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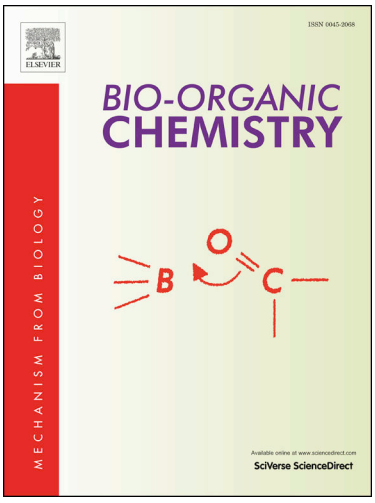

Review article

Study of G protein-coupled receptors dimerization: from bivalent ligands to drug-like small molecules

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Study of G protein-coupled receptors dimerization: from bivalent ligands to drug-like small molecules

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Abstract

In the past decades an increasing number of studies revealed that G protein-coupled receptors (GPCRs) are capable of forming dimers or even higher-ordered oligomers, which may modulate receptor function and act as potential drug targets. In this review, we briefly summarized the design strategy of bivalent GPCR ligands and mainly focused on how to use them to study and/or detect GPCP dimerization *in vitro* and *in vivo*. Bivalent ligands show specific properties relative to their corresponding monomeric ligands because they are able to bind to GPCR homodimers or heterodimers simultaneously. For example, bivalent ligands with optimal length of spacers often exhibited higher binding affinities for dimers compared to that of monomers. Furthermore, bivalent ligands displayed specific signal transduction compared to monovalent ligands. Finally, we give our perspective on targeting GPCR dimers from traditional bivalent ligands to more drug-like small molecules.

Keywords

G protein-coupled receptors

GPCR dimerization

Bivalent ligands

Drug-like small molecules

1. Introduction

G protein-coupled receptors (GPCRs) consist of the largest seven transmembrane receptor family. About one third clinically used drugs on the market act directly or indirectly on GPCRs.[1] Furthermore, almost half of the small molecules being studied preclinically or clinically are targeting GPCRs.

In classical pharmacology allosteric mechanisms were only discussed in terms of intramolecular interactions within a receptor between orthosteric and allosteric sites. Nowadays, there is mounting evidence that also intermolecular receptor-receptor interactions may result in altered receptor recognition, pharmacology and signaling. Bivalent ligands[2,3,4] have been proven useful molecular probes for confirming and targeting dimeric receptors, such as the κ opioid receptor (κOR)-δOR heteromers, μ opioid receptor (μOR)-δOR heteromers, β_2-adrenoceptor (β_2AR)-M_2 muscarinic acetylcholine receptor (M_2R) heteromers and so on.[**Error! Bookmark not defined.**,**Error! Bookmark not defined.**,**Error! Bookmark not defined.**] Bivalent ligands are valuable tools to demonstrate the existence of receptor dimers even in native tissue and can be used to study a specific GPCR dimer behavior without any receptor modification.[5,6,7] Such compounds may also evolve to useful pharmacological agents.[**Error! Bookmark not defined.**]

Bivalent ligands with a spacer of optimal length are anticipated to exhibit a potency that is greater than that derived from the sum of its two monovalent pharmacophores and may allow the targeting of certain dimeric subtypes, thereby increasing the selectivity of drug action.[**Error! Bookmark not defined.**,8,9] Such synergy is based on the assumption that a bivalent ligand will first undergo univalent binding, followed by binding of the second pharmacophore to a recognition site on a neighboring receptor (Figure 1). When the bivalent ligand is in the univalently bound state, the pathway to bivalent binding should be favored over univalent binding of a second ligand because of the small containment volume of the tethered, unbound pharmacophore that is in the region of the unoccupied neighboring receptor site. The situation may be different in case the neighboring receptors are negatively allosterically coupled. In that case the binding enhancement may be mitigated.

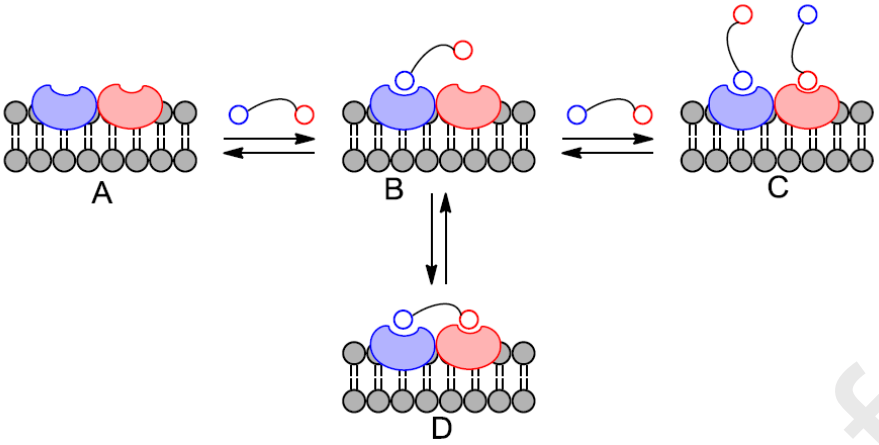


Figure 1. Bivalent ligand concept for bridging a receptor dimer.

The unoccupied dimer (A) undergoes univalent binding that leads to state B. The unoccupied site in B can be "bridged" to give D, which is entropically favored over binding of a second ligand to give the dimer with both sites occupied (C).

2. Concept and design of bivalent GPCR ligands

A typical bivalent GPCR ligand consists of three different components including two pharmacophores, two linking groups and a spacer of optimal length and nature (Figure 2). Specifically, the two pharmacophores are based on GPCR ligands, which could be the same (homo-bivalent ligands) or different (hetero-bivalent ligands). The selected GPCR ligands should be functionalized with a group, e.g. amine, carboxylic acid, alkyne or azide, to allow swift conjugation to the spacer. In addition, the spacer should also be equipped with appropriate ligation handles to couple with GPCR ligands.

Figure 2. General Structure of Bivalent GPCR Ligands

Several recent reviews have revealed how to design the bivalent ligands.[10·11] In brief, pharmacophore identification, linker attachment site, length and composition of linker are significant for construction of bivalent ligands. The selection of the position and nature of the attachment points for linking the two pharmacophore units to the spacer depends on two criteria: the feasibility of the chemical modification and the compatibility of these modifications with receptor binding and intrinsic activity of the pharmacophore.

Preferred reactive groups for connecting two pharmacophoric units and spacers are hydroxyl, amine, and carboxylic groups,[12·13] but also alkynes or azides may be employed to generate 1,4-substituted 1,2,3-triazole linking groups via a copper-catalyzed azide–alkyne cycloaddition (CuAAC) reaction.[14·15·16] The use of azides and terminal alkynes requires additional reaction steps, as these

functional groups are usually not present in most pharmacophores. These functional moieties may be connected to the spacer via ether, amide and triazole moieties. In some studies, ester, amine or thioether functions are also described.

Normally, pharmacophores possess more than one functional group and the spacer has to be attached ideally without significantly disrupting the binding affinity or functionality of the parent compounds. Therefore, the most suitable spacer attachment position is preferentially chosen based on structure-activity relationship (SAR) data.

There are general no rules governing the optimal spacer distances to construct bivalent ligands for various dimeric GPCRs. However, several criteria on spacer length should be carefully considered to determine the optimal length of the connecting entity in a bivalent ligand: the binding mode of the chosen pharmacophore, the respective relative position of the two linker attachment points and the interface between the two protomers.[**Error! Bookmark not defined.**Error! Bookmark not defined.,17] Overall, in the absence of structural information of the targeted dimer, the optimal length of the spacer in a bivalent ligand may vary and needs to be determined empirically for each new pair of target receptors.

In addition to spacer length, both lipophilicity and rigidity of the spacer are important factors when designing a bivalent ligand. Incorporation of piperazine into the spacer group can improve the hydrophilicity of the bivalent ligand, but is slightly less flexible than a polyalkyl chain and PEG spacer, but slightly more than polyamide chains. Here we will not go further for design of bivalent ligands. We will mainly focus on the recent applications of bivalent ligands.

3. Bivalent ligands possessing some specific properties

3.1 Bivalent ligands enhance selectivity and binding affinity

Andersen et al. has designed and synthesized a series of bivalent serotonin ligands (Figure 3) to reveal insight into substrate recognition in the neurotransmitter serotonin (SERT).[18] An optimized bivalent serotonin transporter ligand consisting of a poly(ethylene glycol) spacer binds SERT with >3,700-fold increased binding affinity compared to that of serotonin, indicating that the human serotonin transporter has two distinct substrate binding sites.

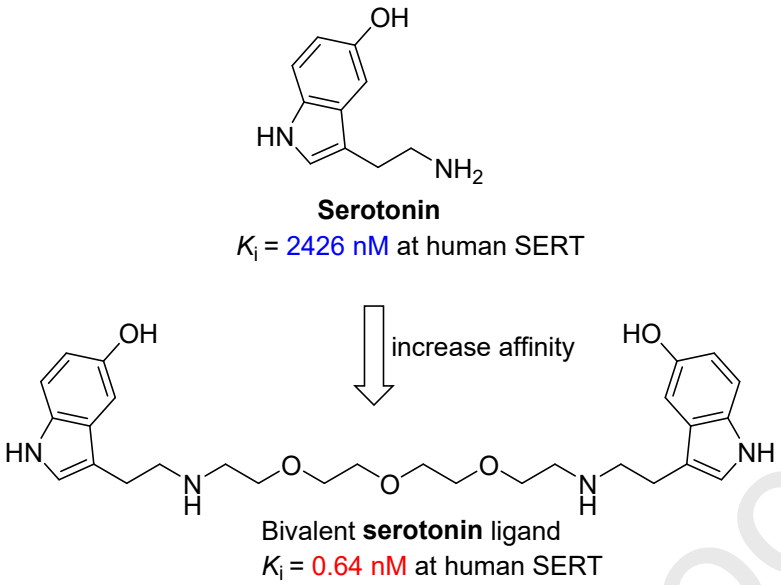


Figure 3. Structure of bivalent serotonin ligand

She et al. synthesized a series of homo- and hetero-bivalent ligands targeting muscarinic acetylcholine receptor (M_1R−M_5R).[19] The heterodimeric ligand UR-SK75 (**2**, Figure 4) containing a MR antagonist (dibenzodiazepinone derivative) and a M_1R/M_4R agonist (xanomeline) revealed a selective and higher binding affinity at M_2R (20-480 folds increase) compared to the other four subtypes. Interestingly, bivalent ligand **2** showed a higher receptor subtype selectivity than compound **1** at M_2R.

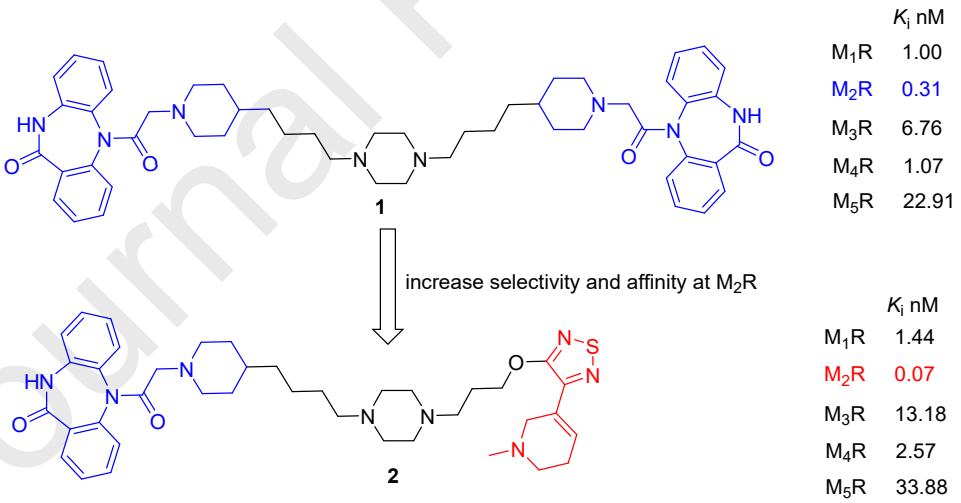


Figure 4. Structure of bivalent muscarinic acetylcholine receptor ligand

To increase the affinity and selectivity of histamine H_2 receptor (H_2R), Pockes et al.[20] prepared bivalent H_2R alkylguanidine ligand **5** (Figure 5) that exhibits significantly higher hH_2R affinity (K_i: 0.047 μM) in binding studies compared to hH_1R, hH_3R, and hH_4R (Table 1). This hH_2R selectivity was much more pronounced than for the monomeric compound **4**. Furthermore, bivalent ligand **5** showed a

higher functional activity (EC_{50}: 0.0042 μM) at guinea pig H_2R (atrium) compared to **3** (EC_{50}: 6.92 μM) and **4** (EC_{50}: 0.27 μM), indicating the potent H_2R agonism.

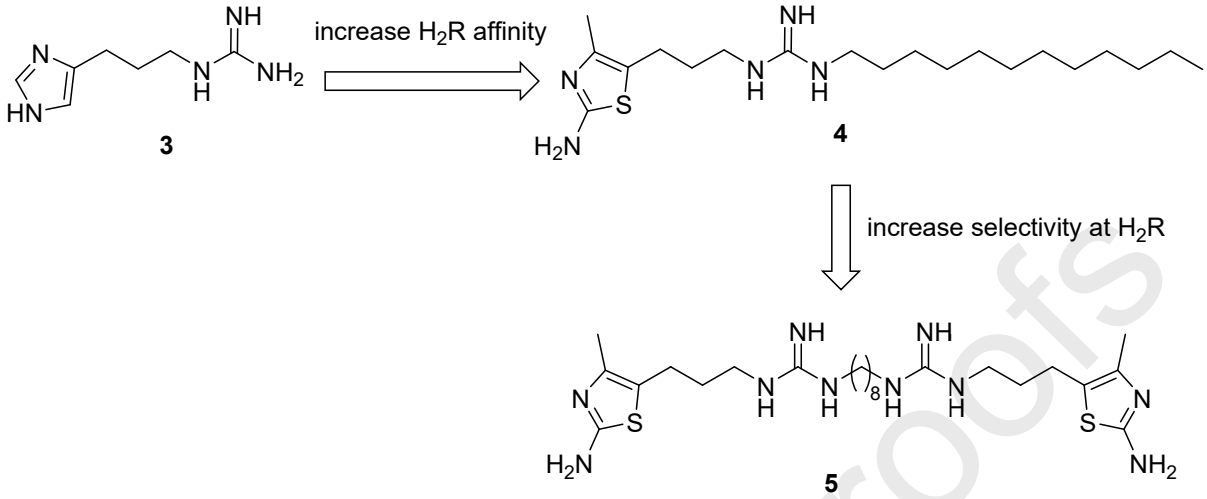


Figure 5. Structure of bivalent histamine H_2 receptor ligand

Table 1. Binding affinities at the human $H_{1,2,3,4}R$ and agonistic activities at the *gp*H_2R (atrium)

Compd	K_i (μM) for *h*H_1R	K_i (μM) for *h*H_2R	K_i (μM) for *h*H_3R	K_i (μM) for *h*H_4R	EC_{50} (μM) at *gp*H_2R (atrium)
3	>100	4.07	0.038	0.0074	6.92
4	>3.3	0.47	2.04	5.62	0.27
5	>3.3	0.047	5.62	10	0.0042

Pulido et al. designed bivalent dopamine D_2 receptor ligand **8** based on *N*-(*p*-aminophenethyl)spiperone **6**, with a high affinity (K_i = 21 pM) for the D_2R homodimer.[21] Bivalent ligand **8** showed a 37-fold lower K_i value compared to its monomeric ligand **7**, suggesting simultaneously binding at both orthosteric binding sites of the D_2R homodimer. This simultaneous interaction with both orthosteric sites of the D_2R homodimer is further confirmed by binding experiments of compound **8** in the presence of TAT-TM6, a TM6 mimetic peptide, which shows an increase in K_d value similar to that of the monovalent ligand. Moreover, they confirmed that transmembrane 6 (TM6) forms the interface of the D_2R homodimer.

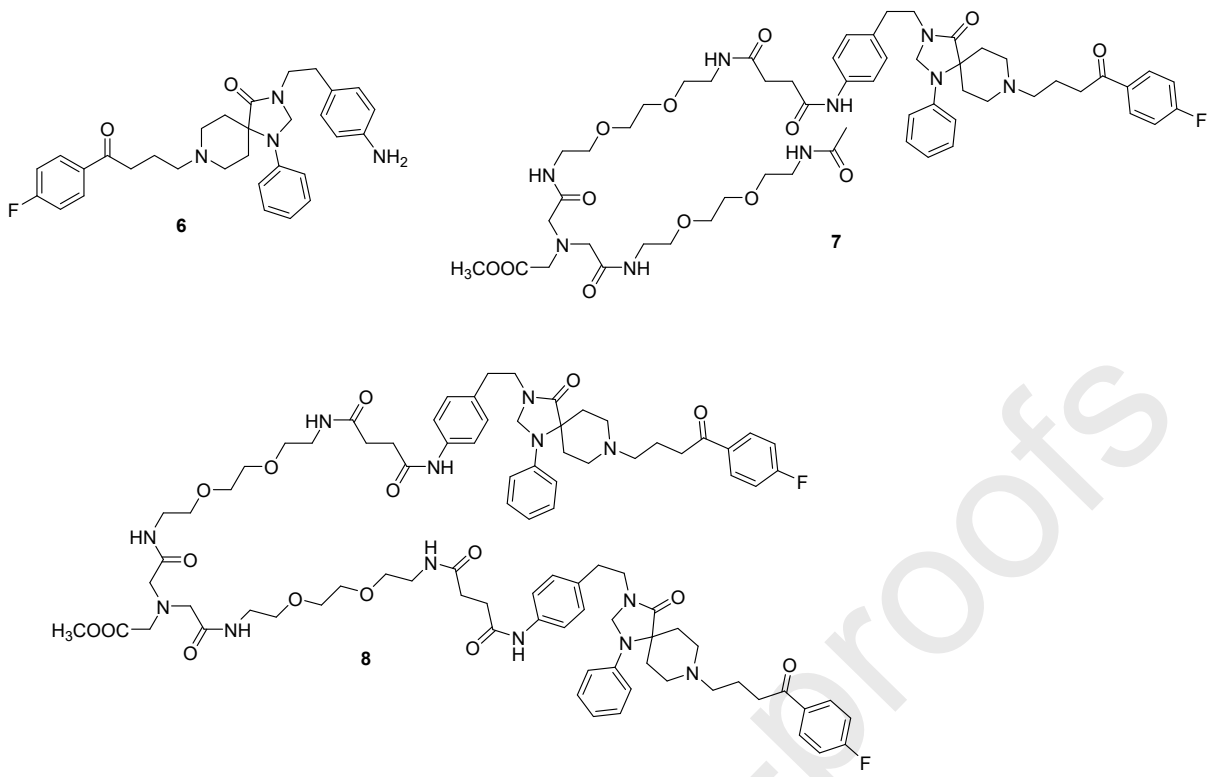


Figure 6. Structure of bivalent dopamine D_2 receptor ligand

3.2 Bivalent ligands display positive cooperativity-high Hill slope

Cross-talk between GPCRs forming a dimer can evoke positive cooperativity, which also can be induced by bivalent ligand owing to the thermodynamic advantage of sequential ligand binding. This effect was observed in the study of bivalent ligands of dopamine D_2 receptor (D_2R) based on 1,4-disubstituted aromatic piperazine (1,4-DAP). Competitive binding curves of monomeric antagonists such as the phenylpiperazine **9[**22**]** (Figure 7) usually show Hill slopes close to 1 (n_H = 0.9~1.2), indicating that monovalent ligand binds to one receptor protomer. Binding assays of the bivalent D_2R antagonist **10a[**Error! Bookmark not defined.**]** displayed remarkably increased Hill slopes of 2.0, suggesting a bivalent binding mode with a simultaneous occupancy of two neighboring binding sites. On the other hand, bivalent ligand **10b** with a 22-atom PEG spacer had a lower Hill slope close to 1.0, while it showed higher binding affinity compared to its monomeric ligand, which possibly indicated this bivalent ligand could bridge the binding sites of the D_2R homodimers.

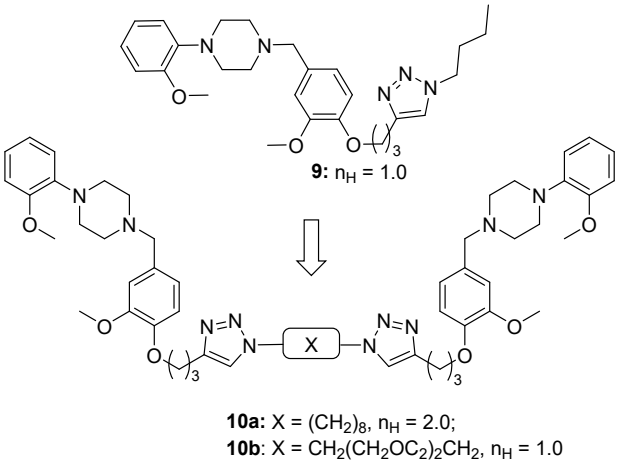


Figure 7. Structures of bivalent ligand **9** and monovalent ligand **10a-b**.

Interestingly, bivalent agonists also exhibited steeper binding curves as compared to their corresponding monovalent ligands. In contrast to antagonists, displacement experiments between monomeric agonists and radiolabeled antagonists reveal shallow curves with Hill slopes of 0.5~0.7. While binding studies of bivalent D_2R agonists revealed significantly increased Hill slopes of 1.3~1.4, indicating that bivalent ligands possibily bridge the dimer. [Error! Bookmark not defined.],[23,24] Therefore, careful analysis of Hill coefficients constitutes a valuable approach to determine a bivalent binding mode.

A following study on bivalent D_2R agonists (aminoindane derivative, Figure 8) by the same group of Gmeiner revealed that bivalent ligand **10c** containing the same spacer as **10a** displayed a steeper binding curve (n_H=1.3) relative to the corresponding monovalent ligand (n_H=0.5), indicating a bivalent binding mode. Nevertheless, the high Hill slope of this bivalent ligand did not affect the functional activity since both the bivalent ligand **10c** and the monomeric ligand showed potent D_2R agonism in inhibiting cAMP formation and inducing D_2R-mediated internalization.[Error! Bookmark not defined.]

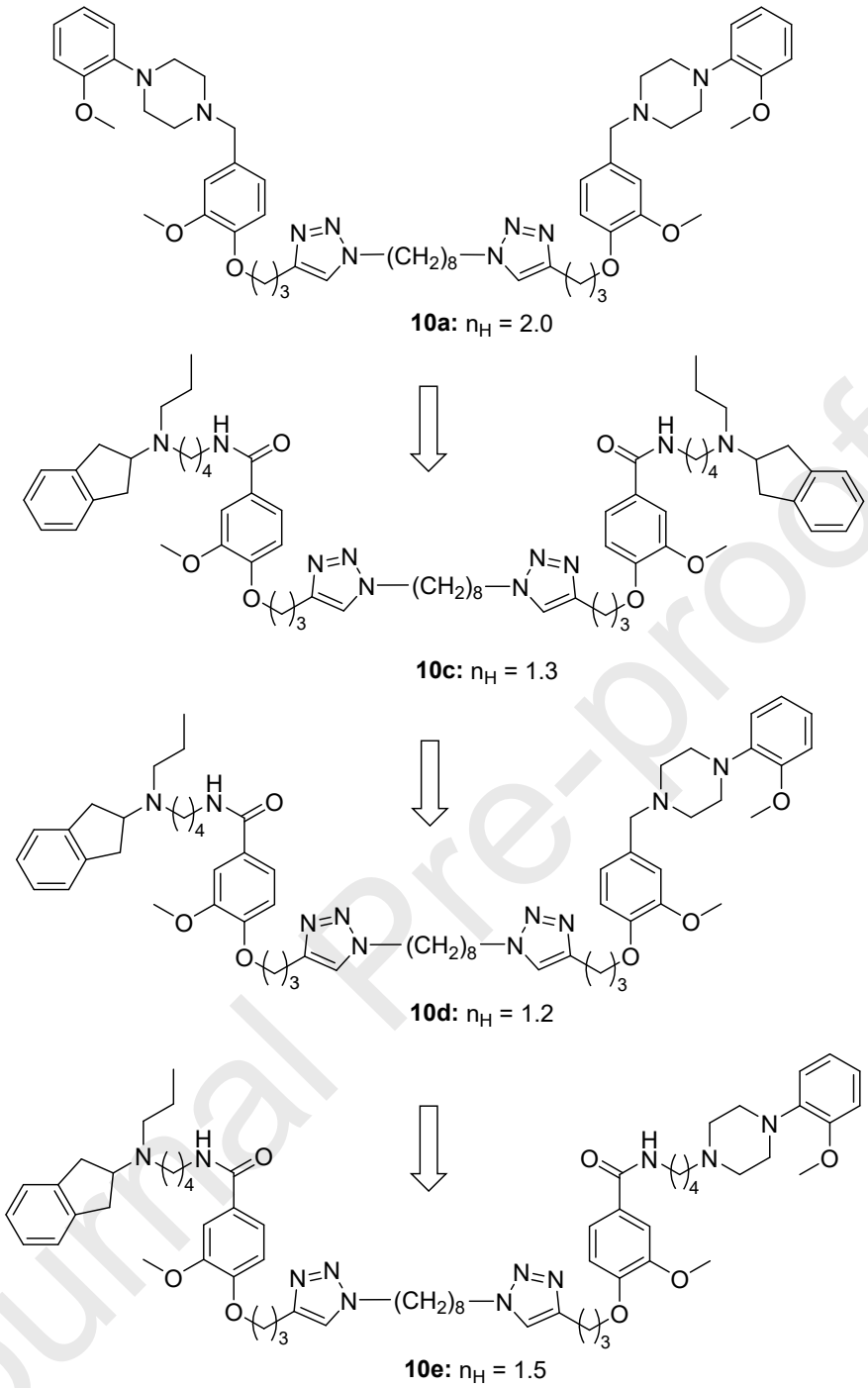


Figure 8. Structure of bivalent D_2R ligands.[Error! Bookmark not defined.]

Meanwhile, the heterobivalent ligands **10d** and **10e** containing a D_2R agonist (aminoindane pharmacophore) and a D_2R antagonist (phenylpiperazine pharmacophore) were also assessed the ability to inhibit cAMP accumulation and induce D_2R internalization.[Error! Bookmark not defined.] The results of cAMP assay revealed that compound **10d** showed weak partial D_2R agonist activity (Emax =13%), whereas **10e** exhibited D_2R antagonism. In addition, neither of the bivalent ligands could activate D_2R-mediated internalization. Therefore, these two heterobivalent ligands did not show the D_2R biased agonisms (cAMP versus internalization).

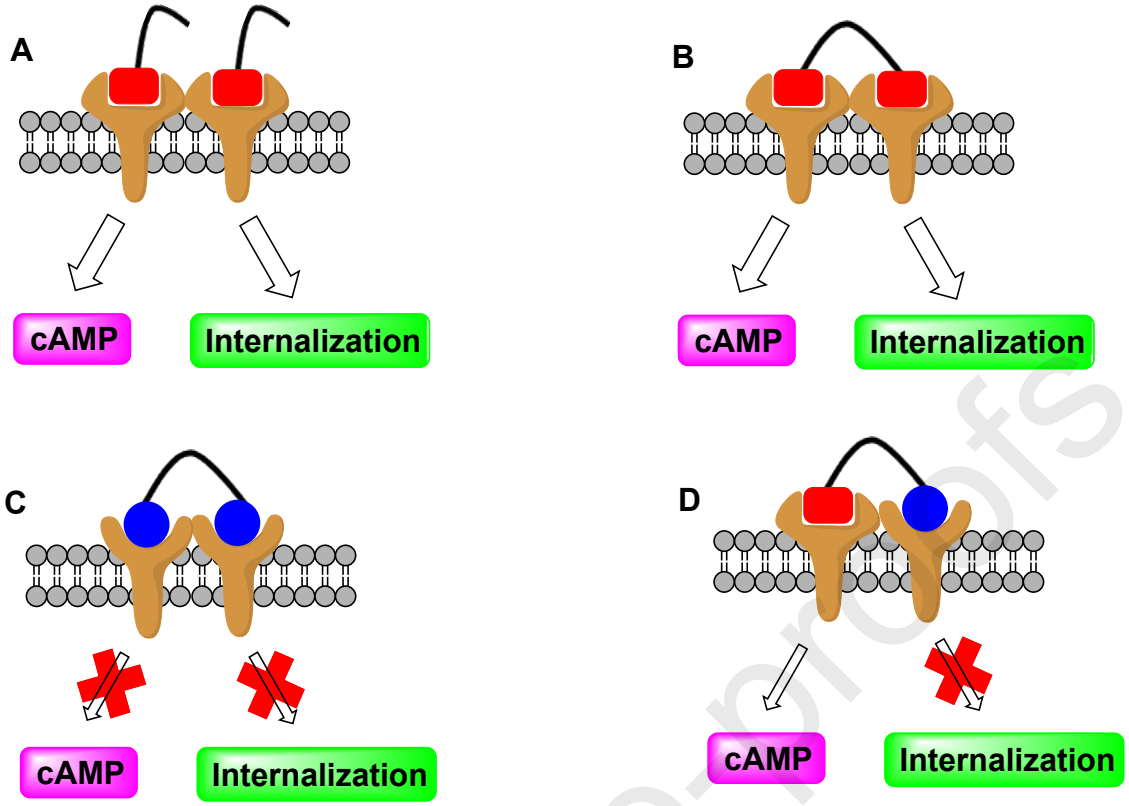


Figure 9. Effect of bivalent D_2R ligands. A cartoon illustrating the effect of bivalent ligands binding to the D_2R dimer on D_2R-mediated cAMP formation and internalization. Red square and blue circle represent D_2R agonist and antagonist pharmacophores, respectively.[Error! Bookmark not defined.] The white arrow is thicker if the signaling is more activated or thinner if it is less activated.

Four cartoons further clarified the interaction of bivalent ligands with signal transduction of D_2R homodimer (Figure 9). Monovalent D_2R agonists binding to the D_2R homodimer could inhibit cAMP accumulation and activate the D_2R-mediated internalization (Figure 9A). Similarly, bivalent D_2R agonists binding could also stimulate the above mentioned signaling (Figure 9B). While bivalent D_2R antagonists binding blocked both cAMP formation and internalization (Figure 9C). Interestingly, heterobivalent ligands comprising a D_2R agonist and a D_2R antagonist binding to the homodimer could lead to a very low efficacy of cAMP (Emax ≤15%), indicating partial D_2R agonism, and inhibit D_2R internalization (Figure 9D).

3.3 Bivalent ligands reveal biphasic binding curve

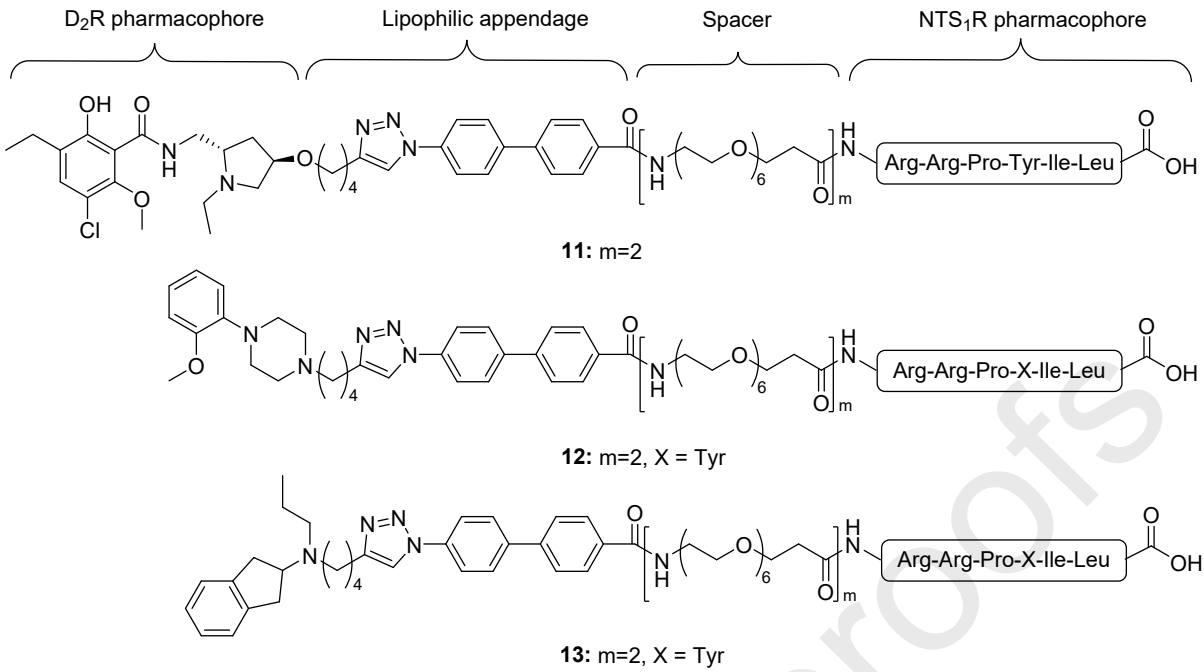


Figure 10. Bivalent ligands (**11-13**) for D_2R and NTS_1R.

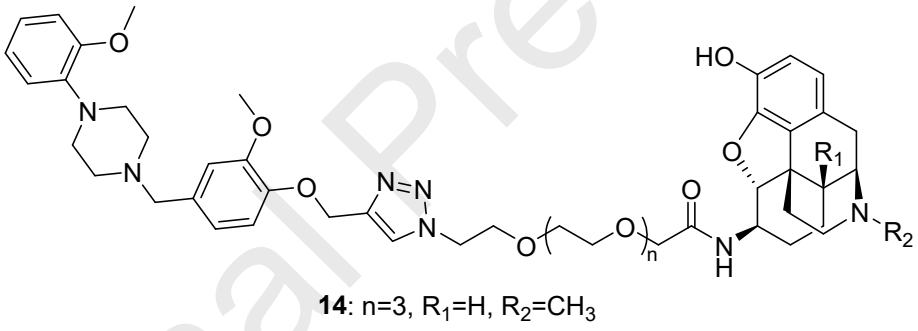
A recent study on bivalent D_2R agonist/antagonist and neurotensin NTS_1 receptor (NTS_1R) agonist ligands (Figure 10) afforded biphasic competition-binding curves when measuring [^{3}H]spiperone displacement.[25] Such biphasic-binding curves (two K_i values) were specifically observed with spacer lengths of 44 (*m*=2), 66 (*m*=3) or 88 (*m*=4) atoms at HEK 293T cell membranes coexpressing D_2R/NTS_1R. The $K_{i\ high}$ values correspond to a bivalent receptor-bridging binding mode to D_2R/NTS_1R heterodimers, while the $K_{i\ low}$ values reveal a monovalent-binding mode to D_2R.

Similar binding assays were performed in the presence of an excess of NTS_1R agonist NT(8-13) (1 µM), which could prevent a biphasic binding mode of the bivalent compounds by hampering binding of their NT(8-13) pharmacophore to the NTS_1R. Indeed, co-incubation prevented high-affinity binding, resulting in typical sigmoidal monophasic curves.[**Error! Bookmark not defined.**]

To confirm the bivalent receptor-bridging binding mode, reciprocal competition experiments were performed with the NTS_1R radioligand [^{3}H]neurotensin. [**Error! Bookmark not defined.**] Employing **13**, a biphasic-binding curve was observed with a $K_{i\ high}$ value of 0.11 pM and a $K_{i\ low}$ at 1.7 nM, which was shifted to a monophasic sigmoidal binding curve in the presence of 1 µM haloperidol (K_i 0.79 nM). Incubation with this monovalent D_2R antagonist thus efficiently prevented the bivalent-binding mode. Affinities for this competition-enforced monovalent-binding mode were found to be in good agreement with results obtained with membranes from CHO-cells stably expressing NTS_1R only (K_i 0.86 nM). To further complement the results obtained with overexpressing heterologous cell lines with results from native brain tissue, competition binding assays with [^{3}H]spiperone and the bivalent ligand **13** were performed with membranes from porcine striatum. Interestingly, compound **13** displayed a biphasic

binding curve with a 140-fold preference for the high-affinity binding site over the low-affinity receptor population ($K_{i\ high}$ 2.8 nM, $K_{i\ low}$ 310 nM, high-affinity fraction 38%). In line with the results from heterologous cell lines, addition of 1 μM NT(8-13) reverted this biphasic binding curve to a sigmoidal binding isotherm with a K_i value of 28 nM. The superior binding of bivalent over monovalent ligands to D_2R/NTS_1R heterodimers was thus not only evident in heterologous cell lines but also in native tissue, though less pronounced, which might be explained by lower receptor expression levels leading to a lower propensity to form D_2R/NTS_1R heterodimers.

Recently, we have successfully designed and synthesized a series of novel heterobivalent ligands (Figure 11) for dopamine D_2-like receptors (D_2-likeR) and the μ-opioid receptor to study the protein-protein interactions.[26] Interestingly, we observed a potent bivalent D_2-likeR/μOR ligand **14** in ligand binding assay, which showed the above mentioned biphasic binding curve in the D_4R/μOR coexpressing cells, suggesting bridging both binding sites of two protomers. Nevertheless, this compound exhibited a one-site binding curve in the cells coexpressing D_2R/μOR, probably indicating that this compound failed to bridge the D_2R/μOR heterodimer. Hence, compound **14** could be used as a pharmacological tool to further study D_4R/μOR dimerization.



14: n=3, R_1=H, R_2=CH_3

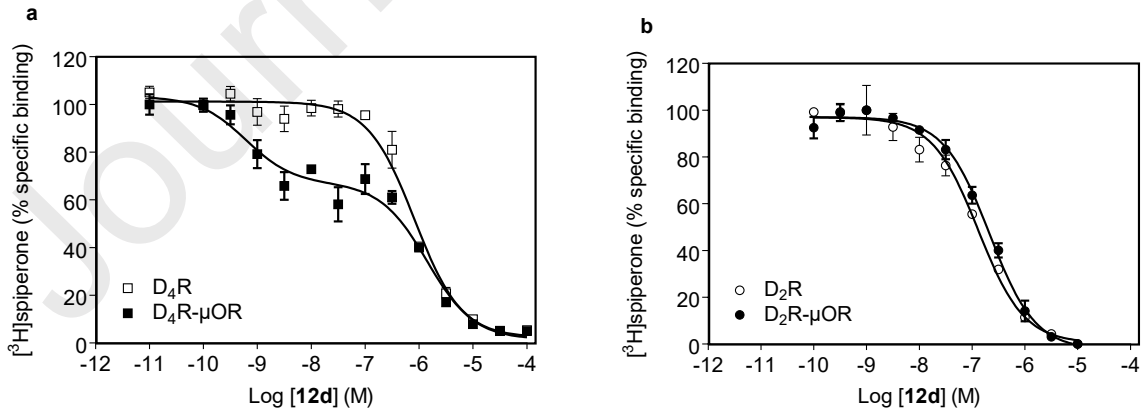


Figure 11. Representative competition curves for bivalent ligand **14** (**12d**). D_2-likeR binding of the bivalent ligand **14** was measured by displacement of [^{3}H]spiperone from membranes of HEK293T cells coexpressing D_4R/μOR (or D_2R/μOR) or monoexpressing D_4R (or D_2R) only. (**a**) D_4R binding of **14** in

the presence (filled squares) or absence (open squares) of μOR. (**b**) D_2R binding of **14** in the presence (filled circles) or absence (open circles) of μOR.

Busnelli et al. reported bivalent ligands **15a-b** comprising two identical oxytocin-mimetics (Figure 12) that induced a three-order magnitude boost in G-protein signaling of oxytocin receptors (OTRs) *in vitro* and a 100- and 40-fold gain in potency *in vivo* in the social behavior of mice and zebrafish.[27]

Through receptor mutagenesis and interference experiments with synthetic peptides mimicking transmembrane helices (TMH), they show that such superpotent behavior follows from the binding of the bivalent ligands to dimeric receptors based on a TMH1-TMH2 interface.[**Error! Bookmark not defined.**]

Furthermore, in this arrangement, only the analogues with a well-defined spacer length (~25 Å) precisely fit inside a channel-like passage between the two protomers of the dimer. The newly discovered oxytocin bivalent ligands represent a powerful tool for targeting dimeric OTR in neurodevelopmental and psychiatric disorders and, in general, provide a framework to untangle specific arrangements of GPCR dimers.

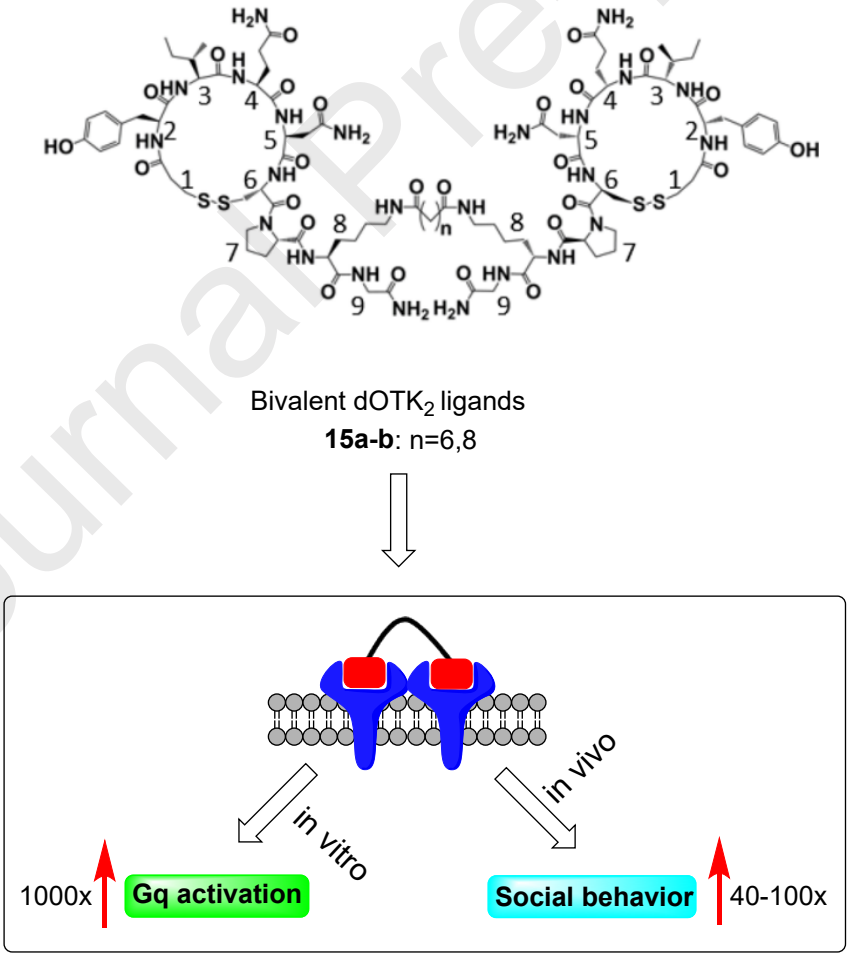
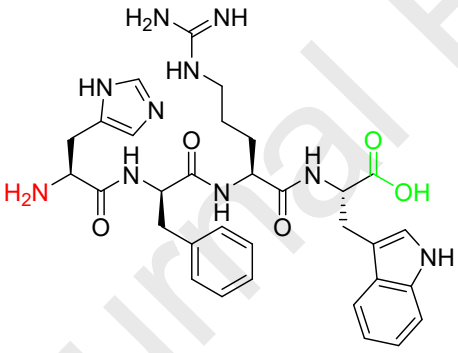


Figure 12. Structure of bivalent oxytocin receptor ligands

3.4 Bivalent ligands show biased agonism

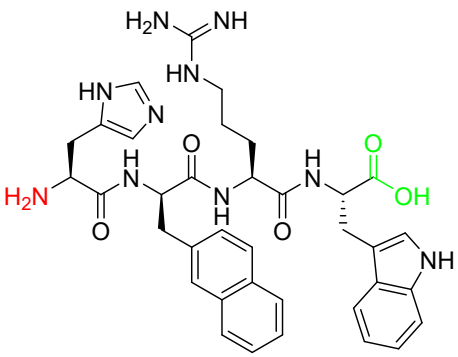
Lensing et al. initiated a study on design and synthesis of homobivalent ligands targeting melanocortin receptor homodimers (Figure 13).[28] The results showed that bivalent melanocortin ligands increased binding affinity and potency of cAMP signaling pathway relative to their monovalent compounds in HEK293 cells stably expressing the mMC1R, mMC3R, mMC4R, and mMC5R. In addition, bivalent ligands with various types of spacers displayed preferences for specific melanocortin receptor subtypes, indicating structural differences between the different dimer subtypes. Interestingly, one of the potent bivalent ligand (Ac-His-DPhe-Arg-Trp-(PEDG20)-His-DPhe-Arg-Trp-NH_2) dramatically decreased *in vivo* food intake in mice after intracerebroventricular administration.

Very recently, Lensing et al. elegantly showed that bivalent ligands may induce biased signaling. They constructed a so-called biased unmatched bivalent ligand (BUmBL) targeting melanocortin receptor homodimers.[29] UmBLs also consist of an agonist (His-DPhe-Arg-Trp) and an antagonist (His-DNal(2')-Arg-Trp) pharmacophore connected via various spacers. A selected BUmBL of the human melanocortin-4 receptor (hMC4R) exhibited biased agonism in HEK293 cells. It potently activated cAMP production (EC_{50} = 1.9−5.9 nM), but minimally stimulated β-arrestin recruitment (⩽ 55% maximum signal at 10 μM). From a medicinal chemistry perspective, this example demonstrates that targeting receptor dimers may become an attractive approach to induce biased signaling.



His-DPhe-Arg-Trp

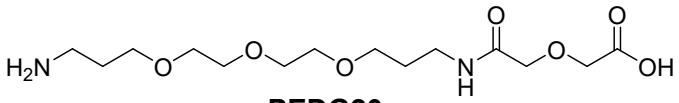
MCR agonist



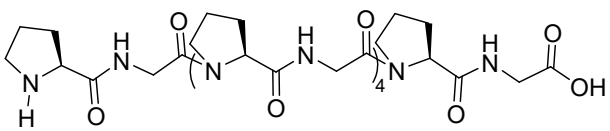
His-DNal(2')-Arg-Trp

MCR antagonist

Linkers



PEDG20



$(Pro\text{-}Gly)_6$

Figure 13. MCR agonist and antagonist pharmacophores and selected linkers were used to construct the bivalent MCR ligands. Red amine and green carboxylic acid represent the attachment points with the linkers.

A cartoon below (Figure 14) illustrated the interaction of ligands with asymmetrically signaling melanocortin homodimers.[Error! Bookmark not defined.] Monomeric agonists (blue circle) could bind both receptors and activate cAMP signal transduction pathway as well as the β-arrestin recruitment (Figure 14A). Homobivalent agonists (blue circle coupled with black spacer) induce similar signaling (Figure 14B). BUmBLs comprising an agonist (blue circle) and an antagonist (red circle) are prone to lead to biased signaling by agonizing the cAMP signaling pathway and antagonizing β-arrestin recruitment upon bound to the asymmetrically signaling homodimer (Figure 14C).

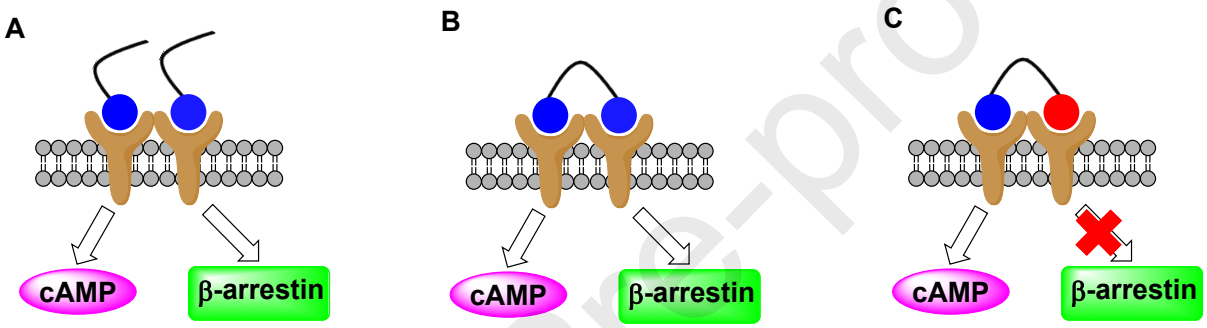


Figure 14. A cartoon illustrating the interaction of ligands with asymmetrically signaling melanocortin homodimers.[Error! Bookmark not defined.]

3.5 Bivalent binding can block internalization

Daniels et al.[30] first synthesized a series of bivalent μOR agonist/δOR antagonist ligands (Figure 15) containing different spacer lengths (16-21 atoms), which were evaluated by intracerebroventricular (i.c.v.) administration in the tail-flick test in mice. Compound **19b** with a 20-atom spacer was able to produce potent antinociception without tolerance, physical dependence, or place preference due to bridging of the μOR-δOR heterodimer, while **19a** and the corresponding monovalent opioid agonist **16**, did show the above-mentioned side effects.

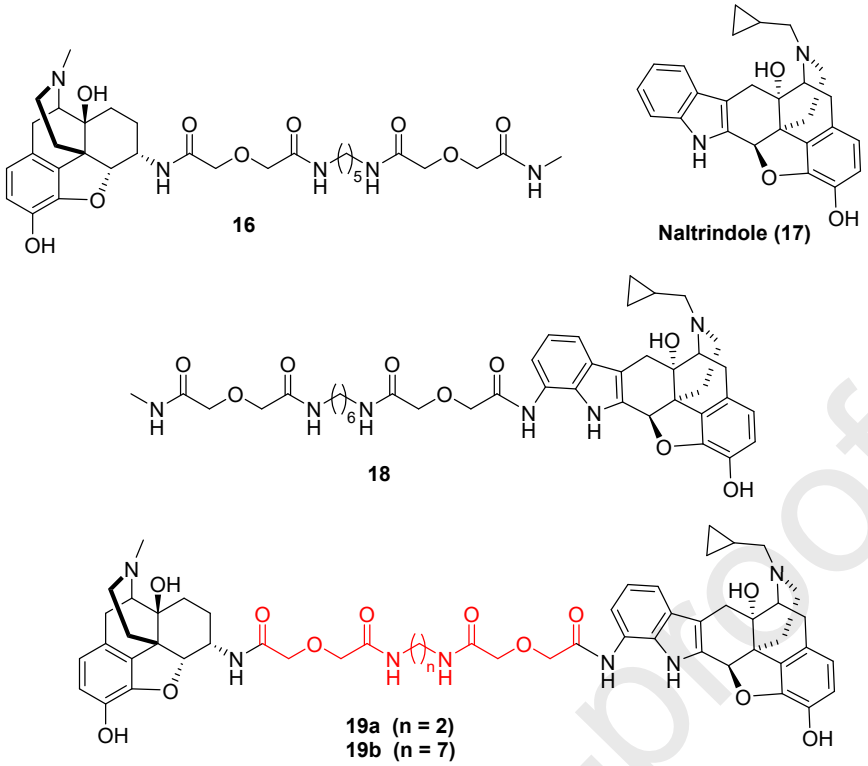


Figure 15. Structures of bivalent ligands (**19a** and **19b**), monovalent ligands (**16** and **18**), and naltrindole (**17**).

In a follow-up study by Yekkirala et al.,[31] an immunocytochemical correlation was employed to support the in vivo biological results of **19b**. They showed that bivalent ligand **19b**, which was free of tolerance due to possible bridging of μOR and δOR protomers, inhibited endocytosis of the heteromeric receptors in HEK-293 cells (Figure 16c), while **19a** and monomeric μOR agonist **16** produced robust internalization (Figure 16a and 16b). Furthermore, due to competition at δOR protomer by **17**, **19b** promotes endocytosis similar to that of **16** and **19a** (Figure 16d). These data strongly indicate that immobilization of proximal μOR and δOR protomers is due to bridging by **19b**. Overall, **19b** and its shorter spacer homologue **19a** possess comparable activity in HEK-293 cells, but generate dramatically different internalization of μOR-δOR heterodimer. Hence, the internalization assay represents a valuable approach for evaluation of agonist−antagonist bivalent ligands bridging GPCR heterodimers.

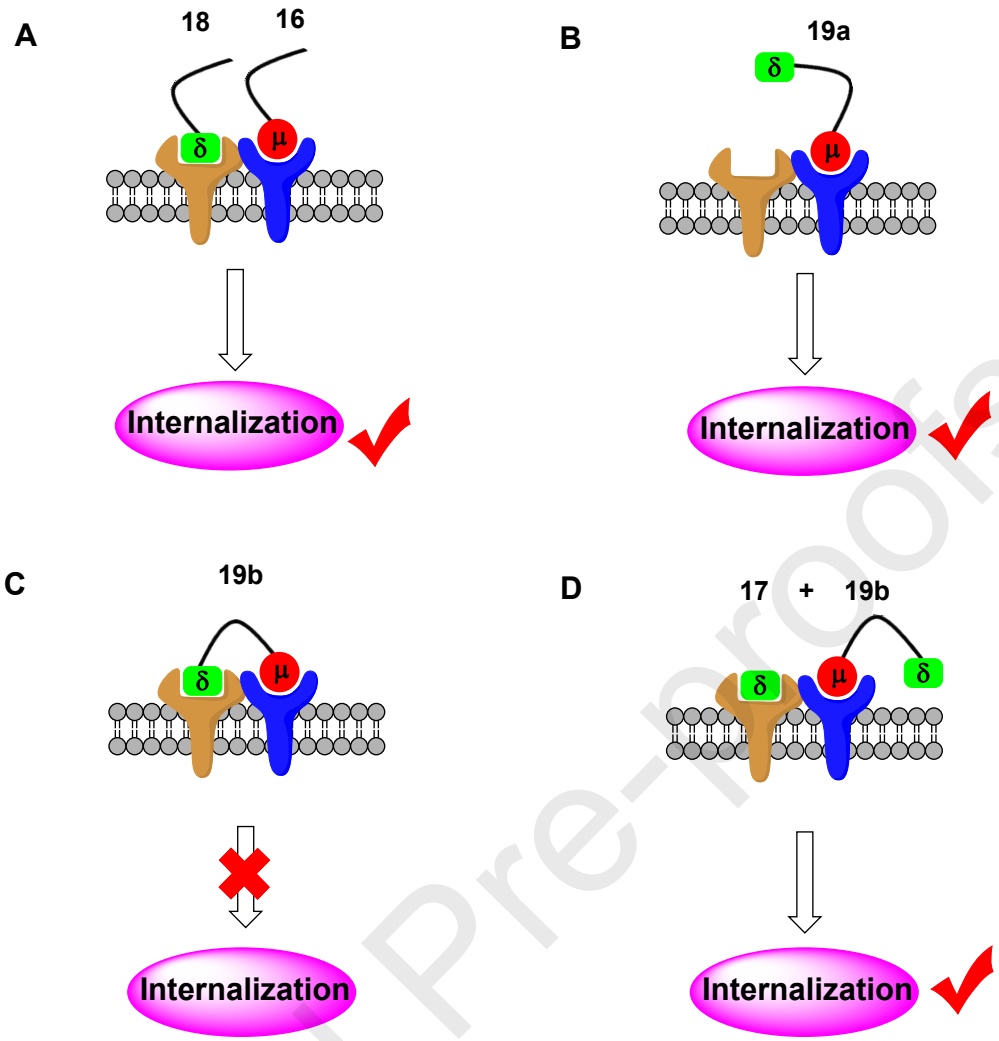


Figure 16. Effect of **17** on trafficking of μ-δ heterodimer by **19b**. A cartoon illustrating the effect of delta antagonist on the disruption of bridging protomers in the μ-δ heterodimer.[Error! Bookmark not defined.]

Lacking μOR/δOR heterodimer-selective antagonists is the major limitation in the study of μOR/δOR dimerization. Hence, Olson et al. synthesized a series of varying length (15−41 atoms) bivalent peptides with selective but moderate/low-affinity pharmacophores for the μOR (**21**) and δOR (**20**).[32] The pharmacological results showed a spacer length dependent μOR/δOR dimer affinity/potency profile *in vitro* ^{35}S-GTPγS coupling (Figure 17A), with the 24-atom spacer length (**D24M**) generating the highest affinity/potency (<1 nM, Table 2) at the μOR/δOR dimer and selectivity (≥89-fold relative to the μOR or δOR monomer). In addition, **D24M** displayed ≥200-fold higher potency than the monomeric compounds at the μOR/δOR heterodimer.

Subsequently, **D24M** was further evaluated in the tail flick test in mice via *icv* injection, which exhibited a dose-dependently antagonized antinociception formed by the μOR/δOR agonists CYM51010 and Deltorphin-II, without antagonizing the monomer agonists DAMGO and DSLET (Figure 17B).[Error!

Bookmark not defined.] Interestingly, **D24M** was also observed to sharply reduce withdrawal behavior in models of acute and chronic morphine dependence.

These data strongly suggest that **D24M** is a first-in-class selective and high affinity/potency μOR/δOR heterodimer antagonist both *in vitro* and *in vivo*.

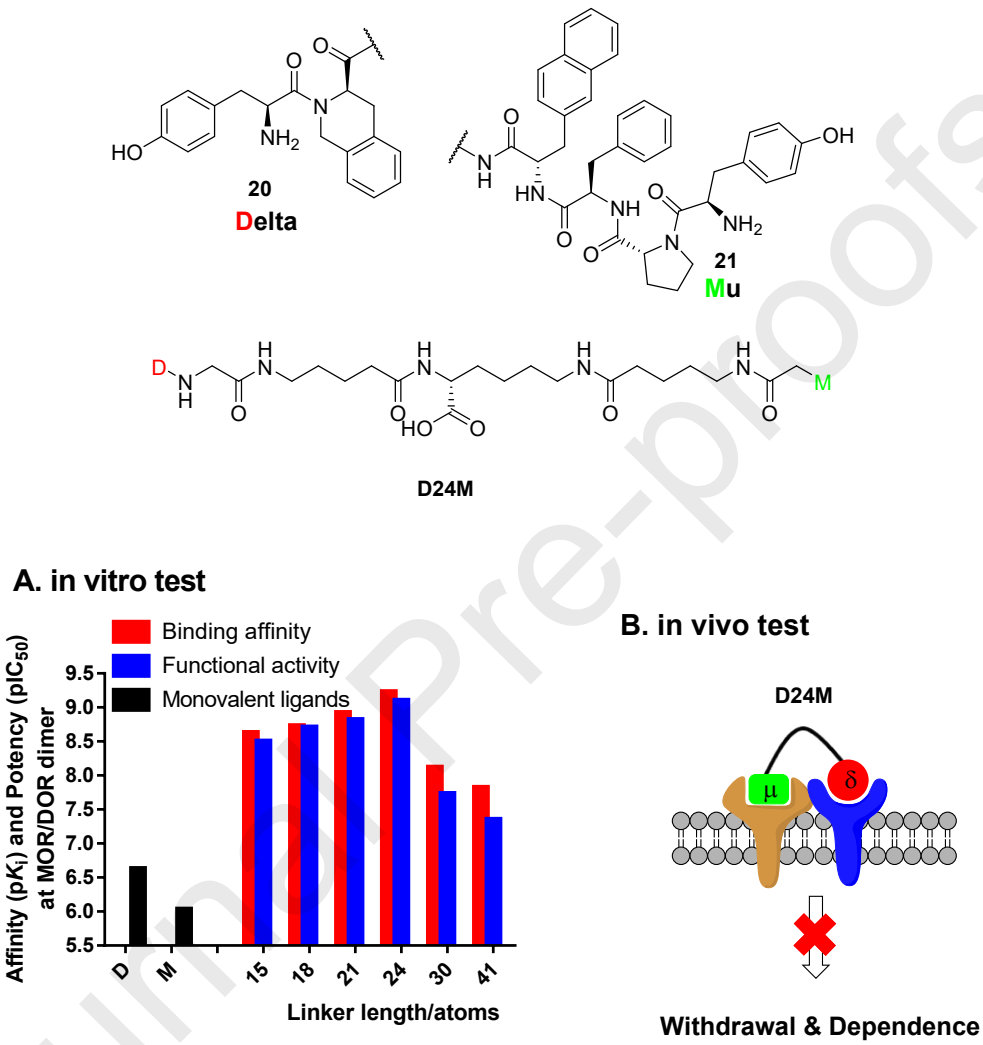


Figure 17. Structures of bivalent μOR/δOR antagonists

Table 2. Binding affinities and functional activities for μOR/δOR

Compd	pK_i for μOR/δOR (K_i nM)	pIC_{50} GTPγS antagonism vs CYM51010 (IC_{50} nM)
D15M	8.60 (2.5)	8.47 (3.4)
D18M	8.70 (2.0)	8.68 (2.1)
D21M	8.89 (1.3)	8.79 (1.6)

D24M	9.20 (0.63)	9.07 (0.85)
D30M	8.09 (8.1)	7.70 (20)
D41M	7.79 (16)	7.32 (48)

Le Naour et al.[33] designed and synthesized bivalent ligands that contain both μOR agonist and CB1 antagonist pharmacophores as tools to study the functional interaction between μOR and CB1 receptors in vivo. This study was based on above mentioned study that demonstrated a lack of internalization of coexpressing μOR and δOR receptors in HEK-293 cells upon treatment with a bivalent μOR agonist/ δOR antagonist ligand **19b**.

The obtained immunofluorescence evidence in HEK293 cells coexpressing μOR and CB1 receptors supported the bridging of protomers in the μOR-CB1 heterodimer by the bivalent ligand **23** (Figure 18) with a 19-atom spacer, as no significant receptor internalization was observed.[Error! Bookmark not defined.] Nevertheless, bivalent ligands with shorter spacer length (≤ 18 atoms) did not share this property. Compound **23** exhibited the highest potency in antinociceptive testing in mice. As neither a combination of monovalent ligands **16** + **22** nor the bivalent ligand **23** produced tolerance in mice, μOR-CB1 apparently is not a significant target for reducing tolerance.

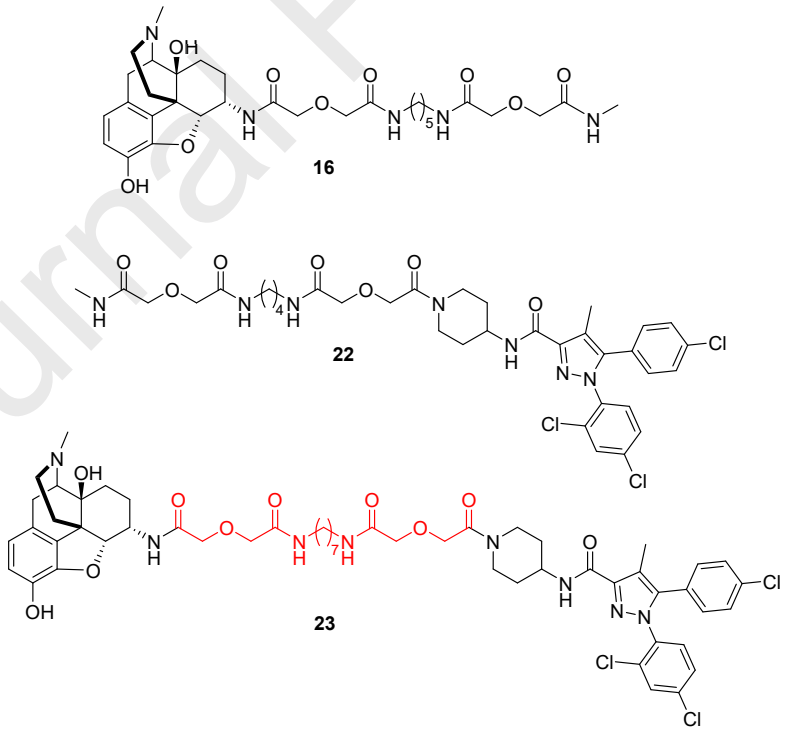


Figure 18. Heterobivalent μOR agonist/CB1R antagonist ligands. Compound **23** with a spacer length of 20 atoms and showing the highest anti-nociceptive potency in a mouse tail-flick assay.[Error! Bookmark not defined.]

3.6 Bivalent ligands boost potency of antinociception *in vivo* without tolerance

Akgün et al.[34] designed and synthesized a series of bivalent ligands that contain a μOR agonist and a mGluR5 antagonist pharmacophore (MPEP) linked through spacers of varying length (10–24 atoms) to study the μOR/mGluR5 receptor interaction *in vivo*. Bivalent ligands were evaluated for antinociception using the tail-flick and von Frey assays in mice pretreated with lipopolysaccharide (LPS) or in mice with bone cancer. In LPS-pretreated mice, **24** with a 21-atom spacer (Figure 19) exhibited the highest potency (intrathecal ED_{50} ~9 fmol per mouse), whereas its ED_{50} value was more than three orders of magnitude higher in untreated mice.

Bivalent ligands with shorter or longer spacers than **24** showed at least a 25-fold higher ED_{50} in LPS-treated mice.[Error! Bookmark not defined.] In addition, a combination treatment of the monomeric μOR agonist and mGluR5 antagonist did not exhibited an increased potency of antinociception. The exceptional potency of **24** may be owning to the optimal bridging of protomers in a putative μOR-mGluR5 heterodimer. Bivalent ligand **24** holds a $>10^6$ therapeutic ratio, which indicates that it may be a promising candidate for treatment of chronic, intractable pain via spinal administration. Furthermore, bivalent ligand **24** was proved to be effective in treating neuropathic pain according to a recent study on spared nerve injury.[35]

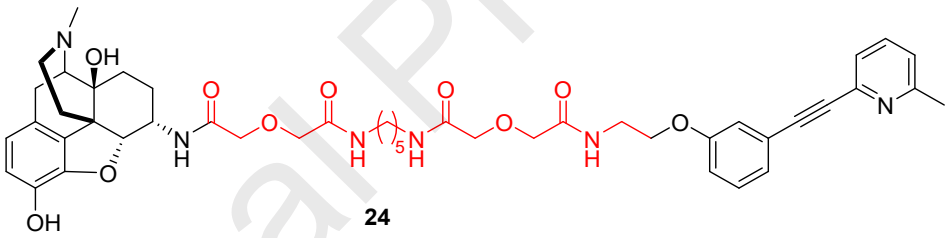


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Figure 19. Bivalent μOR and mGluR5 ligand.[Error! Bookmark not defined.]

Cross-talk between opioid and chemokine (CCR) receptors is initiated by chemokine release which, to some extent, leads to reduced potency of morphine in the treatment of chronic pain. Based on the probability that a μOR-CCR5 heteromer is involved in such cross-talk, Akgün et al.[36] synthesized a series of bivalent ligands that consist of a μOR agonist and a CCR5 antagonist (TAK220) pharmacophores linked through homologous spacers (14−24 atoms).

When tested on the lipopolysaccharide (LPS) inflamed mice, the bivalent ligand **25** with a 22-atom spacer (Figure 20) displayed excellent antinociceptive activity (i.t. ED50 = 0.0146 pmol/mouse) that was 2000-fold higher than morphine. Furthermore, **25** was approximately 3500-fold more potent than a combination treatment of the corresponding μOR agonist and CCR5 antagonist. These results clearly suggest that **25** bridges the protomers of a μOR-CCR5 heterodimer, which was supported by docking studies, and that the μOR-CCR5 heterodimer could act as a novel target for the treatment of chronic pain.

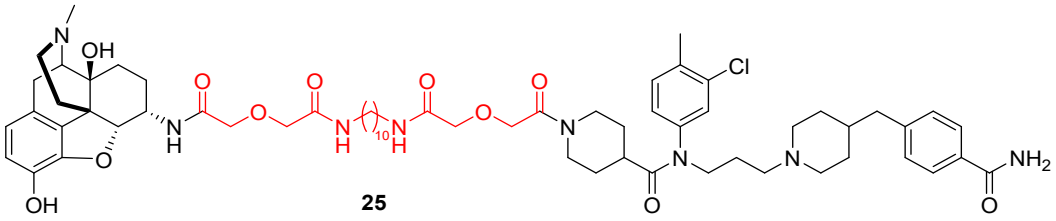


Figure 20. Bivalent ligand **25** bridging μOR and CCR5 receptors.[Error! Bookmark not defined.]

Based on the above studies on opioid receptor, a main difference between the bivalent ligands and clinically used opioid ligands is their remarkable potency in both reducing hyperalgesia and strengthening antinociception without tolerance in inflamed mice. To further identify the targets of bivalent ligands **24** and **25**, Akgün et al. has undertaken a study upon i.t. administration recently.[37] The results showed that the high potency of **24** and **25** in LPS inflamed mice is partially owing to their contributions to directly blocking the activated spinal glia. Specifically, compound **24** displayed the potently enhanced antinociception due principally to selective inhibition of activated astrocytes, while **25** antinociception was owing mainly to blockage of induced spinal microglia.[Error! Bookmark not defined.]

Recently, Zhang's group[38] reported bivalent ligands containing μOR agonist and chemokine receptor CXCR4 antagonist pharmacophores targeting the μOR-CXCR4 heterodimers that produced the largest decrease in the mean number of IP acid-stimulated stretches in ICR mice to test their antinociceptive effectiveness. However, the p value for this effect ($p = 0.11$) did not meet the criterion for statistical significance, further evaluation of these compounds may be warranted.

3.7 Bivalent ligands exhibited higher binding affinity and potency in the cells stably coexpressing both receptors.

Recently, we have synthesized a series of potential bivalent ligands comprising a D_2R agonist and an mGluR5 negative allosteric modulator (NAM) to study the interaction of D_2R and mGluR5.[39] Interestingly, bivalent ligand **26** (Figure 21), with a 20-atom alkylamine spacer, exhibited a 4-fold affinity increase for the D_2R in cells coexpressing the D_2R and the mGluR5, compared to cells only expressing D_2R, suggesting that **26** may bridge the binding sites of the D_2R and mGluR5 receptors. Moreover, **26** shows a 5-fold higher affinity for the mGluR5 than its MTEP azido precursor in D_2R-mGluR5 cells, also pointing towards its capacity to simultaneously occupy both binding sites of the heteromer. From a functional point of view, **26** displays a 7-fold higher potency compared to the monomeric mGluR5 precursor in MAPK phosphorylation in HEK293 cells stably expressing D_2R and mGluR5 receptors. Furthermore, **26** inhibits forskolin stimulated cAMP formation with a 4-fold higher potency compared to its D_2R monovalent alkyne in the coexpressing cells, indicating that the bivalent binding requires a lower receptor occupation to exert signaling. In addition, a docking study reveals that **26** is indeed able to simultaneously bind both receptors by passing between the heterodimeric interface,

comprised of TM5 and TM6 of both receptors, and establishing six protein-ligand H-bonds (Figure 21).[Error! Bookmark not defined.]

Overall, we demonstrated that the length and nature of the spacer between the two receptor pharmacophores strongly affect binding affinity and functional potency both from a D_2R and from an mGluR5 viewpoint. Most importantly, this study allowed us to identify bivalent ligand **26**, which emerges as a promising molecular probe to further investigate D_2R and mGluR5 heterodimerization.

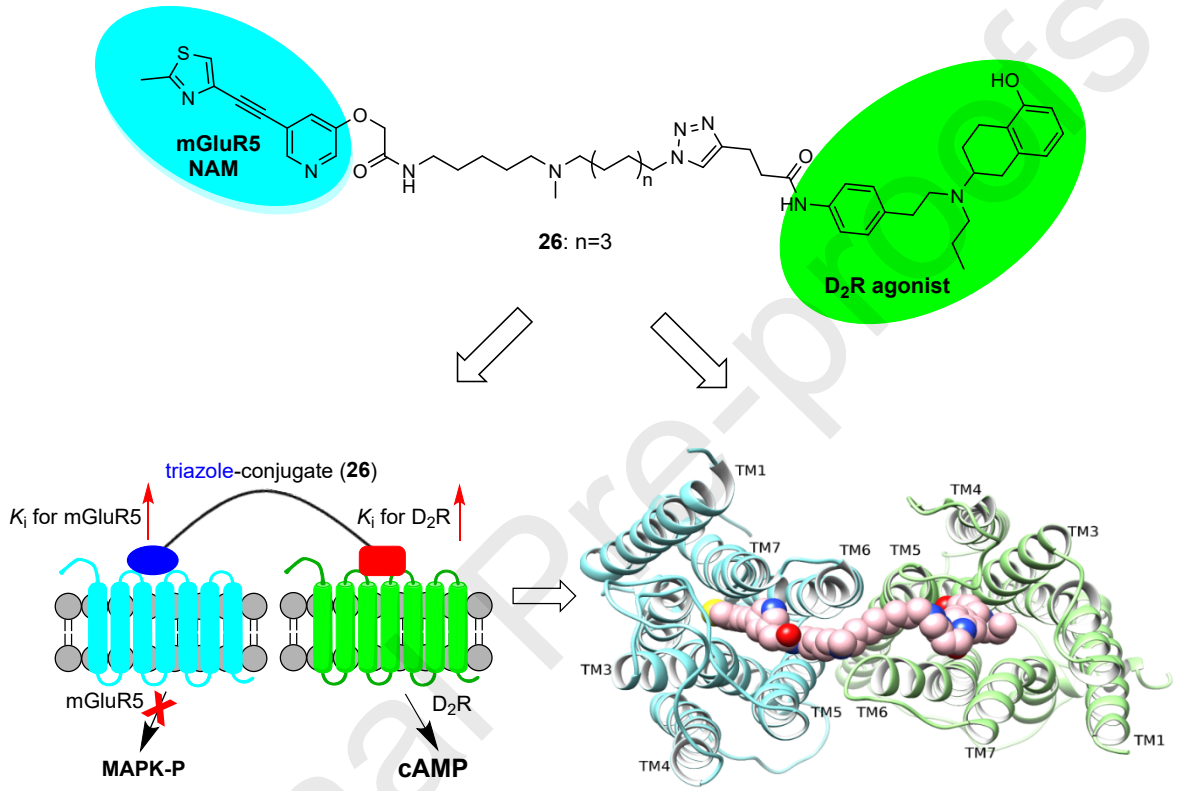


Figure 21. Bivalent ligand **26** targeting D_2R and mGluR5.

In 2020, Poulie et al. designed and synthesized the first series of bivalent ligands **27a-g** targeting the 5-HT_{2A}/$mGlu_2$ heteromer, which comprise the 5-HT_{2A} antagonist MDL-100,907 and the $mGlu_2$ positive allosteric modulator (PAM) JNJ-42491293 (Figure 22).[40] Noticeable functional crosstalk was detected between the two receptors in cells coexpressing 5-HT_{2A}/$mGlu_2$ and 5-HT_{2A}/$mGlu_2$/Gqo5. Whereas the monomeric compounds retained the 5-HT_{2A} antagonist and $mGlu_2$ PAM functional activities, bivalent ligands inhibited 5-HT-induced Ca^{2+} responses in 5-HT_{2A}/$mGlu_2$ cells and both 5-HT- and Glu-induced Ca^{2+} responses in 5-HT_{2A}/$mGlu_2$/Gqo5 cells. Nevertheless, no conclusive correlation between the functional potency and spacer length of the bivalent ligands was observed. In summary, although functional crosstalk between 5-HT_{2A} and $mGlu_2$ was confirmed, it remains unclear how bivalent ligands interact with the 5-HT_{2A}/$mGlu_2$ heteromer.

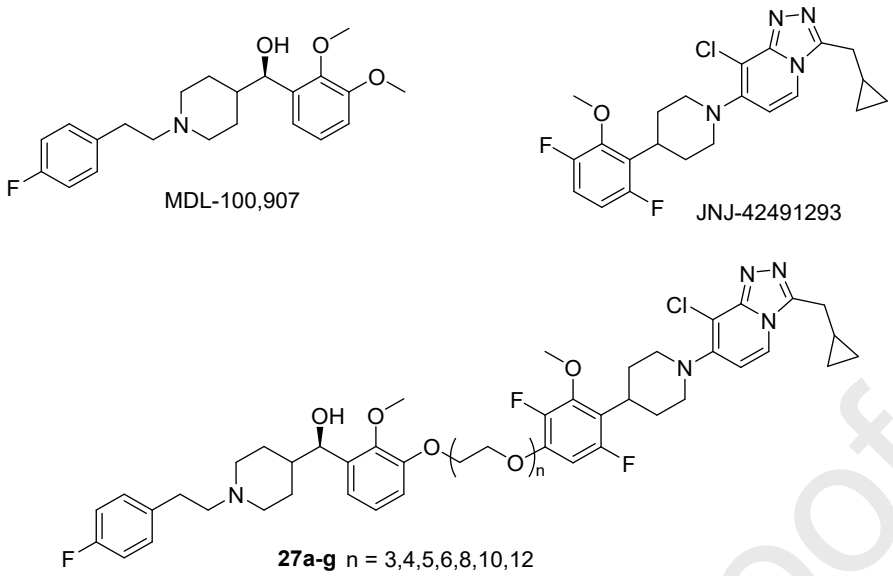
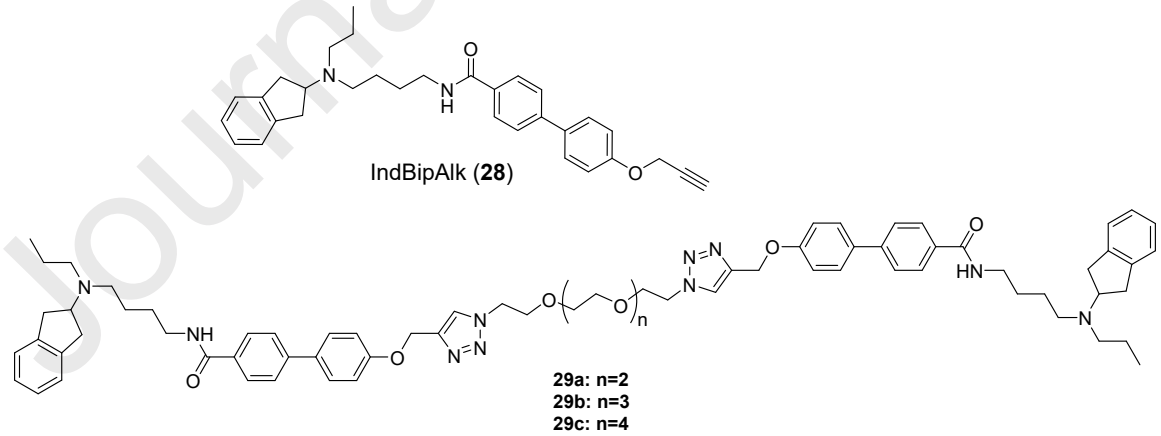


Figure 22. Bivalent ligand **27a-g** for 5-HT_{2A} and $mGlu_2$ receptors.

In 2021, we discovered a true bivalent D_2R agonist **29c** that showed a 16-fold higher binding affinity than the parent monovalent IndBipAlk **28** (Figure 23).[41] Furthermore, compound **29c** displayed a 4-fold higher binding affinity than the shorter bivalent compounds **29a-b** (PEG4 and PEG5), suggesting that the spacer length affects bivalent binding. Compound **29c** (n_H = 1.2) showed a 2-fold steeper binding curve than its monomeric ligand IndBipAlk **28** (n_H = 0.6), suggesting a bivalent binding mode. Bivalent ligand **29c** showed a 5-fold higher potency (EC_{50}=65 nM vs 321 nM for **28**), indicating that it needs a lower concentration to exert signaling for bivalent binding. Molecular docking study reveals that bivalent ligands **29a-c** are able to simultaneously bind the D_2R homodimer in a more relaxed conformation through the interface of TM5-TM6 when increasing the length of linkers (Figure 23).



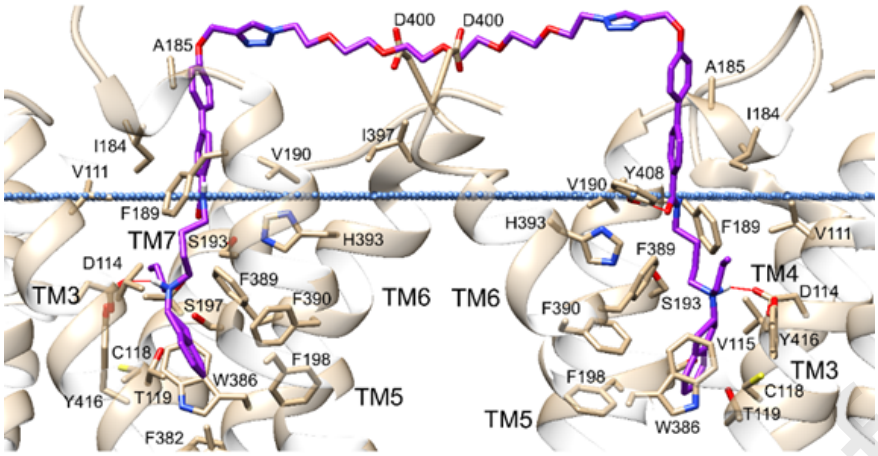


Figure 23. Bivalent ligands **29a-c** for D_2R homodimers and docking pose of **29c** (purple) in the D_2R homodimer. Small blue spheres represent predicted extracellular membrane surface. H-bonds are displayed as solid red lines.

Recently, Zhang's group designed a series of bivalent ligands targeting μOR-CCR5 heteromers[42] and μOR-CXCR4 heteromers[43], which exhibited higher binding affinity and potency in the cells coexpressing both receptors. In addition, both μOR-CCR5 and μOR-CXCR4 heterodimers showed the therapeutic potential to inhibit opioid exacerbated HIV-1 infectivity.

4. Conclusion

Bivalent ligands are defined as compounds that contain two pharmacophores linked by an appropriate spacer. The design of such molecules requires the selection of potent and subtype-selective lead pharmacophores, appropriate attachment points to connect the spacer, and the length and composition of the spacer group. This overview mainly focused on bivalent ligands recently described in the literature as specific pharmacological tools to investigate GPCR dimerization.

The advantages of the bivalent approach are numerous, including enhanced binding affinity, functional potency and receptor subtype specificity. However, the use of bivalent ligands as potential pharmacotherapeutics is limited by problematic molecular properties, such as high molecular weight and lipophilicity. In addition, the linker must be attached to the pharmacophore in a position that tolerates structural modification.

All the examples mentioned above strongly suggest that the length and nature of the linker are crucial factors for the optimal ligand-receptor interactions, which depend on the properties of the selected pharmacophores and binding sites of GPCRs. Furthermore, design of biased blockage of GPCR-mediated internalization or β arrestin recruitment is a promising approach for the bivalent ligands containing an agonist and an antagonist (or NAM) to study the GPCR dimerization. More importantly, bivalent μOR ligands revealed fantastic potency of antinociception without tolerance compared to the monomeric ligands *in vivo*.

Overall, these compounds possess great potential as pharmacological tools to investigate the GPCR dimerization both *in vitro* and *in vivo*.

5. Future perspectives

5.1 Disadvantages of bivalent ligands

The potential of bivalent ligands as possible therapeutics, as opposed to mere pharmacological tools, remains a topic of discussion.[44] Some obvious hurdles exist in the development of bivalent ligands as drugs, including potential absorption, distribution, metabolism, and excretion (ADME) issues, their unlikeliness to cross the blood–brain barrier, and other issues. These issues are related to some general unfavorable physicochemical properties, particularly their high molecular weight, which does not fit Lipinski's rule of 5 and hence limits the future use of bivalent ligands as potential drugs. On the other hand, Portoghese et al. reveals that heteromer induction is a possibility with bivalent ligands, which offers a general approach to unique pharmacology that complements traditional SAR.[45] Although these barriers seem insurmountable, bivalent ligands may potentially become useful therapeutics by alternative/advanced administration routes, or by converting them into integrated dual acting ligands. However, dual acting ligands not only cannot bridge the two binding sites of the GPCRs dimers simultaneously, but also do not allow to distinguish between a given GPCR heteromer and monomers or heteromers with other receptors. More success, however, is expected from "drug-like" compounds that selectively bind and modulate altered binding pockets, which may originate from receptor homodimerization or heteromerization.

5.2 From bivalent ligands to integrated dual acting ligands

A relatively new offshoot from the "classical" bivalent ligands are the so-called integrated dual acting ligands. These molecules are potentially able to interact at two binding sites of a heterodimer, possibly resulting in improved subtype selectivity, higher binding affinity, boosted or modified functional activity, and reduced dependence on multiple drug administration regimens.[46]

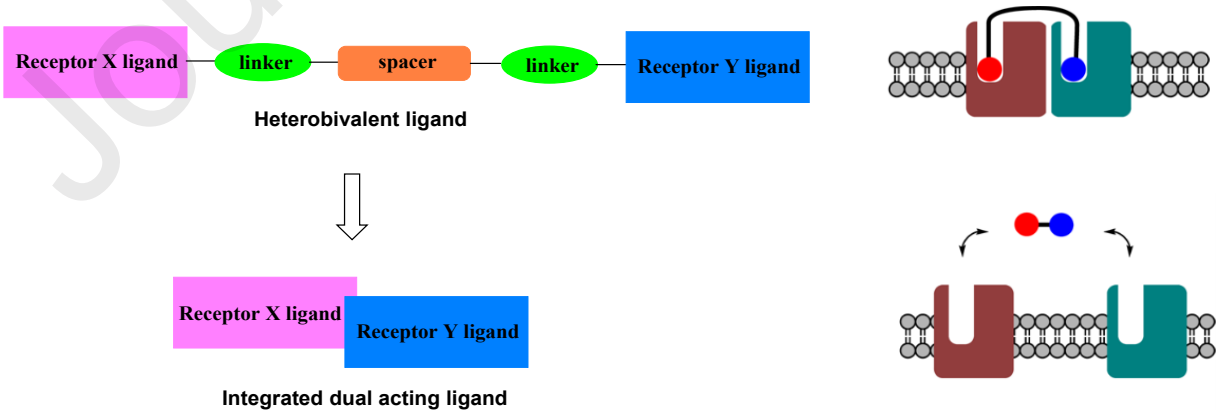


Figure 24. (left) Schematic representation of a heterobivalent ligand and integrated dual acting ligand consisting of a receptor X and a receptor Y ligand. (right) Schematic examples of a heterobivalent ligand binding to a

heterodimer and an integrated dual acting ligand acting at the two orthosteric sites of two different types of receptor monomers.

Jörg et al. pioneered this concept by converting classical heterobivalent ligand **30** consisting the D_2R agonist ropinirole and the $A_{2A}R$ antagonist ZM 241385 into a so-called integrated and more drug-like dual acting ligand **31** (Figure 25), which maintained the potency of the original pharmacophores at both receptors ($A_{2A}R$ and D_2R). [47] Furthermore, preliminary tests suggest that the integrated dual acting ligand is capable of crossing the blood–brain barrier contrary to the original heterobivalent ligands. Although dual acting ligand is more drug-like, it cannot bridge the two binding sites of GPCR dimer simultaneously because of its relatively short spacers.

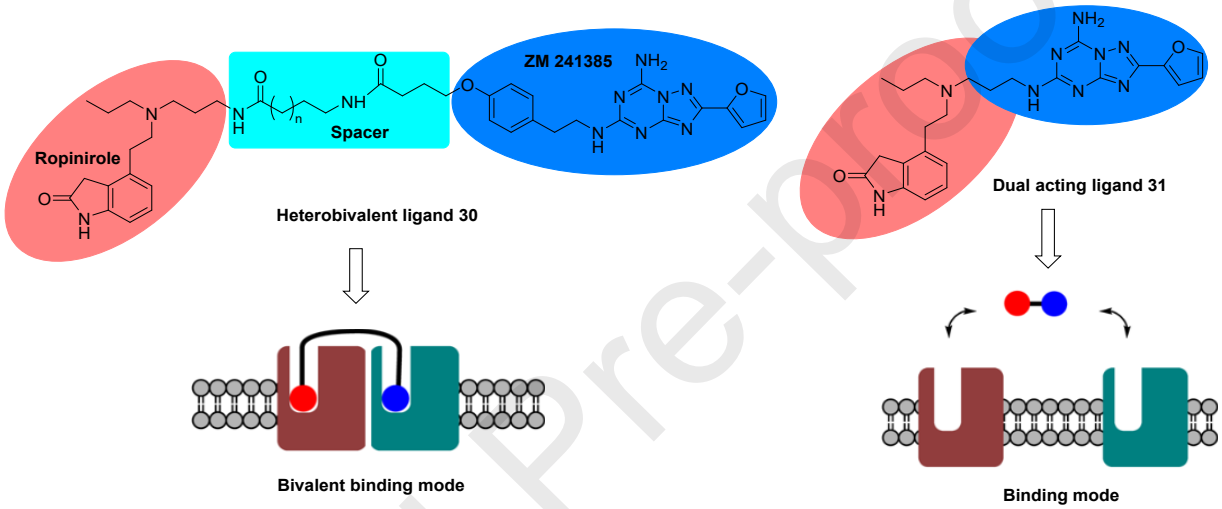


Figure 25. An example of the conversion of a "classical" heterobivalent ligand incorporating the pharmacophores ropinirole and ZM 241385 into an integrated dual acting ligands without the tyramine moiety originally present in ZM 241385.

It is noteworthy that the paradigm of "multiple ligand" approach is not new and many of the drugs originating from screening in animal models, were later found to modulate different targets.[48] Notable examples are the clinically used neuroleptics, which obviously do not obey to the "one-target, one-disease" approach that dominated the pharmaceutical industry for a long time.

5.3 Towards drug-like bitopic ligands that allosterically modulate GPCR homodimer

Recently, SB269652 was defined as the first small molecule negative allosteric modulator (NAM) of the D_2R, which was obtained by virtual screening.[49,50] Structure optimization of SB269652 lead to the bitopic ligands **32**[51] and **33**[52] (Figure 26). Interestingly, when the linker of SB269652 was replaced by *trans*-cyclopropylmethyl group to give compound **32**, which showed 200-fold D_3R selectivity over D_3R/D_2R. In addition, replacing the indole-2-carboxamide with 1*H*-pyrrolo[3,2-*b*]pyridine-3-carboxamide afforded compound **33**, which enhanced 5000-fold potency compared to SB269652.

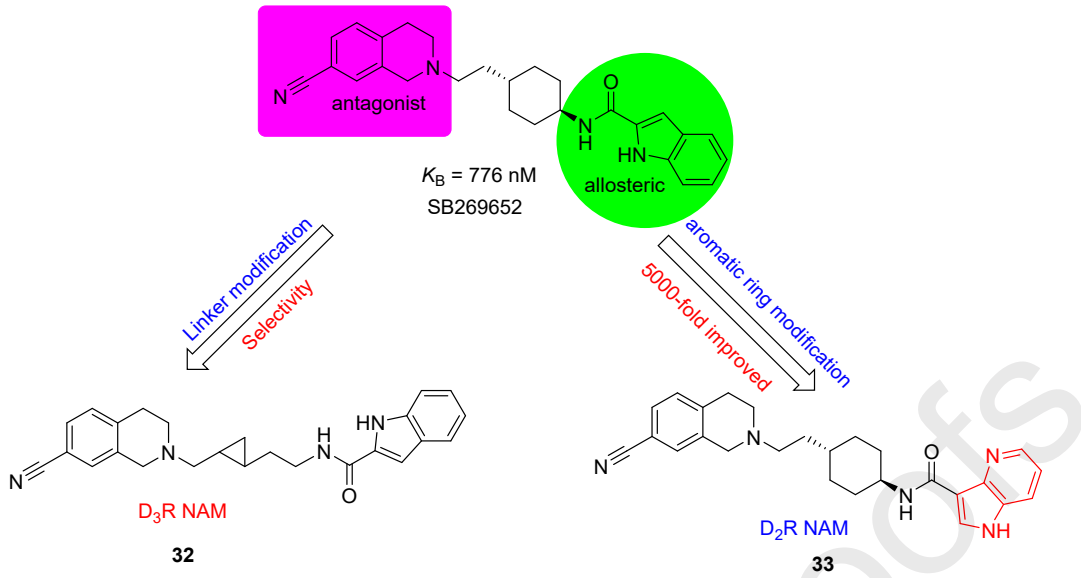


Figure 26. Structures of bitopic ligands **32** and **33** derived from SB269652.

It was proved that compound SB269652 acts through a new mechanism, engaging one protomer of a D_2R homodimer in a dual orthosteric/allosteric (or bitopic) mode, to negatively modulate dopamine binding and function at the other protomer (Figure 27).[53] Hence, SB269652 acts as a so-called bitopic ligand of D_2R. Bitopic ligands contain three components: allosteric and orthosteric heads connected by an optimal length of spacer, which occupy the allosteric and orthosteric binding sites (ABS and OBS) of the target receptor simultaneously. Generally, when the orthosteric binding site of D_2R is occupied by an antagonist head from the bitopic ligand, it will not allosterically modulate the endogenous ligand binding and function. Hence, this indicates a novel mechanism whereby a bitopic ligand binds in an extended pose on one protomer of a GPCR dimer to allosterically modulate the binding of an agonist to the orthosteric site of a second protomer.

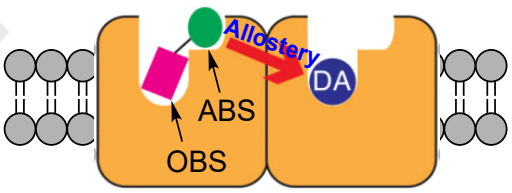


Figure 27. Bitopic ligand SB269652 acts as a negative allosteric modulator (red arrow) across a D_2R homodimer.

5.4 Towards drug-like compounds that selectively activate heterodimeric receptors

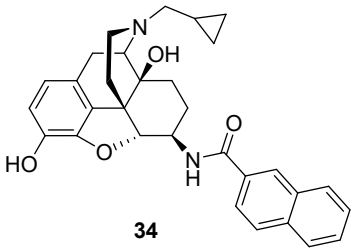


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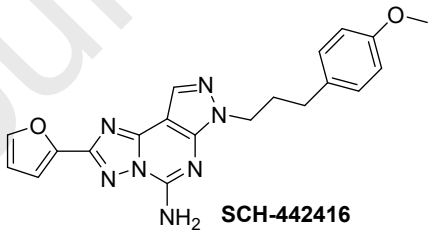
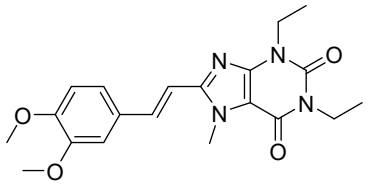
Figure 28. Molecular structure of *N*-naphthoyl-β-naltrexamine (NNTA, **34**).

While the integrated dual acting ligands and bitopic ligands described above still share structural similarities with the monovalent ligands that target both protomers, one step further would be the identification of "monovalent-like" ligands that selectively target heterodimeric receptors. Yekkirala et al.[54] discovered that N-naphthoyl-β-naltrexamine (NNTA, **34**)[55] (Figure 28) selectively activates heteromeric μ/κ-opioid receptors in HEK-293 cells. In a competition binding assay using [^{3}H]diprenorphine, NNTA was found to bind with very high affinity to cells that express μ- (K_i = 0.077 pM) or κ- (K_i = 0.084 pM) opioid receptors. Interestingly, NNTA was found to be a potent antagonist in cells singly expressing μ-opioid receptor, but an exceptionally potent agonist in cells coexpressing μ/κ-opioid receptors. In the mouse tail-flick assay, NNTA exhibited potent antinociceptive activity. Furthermore, it did not produce significant physical dependence in mice.

Likewise, Orru et al.[56] demonstrated in rats that the adenosine A_{2A} receptor ($A_{2A}R$) antagonist **SCH-442416** (Figure 29) has preferential pre-synaptic activity, based on its potency for inhibiting striatal glutamate release. The antagonist **KW-6002** (Figure 29) showed preferential post-synaptic activity, according to its potency for inducing locomotor activation. These behavioral effects may be explained by the higher relative affinity of **SCH-442416** for pre-synaptic adenosine A_1 receptors (A_1R)-$A_{2A}R$ heteromers and the better affinity of **KW-6002** for post-synaptic dopamine D_2 receptor (D_2R)-$A_{2A}R$ heteromers.



SCH-442416



KW-6002

Figure 29. Structures of **SCH-442416** and **KW-6002**.

Taken together, these studies afforded important proof-of-concept that it is possible to identify small molecules that selectively activate heteromeric receptors. In the first particular case it might lead to the development of new potent analgesics with fewer deleterious side effects.

5.5 Towards drug-like compounds that show biased signaling pathway for heteromers

Devi et al. reported a compound CYM51010 (Figure 30) through high-throughput screening of a small-molecule library, which is a β-arrestin2 biased drug-like ligand targeting μOR-δOR heteromers.[57] This compound exhibits activity in μOR-δOR coexpressing cells but not μOR or δOR cells alone. Remarkably, systemic administration of CYM51010 induced antinociceptive activity similar to morphine, whereas chronic administration of CYM51010 led to much lower antinociceptive tolerance than morphine.

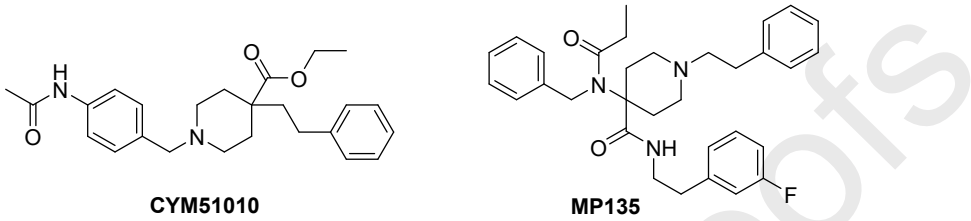


Figure 30. Structures of **CYM51010** and **MP135**.

A following study by the same group identified a drug-like compound MP135 (Figure 30) that exhibits high G-protein biased activity at μOR-δOR heteromers compared to the homomeric δOR or μOR.[58] Pharmacological result of MP135 supports that this molecule could be developed as an antinociceptive agent similar to morphine. However, *in vivo* study reveals that MP135 maintains untoward side effects such as respiratory depression and reward behavior.

Taken together, bivalent ligands are prone to act as molecular probes to study GPCR dimerization both *in vitro* and *in vivo*. Drug-like small molecules discussed above not only selectively target homodimers or heteromers, but also exhibit therapeutic potentials with higher potency and less side effects compared to those compounds only bind monomers. More importantly, drug-like bitopic ligands can be used as tools to study GPCR dimerization through allosteric mechanism. Hence, an increasing number of drug-like small molecules will appear in the study of GPCR dimerization.

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All authors have given approval to the final version of the manuscript.

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Abbreviations

GPCR, G-protein coupled receptor; D_2R, dopamine D_2 receptor; CuAAC, copper-catalyzed azide−alkyne cycloaddition reaction; PEG, poly(ethylene glycol); cAMP, cyclic adenosine monophosphate.

Declaration of interest

The authors declare no competing financial interest.

References

[1] Santos, R.; Ursu, O.; Gaulton, A.; Bento, A. P.; Donadi, R. S.; Bologa, C. G.; Karlsson, A.; Al-Lazikani, B.; Hersey, A.; Oprea, T. I.; Overington, J. P. A comprehensive map of molecular drug targets. *Nat. Rev. Drug Discov.* **2017**, *16*, 19-34.

[2] Daniels, D. J.; Lenard, N. R.; Etienne, C. L.; Law, P. Y.; Roerig, S. C.; Portoghese, P. S. Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc. Natl. Acad. Sci. U S A.* **2005**, *102*, 19208−19213.

[3] Daniels, D. J.; Kulkarni, A.; Xie, Z.; Bhushan, R. G.; Portoghese, P. S. A bivalent ligand (KDAN-18) containing delta-antagonist and kappa-agonist pharmacophores bridges delta2 and kappa1 opioid receptor phenotypes. *J. Med. Chem.* **2005**, *48*, 1713−1716.

[4] Lenard, N.R.; Daniels, D. J.; Portoghese, P. S.; Roerig, S. C. Absence of conditioned place preference or reinstatement with bivalent ligands containing mu-opioid receptor agonist and delta-opioid receptor antagonist pharmacophores. *Eur. J. Pharmacol.* **2007**, *566*, 75−82.

[5] Milligan, G.; Bouvier, M. Methods to monitor the quaternary structure of G protein-coupled receptors, *FEBS J.* **2005**, *272*, 2914−25.

[6] Yekkirala, A. S.; Lunzer, M. M.; McCurdy, C. R.; Powers, M. D.; Kalyuzhny, A. E.; Roerig, S. C.; Portoghese, P. S. N-naphthoyl-beta-naltrexamine (NNTA), a highly selective and potent activator of μ/kappa-opioid heteromers, *Proc. Natl. Acad. Sci. U S A.* **2011**, *108*, 5098−103.

[7] Soriano, A.; Ventura, R.; Molero, A.; Hoen, R.; Casadó, V.; Cortés, A.; Fanelli, F.; Albericio, F.; Lluís, C.; Franco, R.; Royo, M. Adenosine A2A receptor-antagonist/dopamine D2 receptor-agonist bivalent ligands as pharmacological tools to detect A2A-D2 receptor heteromers. *J. Med. Chem.* **2009**, *52*, 5590−602.

[8] Jacobson, K. A.; Xie, R.; Young, L.; Chang, L.; Liang, B. T. A novel pharmacological approach to treating cardiac ischemia. Binary conjugates of A1 and A3 adenosine receptor agonists. *J. Biol. Chem.* **2000**, *275*, 30272−9.

[9] Portoghese, P. S. From models to molecules: opioid receptor dimers, bivalent ligands, and selective opioid receptor probes. *J. Med. Chem.* **2001**, *44*, 2259−69.

[10] Huang, B.; St. Onge, C. M.; Ma, H.; Zhang, Y. Design of bivalent ligands targeting putative GPCR dimers. *Drug Discov Today*. **2021**, *26*, 189−199.

[11] Newman, A. H.; Battiti, F. O.; Bonifazi, A. 2016 Philip S. Portoghese Medicinal Chemistry Lectureship: Designing Bivalent or Bitopic Molecules for G-Protein Coupled Receptors. The Whole Is Greater Than the Sum of Its Parts. *J. Med. Chem.* **2020**, *63*, 1779−1797.

[12] Decker, M.; Fulton, B. S.; Zhang, B.; Knapp, B. I.; Bidlack, J.M.; Neumeyer, J. L. Univalent and bivalent ligands of butorphan: characteristics of the linking chain determine the affinity and potency of such opioid ligands. *J. Med. Chem.* **2009**, *52*, 7389−7396.

[13] Zhang, B.; Zhang, T.; Sromek, A. W.; Scrimale, T.; Bidlack, J.M.; Neumeyer, J. L. Synthesis and binding affinity of novel mono- and bivalent morphinan ligands for kappa, mu, and delta opioid receptors. *Bioorg. Med. Chem.* **2011**, *19*, 2808−2816.

[14] Huber, D.; Hubner, H.; Gmeiner, P. 1,1′-Disubstituted ferrocenes as molecular hinges in mono- and bivalent dopamine receptor ligands. *J. Med. Chem.* **2009**, *52*, 6860−6870.

[15] Bonger, K. M.; van den Berg, R. J.; Knijnenburg, A. D.; Heitman, L. H.; Ijzerman, A. P.; Oosterom, J.; Timmers, C. M.; Overkleeft, H. S.; van der Marel, G. A. Synthesis and evaluation of homodimeric GnRHR antagonists having a rigid bis-propargylated benzene core. *Bioorg. Med. Chem.* **2008**, *16*, 3744−3758.

[16] Weiss, S.; Keller, M.; Bernhardt, G.; Buschauer, A.; Konig, B. Modular synthesis of non-peptidic bivalent NPY Y1 receptor antagonists. *Bioorg. Med. Chem.* **2008**, *16*, 9858−9866.

[17] Hiller, C.; Kühhorn, J.; Gmeiner, P. Class A G-protein-coupled receptor (GPCR) dimers and bivalent ligands. *J. Med. Chem.* **2013**, *56*, 6542−6559.

[18] Andersen, J.; Ladefoged, L. K.; Kristensen, T. N. B.; Munro, L.; Grouleff, J.; Stuhr-Hansen, N.; Kristensen, A. S.; Schiøtt, B.; Strømgaard. K. Interrogating the molecular basis for substrate recognition in serotonin and dopamine transporters with high-affinity substrate-based bivalent ligands. *ACS Chem. Neurosci.* **2016**, *7*, 1406−1417.

[19] She, X.; Pegoli, A.; Mayr, J.; Hübner, H.; Bernhardt, G.; Gmeiner, P.; Keller, M. Heterodimerization of dibenzodiazepinone-type muscarinic acetylcholine receptor ligands leads to increased M_2R affinity and selectivity. *ACS Omega* **2017**, *2*, 6741−6754.

[20] Pockes, S.; Wifling, D.; Keller, M.; Buschauer, A.; Elz, S. Highly potent, stable, and selective dimeric hetarylpropylguanidine-type histamine H_2 receptor agonists. *ACS Omega* **2018**, *3*, 2865−2882.

[21] Pulido, D.; Casado-Anguera, V.; Perez-Benito, L.; Moreno, E.; Cordomí, A.; Lopez, L.; Cortes, A.; Ferre, S.; Pardo, L.; Casado, V.; Royo, M. Design of a True Bivalent Ligand with Picomolar Binding Affinity for a G Protein-Coupled Receptor Homodimer. *J. Med. Chem.* **2018**, *61*, 9335−9346.

[22] Kuhhorn, J.; Hubner, H.; Gmeiner, P. Bivalent dopamine D2 receptor ligands: synthesis and binding properties. *J. Med. Chem.* **2011**, *54*, 4896−4903.

[23] Gogoi, S.; Biswas, S.; Modi, G.; Antonio, T.; Reith, M. E.; Dutta, A. K. Novel bivalent ligands for D2/D3 dopamine receptors: significant co-operative gain in D2 affinity and potency. *ACS Med. Chem. Lett.* **2012**, *3*, 991−996.

[24] Kühhorn, J.; Götz, A.; Hübner, H.; Thompson, D.; Whistler, J.; Gmeiner, P. Development of a bivalent dopamine D2 receptor agonist. *J. Med. Chem.* **2011**, *54*, 7911−7919.

[25] Hübner, H.; Schellhorn, T.; Gienger, M.; Schaab, C.; Kaindl, J.; Leeb, L.; Clark, T.; Möller, D.; Gmeiner, P. Structure-guided development of heterodimer-selective GPCR ligands. *Nat Commun.* **2016**, *7*, 12298.

[26] Qian, M.; Vasudevan, L.; Huysentruyt, J.; Risseeuw, M. D. P.; Stove, C.; Vanderheyden, P. M. L.; Van Craenenbroeck, K.; Van Calenbergh, S. Design, synthesis, and biological evaluation of bivalent ligands targeting dopamine D_2-like receptors and the μ-opioid receptor. *ChemMedChem* **2018**, *13*, 944–956.

[27] Busnelli, M.; Kleinau, G.; Muttenthaler, M.; Stoev, S.; Manning, M.; Bibic, L.; Howell, L. A.; McCormick, P. J.; Di Lascio, S.; Braida, D.; Sala, M.; Rovati, G. E.; Bellini, T.; Chini, B. Design and characterization of superpotent bivalent ligands targeting oxytocin receptor dimers via a channel-like structure. *J. Med. Chem.* **2016**, *59*, 7152−7166.

[28] Lensing, C. J.; Freeman, K. T.; Schnell, S. M.; Adank, D. N.; Speth, R. C.; Haskell-Luevano, C. An in vitro and in vivo investigation of bivalent ligands that display preferential binding and functional activity for different melanocortin receptor homodimers. *J. Med. Chem.* **2016**, *59*, 3112−3128.

[29] Lensing, C. J.; Freeman, K. T.; Schnell, S. M.; Speth, R. C.; Zarth, A. T.; Haskell-Luevano, C. Developing a biased unmatched bivalent ligand (BUmBL) design strategy to target the GPCR homodimer allosteric signaling (cAMP over β-arrestin 2 recruitment) within the melanocortin receptors. *J. Med. Chem.* **2018**, *62*, 144−158.

[30] Daniels, D. J.; Lenard, N. R.; Etienne, C. L.; Law, P. Y.; Roerig, S. C.; Portoghese, P. S. Opioid-induced tolerance and dependence in mice is modulated by the distance between pharmacophores in a bivalent ligand series. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 19208−19213.

[31] Yekkirala, A. S.; Kalyuzhny, A. E.; Portoghese, P. S. An immunocytochemical-derived correlate for evaluating the bridging of heteromeric mu-delta opioid protomers by bivalent ligands. *ACS Chem. Biol.* **2013**, *8*, 1412−1416.

[32] Olson, K. M.; Keresztes, A.; Tashiro, J. K.; Daconta, L. V.; Hruby, V. J.; Streicher, J. M. Synthesis and evaluation of a novel bivalent selective antagonist for the Mu-Delta opioid receptor heterodimer that reduces morphine withdrawal in mice. *J. Med. Chem.* **2018**, *61*, 6075−6086.

[33] Le Naour, M.; Akgün, E.; Yekkirala, A.; Lunzer, M. M.; Powers, M. D.; Kalyuzhny, A. E.; Portoghese, P. S. Bivalent ligands that target μ opioid (MOP) and cannabinoid1 (CB1) receptors are potent analgesics devoid of tolerance. *J. Med. Chem.* **2013**, *56*, 5505−5513.

[34] Akgün, E.; Javed, M. I.; Lunzer, M. M.; Smeester, B. A.; Beitz, A. J.; Portoghese, P. S. Ligands that interact with putative MOR-mGluR5 heteromer in mice with inflammatory pain produce potent antinociception. *Proc. Natl. Acad. Sci. U.S.A.* **2013**, *110*, 11595−11599.

[35] Peterson, C. D.; Kitto, K. F.; Akguen, E.; Lunzer, M. M.; Riedl, M. S.; Vulchanova, L.; Wilcox, G. L.; Portoghese, P. S.; Fairbanks, C. A., Bivalent ligand that activates mu opioid receptor and antagonizes mGluR5 receptor reduces neuropathic pain in mice. *Pain* **2017,** *158*, 2431−2441.

[36] Akgün, E.; Javed, M. I.; Lunzer, M. M.; Powers, M. D.; Sham, Y. Y.; Watanabe, Y.; Portoghese, P. S. Inhibition of inflammatory and neuropathic pain by targeting a mu opioid receptor/chemokine receptor5 heteromer (MOR-CCR5). *J. Med. Chem.* **2015**, *58*, 8647−8657.

[37] Akgün, E.; Lunzer, M. M.; Portoghese, P. S. Combined glia inhibition and opioid receptor agonism afford highly potent analgesics without tolerance. *ACS Chem Neurosci.* **2019**, *10*, 2004−2011.

[38] Ma, H.; Li, M.; Pagare, P. P.; Wang, H.; Nassehi, N.; Santos, E. J.; Negus, S. S.; Selley, D. E.; Zhang, Y. Novel bivalent ligands carrying potential antinociceptive effects by targeting putative mu opioid receptor and chemokine receptor CXCR4 heterodimers. *Bioorg. Chem.* **2022**, *120*, 105641.

[39] Qian, M.; Wouters, E.; Dalton, J. A. R.; Risseeuw, M. D. P.; Crans, R. A. J.; Stove, C. P.; Giraldo, J.; Van Craenenbroeck, K.; Van Calenbergh, S. Synthesis toward bivalent ligands for the dopamine D_2 and metabotropic glutamate 5 receptors. *J. Med. Chem.* **2018**, *61*, 8212–8225.

[40] Poulie, C. B. M.; Liu, N.; Jensen, A. A.; Bunch, L. Design, Synthesis, and Pharmacological Characterization of Heterobivalent Ligands for the Putative 5-HT_{2A}/$mGlu_2$ Receptor Complex. *J. Med. Chem.* **2020**, *63*, 9928−9949.

[41] Qian, M.; Ricarte, A.; Wouters, E.; Dalton, J. A. R.; Risseeuw, M. D. P.; Giraldo, J.; Van Calenbergh, S. Discovery of a true bivalent dopamine D2 receptor agonist. *Eur. J. Med. Chem.* **2021**, *212*, 113151.

[42] a) Huang, B.; Wang, H.; Zheng, Y.; Li, M.; Kang, G.; Barreto-de-Souza, V.; Nassehi, N.; Knapp, P. E.; Selley, D. E.; Hauser, K. F.; Zhang, Y. Structure-Based Design and Development of Chemical Probes Targeting Putative MOR-CCR5 Heterodimers to Inhibit Opioid Exacerbated HIV-1 Infectivity. *J. Med. Chem.* **2021**, *64*, 7702−7723.; b) Arnatt, C. K.; Falls, B. A.; Yuan, Y.; Raborg, T. J.; Masvekar, R. R.; El-Hage, N.; Selley, D. E.; Nicola, A. V.; Knapp, P. E.; Hauser, K. F.; Zhang, Y. Exploration of bivalent ligands targeting putative mu opioid receptor and chemokine receptor CCR5 dimerization. *Bioorg. Med. Chem.* **2016**, *24*, 5969–5987.; c) Yuan, Y.; Arnatt, C. K.; Li, G.; Haney, K. M.; Ding, D.; Jacob, J. C.; Selley, D. E.; Zhang, Y. Design and synthesis of a bivalent ligand to explore the putative heterodimerization of the mu opioid receptor and the chemokine receptor CCR5. *Org. Biomol. Chem.*, **2012**, *10*, 2633–2646.; d) Yuan, Y.; Arnatt, C. K.; El-Hage, N.; Dever, S. M.; Jacob, J. C.; Selley, D. E.; Hauser, K. F.; Zhang, Y. A bivalent ligand targeting the putative mu opioid receptor and chemokine receptor CCR5 heterodimer: binding affinity versus functional activities. *Med. Chem. Commun.*, **2013**, *4*, 847–851.

[43] a) Reinecke, B. A.; Kang, G.; Zheng, Y.; Obeng, S.; Zhang, H.; Selley, D. E.; Anb, J.; Zhang, Y. Design and synthesis of a bivalent probe targeting the putative mu opioid receptor and chemokine receptor CXCR4 heterodimer. *RSC Med. Chem.*, **2020**, *11*, 125–131.; b) Ma, H.; Wang, H.; Li, M.; Barreto-de-Souza, V.; Reinecke, B. A.; Gunta, R.; Zheng, Y.; Kang, G.; Nassehi, N.; Zhang, H.; An, J.; Selley, D. E.; Hauser, K. F.; Zhang, Y. Bivalent Ligand Aiming Putative Mu Opioid Receptor and Chemokine Receptor CXCR4 Dimers in Opioid Enhanced HIV-1 Entry. *ACS Med. Chem. Lett.* **2020**, *11*, 2318−2324.

[44] Shonberg, J.; Scammells, P. J;. Capuano, B. Design strategies for bivalent ligands targeting GPCRs. *ChemMedChem* **2011**, *6*, 963 – 974.

[45] Portoghese, P. S.; Akgün, E.; Lunzer, M. M. Heteromer Induction: An Approach to Unique Pharmacology? *ACS Chem. Neurosci.* **2017**, *8*, 426−428.

[46] Jörg, M.; May, L. T.; Mak, F. S.; Lee, K. C. K.; Miller, N. D.; Scammells, P. J.; Capuano, B. Synthesis and Pharmacological Evaluation of Dual Acting Ligands Targeting the Adenosine A2A and Dopamine D2 Receptors for the Potential Treatment of Parkinson's Disease. *J. Med. Chem.* **2015**, *58*, 718−738.

[47] Jörg, M.; May, L. T.; Mak, F. S.; Lee, K. C. K.; Miller, N. D.; Scammells, P. J.; Capuano, B. Synthesis and pharmacological evaluation of dual acting ligands targeting the adenosine A_{2A} and dopamine D_2 receptors for the potential treatment of Parkinson's disease. *J. Med. Chem.* **2015**, *58*, 718−738.

[48] Morphy, R.; Rankovic, Z. Designed multiple ligands. An emerging drug discovery paradigm. *J. Med. Chem.* **2005**, *48*, 6523-6543.

[49] Stemp, G.; Ashmeade, T.; Branch, C. L.; Hadley, M. S.; Hunter, A. J.; Johnson, C. N.; Nash, D. J.; Thewlis, K. M.; Vong, A. K. K.; Austin, N. E.; Jeffrey, P.; Avenell, K. Y.; Boyfield, I.; Hagan, J. J.; Middlemiss, D. N.; Reavill, C.; Riley, G. J.; Routledge, C.; Wood, M. Design and synthesis of trans-N-[4-[2-(6-cyano-1,2,3,4-tetrahydroisoquinolin-2-yl)ethyl]cyclohexyl]-4-quinolinecarboxamide (SB-277011): a potent and selective dopamine D3 receptor antagonist with high oral bioavailability and CNS penetration in the rat. *J. Med. Chem.* **2000**, *43*, 1878−1885.

[50] Silvano, E.; Millan, M. J.; la Cour, C. M.; Han, Y.; Duan, L.; Griffin, S. A.; Luedtke, R. R.; Aloisi, G.; Rossi, M.; Zazzeroni, F.; Javitch, J. A.; Maggio, R. The tetrahydroisoquinoline derivative SB269,652 is an allosteric antagonist at dopamine D3 and D2 receptors. *Mol. Pharmacol.* **2010**, *78*, 925−934.

[51] Kumar, V.; Moritz, A. E.; Keck, T. M.; Bonifazi, A.; Ellenberger, M. P.; Sibley, C. D.; Free, R. B.; Shi, L.; Lane, J. R.; Sibley, D. R.; Newman, A. H. Synthesis and pharmacological characterization of novel trans-cyclopropylmethyl-linked bivalent ligands that exhibit selectivity and allosteric pharmacology at the dopamine D3 receptor (D3R). *J. Med. Chem.* **2017**, *60*, 1478−1494.

[52] Kopinathan, A.; Draper-Joyce, C.; Szabo, M.; Christopoulos, A.; Scammells, P. J.; Lane, J. R.; Capuano, B. Subtle modifications to the indole-2-carboxamide motif of the negative allosteric modulator N-((trans)-4-(2-(7-cyano-3,4-dihydroisoquinolin-2(1H)-yl)ethyl)cyclohexyl)-1H-indole-2-carboxamide (SB269652) yield dramatic changes in pharmacological activity at the dopamine D2 receptor. *J. Med. Chem.* **2019**, *62*, 371−377.

[53] Lane, J. R.; Donthamsetti, P.; Shonberg, J.; Draper-Joyce, C. J.; Dentry, S.; Michino, M.; Shi, L.; López, L.; Scammells, P. J.; Capuano, B.; Sexton, P. M.; Javitch, J. A.; Christopoulos, A. A new mechanism of allostery in a G protein-coupled receptor dimer. *Nat. Chem. Biol.* **2014**, *10*, 745−752.

[54] Yekkirala, A. S.; Lunzer, M. M.; McCurdy, C. R.; Powers, M. D.; Kalyuzhny, A. E.; Roerig, S. C.; Portoghese, P. S. N-naphthoyl-β-naltrexamine (NNTA), a highly selective and potent activator of μ/κ-opioid heteromers. *Proc. Natl. Acad. Sci. U S A.* **2011**, *108*, 5098-5103.

[55] Li, G.; Aschenbach, L. C.; Chen, J.; Cassidy, M. P.; Stevens, D. L.; Gabra, B. H.; Selley, D. E.; Dewey, W. L.; Westkaemper, R. B.; Zhang, Y. Corrections to design, synthesis, and biological

evaluation of 6α- and 6β-N-heterocyclic substituted naltrexamine derivatives as μ opioid receptor selective antagonists. *J. Med. Chem.* **2009**, *52*, 1416–1427.

[56] Orru, M.; Bakesova, J.; Brugarolas, M.; Quiroz, C.; Beaumont, V.; Goldberg, S. R.; Lluıs, C.; Cortes, A.; Franco, R.; Casado, V.; Canela, E. I.; Ferre, S. Striatal pre- and postsynaptic profile of adenosine $A_{(2A)}$ receptor antagonists. *PLoS One.* **2011**, *6*, e16088.

[57] Gomes, I.; Fujita, W.; Gupta, A.; Saldanha, S. A.; Negri, A.; Pinello, C. E.; Eberhart, C.; Roberts, E.; Filizola, M.; Hodder, P.; Devi, L. A. Identification of a μ-δ opioid receptor heteromer biased agonist with antinociceptive activity. *Proc. Natl. Acad. Sci. USA.* **2013**, *110*, 12072–12077.

[58] Faouzi, A.; Uprety, R.; Gomes, I.; Massaly, N.; Keresztes, A. I.; Le Rouzic, V.; Gupta, A.; Zhang, T.; Yoon, H. J.; Ansonoff, M.; Allaoa, A.; Pan, Y. X.; Pintar, J.; Morón, J. A.; Streicher, J. M.; Devi, L. A.; Majumdar, S. Synthesis and pharmacology of a novel μ−δ opioid receptor heteromer-selective agonist based on the carfentanyl template. *J. Med. Chem.* **2020**, *63*, 13618−13637.

Graphical abstract