Disrupting GPCR Complexes with Smart Drug-like Peptides

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Abstract: G protein-coupled receptors (GPCRs) are a superfamily of proteins classically described as monomeric transmembrane (TM) receptors. However, increasing evidence indicates that many GPCRs form higher-order assemblies made up of monomers pertaining to identical (homo) or to various (hetero) receptors. The formation and structure of these oligomers, their physiological role and possible therapeutic applications raise a variety of issues that are currently being actively explored. In this context, synthetic peptides derived from TM domains stand out as powerful tools that can be predictably targeted to disrupt GPCR oligomers, especially at the interface level, eventually impairing their action. However, despite such potential, TM-derived, GPCR-disrupting peptides often suffer from inadequate pharmacokinetic properties, such as low bioavailability, a short half-life or rapid clearance, which put into question their therapeutic relevance and promise. In this review, we provide a comprehensive overview of GPCR complexes, with an emphasis on current studies using GPCR-disrupting peptides mimicking TM domains involved in multimerization, and we also highlight recent strategies used to achieve drug-like versions of such TM peptide candidates for therapeutic application.

Keywords: peptide therapeutics; transmembrane peptides; GPCR oligomers; non-natural amino acids; cyclic peptides; retro-enanti

1. Introduction

G protein-coupled receptors (GPCRs) constitute the largest and most versatile superfamily of cell membrane-bound proteins, made up of seven trans-membrane α-helices (TM1 to TM7) [1–3] connected by intracellular (IL-1 to IL-3) and extracellular loops (EL-1 to EL-3), and coupled to an intracellular heterotrimeric G protein (e.g., Gs, Gi/o, Gq/11, G12/13) [4]. GPCRs are commonly grouped into six subfamilies (A-F) [5], based on sequence homology and functionality. Despite this apparent diversity, all GPCRs mediate their effects upon agonist-induced activation of the receptor at the extracellular site by a wide variety of ligands and then transduce the signal into intracellular responses [6]. Endogenous GPCR agonists are physically and chemically very diverse, including neurotransmitters (i.e., dopamine, serotonin), hormones (i.e., estrogen, angiotensin), proteins (i.e., chemokines), odors, photons, lipids (i.e., anandamide) or peptides (i.e., bradykinin), among many others [7]. Moreover, and more interestingly, ligand affinity for the GPCR primary (orthosteric) site and efficacy of activation can be increased or decreased by other effectors that bind to a separate (allosteric) site [8].

Given that GPCR signaling is involved in a diverse number of biological processes, GPCRs are considered ideal therapeutic targets [9] for a wide assortment of human diseases ranging from allergic rhinitis to pain, type-2 diabetes mellitus, obesity, depression, insomnia or cancer, to name just a few [10–12]; indeed, 34% of currently FDA-approved small-molecule drugs bind to GPCRs [13]. Originally described as cell-surface monomers that form a ternary complex with the extracellular ligand and the intracellular G protein [14], GPCR higher-order oligomers have in recent years been increasingly recognized as novel signaling units with functional properties distinct from their constituent receptors, thus
opening up a new, only sparingly explored area of study within the GPCR field [15,16]. One possible strategy to probe into GPCR oligomerization and its impact on health conditions would consist in interfering in complex formation by means of exogenous synthetic peptides replicating TM domains involved in helix–helix interactions [17]. In this review, we consider the challenges and opportunities involved in disrupting GPCR oligomer formation by means of TM peptides targeting the complex interface, as a way to regulate oligomerization-dependent functions, and we also discuss strategies reported to improve the druggability of such peptide candidates in the context of cannabinoid-mediated pain management or CNS disorders.

2. GPCR Oligomers

The human genome encodes nearly 1000 different GPCRs, each one highly specific to a signaling pathway [18]. However, growing evidence indicates that many GPCRs can form active higher-order oligomers constituted by equal (homo) or different (hetero) monomers [19–26], with functional properties distinct from their protomer components [27] and generally involved in both healthy and pathological processes [28], thus making them ideal targets for the development and screening of novel drugs [29,30].

One of the first reported GPCR oligomers involved δ- and κ-opioid receptors that, when co-expressed, formed a stable heterodimer with properties not found in cells expressing the same receptor monomers [31]. Subsequently, many other GPCR homo- and/or hetero-complexes have been unveiled, often displaying unique characteristics.

In many of these investigations the importance of TM helices in GPCR oligomerization has been demonstrated, portraying the GPCR complexes as dynamic species in which activation by the agonist induces a realignment of TM dimerization interfaces [32,33]. Indeed, it has been found that a dynamic equilibrium between monomeric and dimeric species can take place [34], modulated by ligand binding, which in turn can enhance or decrease heteromer interaction [35]. Therefore, while the minimal GPCR functional unit can be regarded as constituted by one monomeric receptor and one heterotrimeric G protein (1:1) [36], GPCR dimers can occur when: (i) two G proteins bind both dimer protomers (2:2) [37,38] or (ii) one G protein binds one protomer in the dimer (1:2) [39].

Another distinctive feature of some GPCRs is the switching of the G protein-coupled protomer when dimerization occurs. For instance, serotonin 5HT2A-R couples Gq; however, heteromer formation by cannabinoid CB1-R and 5HT2A-R makes both receptors signal via Gi [40] (Figure 1A). In other words, some GPCR heteromers can couple G protein species different from those favoured by their protomers. Other reported examples are: (i) a heterodimer formed by dopamine D1 and D2 receptors that couples Gq instead of Gs or Gi [41] (Figure 1B); (ii) the heteromer formed by angiotensin AT1 and α2c-adrenergic receptors couples Gs instead of Gi or Gq [42]; and (iii) a melatonin MT1-MT2 receptor dimer that couples Gq instead of Gi [43].

Functionally, GPCR complexes can cause a positive or negative cooperation between promoters, i.e., ligand one binds to protomer one, enhancing or inhibiting, respectively, the affinity of ligand two for protomer two [44]. In general, intermolecular communication between GPCR homo- and heteromers tends to produce synergistic responses (i.e., functional cross-talk) [45]. A more singular phenomenon is cross-antagonism (Figure 1C), which occurs when a protomer antagonist blocks the signal activation of the other protomer [25,40,45]. Such a situation has been described for some GPCR complexes, including the metabotropic Gb1-Gb2 receptors [46], opioid δ-μ receptors [47], somatostatin SST5-dopamine D2 receptors [48], adenosine A2A-dopamine D1 receptors [49], orexin-corticotropin-releasing factor receptor [50] or angiotensin II AT1/dopamine D2 receptor [51].
Despite the extensive literature on GPCR oligomers, in most cases the assessment of their functionality has been only partially addressed and needs further investigation. In this context, chimeric peptide constructs have shown the ability to disrupt homo- and heteromer complexes, altering agonist-induced functionality and providing knowledge on the physiological role of GPCR receptor–receptor interactions [52–55].

3. Synthetic TM Peptides as Tools for GPCR Complex Exploration

The identification of protein–protein interaction interfaces constitutes a fundamental aspect in the study of GPCR complex formation [56], in that it can expand our understanding of the role that receptor oligomerization plays in intercellular communication or in some pathological conditions.

Increasing evidence indicates that specific TM helices are required for oligomerization, and that the synthetic peptides reproducing them are powerful tools to identify sequences essential for GPCR complexation and, by blocking their assembly, gain insights into the functional role of the complex [52,57,58].

For instance, Köfalvi et al. (2020) have recently studied how the adenosine-cannabinoid receptors, specifically the A$_{2A}$R-CB$_{1}$R heterotetramer interface, which also includes A$_{2A}$R-
A$_2$A R and CB$_1$R-CB$_1$R homodimers, is established. To this end they have used computational modelling, with input from several biophysical and biochemical techniques, to design TM interference peptides reproducing each of the A$_2$A R and CB$_1$R TM1-7 helices. The synthetic versions, fused to the cell-penetrating HIV-Tat sequence, were tested by in vitro bimolecular fluorescence complementation (BiFC) experiments. Peptides replicating TM5 and TM6 of both receptors were able to disrupt the heterotetramer; thus, the involvement of their interfaces in the complex formation was confirmed. On the other hand, in the absence of the CB$_1$R receptor, BiFC assays showed that the A$_2$A R-A$_2$A R homodimer was only disrupted by peptide A$_2$A R TM6, while when A$_2$A R was missing, CB$_1$R TM4 was the only peptide disturbing CB$_1$R-CB$_1$R homodimer formation, altogether indicating that TM6 and TM4 sequences are involved in A$_2$A R and CB$_1$R homodimer interfaces, respectively [59].

Once the interfering peptides are identified, they can be used to investigate GPCR complex implications in numerous physiopathological disorders. As an example, Borroto-Escuela et al. (2018) found that rat A$_2$A R TM5 peptide microinjection into the nucleus accumbens causes A$_2$A R-D$_2$R heteromer dissolution plus abrogation of the inhibitory effects of the A$_2$A R agonist CGS21680 on cocaine self-administration, therefore confirming that the A$_2$A R-D$_2$R hetero-complex can be used as a novel target to treat cocaine disorders [53].

More examples where synthetic peptides replicating TM helices involved in dimerization have been shown to be able to split GPCR complex formations are included in Table 1. The in vitro (biophysical and/or biochemical) and in vivo assays used to confirm the existence of GPCR dimers in live cells and their implication (if known) in health disorders, are also presented.
Table 1. GPCR complexes disrupted by synthetic TM peptides.

| GPCR Complex | TMs Involved in Dimerization | Synthetic TM Disruptor Peptide | In Vitro/In Vivo Assays Performed | Patho-Physiological Implication | Ref. |
|--------------|-----------------------------|-------------------------------|----------------------------------|---------------------------------|------|
| A<sub>2A</sub>R-D<sub>2</sub>R | TM4/5 interface | A<sub>2A</sub>R TM5 | • BRET  
• PLA  
• Cocaine self-administration | Cocaine use | [53] |
| APJR-OX<sub>1</sub>R | TM4/5 interface | APJ TM4, TM5 | • BRET  
• Co-IP | - | [60] |
| APJR homodimer | TM1, TM2, TM3, TM4 | TM1, TM2, TM3, TM4 | • BRET  
• FRET  
• TIRFM  
• Co-IP | - | [61] |
| A<sub>2A</sub>R-CB<sub>1</sub>R | TM 5/6 interface | CB<sub>1</sub>R TM5 TM6  
A<sub>2A</sub>R TM5 TM6 | • BiFC  
• BRET  
• CODA-RET  
• Glutamate release | Glutamate release | [59] |
| A<sub>1</sub>R-A<sub>2A</sub>R | TM 5/6 interface | A<sub>2A</sub>R TM4, TM5, TM6  
A<sub>1</sub>R TM5 and TM6 | • BiFC  
• PLA  
• BRET  
• cAMP production  
• DMR | Neurodegeneration  
Neuroinflammation | [62] |
| CB<sub>1</sub>R-5HT<sub>2A</sub>R | TM 5/6 interface | CB<sub>1</sub>R TM5, TM6 | • BRET  
• PLA  
• BiFC  
• NORT  
• Hot plate test | Cognitive impairment | [40] |
| M<sub>3</sub>R homodimer | TM1, TM5, TM7 | TM1-TM5-TM7 | • BRET | - | [63] |
| GPCR Complex       | TMs Involved in Dimerization | Synthetic TM Disruptor Peptide | In Vitro/In Vivo Assays Performed | Patho-Physiological Implication                                      | Ref.  |
|--------------------|-----------------------------|--------------------------------|----------------------------------|---------------------------------------------------------------------|-------|
| CCKR homodimer     | TM6                         | TM6                            | • BRET                           | -                                                                   | [64]  |
|                    |                              |                                 | • FRET                           |                                                                     |       |
| CCR5 homodimer     | TM1, TM2, TM4               | TM1, TM4                        | • FRET Calcium determination     | -                                                                   | [65]  |
| RhoR homodimer     | TM1, TM2, TM4, TM5, H8      | TM1, TM2, TM4, TM5              | • BRET                           | cAMP production                                                    |       |
|                    |                              |                                 | • FRET                           |                                                                      |       |
| β2AR homodimer     | TM1, TM5, TM6, H8           | TM6                            | • Adenyl cyclase activity        | Densitometric analyses                                             |       |
|                    |                              |                                 | •                               |                                                                      |       |
| SCTR               | TM4                         | TM4                            | • FRET                           |                                                                      |       |
|                    |                              |                                 | • BRET                           | Liver diseases                                                     | [55]  |
| AT1aR-SCTR         | TM1/2 interface             | AT1aR TM1, TM4, SCTR TM2, TM4  | • BRET                           |                                                                      |       |
|                    | TM4/4 interface             |                                 | • FRET                           |                                                                      |       |
|                    |                              |                                 | • cAMP                           | Hyperosmolality-induced drinking                                   | [54]  |
| FZD6 homodimer     | TM4, TM5                    | TM4, TM5                        | • FRAP                           |                                                                      |       |
|                    |                              |                                 | • FCCS                           | Cancer and neurologic disorders                                    | [67]  |
| MOR-DOR            | MOR TM1                     | MOR TM1                         | • Co-IP                          |                                                                      |       |
|                    |                              |                                 | • Immunoblotting                 |                                                                      |       |
|                    |                              |                                 | • Tail immersion                 |                                                                      |       |
|                    |                              |                                 |                                  | Morphine tolerance                                                 | [68]  |

Abbreviations: 5HT2A R, serotonin receptor type 2 A; A1 R, adenosine receptor type 1; A2A R, adenosine receptor type 2A; APJR, apelin receptor; AT1a R, angiotensin receptor type 1a; BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; cAMP, cyclic adenosine monophosphate; CB1 R, cannabinoid receptor type 1; CCKR, cholecystokinin receptor; CCR5, chemokine receptor type 5; CODA-RET, complemented donor-acceptor resonance energy transfer; Co-IP, co-immunoprecipitation; D2 R, dopamine receptor type 2; DMR, dynamic mass redistribution; DOR, δ-opioid receptor; FCCS, fluorescence cross-correlation spectroscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; FZD6 R, Frizzled-6 receptor; M1 R, muscarinic acetylcholine receptor type 3; MOR, μ-opioid receptors; NORT, novel object recognition test; OX1 R, orexin receptor type 1; PLA, proximity ligation assay; RhoR, rhodopsin receptor; SCTR, secretin receptor; TIRF, total internal reflection fluorescence; β2AR, adrenergic receptor type β2.
3.1. TM Peptides: Challenges and Opportunities to Drug the Undruggable

Despite presenting great in vitro and in vivo potential in terms of efficacy, selectivity and safety, TM peptide disruptors of GPCR complexes are viewed as undesirable leads for therapeutic application due to their peptide-intrinsic poor pharmacokinetics, including low water solubility, high susceptibility to proteases, poor membrane permeability (including challenging physiological barriers such as the BBB), rapid clearance and immunogenicity [69]. These caveats notwithstanding, an array of peptide engineering strategies have been used over the years to improve druggability (see next paragraph and below) and can be also implemented in this case to develop optimized versions of GPCR complex-disrupting TM peptides and explore their therapeutic applications.

The first useful item in the toolbox is trimming off some N-terminal or C-terminal residues until a minimally active primary structure can be established (Figure 2A). This reductionistic approach is particularly efficacious in the case of TM peptides since, by removing (almost invariably hydrophobic) residues from the cognate TM sequence, water solubility is improved [70–72], while synthesis time and costs are considerably reduced. Another strategy to improve peptide solubility is PEGylation, i.e., attaching several polyethylene glycol (PEG) units to the peptide lead structure (Figure 2B). PEG moieties, apart from being highly hydrophilic, are also good non-immunogenic spacers able to shield the peptide from proteolytic enzymes [73,74]. Additionally, related to this issue, the replacement of natural L- by non-natural D-amino acids is a common manoeuvre to improve peptide stability towards proteases in the digestive tract (e.g., trypsin), plasma and other biological fluids (Figure 2C). Furthermore, non-natural amino acids can enhance target affinity and selectivity by the induction or stabilization of secondary structure motifs (α-helices, β-sheets, β-turns) [75,76]. Along similar lines, the so-called retro-enantio approach can likewise give rise to peptides fully resistant to natural proteases. In a retro-enantio peptide both amino acid sequence and residue chirality are reversed relative to the parent structure, but despite these substantial changes the orientation of the side chains is preserved, hence a resemblance in overall shape (Figure 2D), while the inverted chirality curbs protease degradation, enhancing half-life and thus the potential as a new drug lead [77]. Moreover, the retro-enantio analogue tends to be less immunogenic than the cognate sequence [78]. Lastly, cyclization (head-to-tail (Figure 2E), side-chain-to-tail, side-chain-to-side-chain), including disulfide bond formation from native or (more frequently) engineered cysteine residues, is another quite valuable tool in the box that is regularly shown to reinforce serum stability [79]. Low membrane permeability, poor cellular uptake and inadequate homing specificity [80] are well-recognized snags that seriously jeopardize the success of peptide drugs. In this regard, a variety of drug carriers [81,82], with cell-penetrating peptides (CPPs) as a preeminent, successful example, have emerged as safe and efficient strategies to improve bioavailability. CPPs are a family of short peptide vectors with a remarkable in-built ability to traverse membranes, including important physiological hurdles such as the blood–brain or the skin barriers or the intestinal and nasal mucosae. Acting as drug delivery vectors (Figure 2B), CPPs can deliver into cells a plethora of payloads with therapeutic or diagnostic purposes, overcoming pharmacokinetic limitations and poor access to difficult areas, such as the central nervous system [80]. In sum, CPPs have become a powerful tool to address one of the main bottlenecks in drug development, namely the successful delivery of active compounds to target sites [80]. Moreover, in the specific case of GPCR complexes, CPPs can also determine the trajectory of the peptide disruptor into the membrane [80]. Peptide backbone modification is also an important and widely used approach to improve bioavailability [83,84]. Still another strategy to increase target affinity and cell uptake, as well as to protect against proteolytic degradation, involves stapling, whereby a synthetic brace (staple) introduced between two preestablished sites in the sequence helps to lock the peptide into a specific secondary structure (Figure 2F), thus reducing conformational entropy [85–87]. In the context of CB1R-5HT2AR complex disruption, the stapling approach has been recently applied [88], with preservation of in vitro GPCR oligomer disrupting activity along with improved proteolytic resistance.
work from our group and allied laboratories exploring the possibility of dissociating TM5 and TM6 helices, fused to a cell-penetrating sequence derived from HIV-Tat, to peptides. Two such accounts, dealing with the medical use of cannabinoids to fight pain and peptide with overall shape resemblance; (F) Stapling to constrain the peptide into a specific conformation.

Even though the resources in the peptide engineering toolbox just mentioned have allowed overcoming many of the factors limiting the therapeutic use of natural peptides with moderate success, for GPCR complex-disrupting peptides in particular, the goal of turning a canonical TM sequence into a viable drug lead may still entail a hazardous journey fraught with formidable obstacles. Even so, the latest literature reveals a slowly growing body of reports, including recent work from our group, where some of the aforementioned strategies are creatively applied to improve the druggability of GPCR complex-disrupting peptides. Two such accounts, dealing with the medical use of cannabinoids to fight pain and CNS disorder exploration and treatment, are discussed at some length below, illustrating the design and structural optimization process where TM peptides involved in GPCR dimer interfaces are developed into promising leads in two therapeutic areas with still unmet needs.

3.2. TM Peptides Restricting CB1R-5HT2AR Dimer for Cannabinoid Management in Pain Therapy

While cannabinoid-based therapy has proven effective in alleviating chronic pain [89–91], its psychotropic side effects such as memory loss, disorientation or dizziness are major obstacles in attempts to deploy cannabinoids as analgesics [92–95]. To this day, cannabinoids, both synthetic and naturally occurring, remain at the center of social, legal and medical debates concerning their therapeutic value, while the need for novel pain-alleviating medicines whose beneficial effects outweigh adverse ones is quite obvious in everyday clinical practice.

Various strategies have been investigated to minimize the unwanted outcomes of long-lasting cannabinoid exposure in patients undergoing cannabis-based therapies, so far to little avail. In line with this goal and with the topic of this review, we will now discuss work from our group and allied laboratories exploring the possibility of dissociating Δ9-tetrahydrocannabinol’s (THC) beneficial effects from its detrimental effects by using an optimized TM peptide disruptor to alter the CB1R-5HT2AR complex, i.e., the heterodimer formed by the cannabinoid CB1 and serotonin 5HT2A receptors that is responsible for the undesirable cognitive impairment [40].

In order to identify the functional properties of the CB1R-5HT2AR heterodimer, we first used synthetic peptides reproducing the entire amino acid sequences of the CB1R TM5 and TM6 helices, fused to a cell-penetrating sequence derived from HIV-Tat, to disturb the formation of the heteromer [96]. These peptides were confirmed to be able
to interfere with the CB1R-5HT2A/R complex, both in vitro (BiFC, cAMP assay and p-ERK1/2 signalling) and in vivo (hot plate test and novel object recognition tests, after ICV administration in mice), allowing the selective activation of CB1R by THC [96]. However, their poor pharmacological profiles (i.e., long size, rapid proteolytic digestion, no trans-BBB permeability) were major drawbacks vis-à-vis any medical application. Therefore, with the goal in mind of preserving analgesic properties while minimizing cognitive side effects, our next step was to improve TM5 and TM6 peptide druggability by a combined effort aimed at reducing toxicity, prolonging serum half-life, avoiding immunogenicity, achieving BBB permeability and generally enhancing bioavailability, with oral activity as the final goal [96] (Figure 3).

![Figure 3](https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00484 (accessed on 27 December 2021). Further permissions related to the material excerpted should be directed to the American Chemical Society).

Figure 3. Development of a downsized, protease-resistant, orally available peptide compound, fused to an enhanced BBB-crossing CPP, which restricts the CB1R-5HT2A/R heterodimer formation correlated to THC’s unwanted effects. (Reproduced with permission from [96]. American Chemical Society, 2021. Copyright © 2021 American Chemical Society, https://pubs.acs.org/doi/10.1021/acs.jmedchem.1c00484 (accessed on 27 December 2021). Further permissions related to the material excerpted should be directed to the American Chemical Society).

After in silico identification of hotspots (i.e., close contact residues) in the CB1R-5HT2A/R heterodimer interfaces, streamlined versions of the TM5 and TM6 peptides, i.e., containing mainly those amino acid residues predicted as involved in the interaction domain, were designed, synthesized and assayed, again juxtaposed to a CPP shuttle sequence (several size-optimized options tested). The results indicate that: (i) the downsized versions were as efficient as the original TM sequences in disrupting the CB1R-5HT2A/R heteromer, and (ii) the previously used HIV-Tat CPP motif could be replaced, without loss in disruptive capacity, by a BBB shuttle peptide (BBBnP) sequence that ensured bioavailability of the novel analogs into their brain target [96].

In a last optimization round, candidates with reversed (i.e., non-natural D-residue) chirality were generated as the retro-enantiomeric versions of the previous, shortened interfering peptides, again fused to a BBBnP motif also made up of D-residues. As mentioned earlier, by virtue of the retro-enantiomeric approach the spatial orientation of the TM peptide side chains, hence the global shape, roughly resembles the canonical versions, and in tune with this, remains able to disturb the CB1R-5HT2A/R heterodimer (in the BiFC assay). In addition, the BBBnP tag ensured efficient crossing of an in vitro BBB model and, given the all-D configuration, the peptide turned out to be highly resistant to serum proteases [96].

The last stage in our approach was evaluation in vivo: the two best-performing candidates were given intravenously to mice, co-administered with THC. While analgesic effects were observed for both peptides, only one of them was able to prevent THC-induced
cognitive impairment. This peptide, wliymyayvaGilkrw (lower case one-letter notation for D-residues), was additionally shown to be orally available and non-immunogenic and is hence regarded as a highly promising lead in therapeutic approaches involving cannabinoid pain management without undesirable cognitive side effects [96].

3.3. TM Peptide Restricting A2A R-A2A R Dimer for CNS Disorders

Several functional G-protein heterodimers (Table 2, entries 1–7) and heterotrimers (Table 2, entries 8–10) with other GPCRs have been observed and described for the adenosine A2A receptor (A2A,R), playing significant roles in neurodegenerative diseases or drug abuse. Apart from heteromerization, A2A,R homodimerization is well-recognized and raising attention as A2A-R-A2A,R homodimer inhibition has been recently reported to be involved in CNS disorders [97].

Table 2. A2A,R complexes with other GPCRs and their implications.

| Heteromer     | Ligand                              | Implication                                      | Ref.  |
|---------------|-------------------------------------|--------------------------------------------------|-------|
| A1 R-A2A R    | Caffein (A1 R, A2 R antagonist)     | Drug tolerance                                   | [98]  |
| A2A R-D2 R    | A2 A,R antagonists, D2 R agonists   | Parkinson’s disease, schizophrenia, drug addiction | [99,100] |
| D3 R-A2A R    | CGS-21680 (A2A,R agonist)          | Schizophrenia                                    | [101] |
| CB1 R-A2A R   | CBD (CB1 R agonist)                | Cognitive impairment                             | [102,103] |
| A2A R-mGlu5 R | CHPG (mGlu5 R agonist)             | Parkinson’s disease                              | [23]  |
| A2A R-H3 R    | RAMH (H3 R agonist)                | Autism, obsessive and compulsive disorder        | [104] |
| A2A R-5HT1A R | CGS 21,680 (A2A,R agonist), 8-OH-DPAT (5HT1A R agonist), SCH 58,216 (A2A,R antagonist), methysergide (5HT1A R antagonist) | Dyskinesia                                       | [105] |
| A2A R-D2 R-mGlu5 R | A2 A,R antagonists, D2 R agonists, D2 R antagonists, mGlu5 R agonists | Psychosis, Parkinson’s disease, drug abuse | [106] |
| CB1 R-A2A R-D2 R | TBD                                 | Endocannabinoid modulation                       | [107] |
| A2A R-D2 R-NMDAR | α-synuclein                           | Neurodegeneration, neuroinflammation             | [108] |

Abbreviations: 5HT1A R, serotonin receptor type 1 A; 5HT2A R, serotonin receptor type 2 A; A1 R, adenosine receptor type 1; 8-OH-DPAT, 8-Hydroxy-2-(di-n-propylamino)tetralin; A2A R, adenosine receptor type 2A; CBD, cannabinoid; CB1 R, cannabinoid receptor type 1; CHPG, (R,S)-2-chloro-5-hydroxyphenylglycine; CG6-21680, 4-[2-[6-amino-9(N-ethyl-beta-d-ribofuranuronaminoamidosyl)-9H-purin-2-yl]aminojethyl]benzenepranoic acid; D2 R, dopamine receptor type 2; D3 R, dopamine receptor type 3; mGlu5 R, metabotropic glutamate receptor type 3; H3 R, histamine receptor type 3; NMDAR, N-metil-D-aspartate receptor; RAMH, (R)-alpha)-(−)-methylhistamine dihydrobromide; SCH 5826, [5-amino-7-(2-phenylethyl)-2-(2-furyl)-pyrazolo[4,3-c]1,2,4-triazolo[1,5-c]pyrimidine]; TBD, to be determined.

One strategy to explore A2A,R-A2A,R homodimer expression and its impact on brain disorders would consist, as in previous accounts above, in interfering in their formation by means of synthetic peptides replicating TM domains involved in helix–helix interactions. Thus, our laboratory recently ventured to assess A2A,R- A2A,R homodimer functionality using a peptide comprising the A2A,R TM5 sequence, involved in the homodimer interface, fused to a linear HIV-Tat (47–57) CPP. Unfortunately, the outcome was unclear due to the fast in vivo degradation of the peptide.

Different strategies to boost CPP protease resistance while preserving cell penetration efficiency have been developed [109]. One noteworthy approach is that afforded by cyclic versions of CPPs (CCPPs for short) such as cyclo Tat [110] or CPP12 [111]. These novel platforms have shown superior translocation ability over the linear versions and, importantly, a higher imperviousness to protease degradation. Based on such precedents, we set out to develop a construct where the TM5 peptide disruptor was linked to a cyclic Tat-like construct (Figure 4), with a view of ensuring an extended lifespan that might prove
useful for future in vivo A2AR-A2AR homodimer studies. The hypothesis proved correct, and the CCPP afforded higher A2AR-A2AR disrupting capability than the linear HIV-Tat counterpart. Moreover, by including non-natural amino acids, the desired high proteolytic stability under physiological conditions was also achieved [97].

![Diagram of A2AR-A2AR complex disruptor](image)

**Figure 4.** Design of an efficient A2AR-A2AR complex disruptor by combining a homodimerization-blocking sequence with a modified CCPP with improved pharmacokinetics properties (Figure adapted from [97]. MDPI, 2019). The Tat-like construct combines both L- and D-amino acids, and a side-chain-to-side-chain cyclization between the ε--amino group of the N-terminal Lys and the γ-carboxyl of C-terminal Glu (marked with an asterisk).

4. Conclusions

G protein-coupled receptors are preeminent among drug targets and, given the intensive research efforts devoted to them, are arguably likely to remain valuable sources of future pharmaceutical leads with important therapeutic indications. In this context, the emerging evidence of naturally existing GPCR oligomers has upended the conventional wisdom of the GPCR monomer as functional unit, opening up new horizons for pharmacological intervention. However, in contrast to well-studied GPCR monomer transduction, the mechanisms of GPCR oligomerization and, more importantly, their implication in health or disease, remain in most cases only partially elucidated and need to be studied further.

Several approaches have been made to gain insights into the functional relevance of GPCR oligomerization. For example, bivalent ligands that simultaneously bind the two physically interacting GPCRs orthosteric sites can be used as valuable pharmacological tools to study the quaternary structure of receptor dimers. Alternatively, mutant receptors that do not dimerize can be applied to probe the role of oligomers in the modulation of
signal transduction. In this scenario, GPCR complex disruption by peptides reproducing TM sequences involved in dimer interfaces is increasingly recognized as a fruitful method for GPCR oligomer functional exploration, thus holding significant promise for the rational design of new GPCR-homing peptide drugs.

Despite their undisputed potential in medicinal applications, naturally occurring peptides have long been singled out as therapeutically problematic for intrinsic weaknesses such as low bioavailability and/or short in vivo half-life. To redress these shortcomings, an extensive array of structural modifications including C- or N-terminal truncation, use of non-natural amino acids, PEGylation or different cyclization tactics, to name just a few, have been successfully deployed over the years to fulfil the switch from natural (vulnerable) to engineered (drug-like), best-performing peptide leads. In the specific realm of TM peptides targeting GPCR oligomers, increasing research shows how, contrary to deeply rooted prejudice, educated structural elaboration of an initially naive sequence can enact its evolution into a full-fledged medicinal peptide entity. The first example of such transition shows that the analgesic properties of cannabinoids can be exploited while keeping their adverse side reactions (i.e., psychoactive effects and cognitive impairment) at bay by co-administration with a novel optimized CB1-R-5HT2A-R heterodimer-disrupting peptide. Such an approach has predictable potential in alleviating the plight of patients undergoing chronic pain medication. In this study, inclusion in the late-stage candidate of a substantially engineered CPP tag has turned out to be decisive, by endowing the peptide with BBB-crossing properties and hence access to brain cells where it can productively disrupt the CB1-R-5HT2A-R heterodimer. Similarly, an A2A-R homodimer-disrupting construct, efficiently delivered by a protease-resistant cyclic CPP, was designed and produced for in vivo studies on the A2A-R-A2A-R homodimer implication in CNS disorders, including schizophrenia and Parkinson’s disease. Understanding its CNS delivery may pave the way to eventually harnessing some of the most challenging problems faced in the treatment of neurodegeneration. These encouraging developments are to be viewed as the first fruits in the quest for GPCR complex-disrupting agents, a field holding undoubtable promise for therapeutic application but where much work remains to be done.

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