 of the MOR and terminal amino group of peptide ligand is observed in 6 out of 9 ligands. For ligand **7**, the benzo moieties and rest of the molecule were found to lie within a hydrophobic contact to Ile¹⁴⁴, Ile²⁹⁶, Trp²²⁶, Val³⁰⁰ and Trp³¹⁸. All the other ligands have shown decreased binding affinities (Table 5 and see Supporting Information: Figure S1) relative to ligands **7** and **11**.

If we correlate the G_{bind} and K_i values of ligand **3** and **4**, it showed that in ligand **3** phenylalanine replacement with 4-Cl-phenylalanine ligand **4** formed and it exhibited higher G_{bind} (−4.29 kcal/mol) and K_i (7.0 nM) than ligand **3** G_{bind} (−4.80 kcal/mol) and K_i (88 nM). This may be due to the extensive hydrogen bonding between the amino group of Tyr and Asp¹⁴⁷ (1.47 Å) and other hydrogen bonding between NH group of 4-Cl-phenylalanine and Glu²²⁹ (1.35 Å) amino acid. Similarly, oxygen atom of furan ring accepts a hydrogen bond from Lys²⁰⁹ (2.62 Å) amino acid residue while rest of the molecule was found to lie within a hydrophobic contact to Val³⁰⁰, Trp²⁹³, Trp³¹⁸, Ile²⁹⁶, Ile³²² and Leu²¹⁹ residues.

Similarly, correlation of ligand **6** and **9** revealed that substitution of alanine with tetrahydroisoquinoline of ligand **6** and truncation of glycine it formed ligand **9**, and exhibited the greater G_{bind} (−9.34 kcal/mol) and K_i (10 nM) values as compared to ligand **6** G_{bind} (−6.01 kcal/mol) and K_i (12 nM). This is due to the hydrogen bonding between the terminal amino group of Dmt and Asp¹⁴⁷ (1.60 Å) and a hydrogen bond acceptance of most electronegative oxygen of furan ring and Thr²¹⁸ (3.27 Å) amino acid. Moreover, some hydrophobic contacts could also be seen between the ligand and binding pocket such as Trp³¹⁸, Trp²⁹³, Val³⁰⁰, Leu²¹⁹, Ile¹⁴⁴, Ile³²² and Ile²⁹⁶ amino acid residues and also showed arene-cation contact potential between phenyl ring of Dmt and Tyr³²⁶.

It is worth noting that these ligands (**3-11**) showed lower binding affinities *in vitro* and higher G_{bind} (−9.54 to −15.02 kcal/mol) within 5-HT_{2B} receptor binding pocket *in silico* probably due to bulky substitution at desmethylescitalopram moiety. This moiety was found to be oriented differently (compared to μ -opioid binding pocket) in an unfavorable conformation within the binding pocket.

3. Conclusion

In the current investigation, bifunctional ligands have been designed, synthesized and tested for their activities at the opioid and SSRIs receptors for the treatment of pain and depression. These bifunctional ligands produced potent δ , μ and κ -opioid activities and minimal SSRIs activities. The rationale behind this opioid/SSRIs bifunctional ligands concept was that the opioid would decrease the release of neurotransmitters in the synapse while the SSRIs moiety increase the selective reuptake inhibition of serotonin only. The results showed that our novel ligands exhibit excellent binding to opioid receptors relative to SSRIs. These ligands could be good analgesics. To gain insight into the binding of these ligands at the μ -opioid receptor, molecular modeling studies were performed. As the result, ligands **7** and **11** were found to have the best μ -opioid binding profiles in the binding pocket with −12.14 kcal/mol of G_{bind} and −12.41 kcal/mol of G_{bind} , respectively, which are a good match of *in vitro* binding affinities (K_i = 1.0 nM for ligand **7**, 0.4 nM for ligand **11**).

4. Material and Methods

All chemicals were purchased from Novabiochem, ChemImpex International, and RSP and were used as received. ¹H NMR and ¹³C spectra were recorded on a Bruker Advance-400 and Bruker DRX-600 spectrometer equipped with 5-mm Nalolac triple-resonance single z-axis gradient probe at 25 °C with XW in NMR. All NMR chemical shifts are reported as ppm relative to tetramethylsilane (TMS: δ =0.00). Coupling constants (J) are reported in hertz. Spectra were processed with Mestre Nova and Mestre C softwares. Mass spectra were taken in the positive ion mode under ESI methods. High resolution mass spectra were obtained on the 9.4 T Bruker FT-ICR-MS spectrometer in CH₃OH: CH₃CN (1: 1). The purity of compounds was determined by HPLC and TLC on silica gel plates (Analtech 02521), solvent system EtOAc: hexane (8: 2) and CH₂Cl₂: MeOH: AcOH(9: 1: 0.1).

4.1.1 (S)-1-(4-Fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile (desmethylescitalopram) (1)

(S)-1-(4-Fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile (desmethylescitalopram) (**1**) was prepared by following the reported methods with a modification.^{37,38} Escitalopram oxalate (12 mmol) was dissolved in EtOAc (50 mL) and stirred for 5 min. Aqueous ammonia (30 %, 50 mL) was added and stirred for further 30 min. The organic layer was separated and the aqueous layer was further extracted with EtOAc (2 × 25 mL). The combined organic extract was dried over anhydrous MgSO₄ overnight. EtOAc was evaporated under reduced pressure. The residue was dissolved in toluene (20 mL) and α -chloroethylchloroformate (2.6 mL, 24 mmol) was added, and refluxed for 6 h at 110 °C. After cooling, the reaction mixture was diluted with CH₃OH (10 mL) and refluxed for further 5 h. The solvents were removed under reduced pressure. TLC (CH₃OH: CHCl₃ = 1:1) showed that the residue is a mixture of two components. The two components were separated via column chromatography using (CH₃OH: CHCl₃ = 1:1) as eluent. The component with R_f value (0.2) was isolated as light brown oil (0.2 g, 4 %) and found to be dimer of desmethylescitalopram (**1**). IR (Nujol/cm⁻¹) $\nu_{\max}$ 1620 (C=N). MS (ESI): m/z 621 [M+H]⁺. HRMS calcd for C₃₈H₃₉F₂N₄O₂: 621.2972 and found (ESI, [M+H]⁺): 621.2974. The component with R_f value (0.7) was the desired product; light brown oil (4.8 g, 96 %). MS (ESI): m/z 311 [M+H]⁺. HRMS calcd for C₁₉H₂₀FN₂O: 311.1559 and found (ESI, [M+H]⁺): 311.1554. The desmethylescitalopram (**1**) was kept under the atmosphere of argon in order to avoid oxidation. IR (Nujol/cm⁻¹) $\nu_{\max}$: 2260 (CN); ¹H NMR (400 MHz, CDCl₃) δ_H : 9.00 (br s, 1H), 7.70 (t, J = 12.1 Hz, 3H), 7.60 (q, J = 8.0 Hz, 2H), 7.10 (t, J = 12.1 Hz, 2H), 5.19 (dd, J = 12.0, 4.0 Hz, 2H), 2.74-2.70 (m, 2H), 2.25 (d, J = 4.0 Hz, 3H), 2.20 (t, J = 12.0 Hz, 2H), 1.51-1.59 (m, 2H). ¹³C NMR (100 MHz, CDCl₃) δ_C : 163.20, 161.03, 149.03, 139.93, 132.10, 126.44, 125.22, 122.99, 118.33, 115.17, 112.08, 90.71, 71.46, 48.97, 38.00, 32.63, 20.46. MS-(ESI) m/z : [M+H]⁺, 311; HRMS: Calcd. for C₁₉H₂₀FN₂O: 311.1559; found: 311.1554; Physical state: Light brown oil; Yield: 4.8 g (96 %).

4.2. Synthesis of (S)-1-(4-fluorophenyl)-1-(3-[methylamino]propyl)-1,3-dihydroisobenzofuran-5-carbonitrile (1)-conjugated peptide ligands (3-11)

General Procedure—Ligands **3-11** were prepared by stepwise synthesis using N^α-Boc chemistry starting from (S)-1-(4-fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile (**1**).¹² (S)-1-(4-Fluorophenyl)-1-[3-(methylamino)propyl]-1,3-dihydroisobenzofuran-5-carbonitrile (**1**, 1 mmol) and N^α-Boc-amino acid (1.1 mmol) were dissolved in DMF (15 mL) and cooled in an ice bath for 10 minutes. HBTU (1.2 mmol) and DIEA (2 mmol) were added to the reaction mixture and stirred for 1–2 h at room temperature. The disappearance of the starting amine (**1**) was monitored by TLC. After completion of the reaction, saturated solution of NH₄Cl (15 mL) was added into the reaction mixture and extracted with EtOAc (3 × 15 mL) and washed with aqueous NaHCO₃ (5 %, 3 × 15 mL), brine (1 × 15 mL), and water (1 × 15 mL), consecutively. The organic extract was dried over anhydrous MgSO₄ and filtered. The EtOAc was evaporated under reduced pressure and triturated with diethyl ether to give a solid. The N^α-Boc group was deprotected by 80% TFA in dichloromethane at 0 °C for 30

min. After completion of the chain elongation by subsequent couplings and deprotections, the mixtures were evaporated under reduced pressure. The residues were triturated with diethyl ether and purified by preparative RP-HPLC (20–80 % acetonitrile in aqueous 0.1 % TFA within 20 min for ligands (**3-11**) to give pure ligands as white powders in overall yields of 44–56 %. The purity of the ligands was determined as 97 % by analytical HPLC (i) 10–90 % acetonitrile in aqueous 0.1 % TFA in aqueous 0.1 % TFA within 40 min, ii) 25–65 % acetonitrile in aqueous 0.1 % TFA in aqueous 0.1 % TFA within 20 min). The purity of the ligands were also checked by TLC using three different solvent systems (i) CHCl₃/MeOH/AcOH = 90:10:3, (ii) EtOAc/BuOH/H₂O/AcOH = 5:3:1:1, (iii) BuOH/H₂O/AcOH = 4:1:1. The sequence was repeated to insert other required amino acids in the desired peptide.

(S)-2-(2-((R)-2-((S)-2-Amino-3-(4-hydroxyphenyl)propanamido)propanamido)acetamido)-N-(3-((S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl)propyl)-N-methyl-3-phenylpropanamide (3): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 8.44-8.34 (m, 3NH), 8.16-8.07 (m, 2H), 7.81-7.78 (m, 3H), 7.72-7.70 (m, 1H), 7.58-7.55 (m, 2H), 7.18-7.17 (m, 3H), 7.152 (s, 2H), 6.42 (d, *J* = 3.2 Hz, 3H), 5.14 (s, 1H), 2.77 (s, 2H), 2.67 (d, *J* = 1.6 Hz, 2H), 2.51-2.49 (m, 3H), 2.25-2.23 (m, 2H), 2.20-2.19 (m, 1H), 2.18 (s, 1H), 2.17-2.13 (m, 8H), 2.06-2.02 (m, 2H), 1.77 (s, 1H), 0.90-0.86 (m, 3H). Yield: 0.42 g (56 %).

(S)-2-(2-((R)-2-((S)-2-Amino-3-(4-hydroxyphenyl)propanamido)propanamido)acetamido)-3-(4-chlorophenyl)-N-(3-((S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl)propyl)-N-methylpropanamide (4): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 7.64-7.59 (m, 2H), 7.53-7.51 (d, *J* = 8.0 Hz, 1H), 7.47 (s, 2H), 7.44-7.42 (m, 1H), 7.40-7.38 (m, 2H), 7.28 (s, 1H), 7.27 (d, *J* = 7.2 Hz, 1H), 7.21-7.17 (m, 4H), 7.16 (s, 3H), 7.02-7.00 (m, 1H), 5.31 (s, 1H), 5.24-5.13 (m, 2H), 3.41-3.30 (m, 1H), 3.07-3.06 (m, 1H), 3.02-3.00 (m, 1H), 2.89-2.88 (m, 4H), 2.70 (s, 3H), 2.76-2.72 (m, 2H), 2.58-2.51 (m, 2H), 2.26 (t, *J* = 6.8 Hz, 2H), 2.16-2.09 (m, 2H), 1.82-1.79 (m, 2H), 1.50-1.36 (m, 3H). Yield: 0.35 g (47 %).

(R)-2-((S)-2-Amino-3-(4-hydroxyphenyl)propanamido)-N-(2-((S)-1-((3-((S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl)propyl)(methyl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl)amino)-2-oxoethyl)hexanamide (5): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 7.65-7.60 (m, 2H), 7.53 (d, *J* = 6.4 Hz, 1H), 7.45 (s, 2H), 7.42-7.40 (m, 5H), 7.28-7.27 (m, 3H), 7.20-7.15 (m, 1H), 7.13-7.11 (m, 1H), 7.06-7.03 (m, 1H), 7.02-7.00 (m, 2H), 5.25-5.12 (m, 2H), 4.03-4.02 (m, 2H), 4.00-3.97 (m, 1H), 3.79 (s, 1H), 3.42-3.37 (m, 1H), 3.05-3.00 (m, 1H), 2.88-2.86 (m, 6H), 2.81 (s, 3H), 2.75-2.55 (m, 2H), 2.22-2.13 (m, 2H), 2.09-1.81 (m, 8H), 1.54-1.40 (m, 3H). Yield: 0.34 g (48 %).

(S)-2-(2-((R)-2-((S)-2-Amino-3-(4-hydroxy-2,6-dimethylphenyl)propanamido)propanamido)acetamido)-3-(4-chlorophenyl)-N-(3-((S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl)propyl)-N-methylpropanamide (6): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 8.78-8.71 (m, 1H), 8.37 (br s, 3H), 8.10-8.01 (m, 2H), 7.74-7.72 (m, 1H), 7.70-7.63 (m, 1H), 7.52-7.48 (m, 2H), 7.19-7.16 (m, 1H), 7.12-7.02 (m, 5H), 6.45-6.28 (m, 2H), 5.15-5.04 (m, 2H), 4.74-4.64 (m,

1H), 4.17-4.15 (m, 2H), 3.80-3.76 (m, 2H), 3.63-3.50 (m, 2H), 3.36-3.22 (m, 1H), 3.13-2.91 (m, 2H), 2.85-2.77 (m, 1H), 2.71 (s, 3H), 2.45-2.43 (m, 1H), 2.11 (s, 6H), 2.07-1.94 (m, 2H), 1.29-1.16 (m, 2H), 0.84-0.57 (m, 3H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_C : 172.07, 171.23, 170.63, 168.55, 158.69, 156.14, 141.39, 140.34, 138.84, 134.43, 132.51, 132.31, 131.98, 131.60, 131.44, 129.90, 129.01, 128.78, 128.40, 117.67, 115.69, 114.78, 111.03, 90.90, 71.45, 59.86, 57.76, 56.49, 51.85, 47.68, 37.67, 36.45, 35.09, 21.85, 20.31, 18.30. Yield: 0.31 g (46 %).

(R)-2-[(S)-2-Amino-3-(4-hydroxy-2,6-dimethylphenyl)propanamido]-N-(2-[(S)-1-[(3-[(S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]propyl](methyl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl]amino)-2-oxoethyl)hexanamide (7): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 8.39 (br s, 3H), 8.27 (br s, 1H), 8.19-8.12 (m, 2H), 7.86-7.82 (m, 3H), 7.76 (d, *J* = 8.0 Hz, 1H), 7.63-7.60 (m, 2H), 7.25-7.19 (m, 3H), 7.08-6.97 (m, 2H), 6.45 (br s, 2H), 5.25-5.16 (m, 2H), 4.86-4.77 (m, 1H), 4.18-4.15 (m, 2H), 3.72-3.63 (m, 2H), 3.45-3.32 (m, 1H), 3.23-3.06 (m, 2H), 2.95-2.86 (m, 2H), 2.82 (s, 3H), 2.72-2.70 (m, 1H), 2.57-2.56 (m, 2H), 2.25 (s, 6H), 2.18-2.03 (m, 2H), 1.46-1.21 (m, 2H), 1.20-1.07 (m, 2H), 0.82-0.79 (m, 5H). ¹³C NMR (150 MHz, DMSO-*d*₆) δ_C : 171.60, 170.76, 168.89, 168.51, 160.74, 159.14, 156.14, 141.23, 140.27, 138.71, 133.75, 133.67, 132.49, 131.63, 131.51, 131.35, 129.99, 128.80, 119.24, 117.51, 115.37, 114.70, 110.98, 90.89, 71.45, 53.59, 51.88, 50.46, 49.18, 47.52, 37.62, 35.07, 33.64, 31.88, 27.22, 22.33, 21.84, 20.41, 14.09. Yield: 0.35 g (47 %).

(S)-2-[(S)-2-Amino-3-(4-hydroxy-2,6-dimethylphenyl)propanoyl]-N-(2-[(R)-3-(4-chlorophenyl)-1-[(3-[(S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]propyl)(methyl)amino)-1-oxopropan-2-yl]amino)-2-oxoethyl)-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (8): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 8.35 (s, 2H), 7.30-7.29 (m, 2H), 7.27-7.26 (m, 1H), 7.23-7.20 (m, 4H), 7.15-7.12 (m, 1H), 7.09-7.08 (m, 1H), 7.06-7.02 (m, 4H), 6.86-6.79 (m, 1H), 6.76-6.75 (m, 1H), 6.57 (dt, *J* = 4.0, 8.0 Hz, 1H), 6.29-6.26 (m, 2H), 4.89 (s, 1H), 4.54-4.44 (m, 2H), 4.17-4.12 (m, 1H), 3.12-3.09 (m, 2H), 3.06-3.02 (m, 2H), 2.99-2.80 (m, 2H), 2.78-2.76 (m, 1H), 2.61-2.55 (m, 1H), 2.48 (s, 6H), 2.20-2.19 (m, 1H), 2.13-1.82 (m, 2H), 0.92-0.88 (m, 2H). Yield: 0.34 g (46 %).

(S)-2-[(S)-2-Amino-3-(4-hydroxy-3,5-dimethylphenyl)propanoyl]-N-[(R)-3-(4-chlorophenyl)-1-[(3-[(S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]propyl)(methyl)amino)-1-oxopropan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (9): ¹H NMR (400 MHz, DMSO-*d*₆) δ_H : 8.30 (d, *J* = 12.0 Hz, 3H), 7.34-7.29 (m, 1H), 7.27-7.25 (m, 1H), 7.23-7.20 (m, 1H), 7.19-7.15 (m, 3H), 7.09-7.03 (m, 4H), 6.92-6.86 (m, 1H), 6.79-6.75 (m, 1H), 6.72-6.65 (m, 1H), 6.56-6.52 (m, 1H), 6.28 (d, *J* = 3.2 Hz, 2H), 6.22 (d, *J* = 5.6 Hz, 1H), 4.97 (s, 1H), 4.86-4.77 (m, 1H), 4.67-4.54 (m, 1H), 4.48-4.41 (m, 2H), 4.31-4.06 (m, 1H), 3.04-2.99 (m, 4H), 2.86-2.76 (m, 2H), 2.73-2.50 (m, 2H), 2.48 (s, 6H), 2.19-2.18 (m, 9H), 0.88-0.87 (m, 2H). Yield: 0.33 g (45 %).

(S)-2-[(S)-2-Amino-3-(4-hydroxy-3,5-dimethylphenyl)propanoyl]-N-(2-[(R)-1-[(3-[(S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]propyl](methyl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl]amino)-2-oxoethyl)-1,2,3,4-

tetrahydroisoquinoline-3-carboxamide (10): ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ_{H} : 8.36 (br s, 3NH), 8.33 (s, 2H), 8.12-8.09 (m, 1H), 7.80-7.79 (m, 2H), 7.71-7.69 (m, 1H), 7.58-7.54 (m, 2H), 7.20-7.17 (m, 2H), 7.15 (s, 1H), 7.12-7.08 (m, 1H), 7.02-6.91 (m, 1H), 6.40-6.39 (m, 4H), 4.28 (s, 1H), 4.14-4.11 (m, 4H), 3.71-3.61 (m, 4H), 3.54 (s, 1H), 2.75-2.73 (m, 4H), 2.66-2.64 (m, 1H), 2.51-2.2.49 (m, 3H), 2.17-2.16 (m, 8H), 1.35-1.06 (m, 1H), 0.77-0.74 (m, 6H). Yield: 0.31 g (54 %).

(S)-2-[(S)-2-Amino-3-(4-hydroxy-3,5-dimethylphenyl)propanoyl]-N-[(R)-1-({3-[(S)-5-cyano-1-(4-fluorophenyl)-1,3-dihydroisobenzofuran-1-yl]propyl}(methyl)amino)-3-(4-fluorophenyl)-1-oxopropan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide (11): ^1H NMR (400 MHz, $\text{DMSO}-d_6$) δ_{H} : 8.35 (br s, 3NH), 7.92-7.70 (m, 1H), 7.23-7.20 (m, 3H), 7.06-7.02 (m, 7H), 6.87-6.83 (m, 1H), 6.75 (s, 1H), 6.56-6.53 (m, 1H), 6.29-6.27 (m, 3H), 5.08 (s, 1H), 4.89-4.85 (m, 4H), 4.55-4.44 (m, 2H), 3.11-3.04 (m, 6H), 2.95-2.86 (m, 2H), 2.80-2.74 (m, 1H), 2.62-2.58 (m, 1H), 2.48 (s, 1H), 2.20-2.18 (m, 7H), 2.13-2.12 (m, 1H), 1.82-1.80 (m, 2H), 0.89-0.86 (m, 2H). Yield: 0.34 g (51 %).

4.3. Radioligand labeled binding assays and functional assays

Ligands were tested for their binding affinities at opioid receptors, as previously described.^{12,14,39,40} Initial screens for various receptors and transporters and functional assays for serotonin (5-HT) 5-HT_{2A}, 5HT_{2B} and 5HT_{2C} receptors were determined in Roth lab.^{31,41} The results of opioid binding affinities obtained from the Roth lab were consistent with our findings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We gratefully acknowledge a grant from the Higher Education Commission of Pakistan under the IRSIP program to support Mehr-un-Nisa. This work was partially supported by grants from The U.S. Public Health Services, NIH, and NIDA P01DA006284 and R01DA013449.

Abbreviations

AcOH	acetic acid
Boc	<i>tert</i> -butoxycarbonyl
CHO	Chinese hamster ovary
DALEA	[D-Ala ² , Leu ⁵] enkephalin amide
DIEA	diisopropylethylamine
DMF	<i>N,N</i> -dimethyl formamide
hDOR	human δ -opioid receptor
DPDPE	c[D-Pen ² , D-Pen ⁵]enkephalin

DAMGO	[D-Ala ² , NMePhe ⁴ , Gly ⁵ -ol]enkephalin
Dmt	2,6-dimethyltyrosine
EtOAc	ethylacetate
HBTU	<i>O</i> -(benzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
5-HT	5-hydroxytryptamine/serotonin
hKOR	human κ -opioid receptor
MeOH	methanol
rMOR	rat μ -opioid receptor
NE	norepinephrine
NET	norepinephrine transporter
RCSB	The Research Collaboratory for Structural Bioinformatics
RP-HPLC	reversed-phase high performance liquid chromatography
SAR	structure-activity relationship
SNRIs	serotonin/norepinephrine re-uptake inhibitors
SSRIs	selective serotonin reuptake inhibitors
TMH3	transmembrane helix 3
TCA	tricyclic antidepressants
TFA	trifluoroacetic acid
Tic	1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid
TLC	thin layer chromatography

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Figure 1.
Design of opioid/SSRIs bifunctional ligands.

(a)

(b)

Ligand 7

(c)

(d)

Ligand 11**Figure 2.**

(a) and (c) represent 2D while (b) and (d) represent 3D view of ligand **7** and ligand **11** μ -opioid receptor complex, respectively. Dotted lines show the hydrogen bond interactions.

Scheme 1. Synthesis of bifunctional ligands of opioid and SSRIs

Reagents and conditions: (i) HBTU, DMF, DIEA, rt. 1 h, (ii) TFA (50 %) in CH_2Cl_2 , rt. 30 min.

Table 1

Analytical data of opioid/SSRIs bifunctional ligands (**3-11**)

no	Molecular Formula	Low MS ^a		HRMS ^b		HPLC t _R /purity (min/%) ^c			TLC, R _f ^d		
		Obsd.		Obsd.	Calcd.	(A)	(B)	(I)	(II)	(III)	
3	C ₄₂ H ₄₆ FN ₆ O ₆	749		749.3457	749.3455	22.4/98	34.2/98	0.82	0.47	0.77	
4	C ₄₂ H ₄₅ ClFN ₆ O ₆	783		783.3807	783.3811	23.1/99	35.2/99	0.79	0.39	0.61	
5	C ₄₅ H ₅₁ F ₂ N ₆ O ₆	809		809.3833	809.3831	20.3/100	32.2/100	0.75	0.31	0.59	
6	C ₄₄ H ₄₉ ClFN ₅ O ₆	811		811.3379	811.3381	24.6/100	35.2/100	0.74	0.37	0.55	
7	C ₄₇ H ₅₅ F ₂ N ₆ O ₆	837		837.4146	837.4156	21.6/99	32.1/99	0.72	0.21	0.56	
8	C ₅₁ H ₅₃ ClFN ₆ O ₆	899		899.3449	899.3457	23.2/100	34.3/100	0.77	0.41	0.67	
9	C ₄₉ H ₅₀ ClFN ₅ O ₅	842		842.3479	842.3479	24.1/98	33.2/98	0.83	0.44	0.75	
10	C ₅₁ H ₅₃ F ₂ N ₆ O ₆	883		883.3989	883.3999	20.2/100	31.5/100	0.78	0.25	0.49	
11	C ₄₉ H ₅₀ F ₂ N ₅ O ₅	826		826.3774	826.3785	20.7/100	31.2/100	0.80	0.43	0.62	

^a (M – TFA + H)⁺, ESI method [Finnigan, Thermoelectron, LCQ classic].

^b (M – TFA + H)⁺, FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF (Bruker Ultraflex III).

^c Completed on a Hewlett Packard 1100 [C-18, Microsorb-MVTM, 4.6 mm × 250 mm, 5 μm]. (A) 10–90 % of acetonitrile with 0.1 % TFA within 30 min and up to 100 % within extra 5 min, 1 mL/min, (B) 25–65 % of acetonitrile having 0.1 % TFA within 40 min and equal to 100 % within additional 5 min, 1 mL/min.

^d (I) CHCl₃/MeOH/AcOH = 90/10/3, (II) EtOAc/BuOH/water/AcOH = 5/3/1/1, (III) BuOH/water/AcOH = 4/1/1.

Table 2

Binding affinities of the opioid/SSRIs bifunctional ligands at the opioid receptors.

no	peptide structure	rMOR ^a , [³ H]DAMGO ^b		hDOR ^a , [³ H]DPPDPe ^c		hKOR ^a , [³ H]U69,593 ^d	
		LogIC ₅₀ ^f g	K _i ^h (nM)	LogIC ₅₀ ^f g	K _i ^h (nM)	LogIC ₅₀ ^f g	K _i ^h (nM)
3	Tyr- <i>D</i> -Ala-Gly-Phe-	-6.70 ± 0.13	88	-6.90 ± 0.03	57	-6.52 ± 0.07	86
4	Tyr- <i>D</i> -Ala-Gly-Phe(4-Cl)-	-7.84 ± 0.10	7.0	-8.47 ± 0.06	1.6	-6.82 ± 0.08	43
5	Tyr- <i>D</i> -Nle-Gly-Phe(4-F)-	-7.46 ± 0.03	16	-8.50 ± 0.05	1.4	-6.61 ± 0.09	68
6	Dmt- <i>D</i> -Ala-Gly-Phe(4-Cl)-	-7.57 ± 0.35	12	-8.09 ± 0.09	4.0	-6.81 ± 0.18	37
7	Dmt- <i>D</i> -Nle-Gly-Phe(4-F)-	-8.65 ± 0.23	1.0	-8.77 ± 0.12	0.8	-7.92 ± 0.11	3.0
8	Dmt- <i>D</i> -Tic-Gly-Phe(4-Cl)-	-7.13 ± 0.09	35	-7.36 ± 0.03	20	-6.49 ± 0.08	91
9	Dmt- <i>D</i> -Tic-Phe(4-Cl)-	-7.69 ± 0.08	10	-8.70 ± 0.06	0.88	-7.34 ± 0.07	13
10	Dmt- <i>D</i> -Tic-Gly-Phe(4-F)-	-7.84 ± 0.05	7.0	-8.70 ± 0.04	0.91	-7.50 ± 0.05	9.0
11	Dmt- <i>D</i> -Tic-Phe(4-F)-	-9.13 ± 0.14	0.4	-7.55 ± 0.06	13	-6.09 ± 0.05	230
	YaGF-NH ₂	-8.24 ± 0.13	2.8	-6.14 ± 0.16	300	-	-
	DALEA	-	7.7	-	2.4	-	-

^a Competition analyses were determined using membrane preparations from transfected HN9,10 cells that constitutively expressed the respective receptor types.

^b K_d = 0.50 ± 0.1 nM.

^c K_d = 0.85 ± 0.2 nM.

^d K_d = 1.5 nM.

^f Logarithmic values analyzed from the nonlinear regression analysis of data collected from as a minimum two independent experiments.

g Competition against radiolabeled ligand.
 h Calculated by the Cheng-Prusoff equation.

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Table 3

Binding affinities of the Opioid/SSRIs bifunctional ligands at various receptors and transporters

No	K_i (μ M) ^{a,b}									
	Adrenergic receptors ^c			Dopamine receptors ^d		Monoamine transporters ^c		Sigma receptor ^c		σ_1
	α_{1A}	α_{2A}	α_{2C}	D ₂	D ₃	NET	SERT	SERT		
	[¹²⁵ I]HEAT	[¹²⁵ I] iodoclonidine	[¹²⁵ I] iodoclonidine	[³ H]N-methylspiperone	[³ H] niosoxetin	[³ H] citalopram	[³ H] pentazocine			
3	6.8	n.d.	n.d.	n.d.	>10	0.75	0.28			0.41
5	6.6	n.d.	n.d.	n.d.	4.3	2.4	n.d.			0.047
6	4.6	4.7	0.24	2.2	0.89	n.d.	n.d.			>10
7	>10	n.d.	0.28	2.7	2.0	n.d.	n.d.			1.9
10	n.d.	n.d.	n.d.	n.d.	6.7	n.d.	5.7			n.d.
11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.4			n.d.

^a Competition analyses were determined using membrane preparations from transiently or stably transfected cell lines.

^b Standards used for different receptors: chlorpromazine (K_i = 44 nM) for α_{1A} ; oxymetazoline (K_i = 2.0 nM) for α_{2C} ; prazosin (K_i = 50 nM) for α_{2C} ; diazepam (K_i = 1.2 nM) for Rat Brain Site BZP; haloperidol (K_i = 4.5 nM) for D₂; chlorpromazine (K_i = 3.2 nM) for D₃; desipramine (K_i = 12 nM) for NET; amitriptyline (K_i = 19 nM) for SERT; haloperidol (K_i = 7.0 nM) for Sigma I

^c Tris-HCl assay buffers.

^d HEPES-HCl assay buffers.

n.d.: not determined due to a lower (<50%) inhibition in an initial screen at 10 μ M.

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Table 4

Functional Assay results for the Opioid/SSRIs bifunctional ligands at serotonin receptors (5-HT₂).

no	5-HT _{2A} (%) ^{a,b}		5-HT _{2B} ^{a,b}		5-HT _{2C} ^{a,b}	
	agonist ^c	antagonist ^d	agonist ^c	antagonist ^d	agonist ^c	antagonist ^d
6	-0.1	20	0.10	-6.1	-0.3	6.0
7	-0.1	23	0.10	4.7	-0.3	11
10	34	94	0.20	49	2.8	5.2
11	0.7	99	0.20	43	0.2	10

^aFunctional assays were performed on transiently or stably transfected cell lines using Hanks balanced salt solution.

^b5HT was used as a standard for 5HT₂ receptors; EC50 = 250 nM for 5HT_{2A} receptor; EC50 = 1.6 nM for 5H₂B receptor; EC50 = 1.7 nM for 5HT_{2C} receptor.

^cNormalized and transformed to percentage values. The reference agonist activity at 10 μM is set as 100% and basal activity with buffer as 0%.

^dThe basal activity with buffer only is set as 100% inhibition and the activity of the EC80 of the reference agonist as 0% inhibition.

Table 5Results of top scoring complex for each molecule with μ -opioid activity.

No. of Compds.	G_{bind}^a (kcal/mol)	K_i^b (nM)	Amino acids showing hydrogen bond contacts	Distance ^c (Å)
3	-4.80	88	Asp ¹⁴⁷	1.44
			Gly ³²⁵	1.75
			Lys ³⁰³	2.93
4	-4.29	7.0	Glu ²²⁹	1.35
			Asp ¹⁴⁷	1.47
			Lys ²⁰⁹	2.62
5	-7.85	16	Arg ²¹¹	3.36
			Asp ¹⁴⁷	1.67
6	-6.01	12	Gln ¹²⁴	2.08
			Gln ¹²⁴	2.58
			Tyr ¹²⁸	2.58
			Asp ¹⁴⁷	1.74
			Arg ²¹¹	2.40
7	-12.14	1.0	Asp ¹⁴⁷	1.55
8	-6.26	35	Arg ²¹¹	2.66
			Thr ²¹⁸	2.80
9	-9.34	10	Asp ¹⁴⁷	1.60
			Thr ²¹⁸	3.27
10	-10.65	7.0	Tyr ¹²⁸	2.67
			Ile ²¹⁵	1.69
			Thr ²¹⁸	2.47
11	-12.41	0.4	Asn ¹²⁷	2.45
			Asn ¹²⁷	2.74
			Asp ²¹⁶	1.78

^aEstimated MM/GBVI binding free energy(Kcal/mol) calculated as “S score” by MOE.^bPercent inhibition of radiolabeled ligand binding at 10 μ M concentration of test ligands.^cHydrogen bond lengths calculated from docked pose by using *Ligand interaction* tool of MOE.