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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

Amy Hauck Newman[#†,*], **Francisco O. Battiti**[#], **Alessandro Bonifazi**[#]

Medicinal Chemistry Section, Molecular Targets and Medications Discovery Branch, National Institute on Drug Abuse – Intramural Research Program, National Institutes of Health, 333 Cassell Drive, Baltimore, Maryland 21224, United States

[#] These authors contributed equally to this work.

Abstract

The genesis of designing bivalent or bitopic molecules that engender unique pharmacological properties began with Portoghese's work directed toward opioid receptors, in the early 1980's. This strategy has evolved as an attractive way to engineer highly selective compounds for targeted G-protein coupled receptors (GPCRs) with optimized efficacies and/or signaling bias. The emergence of X-ray crystal structures of many GPCRs and the identification of both orthosteric and allosteric binding sites have provided further guidance to ligand drug design that includes a primary pharmacophore (PP), a secondary pharmacophore (SP) and a linker between them. It is critical to note the synergistic relationship among all three of these components as they contribute to the overall interaction of these molecules with their receptor proteins, and that strategically designed combinations have and will continue to provide the GPCR molecular tools of the future.

Graphical Abstract

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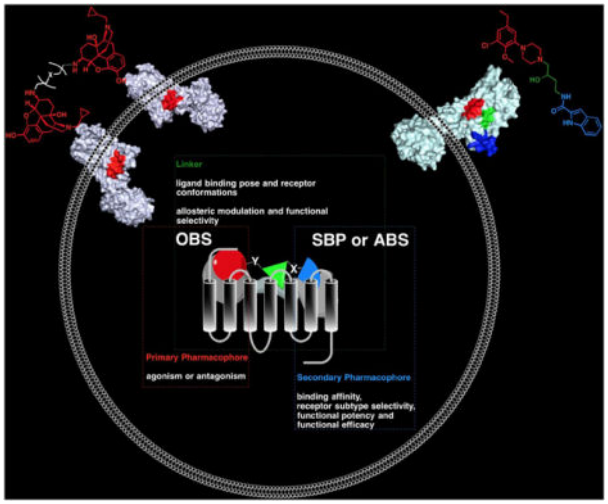
[*]Corresponding author: Amy H. Newman: Phone: (443)-740-2887. Fax: (443)-740-2111. anewman@intra.nida.nih.gov.



INTRODUCTION: BIVALENT LIGANDS

Bivalent ligands directed toward opioid receptors were first introduced into the literature by none other than Phil Portoghese, in 1982.[1] These molecules were defined as having two pharmacophores linked by a “connecting chain” or “spanner” which in this Perspective will be referred to as a “linker”.[2] Specifically, these pharmacophores are what we now designate as primary pharmacophores (PP), with each terminus targeting the orthosteric binding site (OBS) of the targeted receptor – in this early case, opioid receptors. Portoghese posited that these bivalent molecules would exhibit increased potency over the monovalent analogue due to a gain in entropic energy provided the linker was sufficiently long to reach the OBS of two adjacent opioid receptors (Figure 1). Further, it was envisioned that the linker length could serve as a molecular yardstick to determine the distance between these two sites when bound by the bivalent ligand.

In this Perspective we will not attempt to review every bivalent or bitopic ligand designed and investigated, but rather start with a historical view of how the pioneering efforts of Portoghese have influenced an enormous and exciting field of drug design. We will provide the rationale for designing bivalent and/or bitopic molecules and hone into how these early discoveries have influenced our own drug design on dopamine D_2-like compounds. Specifically, we will discuss the importance and influence of the PP, the secondary pharmacophore (SP) and the linker between them. Ultimately, we will propose that it is the fine-tuned design of the entire molecule, which has ultimately lead to remarkable tools that have helped shape our thinking regarding G-protein coupled receptor (GPCR) structure and function and will undoubtedly inform the next generation of therapeutic agents.

Of note, for the purpose of this perspective, the term “bivalent” molecule will refer to a molecule that has a PP on both ends of a linker that will vary in size/length and may be rigid or flexible. It is worth noting that the PPs maybe be identical (homobivalent) or distinct (heterobivalent). In contrast, “bitopic” molecules consist of a PP and a SP that is structurally different and is expected to interact with a secondary binding pocket (SBP) that results in a unique pharmacological profile of the molecule, either through receptor selectivity,

functional selectivity and/or allostery. The PP and SP are connected by a linker of defined length and molecular composition. The role of the linker once was considered to only keep the PPs, in the case of bivalent molecules, or the PP and the SP, in the case of bitopic molecules, at a specific distance from one another. However, herein we propose that the linker not only plays a critical role in the proximity of the two pharmacophores, but its chemical composition is also fundamental in the overall pharmacological profile of the molecule.

History: the Portoghese opioid receptor bivalent ligand legacy

The initial work on bivalent molecules directed toward opioid receptors was done on a 6-amino-derivative of naltrexone (**1**), naltrexamine (**2**), and varying length polyethyleneglycol linkers.[1] Using the guinea pig ileum and mouse vas deferens models as measures of "narcotic antagonistic activity" against the opioid agonists morphine (mu), ethylketocyclazocine (kappa) or DADLE (delta), the authors concluded that the potencies they measured with two of the bivalent ligands exceeded those of the monovalent ligands and were dependent on the "spanner" length.[1] Subsequently, Portoghese expanded this idea to agonists with different linkers and began to see significant differences in potencies and efficacies.[3–5] It must be noted that these compounds were designed before the concepts of receptor oligomers had been proposed as functional units. Clearly, over time the conceptual framework has evolved, but the practice of using bivalent (and bitopic) molecules to tweak affinities and efficacies remains relevant.

Early on, the opioid receptor subtypes (mu, kappa, delta) posed a medicinal chemistry challenge of determining structure-activity relationships (SAR) that could be delineated between receptor subtypes, so as to synthesize highly subtype selective ligands. One approach to this challenge was to design bivalent, non-peptide opioid antagonists.[6] The concept that high receptor subtype selectivity could be achieved with bivalent molecules was predicated on a critical role of the linker. Even before data emerged supporting the existence of homomers (sometimes referred to as homodimers and existing as two copies of the same protein that are functionally linked) or heteromers (sometimes referred to as heterodimers and consist of one copy of two different proteins that are functionally linked), Portoghese hypothesized that close spatial arrangements of neighboring OBSs could be bridged with the binding of a bivalent ligand, and when these vicinal sites were both bound by the same ligand, affinity would be improved (Figure 1). Indeed, a second potential way of binding would be where one terminus binds to the OBS and the other terminus, despite being structurally a PP, binds to a secondary site, within the same receptor, which would also convey higher affinity binding due to additional contact points. The clever approach to testing this hypothesis was to synthesize two bivalent ligands for comparison, one with both termini having the active (−)-stereochemistry, one with an active (−)-**1** terminus and one inactive (+)-**1** terminus. Although the latter molecule had higher affinity for the mu opioid receptors than the monovalent compound, it was 1/30th as active as the (−)/(−) bivalent ligand, suggesting that the (+)-terminus was interacting with another site, which conveyed favorable interactions, but was not as favorable as the (−)/(−) bivalent molecule. At this time, another scenario was not contemplated and that is if one terminus binds to the OBS, by virtue of being tethered, the second terminus is in close proximity to the OBS, so that when

one pharmacophore dissociates from the receptor, the other could readily bind. In this way, affinity for the receptor in the (−)/(−) bivalent molecule would also be improved entropically (Figure 1).

Interestingly, when (−)-**1** was fused to another (−)-**1** via a pyrrole ring to give norbinaltorphimine (**3**; Figure 1), a highly potent and selective kappa receptor antagonist resulted.[6] Cleverly, the meso isomer was also synthesized, in which (−)-**1** was fused identically with (+)-**1**, and discovered to be 5 times as potent as **3** (norbinaltorphimine) in the smooth muscle preparations, suggesting that both termini were not binding to vicinal receptors, but rather the whole molecule that combined both termini with the rigid linker conferred a highly selective and potent kappa antagonist.[7–8] It was concluded that one end binds to what we now refer to as the OBS and the other end binds to an SBP that is unique to kappa receptors. Portoghese suggested that this model resembled the “message-address” concept first presented by Schwyzer[9] wherein the “message” is the PP, binding to the OBS, whereas the “address” is the SP and confers additional binding interactions that can improve affinity and selectivity for the target kappa opioid receptor. He went on to discover naltrindole (**4**) (Figure 1), a highly selective and potent bivalent delta antagonist.[10] Remarkably, these tools are still used today.

BIVALENT MOLECULES FOR OTHER GPCRs TO STUDY HOMOMERS AND HETEROMERS

GPCR oligomers, in the form of dimers (homomers or heteromers) have been proposed as the “actual targets” of neurotransmitters and molecules that bind to the OBS.[11] Although biochemical evidence suggested the existence of receptor dimers, it was not until the 1990’s that this concept gained traction,[12] well after the first bivalent ligands were described. Indeed, the first opioid receptor dimerization was described for the delta opioid receptor subtype in 1997[13] and later confirmed using time resolved fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET) techniques.[14] Shortly after the Devi group described delta opioid receptor dimers, they discovered that heteromers consisting of delta and kappa opioid receptors form functional heteromers, opening a whole new vista for GPCR research,[15] but also the possibility that bivalent molecules could be designed to specifically target receptor heteromers that would only exist in discrete brain regions, hence potentially affording site-directed therapeutic agents.[16] The notable success of Portoghese’s pioneering bivalent approach with the opioid receptors prompted multiple efforts to find the applicability of these bivalent molecules in various different GPCR systems. GPCRs functioning in these oligomeric states produce distinct signaling responses from the monomers alone,[17] consequently bivalent molecules have provided powerful molecular tools for the study of these GPCRs.[18–19]

Following Portoghese’s footsteps, LeBoulluec *et al.* reported the first bivalent ligands targeting serotonin (5-HT) receptors using the 5-carboxamidoindole moiety as the PP in a series of homobivalent ligands as early as 1995, reporting increased affinity towards the 5-HT_{1A} and 5-HT_{1D} receptors and increased selectivity for the 5-HT_{1D} receptor over the PP alone.[20] At this time the improved affinity was not attributed to the targeting of receptor

dimers, likely due to the lack of evidence for GPCR dimerization available at the time. These efforts were quickly driven forward by Halazy *et al.* to expand the use of bivalent molecules in the study of the serotonergic system[21] developing a vast array of high affinity selective ligands. Since then, evidence of oligomerization among the 5-HT receptors has become increasingly available[22] leading to increased efforts toward discovering novel bivalent ligands with increased subtype selectivity capable of discriminating among receptor subtypes.[23–29] Likewise, bivalent ligands have also played a prominent role in the study of cannabinoid receptors (CB1/2), starting with the efforts of Zhang *et al.*[30] a large library of bivalent ligands have been developed to study homo- and heteromers formed by the cannabinoid receptors.[31–33] Despite remaining unclear whether current bivalent ligands of the cannabinoid receptor system do indeed simultaneously bind two receptors within a dimer as postulated,[34] bivalent ligands remain attractive tools in the study of these receptor complexes.[35–38]

Heteromers of neurotransmitter receptors are thought to play an important role in neurotransmission within the central nervous system (CNS),[39–40] as a result, these can be potential targets for a variety of therapeutic applications. Indeed, the prevalence of heteromers within the adrenergic receptor systems[41–44] make them interesting targets for the treatment of various cardiovascular ailments.[45–47] For example, Karellas *et al.* introduced heterobivalent molecules targeting beta2-adrenergic receptor/adenosine A_1 receptor (β2AR/A_1AR) heteromers in an effort to understand the cross talk among these two cellular cAMP regulating receptors;[48] it was proposed that careful balance between the signaling pathways generated by the two receptor monomers conforming this heteromer could aid in the prevention of certain cardiac arrhythmias.[47] Due to the importance of functions regulated by this receptor system, it has become the focus of significant analysis via heterobivalent ligands.[49]

The study of dopamine receptor heteromers with the use of heterobivalent ligands is equally promising. Adenosine A_{2A} receptor (A_{2A}R) and dopamine D_2 receptor heteromers (D_2R/A_{2A}R) are of particular interest due to their purported role in Parkinson's Disease.[50–51] Indeed, the potential of dopamine receptor agonists as agents to treat Parkinson's disease is well documented.[52] Moreover, it has been hypothesized that antagonism at A_{2A}R in conjunction with agonism at the D_2R has the potential to increase the therapeutic utility of the D_2R agonists by counteracting some of the common side effects and helping prevent disease progression.[53–55] As a result, significant focus has been directed at heterobivalent ligands that can target these heteromers. For example, Soriano *et al.* reported the synthesis of heterobivalent ligands utilizing XCC (2-(4-(2,6-dioxo-1,3-dipropyl-2,3,4,5,6,7-hexahydro-1*H*-purin-8-yl)phenoxy)acetic acid) as the A_{2A}R antagonist pharmacophore and (±)-PPHT-NH_2 (6-((4-aminophenethyl)(propyl)amino)-5,6,7,8-tetrahydronaphthalen-1-ol) as the D_2R agonist.[56] Jörg *et al.* later reported excellent affinities in a series of bivalent ligands containing the canonical ropinirole as the D_2R agonist and ZM 241385 (4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol) as A_{2A}R antagonist.[57] Similar efforts have been aimed at targeting Adenosine A_1 receptor-dopamine D_1 receptor (A_1R-D_1R) heteromers.[58] More recently heterobivalent ligands were used to target D_2R and neurotensin NTS1 heteromers, with affinities up to three orders of magnitude higher and selectivity greater than seen in cells expressing solely D_2R,[59] as well as the dopamine D_2R-

metabotropic glutamate 5 (D_2R/mGluR5) heteromer,[60] yet another testament to the potential of heterobivalent ligands in the study of heteromers.

Due to the extensive amount of homo- and heteromers observed among dopamine receptors,[61–64] many of which are directly linked to key roles in the pathology of substance use disorders,[62, 65–67] significant effort has been directed toward developing bivalent ligands that effectively target dopamine receptor oligomers selectively. Several research groups have focused on the synthesis of bivalent ligands to explore the pharmacological implications of targeting dopamine receptor dimers. The Gmeiner group has published several papers reporting the synthesis of a variety of bivalent probes, connecting two agonist or antagonist PPs, binding in the OBS with polyethylene glycol or polymethylene unit linkers. Their main goal was to study receptor dimerization and target dopamine receptor homo- and heteromers to evaluate their role in schizophrenia and psychotic behaviors.[68–71] Despite the high potential of this drug design, it still presents several limitations, especially in the ability to fully understand the binding modes and kinetics of these highly flexible and high molecular weight compounds. Moreover, when designing these bivalent ligands targeting receptor dimers, the optimum length for the linkers becomes even more important, in order to be able to effectively target a dimer entity and minimize the “flip-flop” binding mechanism where only the PP or the SP binds alternatively to the OBS or allosteric binding site (ABS), within monomers. Interest in the study of the D_2-D_2 dimer has led to various other efforts in developing bivalent ligands,[72] which can target this homomer, as also seen in Gogoi *et al.* with the synthesis of a homobivalent ligands using 5-OH-DPAT as the PP,[73] and McRobb *et al.* who report a clozapine based homobivalent ligand, which results in increased affinity and functional potency over the monomer parent drug.[74]

BITOPIC LIGANDS: MOLECULES TO STUDY ALLOSTERISM AND FUNCTIONAL BIAS OF GPCRS

General Drug Design and Pharmacological Implications

GPCRs are one of the most targeted family of receptors for drug design and development of novel therapeutics and pharmacological tools. GPCRs can be activated, and their signaling modulated, by several endogenous neurotransmitters, as well as synthetic ligands. Their predominant role in regulation of neurological and physiological processes, centrally or peripherally mediated, make them interesting targets for studying signaling mechanisms in cellular processes. Clearly, improving our understanding of the mechanisms underlying these signaling processes can lead to the development of safer and more specifically directed therapeutics. Roth and colleagues[75] recently summarized how advances in molecular pharmacology are expanding the common knowledge of GPCRs. Combination of molecular studies and receptor pharmacology are shedding light on unique mechanistic consequences of structurally, chemically and functionally specific ligand-receptor interactions from carefully designed synthetic molecules. Over the last decade, the Christopoulos research group has studied the concept of GPCRs as “dynamic proteins” and has investigated the implications of multiple receptor active-states linked to distinct functional responses and pathophysiological outcomes.[76]

Thanks to the recent advances in X-ray crystallographic techniques and cryo-microscopy technology, the number of GPCR structures being resolved is exponentially increasing. Advanced cryo-microscopy is pushing the boundaries of GPCR structural studies, allowing visualization of protein conformational changes upon ligand binding, and how those adjustments can impact the ability to recruit/activate second messengers. Indeed, GPCRs are known to possess multiple binding sites,[76] with the OBS being the most targeted for development of agonists or antagonists alike.[77] However, allosteric ligands, binding at spatially distinct and less structurally conserved sites, can also stabilize unique receptor states, and exploit key pharmacological responses modulating the functional profiles of orthosteric ligands. GPCR allostery, or more generally targeting SBPs within receptors, is becoming the key to enhance receptor-subtype specificity, study the kinetic processes behind ligand-receptor interactions, and understand receptor functional cooperativity.

Improved understanding of GPCR functional states, drug development guided by computational chemistry, structure-activity and structure-function relationship studies (SAR and SFR), combined with advances in analytical pharmacology models are “bridging the gap”[77] towards the design of complex bitopic molecules.[78] Bitopic orthosteric-allosteric ligands can be seen as a natural extension of the classic bivalent drug design approach. Linking PPs that bind to the OBS, with SPs that bind to an allosteric binding site (ABS), or more generally an SBP, merge and combine the pharmacological properties of otherwise independent ligands in a new “hybrid” molecule with a unique pharmacological profile (Figure 2).

The main pharmacological consequences of bitopic ligand effects are not just an increased affinity and selectivity for the target receptor, but often their highly specific binding interactions translate into biased stimuli (functional selectivity), positive or negative allosteric modulation or cooperativity. Biased agonism or functional selectivity, is defined as the ability of a ligand to activate only discrete functional pathways associated with receptor signaling machinery, as a consequence of uniquely induced protein conformations. This can be seen as a process starting with the bitopic ligand concomitant interactions with the OBS and SBP, followed by structural changes in the protein conformation, leading to more favorable recruitment of either G-proteins or β-arrestins, thereby enacting a functionally selective signaling response. Christopoulos defines the phenomenon of biased signaling as an extension of the allosteric nature of all the GPCRs seen as “signaling machines”.[78] Particularly, he describes how the binding of extracellular ligands “transfers” structural information to distinct intracellular sites, which then are responsible for propagating functional responses through specific signaling proteins.

Developing agonists that selectively activate a GPCR signaling pathway over another is essential to identifying which signaling cascade might be responsible for altered physiological responses and drug “on-target” side effects, and consequently how to develop safer treatments by minimizing these undesired responses. Due to their complex structural nature, the study of the pharmacological effects of bitopic ligands and the meaningful evaluation of their bias selectivity is still a significant challenge. In order to assess functional selectivity, drug-elicited effects need to be tested in multiple parallel functional assays for the GPCR target of interest, and the results compared with reference agonists to properly

perform bias factor analyses, taking into account assay sensitivities and receptor expression levels. Lane has been at the forefront of developing mathematical models for cooperativity and operational models for functional selectivity studies.[79–82] He has described several analytical considerations to be taken into account when working with bitopic ligands: in particular, he describes how bitopic ligands binding can occur via a “flip-flop” mechanism (a mechanism almost impossible to identify in normal experimental conditions). However, when the bitopic ligands engage the receptor simultaneously in OBS and ABS/SBP, we should expect cooperative binding, with the strength of equilibrium constant and functional responses different compared to a pure allosteric or orthosteric binding mode. From our work, and extensive characterization of our dopaminergic bitopic ligand library, it is evident how a bitopic interaction mode induces functional responses, binding affinities and subtype receptor selectivity significantly different from the PP, SP, or allosteric modulators, when tested alone.[83–87]

Though in this Perspective we will primarily focus on the development of bitopic ligands for the dopaminergic system, this strategy has played an important role in the development of ligands for a variety of GPCRs. Among the first receptor systems to be studied with bitopic molecules were the muscarinic acetylcholine receptors. The extensive body of literature detailing the allosteric pharmacology of these receptors[88] make them suitable targets for rational bitopic ligand design, furthermore, they pose an attractive target for medicinal chemists due to the pivotal role they play in multiple body functions and their distribution throughout the nervous system.[89–91]

One of the first muscarinic bitopic molecules reported is methoctramine, a polymethylene tetraamine first reported by Melchiorre *et al.*[92] as a selective cardiac M2 receptor antagonist. A homobivalent ligand with two 2-methoxybenzylamine moieties at either end of an extended linker, the ligand was found to be a potent M2 antagonist as a result of its bitopic binding mode, binding both to the OBS as well as an ABS in the outer loop of the receptor.[93] Over the last three decades, a remarkable increase in bitopic ligands with ever improving affinities and selectivities, ranging from agonists,[94–95] partial agonists[96–98] and antagonists[99–103] have been developed to study the family of muscarinic receptors.

Another receptor system gaining attention in recent years are the sphingosine-1-phosphate GPCRs ($S1P_1$ through $S1P_5$), largely due to the relevant role they play in the regulation of the cardiovascular, lymphatic, and nervous systems.[104] They are considered essential to key features of vascular development, maintenance of vasculature, and lymphocyte migration.[105] As a result, this receptor system has been an attractive target for the treatment of a variety of ailments ranging from autoimmune and cardiovascular pathologies to cancer and inflammatory diseases.[104, 106] Achieving ligands with high degrees of subtype selectivity among these receptors has been a challenge due to the significant homogeneity shared among them.[107] However, in an effort to develop subtype selective ligands capable of elucidating the different functions of the S1P receptors, Kohno *et al.*[108] synthesized multiple bitopic ligands among which SPM-242 ((*S*)-2-amino-4-(2-chloro-4-((3-hydroxyphenyl)thio)phenyl)-1-hydroxybutan-2-yl-dihydrogen phosphate), containing a phosphate moiety to recognize the OBS and a 2-[(3-chlorophenyl)thio]-phenol secondary pharmacophore, has raised considerable interest. Biological evaluation of this lead molecule

showed it functions via a bitopic binding mode and is a selective antagonist for the $S1P_3$ subtype.[107] Chemical manipulation of this scaffold, reducing the 2-hydroxymethyl moiety in the linker to an ethyl chain and *ortho*-trifluoromethylation of the phenol in the secondary group, engendered ligand SPM-354 ((*S*)-2-amino-4-[2-chloro-4-(2-hydroxy-5-trifluoromethylphenylthio)phenyl] -2-propylbutylphosphoric acid monoester) which exhibited significantly increased selectivity for $S1P_3$ over other subtypes relative to parent molecule, improved potency and efficacy as well as improved metabolic stability.[109] The results observed by these bitopic molecules were unparalleled by hitherto available S1P receptor ligands and provide an avenue for the study of the $S1P_3$ receptor subtype and its role in *in vivo* cardiovascular function.[109]

The adenosine receptor (AR) tells another success story for bitopic ligands. As mentioned earlier, the adenosine receptors have long been an attractive target for medicinal chemists due to their active role in mediating a variety of biological processes. The A_1AR receptor plays an important physiological role in the prevention of ischemia/reperfusion damage to heart tissue,[110] and while adenosine receptor agonists have shown promising results as treatments[111–112] their use as clinical tools has been encumbered by numerous deleterious side effects at pharmacologically active doses, the most commonly cited being bradycardia.[113] It was consequently postulated that a rationally designed biased agonist may favor activation of the cellular pathway that promotes cardio-protection while avoiding stimulation of the pathways which may lead to adverse side effects. Valant *et al.* reported the first A_1AR receptor bitopic biased agonist, VCP746 (5-amino-4-benzoyl-*N*-(6-((9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-6-yl)amino)hexyl)-3-(3-(trifluoromethyl)phenyl)thiophene-2-carboxamide) constructed from adenosine (as primary pharmacophore) and VCP171 ((2-amino-4-(3-(trifluoromethyl)phenyl)thiophen-3-yl)(phenyl)methanone), a known AR positive allosteric modulator.[114] The resulting bitopic molecule was found to induce the cardioprotective effects of AR agonism while not inducing bradycardia; agonism at the A_1AR biased away from calcium mobilization relative to all other pathways contributes to prevention of the cytotoxic levels of calcium observed during ischemia/reperfusion injury events.[115] This ligand effectively activates the therapeutic pathways of the A_1AR receptor while remaining inactive towards the pathways responsible for the undesired side effects. Following the original report, a series of strategically designed bitopic biased ligands has provided additional tools for medicinal chemists to further investigate the effect of the A_1AR downstream pathways.[116]

Dopamine receptor bitopic ligands: application to receptor selectivity, allostery and functional bias

Dopamine receptors, particularly the D_2-like family that includes subtypes D_2R, D_3R and D_4R, are some of the most studied and targeted GPCRs. They are implicated in many physiological processes such as reward, addiction, locomotion and movement coordination, as well as metabolism and hormonal regulation.[117] They all share high homology in their OBS, making the development of selective agonists/antagonists a major challenge. The crystal structures for the D_3R was resolved in 2010 in complex with the D_2R/D_3R antagonist/inverse agonist, eticlopride (**5**) and has significantly guided the effort towards the design of more selective D_3R antagonists.[118] More recently, in 2017 and 2018, the Roth

research group crystallized and resolved the structures of D_2R and D_4R in complex with risperidone and nemonapride, respectively.[119–120] These findings have illuminated the dopamine D_2-like receptor structures and enabled structure-based campaigns for the design and synthesis of novel D_2-like subtype selective ligands.[121–122]

In addition to developing SAR for the optimization of the PP and SP in our drug design, we also focus on the most understudied component of bitopic molecules, which we will highlight in this perspective: the linker. Often seen as a mere connecting portion of the molecule, we have pushed our drug design in the direction of evaluating linkers with different length, substituents, polarity, rigidity and chirality. We have demonstrated how properly designed linkers can create specific interactions with amino acid residues and guide the favorable (or unfavorable) binding poses of the ligands, inducing unique bias and allosteric pharmacological responses, together with unprecedented receptor-subtype affinity and selectivity. The following discussion will mainly focus on our successful drug design of highly selective bitopic dopaminergic agonists and antagonists, highlighting how the advances in molecular and structural pharmacology inspired the discovery of these new classes of ligands.

When Chien *et al.* published the D_3R crystal structure,[118] they also reported computational studies performed using our ligand, ((*R*)-PG648 (**6**) R-22; Figure 3), to show how the 2,3-dichloro-phenylpiperazine PP and the 1*H*-indole-2-carboxamide SP were binding in two distinct sites. As such, the PP recognized the OBS, and nicely overlapped with eticlopride (**5**), whereas the SP was posed in the less conserved SBP. The presence of a tractable SBP is now well established for these D_2-like receptor subtypes and can be targeted to improve affinity and subtype selectivity.[122–123] In addition, the crystal structure and subsequent molecular dynamics studies provided the first indication that a bitopic binding mode was important for the design of ligands with higher D_3R subtype affinity and selectivity. Furthermore, our attention was directed to the key role played by the linker, the 3-OH substituent in particular, whose interactions oriented the molecule towards its optimal pose. These observations prompted us toward significant efforts in designing new bitopic D_3R agonists and antagonists, exploring structural features of PP, SP and linker substitutions, leading to successful SAR campaigns in preparing highly subtype selective ligands and small molecule tools with unique pharmacological profiles.

Identification of the D_3R PP and SP leading to discovery of their bitopic nature

When we first embarked on the discovery of novel and D_3R selective antagonists,[124–127] we were frankly not thinking of these molecules as being bitopic. Based on the structures of NGB2904 (**7**)[128] and BP897 (**8**),[129] early D_3R-selective antagonists/partial agonists (Figure 3A), we first attempted to identify novel scaffolds based on fragments of these lead molecules that appeared in other compounds in our in-house library. We settled for optimizing **6**, retaining the 2,3-dichloro-phenylpiperazine motif, modifying the tricyclic terminus and investigating optimal linker length between the two. Our early efforts[124] identified key SAR for high affinity at D_3R and modest selectivity over the homologous D_2R and we and others continued to build on this scaffold in following years.[126, 130–133]

As mentioned, the advance of the D_3R crystal structure in the presence of the D_2R/D_3R antagonist/inverse agonist, eticlopride (**5**), confirmed what we had suspected: the 2,3-dichloro-phenylpiperzine served as the PP of our molecules,[118] whereas the 1*H*-indole-2-carboxamide terminus of the lead compound **6**,[134] serving as the SP, occupied a SBP that was unique to D_3R and conveyed ~400-fold D_3R selectivity over D_2R (Figure 3B). Subsequently, we deconstructed our D_3R scaffold into its "synthons" and confirmed that the PP indeed showed essentially no D_3R-selectivity over D_2R until it was linked to the SP.[135] In addition, the affinity and efficacy of this scaffold appeared to be primarily attributed to the PP, whereas the SP was responsible for D_3R selectivity. Further elucidation of the SBP identified a single Glycine (Gly98) residue in extracellular loop 1 (EL1) to be a key contributor to D_3R selectivity.[136] Importantly, we found the SP had virtually no binding affinity to the D_3R, as measured with radioligand binding assays, until it was linked to the PP. Moreover, as suggested in the D_3R crystal structure paper, the role of the 3-OH substituent in the linker became apparent in further conveying D_3R selectivity to these molecules.[136]

Role of the linker in D_3R selectivity: partial agonists/antagonists

Early on in our SAR campaign, we took a page out of the "Portoghese play book" and began to explore functionalization of the 4C-linker between the PP and SP. The possibility that these compounds were bitopic had begun to emerge, but the role of the linker besides being key to keeping the PP and SP at an optimal distance from one another had not been investigated. We discovered, for example that a *trans* olefin could replace the saturated butyl linking chain and retain high affinity D_3R binding (e.g., PG01037, (**9**) K_i= 1 nM, Figure 4) and D_3R/D_2R selectivity of >100[125, 137–138] but that a rigid alkyne was not tolerated.[137]

In order to reduce the lipophilicity of these molecules to improve their *in vivo* profiles, we began to explore the addition of an -OH group in either the 2- or 3-position of the linking chain.[134] Indeed, we discovered that these analogues were well tolerated at D_3R but notably their D_3R selectivity was further improved with the addition of the 3-OH (e.g., rac-**6**, D_3R/D_2R ~400-fold)[134] and not the 2-position (Figure 3). These observations were confirmed computationally and a Tyrosine residue on trans-membrane helix 7 (TM7) was identified as a contributor to the D_3R selectivity observed[118] providing the first clue that not only was the SP important for D_3R selectivity, but functionalization of the linker could also play a role (Figure 3, 4). Subsequent separation of the enantiomers of rac-**6** identified enantioselectivity at D_3R, without concomitant enantioselectivity for D_2R. The *R*-enantiomer, **6**, was identified as the eutomer in this series, further highlighting the importance of the linker composition and the subtle but tractable differences in these binding sites.[134] Of note, this substitution was limited, as for example acetylation[134] at the 3-position was not tolerated. Importantly, other groups subsequently showed that hydroxylation as well as other structural modifications to the linking chain were well tolerated at D_3R, in this scaffold.[139]

More recently, capitalizing on the D_3R crystal structure and incorporating it into the small molecule SAR that has been well established, we have discovered a new series of highly D_3R selective antagonists/partial agonists, namely VK4-116 (**10**) and VK4-40 (**11**) (Figure

4.)[87, 123] In this series, by simply modifying the substitution on the 4-phenylpiperazine, retaining the 3-OH linker and the privileged 1*H*-indole-2-carboxamide SP, highly D_3R selective analogues with high affinity emerged.[87] This investigation further highlights the importance of the PP, SP, and the length and composition of the linker, substantiating the concept that the "whole is greater than the sum of its parts." Importantly, **10**, one of the most D_3R selective compounds reported to date, demonstrated remarkable metabolic stability and early *in vivo* profile to support further pursuit as a lead molecule for the prevention and treatment of opioid use disorder, which will be described in further detail below.

Given fluorine (-F) is often regarded as a bio-isostere to a hydroxyl (OH) group[140–142] we went on to explore the linker substitution with a 3-F (Figure 3C). This SAR campaign led to some of the most D_3R selective ligands reported at that time[143–144] and we have further pursued these leads to discover new and improved molecules for *in vivo* study.[145] Importantly, we determined that the amide linked heterocyclic terminus was critical for this scaffold to bind to D_3R, as reducing this to the amine, significantly decreased D_3R binding affinity.[143] However, we subsequently showed that replacing the amide with a triazole scaffold using click chemistry was also well-tolerated.[146] In this series, the lead compound, which shares the PP, SP and 3-OH linker with our previous lead compound rac-**6** (PG648), demonstrated D_3R K_i=6 nM with a D_3R/D_2R selectivity of 165. Importantly, other compounds in that series, which also possessed the 3-OH linker, demonstrated even more robust D_3R/D_2R selectivity, albeit at lower affinities for D_3R, a trend we have also observed in the 3-F series.[144–145]

As previously mentioned, Lane and his research group embraced this same bitopic drug design approach and have devoted extensive efforts in developing analytical pharmacology methods to fully characterize the binding and functional profiles of small molecules simultaneously binding the OBS and SBP within the same receptor. In 2015, in back to back papers, Shonberg and Mistry reported how the well-known bitopic ligand SB269652 (**12**) acts as a negative allosteric modulator of the dopamine D_2R receptor (Figure 5).[147–148] They described, for the first time, a small molecule negative allosteric modulator for D_2R engaging the receptor in a bitopic mode. They also studied how the bitopic engagement of a D_2R protomer can limit dopamine (DA) binding in the potential formation of dimers. They performed extensive SAR studies to determine the role played by each portion of the **12** bitopic molecule in its biological profile. Shonberg reported that the 1,2,3,4-tetrahydroisoquinoline PP with its basic tertiary amine is important to maintain high affinity, overall allostery and functional profiles, since it is the "head" or PP of the molecule competing with DA for the OBS. He also described the 1*H*-indole-2-carboxamide SP, as a "tail" group whose lipophilicity is a common structural feature in numerous D_2-like subtype selective bitopic ligands. Moreover, SAR based on the linker portion of the molecule provided the observation that subtle changes in the alkyl chain length and rigidity completely alter the orientation of SP causing a switch from negative allosterism to competitive antagonism. Mistry generated new SAR based on the fragmentation of the **12** structure, identifying D_2R allosteric fragments based on the 1*H*-indole-2-carboxamide SP scaffold. In this report, an allosteric pocket in the D_2R between TM2 and TM7 was discovered, which can be targeted to design novel allosteric modulator and bitopic ligands.

However, the most important conclusion from these studies is the observation that despite the role played by the single pharmacophore, in order to simultaneously bind both OBS and ABS, PP and SP need to be connected in close proximity, and perfect alignment of all the fragments by the linker is essential. These studies further highlight the importance of the linker and how structural modifications generate preferable SAR. Based on these findings Capuano, Lane and Christopoulos recently published a new generation of analogues of **12**, where changes in the 1*H*-indole-2-carboxamide SP dramatically affected functional affinity, cooperativity and overall pharmacological properties with respect to the parent molecule.[149] This underscores again how bitopic ligands are composed of interconnected structural scaffolds, and how modifications in a part of the molecule directly affects the overall structural pose within the receptor, recognition of receptor binding sites, and ultimately translation to changes within the intracellular signaling machinery.[79–80, 149]

Parallel studies from our own lab also used the **12** scaffold, but in comparison to its analogue SB277011A (**13**; Figure 4), a classic D_3R antagonist used in many *in vivo* studies that has not been shown to have any allosteric properties at either D_2R or D_3R. We set out to better understand the structural basis of these observations at D_3R, carefully deconstructing each molecule and then reconstructing novel analogues, including the addition of a cyclopropylated linker.[84] Once again, we saw that the PPs had modest binding affinities and selectivities for D_3R over D_2R and showed competitive interactions at D_3R. Likewise, the SP did not bind to the OBS at concentrations of up to 100 μM. However, depending on the length and composition of the linker, several analogues with the same PP as **12** showed allosterism at D_3R. Furthermore, these data suggest that the specific orientation of the SP within the SBP can confer noncompetitive or allosteric modes of interaction but that relatively subtle changes to structure and orientation of the SP, likely due to the composition of the linker, can cause a switch between apparently allosteric and competitive antagonism (Figure 4).[84] In addition, compound **14 (18a** from reference 84; VK4-87, Figure 4) demonstrated ~4-fold higher D_3R affinity than the parent compound (K_i=0.89 nM) and >200-fold D_3R/D_2R selectivity, further exemplifying the importance of its bitopic nature not only for functional allostery but for improving affinity and selectivity at the target receptor.[84] In contrast, compound **15 (25a** from reference 84; Figure 4) also showed negative allosterism at D_3R, but had both lower affinity and selectivity for D_3R than either the parent compound or **14**. Of note, the only difference between these two molecules is the location of the cyclopropyl group in the linker. Additional investigation of compound **14** is under way.

Role of the linker in D_3R selectivity: Agonists

As we previously described, the pharmacological effects of bitopic ligands are not limited to allosteric modulation, but growing evidence points to how specific ligand-receptor interactions, particularly regarding bitopic agonists, translate into functional selectivity or bias stimuli. Recent efforts from different research groups have converged toward the design and synthesis of D_2R/D_3R agonists to identify structural requirements necessary to selectively activate either the G-proteins or the β-arrestin signaling pathways. For example, characterization of the physiological responses associated with activation of independent GPCR pathways has been extremely successful in the opioid research field. Understanding the physiological importance of activation of the mu–opioid receptor G-protein pathway, in

comparison to the potentially undesired effects mediated by the β-arrestin pathway, is leading to the development of promising new agonists for pain management.[150–154] However, for the DA receptors, the link between signaling pathways and biological and behavioral responses remains elusive. Nevertheless, with the emerging toolbox of bitopic ligands with functionally selective profiles, promise to overcome these challenges is on the horizon.

SAR campaigns to identify highly selective D_2R or D_3R-subtype selective agonists have remained largely unsuccessful, likely due to these molecules occupying the highly homologous OBS. The use of the bitopic molecule strategy to identify functionally biased and full agonists has grown more recently. Indeed, in the past few years there have been several published studies on D_2R bitopic biased agonists selective for G-protein or β-arrestin activation. Most of the published structures rely on common scaffolds or molecular fragments often based on the partial agonist aripiprazole (**16**) or cariprazine (**17**).[155–157] Despite the availability of many new compounds, the D_2-like bias agonism research field still presents several limitations. Inconsistencies in (i) data analyses and assessment of bias factor comparisons, (ii) multiple differing functional assays, and (iii) overall medicinal chemistry has made generating highly subtype selective full agonists challenging. As such, a clear translation of small molecules *in vitro* results to *in vivo* animal models of pathophysiological behaviors has thus far been impeded. Indeed, most of the studies aimed to dissect the role of DA signaling pathways in the central nervous system *in vivo* are still relying on genetic manipulations rather than pure pharmacological approaches or a combination thereof.[158–160]

Novel and bitopic D_2R agonists based on sumanirole

In our first attempt to design selective D_2R agonists, Zou and Keck *et al.*,[161] reported initial SAR based on the D_2R/D_3R-preferential agonist, sumanirole (**18**; Figure 5) as the PP scaffold. This work was focused on understanding how regiochemical substitutions in positions 1-, 5-, and 8- of the sumanirole pharmacophore would affect its binding poses, and consequent affinity and agonist potency profiles. The initial SAR investigation built the foundation for follow up studies directed toward exploring the chemical space around the D_2R OBS, targeting the SBP, and studying ligand-receptor interactions that are mediated by the linker portion of the molecule. Zou and Keck reached the important conclusions that i) simple sumanirole *N*-1 alkylation was tolerated and produced compounds with overall reduced potency at D_3R, but not significant changes in D_2R vs. D_3R subtype selectivity, suggesting that the *N*-1 interactions at the OBS might translate in different functional activations for the highly homologous D_2R and D_3R; ii) *N*-5 substitution with the canonical *n*-butyl-arylamide linkers and SP, found in classic D_3R antagonists described above, induced significant increases in affinity and full agonist potency for the D_2R, despite having a relatively nonselective subtype binding profile; iii) it is important to use the agonist [^{3}H]-7-OH-DPAT radioligand when testing binding affinities for D_2R and D_3R agonists. Agonist competition experiments performed in the presence of an antagonist radioligand (e.g., [^{3}H]-*N*-methylspiperone) tend to underestimate affinities and dramatically shift selectivity ratios.

These initial observations on how *N*-1 and *N*-5 substitutions can be responsible for changes in either affinity or potency of new sumanirole (**18**) analogues inspired us to synthesize a new library of bitopic molecules and further characterize the structural requirements for both SP and linkers to optimally target the SBP. In 2017, Bonifazi and Yano *et al*. published the design and synthesis of complex bitopic analogues (Figure 5), as well as their functional profiles, to identify biased agonism among the possible D_2R-mediated signaling pathways analyzed.[162] In particular, they explored the structure-activity and structure-function relationships of the new analogues via bitopic substitution on **18**'s *N*-5 and/or *N*-1 position mediated by linkers with different lengths and polarities. In agreement with the literature previously described, they confirmed how bitopic interactions often translate into highly functionally selective profiles. For example, it was observed that *N*-5 alkylation led to low nanomolar affinity (K_i) at D_2R and enhanced sub-nanomolar potency (EC_{50}) toward the G-protein signaling (cAMP inhibition) pathway, identifying highly G-protein biased agonists based on potency. In contrast, when studying the effect of *N*-1 bitopic alkylation on functional efficacy, they reported that despite a markedly reduced D_2R affinity, the analogues presented lower potency, but near full activation (E_{max}) of the G-protein pathway, obtaining G-protein biased agonists based on efficacy. Overall, this work highlighted the flexibility of the sumanirole *N*-5 position to increase D_2R affinities, potencies and push the boundaries of biased agonism towards new limits previously unexplored. Moreover, they identified the optimal length and composition (polarity) of the linker.

After studying the regiochemical requirement of the sumanirole PP, in 2019, Bonifazi and Yano *et al*. published a follow up study where they explored new allosteric aromatic SP structures, chirality in the SP, and carefully analyzed the functional effects of a bitopic ligand presenting more rigid cyclopropyl-containing linkers (Figure 5).[86] The *trans*-cyclopropyl linkers used were inspired by the previously reported D_3R non-competitive antagonist **14** (**18a** from reference 84; Figure 4).[84] As described above, the non-competitive nature of this compound was exclusively dependent on the length and regiochemistry of the cyclopropyl moiety embedded in the linker. They subsequently showed that by introducing subtle modifications, such as a 2-methyindoline-amide allosteric SP (**20**) or *trans*-cyclopropyl indole-amide linker (**21**) to the *N*-5 sumanirole (**18**) bitopic analogues, they could achieve the highest D_2R G-protein biased agonism observed to date (bias factor >1000 fold over β-arrestin recruitment).[86] In addition, the presence of the *trans*-cyclopropyl linker provided privileged recruitment and activation of G_o-protein over G_i-protein (bias factor >10 fold). This was the first time that biased agonism was observed within D_2R coupled G-proteins belonging to the same sub-family, and it was the first time where the linker was deemed a critical mediator of such an extraordinary bias response.

Over the years, our lab has meticulously established how PPs are responsible for maintaining the agonist or antagonist profile of the bitopic molecules,[85, 135] meanwhile the SPs modulate receptor subtype selectivity and functional potency of these bitopic molecules. We have also provided structural evidence showing how the linker plays a key role in inducing functional selectivity and allosteric modulation, likely due to its ability to effect specific ligand-receptor conformations. This underscores how bitopic ligands combine the

best properties of single synthons into new complex structures, and ultimately affects downstream signal messengers through discrete drug-protein interactions.

Novel and bitopic D_3R agonists based on PD128,907 and PF592,379

The SAR story that we have developed over the years regarding D_3R antagonist selectivity and D_2R biased agonism inspired us to take the challenge of designing D_3R selective agonists and provide the pharmacology community with an essential counterpart tool to the highly selective antagonists already generated.[84–85, 87, 123, 163] Recently, Battiti, Cemaj and Guerrero *et al.*,[164] published a comprehensive SAR study aimed at identifying the optimal structural features for the PP and linker to optimize affinity and selectivity in D_3R agonist drug design. Our chemical campaign was particularly focused on the significance of chirality and aimed to explore chiral combinations between PP and linker for optimal D_3R binding (Figure 6).

We designed our new bitopic ligands based on the well-known D_3R agonists, (+)-PD128907 (**22**) and PF592379 (**23**) as the PPs (Figure 6). Both compounds present two chiral centers in their core structures, and both present multiple positions for potential alkylation and chemical modification. Despite the intractable nature of the **22** scaffold (e.g., all chemical modifications resulted in lower affinities and D_3R selectivities than the parent molecule), **23** proved to be an extremely interesting scaffold for chemical manipulation.

While **23** is reported as the (2*R*,5*S*)-eutomer at the morpholine ring (Figure 6), we demonstrated that, when assembled into a bitopic configuration, the (2*S*,5*S*) conformation becomes the privileged architecture, with increased affinity and selectivity for the D_3R. When bitopic ligands presenting (2*R*,5*S*)- and (2*S*,5*S*)- morpholine isomers were tested in parallel, we observed >1000 fold diastereospecificity in D_3R binding for the (2*S*,5*S*)-enantiomer. Moreover, the (2*S*,5*S*) bitopic conformation significantly improved the subtype selectivity, reducing the overall D_2R affinity. In addition, a cyclopropyl moiety was incorporated in the linker as previously described for compound **14** (**18a** from reference 84; Figure 4) and we achieved full resolution of the four chiral centers. The identified lead eutomer demonstrated significantly higher D_3R selectivity than PF592379 (**23**) and is among the highest D_3R selective agonists described in the literature to date. We found that a very specific chiral combination is necessary for the *trans*-cyclopropyl function, since inversion in configuration toward the less active isomer decreased the affinity >150 fold (compounds **24**-**27**; Figure 6). Moreover, this is the first time it has been observed that the PP and linker are structurally and conformationally dependent on one another in achieving the optimal ligand binding pose at D_3R for an agonist. We demonstrated that the right geometry for the linker, not only changes the stereochemical requirements of the PP, but it might also direct the SP toward different SBP interactions, opening the opportunity of a completely new range of possible ligand-receptor conformations, which can translate to yet unexplored intracellular functional activities. This work is just the first step in understanding how linkers, when optimized in their length, relative regiochemical positions of the PP and SP, and relative and absolute stereochemistry can dramatically modulate pharmacological profiles. Due to the high degree of homology among the D_2-like dopamine receptors, exploring less studied chemical spaces and binding sites different from OBS and SBP might

be the key to achieving high subtype selectivity. Even in the presence of highly homologous and structurally conserved receptors, subtle differences in amino-acid residue spatial orientation can be targeted with carefully designed ligands presenting suitable stereochemistry. We expect that future computational studies will aid in better understanding D_3R agonist-receptor interactions, as well as inspire new studies on the significance of specific ligand induced receptor conformations to elicit desirable pharmacological profiles.

IN VIVO STUDIES WITH LEAD D_3R SELECTIVE LIGANDS – RELEVANCE TO DRUG DEVELOPMENT

Thus far in this perspective, we have focused on the roles and importance of the PP, SP and linker between them for affinity, receptor subtype selectivity, allostery and functional selectivity or signaling bias. However, beyond discovering how each of these pieces works in concert to bind GPCRs in specific conformations that elicit these profiles, we ultimately want to know what these compounds do *in vivo* and if we can relate these profiles to behavior. In this pursuit, the PP, SP and linker also play a critical role as these exquisitely designed small molecules ultimately have to possess the appropriate biophysical properties to penetrate the blood brain barrier (in the case of our D_2R/D_3R ligands), be metabolically stable, have appropriate pharmacokinetics, including oral bioavailability and to not have off-target effects that can pose behavioral or other toxicities. Indeed, we can develop an interesting compound that binds with high affinity and selectivity to our desired receptor subtype, with the *in vitro* efficacy we also desire, however it might be inactive *in vivo* because of structural flaws that make it a poor drug. This challenge has been particularly difficult with the bitopic molecules directed toward the D_3R due to their lipophilicity, metabolic instability, short half-lives and propensity to be P-glycoprotein (PGP) transported-substrates.[130, 165–166] Keseru has described highly “decorated” and potent compounds through target-based SAR have resulted in an abundance of compounds with high affinity and/or selectivity for their biological target, but that are large and lipophilic, resulting in a poor absorption, distribution, metabolism, excretion, and toxicity (ADMET) profiles and ultimate failure preclinically or in the clinic.[167–168]

There are many reviews in the literature[123, 168–171] addressing Lipinski’s Rule of 5 and more recent permutations and computational models used to predict these physical properties to be used in drug design; these will not be reviewed here. Instead, a quick review of our own program developing high affinity and selective D_3R antagonists/partial agonists toward substance use disorders will be used as an illustration of our evolution in drug design and again how the combination of SP, PP and linker played a role in developing these *in vivo* tools.

As described above, our starting lead molecule, NGB2904 (**7**; Figure 3A), came out of the lab of the late Andrew Thurkauf at Neurogen.[128] We collaborated with Thurkauf briefly[124] and discovered some early analogues, but none that were of particular interest, until we started to modify the linker,[137] incorporating a *trans* olefin to give PG01037 (**9**; Figure 4).[125, 130, 138] To date, there are nearly 40 papers cited in PubMed using **9** as a tool for both *in vitro* and *in vivo* studies and it has been commercially available for several years. Indeed, its

debut in the behavioral literature as a selective D_3R antagonist, was in a series of papers from the Woods lab showing that it selectively blocked the yawning effect of several D_3R agonists in rats.[172–174] These studies were replicated in male and female rhesus monkeys under various drug combination conditions.[175–176] Subsequently, several other labs have used this compound and its close analogues in models of L-DOPA-induced dyskinesia,[177–178] cocaine or methamphetamine use disorders and impulsivity.[126, 179–190] The key to the success of **9** was that we were able to improve its D_3R selectivity and also reduce its lipophilicity over **7**, via incorporating that *trans* olefin linker and modifying the SP, by replacing the tricyclic fluorenyl function with a 2-pyridyl-phenyl group.[125, 138] Nevertheless, its subsequent characterization as a PGP transported substrate[191] dampened enthusiasm for taking this compound into further development for human use.

While **9** proved to a good *in vivo* tool, subsequent drug design led to compounds with subnanomolar affinity and/or >300-fold D_3R/D_2R selectivity. Importantly, a seminal modification was the addition of a 3-OH into the linker to give the next lead molecule rac-**6** (PG648; Figure 4).[134] As described above, rac-**6** and its (*R*)-enantiomer (**6**) have binding affinities for D_3R of ~1 nM and are ~400-selective over D_2R, as well as having a clean off target profile.[123, 127, 134] In this molecule, we retained the 2,3-dichlorophenylpiperazine PP, but modified the SP to the recently identified D_2R/D_3R privileged 2-indoleamide. We showed that rac-**6** was indeed effective in rodent models of methamphetamine use disorders, but to our disappointment, it was not effective in similar models in nonhuman primates.[127] There may be many reasons for this, not the least of which is that nonhuman primates, like humans, are far more diverse in their response to drugs of abuse and potential medications and the n's are always low, making statistical significance more challenging to obtain than in rodent studies. Nevertheless, one factor that may have contributed to the lack of effectiveness in these models was that we later discovered rac-**6** was metabolically unstable in rhesus monkey liver microsomes (unpublished data) providing an example of species differences that one also has to consider when developing lead molecules. Again, different functional groups are more metabolically vulnerable than others across species and the key is to find compounds that are predicted to be stable across species, before spending resources developing compounds that are only effective in rodents. This is of course not always easy in academic labs, where resources like these are not as common as in the pharmaceutical industry. Nevertheless, our own experiences have led us to be more thoughtful of metabolism across species earlier in our drug discovery process, which is not always predictable based on structure. Indeed, we originally thought that the 3-OH group may be metabolically vulnerable, which lead us to synthesize the 3-F analogues.[143–144] Subsequent metabolic ID in our newer lead compounds suggest that in fact, the 3-OH does not appear to be metabolically vulnerable.[87, 192]

Although we have synthesized many analogues more recently, further examining all parts of these bitopic molecules and developing both SAR as well as identifying *in vivo* tools, our efforts have now directed us toward the overall profile of our new lead molecules, **10** (VK4-116) and **11** (VK4-40; Figure 4). These two analogues share the 3-OH linker and the privileged indole-2-carboxamide, but differ in the PP, which was inspired by the D_2R/D_3R antagonist/inverse agonist eticlopride (**5**), as described above. We posited that the SP, PP and

linker of these molecules had proven to have both the pharmacological specificity and metabolic stability in other molecules from our lab and others and hoped that the combination would lead to new molecules with outstanding D_3R affinity, selectivity and ADMET properties that predict *in vivo* efficacy.[87, 145] We have synthesized the enantiomers and discovered the *R*-enantiomer for both to be the eutomer. Extensive behavioral testing of **10** and **(*R*)-11** has been done and further studies are underway.[192–193] The *R*-enantiomers of these two lead molecules have been tested in telemeterized rats alone and in the presence of either cocaine or oxycodone, with impressive results showing neither compound exacerbates the cardiovascular effects of these drugs of abuse and, in some cases, serve to reduce blood pressure and/or heart rate which is increased in their presence.[194] Further development of these lead molecules is underway in hopes that at least one will be safe enough for clinical trials. This is especially important, not only for the hope of development as a non-opioid therapeutic for the treatment and possibly prevention of opioid use disorder, but to also determine if the preclinical models we are testing these compounds in (rodents and nonhuman primates) translate into humans as, although we know that many of these models have translational validity based on the success of the opioids, namely methadone and buprenorphine, we do not know if the non-opioid treatments that are being developed will show the same profile[195] and this is critical to our success.

SUMMARY: THE WHOLE IS GREATER THAN THE SUM OF ITS PARTS

In summary, from the seminal work of Portoghese through the contributions of many, the discovery of bivalent and bitopic molecules has provided a fantastic toolbox of molecules with which to understand basic biology of the GPCRs we have targeted. As X-ray crystal structures of receptor targets are becoming increasingly available, identification of potential SBPs which can be exploited with bitopic molecules opens an avenue for ever more selective and potent rationally designed small molecules. Moreover, the promise that some of these molecules may make it to the clinic because of their unique ability to bind the targeted receptors in such a way that a therapeutic arises with all (or many) of the right attributes to be an effective drug exists. Importantly, while we have focused on the structures of the PPs, SPs, and the linker, it is undoubtedly the combined effect of these strategically connected pieces that contribute to the overall pharmacological profile of the drug, including its drug-like properties, which are critical to its success. Thus, it is paramount to consider the whole molecule as not just the sum of its parts, but that the right combination can result in molecules that are greater/better/more promising leads. Our adventure into bivalent and bitopic molecules, inspired by Portoghese and beyond, will continue. We hope that others will follow in these footsteps when discovering molecules for their target of choice as the fundamental principles will be the same. There is no doubt that these bitopic and bivalent molecules will provide the tools necessary to parse signaling bias and relate those signaling pathways to behaviors that can be targeted or avoided, depending on the outcome. Paying attention to the chemical properties of the PP and SP and the length and composition of the linker between them has proven fruitful for our work on the D_3R and D_2R and we look forward to the discoveries of others using these medicinal chemistry principles.

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BIOGRAPHIES

Amy Hauck Newman received her doctorate in Medicinal Chemistry from the Medical College of Virginia, Virginia Commonwealth University. After postdoctoral studies at the NIH and a brief time at WRAIR, she joined the National Institute on Drug Abuse-Intramural Research Program, NIH. She is currently Acting Scientific Director, Chief of the Molecular Targets and Medications Discovery Branch and Director of the NIDA-IRP Medications Development Program. She has coauthored more than 280 original articles and reviews on the design, synthesis, and evaluation of CNS active agents, with an emphasis on selective ligands for the dopaminergic system, as potential treatment medications for substance use disorders. She is also an inventor on several licensed NIH patents. She was the first woman to receive the Philip Portoghese Lectureship Award.

Francisco O. Battiti received his BS in Chemistry and BSLA in Physics from the University of Illinois at Urbana-Champaign in 2018, conducting research on functionalization in dearomatization strategies for natural product synthesis under the mentorship of Prof. David Sarlah. Francisco joined the Medicinal Chemistry Section of the Molecular Targets and Medications Discovery Branch as a post-baccalaureate IRTA Fellow and is a recipient of the Scientific Director’s Diversity in Research Fellowship, in the laboratory of Dr. Amy H. Newman, in 2018.

Alessandro Bonifazi received his M.S., Pharm. D. and doctorate degree in Pharmaceutical Sciences at the University of Camerino, Italy (School of Advanced Studies) – under the mentorship of Prof. Wilma Quaglia, in 2014. He joined the Medicinal Chemistry Section in the Molecular Targets and Medications Discovery Branch, as a Postdoctoral Fellow in the laboratory of Dr. Amy H. Newman in 2014. In 2019 he was appointed Research Fellow in the same laboratory. In addition to performing and developing multiple cell- and tissue-based radioligand binding assays, his research interests include the structure-based rational design and synthesis of highly selective D_2 and D_3 receptor agonists with the aim to elucidate ligand-receptor interactions necessary to activate specific signaling pathways.

Abbreviations Used:

GPCR	G-protein coupled receptor
D_3R	dopamine D3 receptor
D_2R	dopamine D2 receptor
PP	primary pharmacophore

SP	secondary pharmacophore
OBS	orthosteric binding site
SBP	secondary binding pocket
ABS	allosteric binding site
TM	transmembrane
ADMET	absorption, distribution, metabolism, excretion, toxicity

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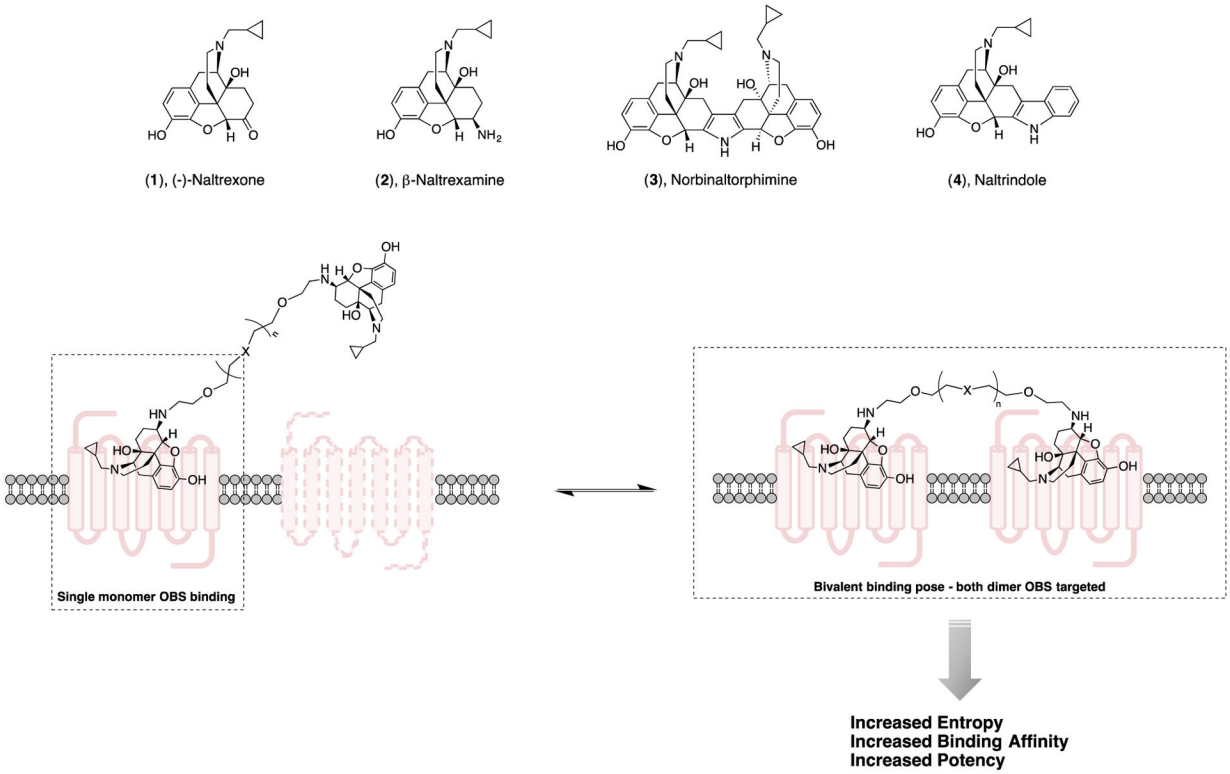

Figure 1.

Bivalent ligand cartoon with naltrexone (**1**), β-naltrexamine (**2**), norbinaltorphimine (**3**) and naltrindole (**4**). The bivalent ligand is represented while engaging in binding with GPCR monomer and homo/hetero-dimer.

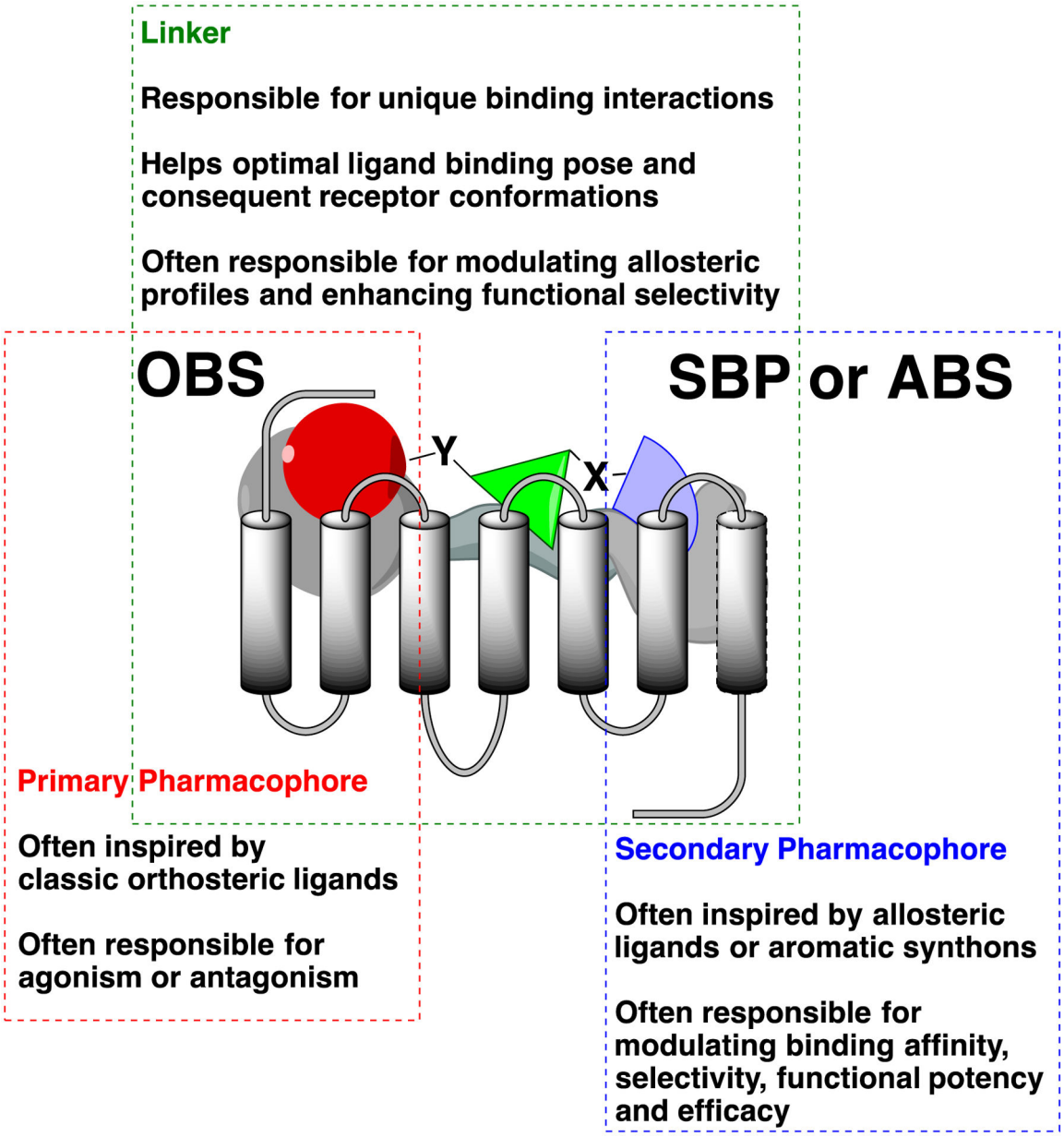

Figure 2.
Conceptual framework of bitopic ligands and the role of each segment.

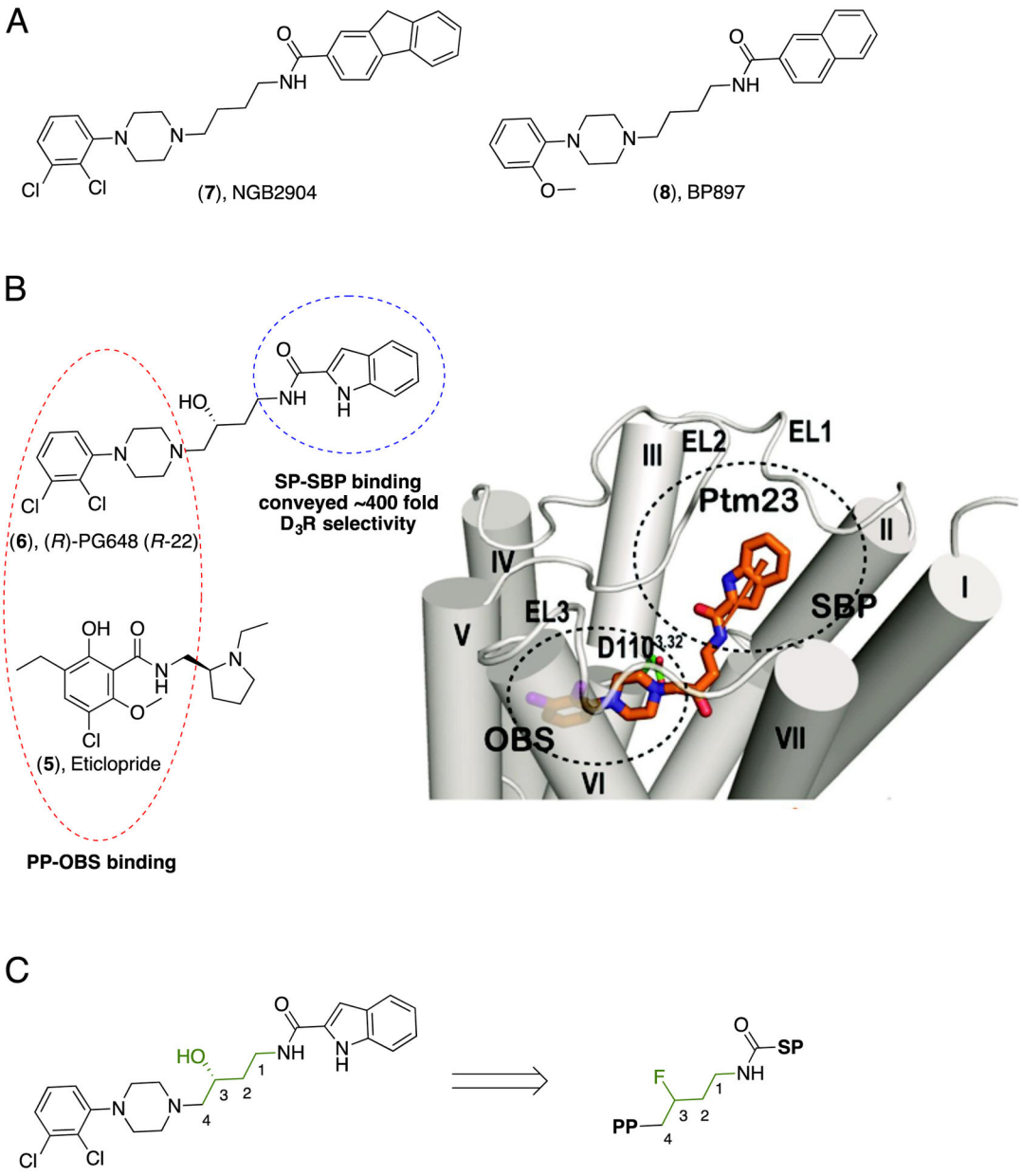

Figure 3.

A) Structures of NGB2904 (**7**) and BP897 (**8**) representing aryl-phenylpiperazine as the PP. **B)** Structures of eticlopride (**5**) and (*R*)-PG648 (**6**) and highlighting the overlap between their PP binding in the OBS. Docking pose of **6** in D_3R showing its bitopic binding mode, with simultaneous interactions with both OBS and SBP. Figure published in Newman, AH et al. *J. Med. Chem.* **2012**, 55, 6689–6699 [Copyright © 2012 American Chemical Society]. **C)** Structural investigation of the linker, exploring the bioisosteric substitution of the -OH with a -F atom.

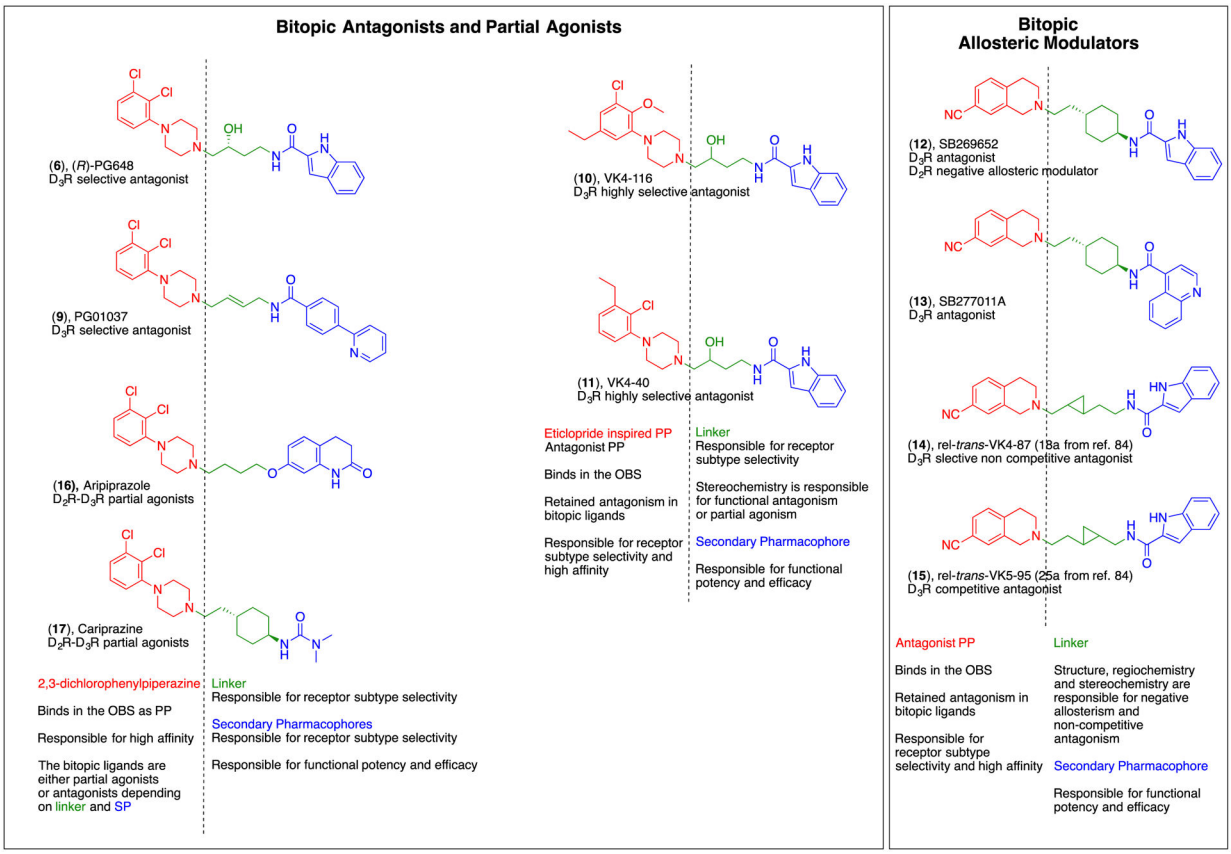

Figure 4.

Summary of the main dopaminergic bitopic compounds, studied over the years, which inspired SAR and drug design to generate highly selective agonists, partial agonists and antagonists. The PP are highlighted in red, in linkers in green and the SP in blue. The descriptions below every structure highlight the primary role played by each fragment of the bitopic molecules toward unique pharmacological profiles.[84, 87, 148, 149]

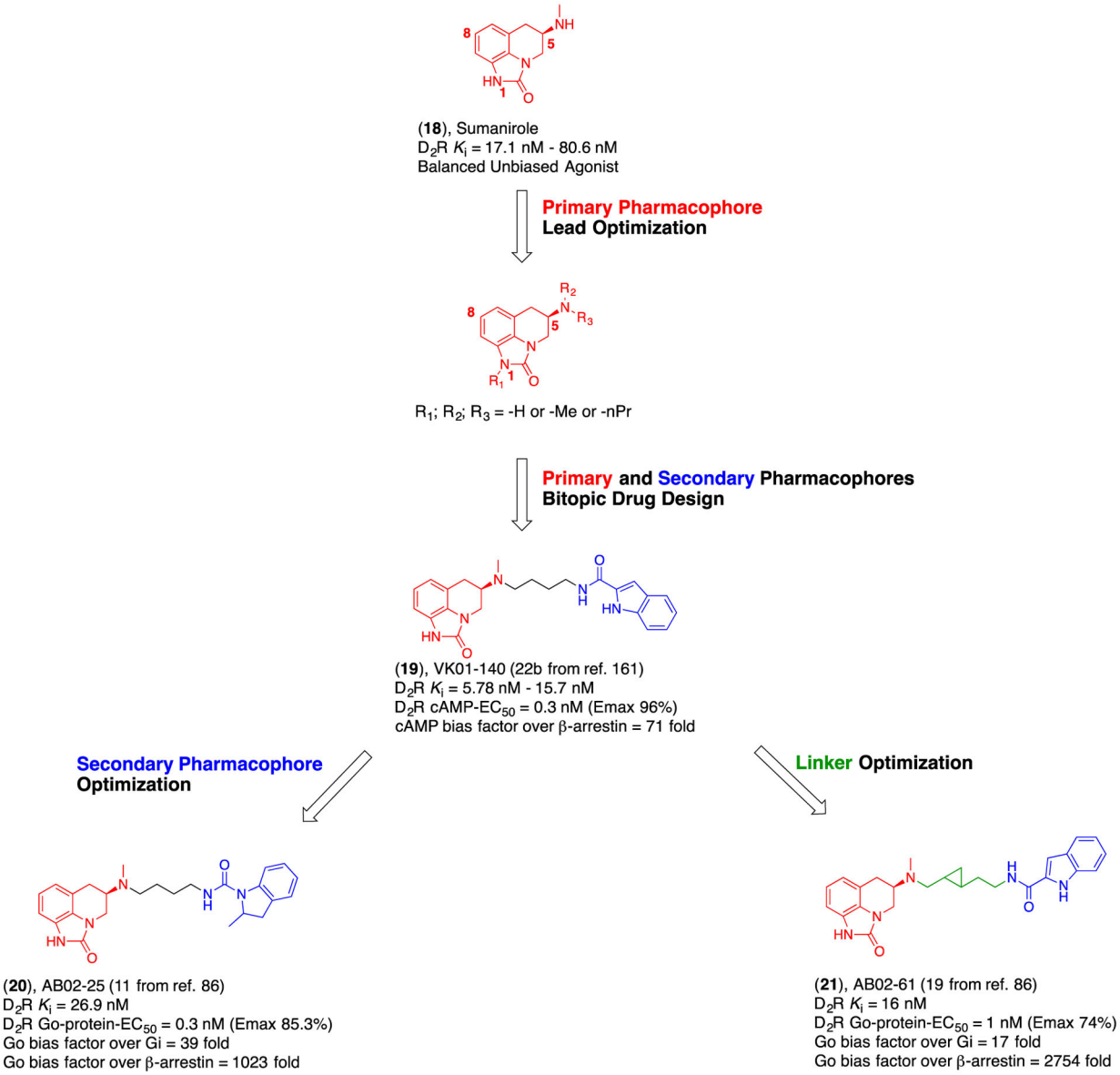

Figure 5.

Drug design strategy, from study of the PP, to structural optimization of SP and linker to improve D_2R affinity, selectivity and specific pharmacological profiles achievable only with bitopic ligands.[86, 161, 162]

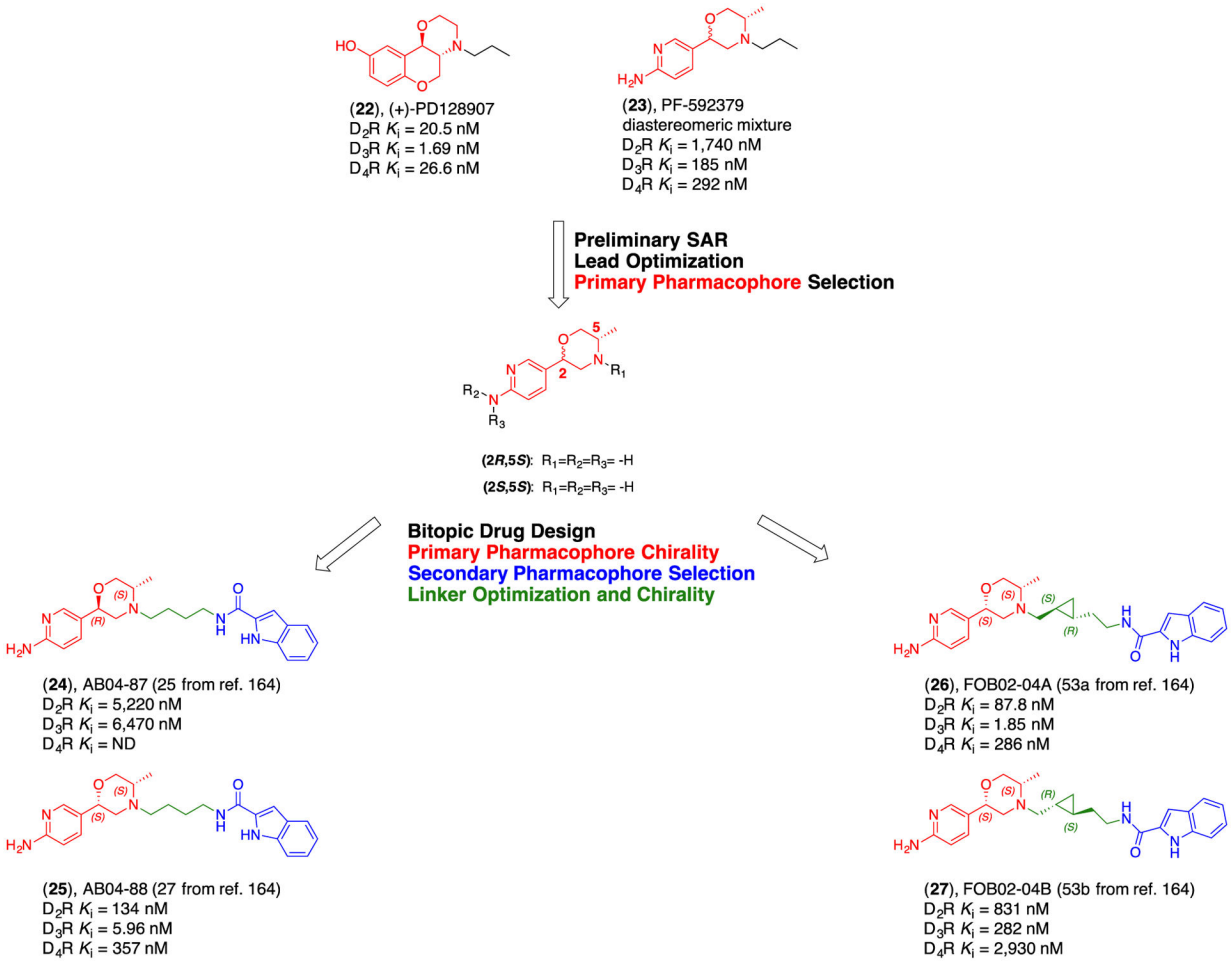

Figure 6.

Drug design strategy, from study of the PP, to structural optimization of SP and linker to improve D_3R affinity, selectivity and specific pharmacological profiles achievable only with bitopic ligands.[164]