HHS Public Access

Author manuscript

J Med Chem. Author manuscript; available in PMC 2019 June 09.

Published in final edited form as:

J Med Chem. 2019 January 10; 62(1): 88–127. doi:10.1021/acs.jmedchem.8b00875.

Allosteric Modulation of Class A GPCRs: Targets, Agents, and Emerging Concepts

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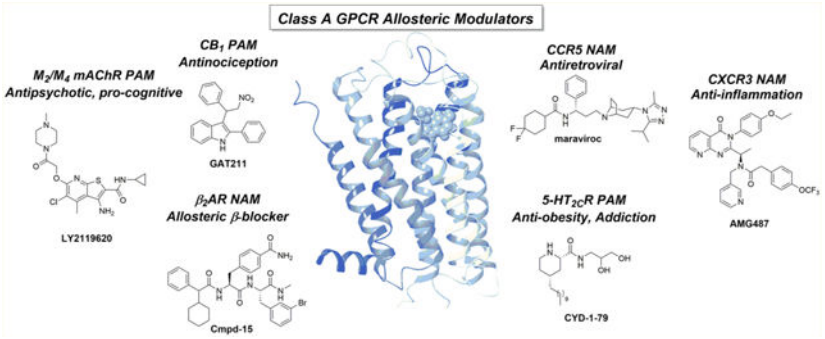
Abstract

G-protein-coupled receptors (GPCRs) have been tractable drug targets for decades with over one-third of currently marketed drugs targeting GPCRs. Of these, the class A GPCR superfamily is highly represented, and continued drug discovery for this family of receptors may provide novel therapeutics for a vast range of diseases. GPCR allosteric modulation is an innovative targeting approach that broadens the available small molecule toolbox and is proving to be a viable drug discovery strategy, as evidenced by recent FDA approvals and clinical trials. Numerous class A GPCR allosteric modulators have been discovered recently, and emerging trends such as the availability of GPCR crystal structures, diverse functional assays, and structure-based computational approaches are improving optimization and development. This Perspective provides an update on allosterically targeted class A GPCRs and their disease indications and the medicinal chemistry approaches toward novel allosteric modulators and highlights emerging trends and opportunities in the field.

Graphical abstract



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The authors declare no competing financial interest.

1. INTRODUCTION

G-protein-coupled receptors (GPCRs) are seven-transmembrane proteins that have been of high pharmaceutical interest for decades due to their physiological importance and accessibility for small molecule targeting. GPCRs are integral for physiological responses to a variety of stimuli, which span photons, ions, small molecules, macromolecules, peptides, and proteins. These diverse stimuli, environmental and endogenous, are in accordance with the vast functions mediated by GPCRs. Aspects of cognition, immune response, and cellular organization, among many others, are regulated by GPCR signaling. The pharmacological modulation of GPCRs provides leverage for the treatment of diseases of the central nervous system (CNS), cancer, viral infections, inflammatory disorders, metabolic disorders, and others. Additionally, the location of GPCRs in the cellular membrane allows unique pharmacological access to these proteins and the effectors and second messenger systems coupled to the receptors allow for efficient drug action. The location, topology, and physicochemical attributes of many GPCR binding pockets have resulted in the discovery of numerous small molecule drugs that have been in clinical use for decades.[1]

GPCRs represent the largest family of druggable proteins in the human genome and unsurprisingly are targeted by more than 30% of marketed drugs in the United States.[2] Despite this large percentage, recent studies highlight that a small fraction of the possible druggable GPCRome has been exploited by approved drugs.[3] Multiple reasons persist for the relatively low number of GPCRs targeted by FDA-approved drugs. Foremost, the biological functions of many potentially druggable GPCRs remain unclear, as seen in the understudied orphan GPCRs (oGPCRs), and new biological complexities remain to be added to established targets.[4] Another reason is several disease indications with established GPCR targets have not progressed drugs into the clinic when targeted by traditional agonists or antagonists, which bind at highly conserved orthosteric sites and may produce off-target effects.[5,6] Undeniably, most of GPCR-targeted FDA-approved drugs bind to orthosteric sites; however, when therapeutic efficacy or safety hinges on distinguishing between highly homologous receptor subtypes, other modes of modulation, such as allosteric modulation, confer specific advantages.[7]

Allosteric modulation of protein function was first described in enzymes and is now understood to be an integral aspect of functionality in other protein types, including GPCRs.[8] A GPCR allosteric modulator is generally defined as a modulatory ligand that does not occupy the orthosteric binding site and binds to a spatially and topologically distinct (allosteric) site on the receptor. It is now known that endogenous allosteric modulators are ubiquitous among GPCRs, the most apparent being the heterotrimeric G proteins, and many synthetic ligands may exploit these sites.[9] Other examples of allosteric sites seem to be unrelated to endogenous molecules yet display topologically favorable features due to the receptor folding and assembly. Nevertheless, allosteric site residues tend to be less conserved among receptor subtypes and can offer unparalleled subtype specific targeting. Other advantages of allosteric ligands are fundamental to their mode of action, including the ability to fine-tune the response to an orthosteric ligand in a time and spatially dependent manner, a feature that holds promise for immune and CNS targets.[10] Additionally, allosteric

modulators may also confer signaling bias and probe dependence, further contributing to the possibility for remarkably precise pharmacological modulation.[11]

The GPCR superfamily is subdivided into divergent groups (classes) based on homology and function including class A (rhodopsin-like receptors), class B (the secretin family), class C (metabotropic glutamate receptors), class D (fungal mating pheromone receptors), class E (cyclic adenosine monophosphate (cAMP) receptors), and class F (Frizzled and Smoothened receptors).[12–14] Class A represents the largest class of GPCRs and contains further classifications for members based on the type of endogenous signaling molecules (Figure 1). This Perspective will focus on recent medicinal chemistry advances for allosteric modulators across a selection of class A GPCRs with diverse signaling molecules, including small molecules, peptides, proteins, and lipids. Due to the size of class A and the historical importance of many of its members, these receptors make up most of the current receptor drug targets.[15] Likewise, industry and academic programs have been established to discover allosteric modulators of class A receptors for the treatment of mental health disorders, viral infection, inflammation, and other indications.

Mechanistically, allosteric modulators can increase the functional response to an orthosteric agonist, acting as a positive allosteric modulator (PAM), or inhibit the functional response to an orthosteric agonist, acting as a negative allosteric modulator (NAM). There have been reported neutral allosteric ligands (NALs), which do not modulate the receptor function but compete with other PAMs or NAMs at the allosteric binding site. Additionally, for many targets included in this Perspective, allosteric ligands may possess an intrinsic agonist profile in the absence of an orthosteric ligand, despite binding to the allosteric site. In such instances, allosteric ligands that potentiate agonists as well as display intrinsic efficacy are termed ago-PAMs. Further, NAMs may act directly via allosteric antagonism of an orthosteric agonist's stimulation or they may assert antagonism by binding to an allosteric site and inducing changes to the receptor's structure or "life cycle" that prohibit eventual receptor activation by an orthosteric agonist. The latter has been observed in cases where NAMs alter receptor trafficking or, in the case of the CCR5 NAM maraviroc, dimer populations of CCR5 are altered.[16] New insights have shown these allosteric alterations can be mediated by various components of the receptor, lipid membrane, orthosteric ligand(s), and effector proteins, ultimately providing a more complete, and complex, view of the potential for allosteric modulation.[8,17] These modes of allosteric modulation are conferred via diverse allosteric binding sites that include extracellular regions, the interior, and lipid-facing exterior of the transmembrane (TM) helix bundle and intracellular regions. Elegant structural studies have confirmed these interesting binding poses and provide the framework for modulating new targets with notable precision. Figure 2, containing three GPCR-allosteric modulator cocomplex crystal structures, illustrates the concept of allosteric modulators functioning in diverse modes and binding to class A GPCRs in diverse structural regions. The structural interactions between class A GPCR residues and the corresponding allosteric modulators have been reviewed by Lu and Zhang, also included in the Allosteric Modulators special issue.[18] This Perspective will review key concepts for allosteric modulator discovery and optimization, provide a thorough overview of the recent medicinal chemistry efforts for class A GPCR allosteric modulation, and highlight diverse applications of allosteric modulators across the class A GPCR family.

2. OVERVIEW OF THE ALLOSTERIC MODULATION OF CLASS A GPCR DRUG TARGETS

GPCRs are the most targeted protein class by modern pharmacotherapies due to their innate capability of transducing extracellular signals into wide-ranging cellular responses. In a growing number of molecular and structural studies, activated GPCRs are increasingly appreciated to transduce their signal through structural alterations in the TM domains that not only result in the association of effector heterotrimeric G proteins but also lead to association of β-arrestins, scaffolding proteins, and various kinases.[4] Due to the known complexities of GPCR signaling and the added intricacy of measuring allosteric modulation, targeted discovery of allosteric modulators has been enabled only in recent decades by technological improvements in ligand screening assays.[22] A common primary screening assay employed in allosteric modulator discovery is a functional assay in which the effects of allosteric modulators can be observed by alterations to orthosteric agonist or antagonist potency and/or efficacy. An orthosteric agonist or antagonist with known and reproducible activity, along with the potential allosteric ligand, is added to a system in which a functional output can be measured, as in a calcium-mobilization measurement. Since GPCR activity is not a simple on/off mechanism, it is important that the assay be able to measure the magnitude of agonist-induced functional response, as well as the magnitude of functional modulation from an allosteric modulator. The resultant concentration—response curve can provide quantitative measures of orthosteric ligand efficacy (E_{max}), potency (EC_{50}), and the potency estimate for an allosteric modulator (e.g., PAM EC_{50}). When fit to the operational model of allosterism, the unique behavior of the receptor induced by the allosteric ligand can be quantified. The allosteric modulation of orthosteric ligand affinity is denoted as the cooperativity factor (α), and the allosteric modulation of efficacy is described by the value β. These influences may be described by a composite metric of cooperativity denoted by log $\alpha\beta$. According to this model, the intrinsic efficacy of the allosteric ligand can be described by the factor τ_B and the intrinsic efficacy of the orthosteric ligand is described by the value τ_A. Excellent reviews have been published and should be consulted on this model, the contributing factors, and the importance of the quantification of allosteric modulator activities.[23–25] It is important to note that historically much of the medicinal chemistry and corresponding structure—activity relationships (SARs) in the field of allosteric modulator discovery has relied on allosteric modulator potency estimates obtained from concentration—response curves, as seen in this Perspective. While useful in screening, potency estimates have limitations and have been shown to diverge from the allosteric modulator affinity measured in binding experiments.[26]

Binding assays with radioligands are also commonly employed to understand allosteric modulation and report kinetic inhibition constants (K_i). These competition or saturation binding assays are employed on receptor-expressing membrane preparations. Membrane preparations may also be used for functional assays via the quantification of [^{35}S]-guanosine-5'-*O*-(3-thio) triphosphate (GTPγS) binding to the G protein α subunit upon receptor activation. Finally, bioluminescence resonance energy transfer (BRET) biosensors have been increasingly utilized in cells to study the protein—protein interactions that occur upon receptor activation, specifically between GPCRs and signaling proteins such as G

proteins or β-arrestins. Throughout the following Perspective, these terms will be used to describe the activity of allosteric modulators as reported in the corresponding original manuscript. Additionally, observations should be made regarding the importance of quantitatively characterizing allosteric modulators in multiple dimensions of their effect to thoroughly inform SARs.

Traditionally, allosteric modulators have displayed divergent physicochemical properties from those of class A GPCR orthosteric ligands, exemplified by higher lipophilicity, rigidity, and typically reduced affinity for their binding site.[38] Due to years of careful optimization and structurally informed design, the development of allosteric modulators has significantly improved in achieving druglikeness and is now progressing candidates forward in preclinical development and human clinical trials as well as in the market for clinical use (Table 1). A contributing factor to the optimization of allosteric ligands is the recent availability of 10 high resolution crystal structures displaying allosteric binding sites and the corresponding receptor activation states (Table 2). These structures provide information on binding sites as well as explanation toward differential allosteric modulation of downstream signaling pathways. The latter phenomenon has been termed signaling bias (also functional selectivity) and is characterized by potentiation or inhibition of a selected signaling cascade(s) over other signaling cascades activated by the GPCR.[11] Class A GPCRs translate signaling through association with effector proteins, predominately heterotrimeric G proteins, from whom the receptor class derives its name, and arrestins. Signaling bias, as reported in this Perspective, primarily reflects selective or biased modulation of either G protein-mediated signaling or β-arrestin-mediated signaling, although biased receptor interactions within the G protein and other proteins have been shown.[39,40] Selectively developing ligands that display a marked signaling bias in accordance with target-specific underlying biology may produce more efficacious drug candidates or may produce candidates with decreased adverse effects. Currently, this trend has been predominantly explored by research groups developing orthosteric ligands for class A GPCRs, but it is also a consideration for allosteric ligand development, as shown by selected allosteric modulators in this Perspective.

Interest in class A GPCR allosteric modulators initially grew because of a theoretical improvement in target selectivity, especially among receptor subtypes displaying high degrees of homology in the orthosteric site.[41] Since then, subtype selectivity has been shown to be one of many advantages, including the preservation of spatial and temporal dynamics of cell signaling. These benefits are often highlighted with respect to targeting GPCRs within the CNS in which, for example, neurotransmitter release and receptor activation are region- and circuit-specific with a high degree of temporal regulation.[10] In such a case, an allosteric modulator may preserve these important characteristics and avoid receptor desensitization by relying on endogenous activation events. Additionally, allosteric modulators have a “ceiling effect”, driven by the saturable nature of allosteric interactions and whereby the extent of their activity is dictated by the concentration of orthosteric ligand present, possibly decreasing overdose concerns. Many of these advantages have been exploited by allosteric modulators targeting receptors in the periphery. For example, the chemokine receptors are an integral component of the immune system and inflammatory response, and one receptor subtype may respond to numerous chemokine ligands (and vice

versa) in a receptor-, agonist-, tissue-, and time-dependent manner.[42–44] Thus, allosteric modulation is an attractive strategy for developing precise therapeutics for both the CNS and periphery.

3. RECENT ADVANCES IN THE DISCOVERY AND DESIGN OF CLASS A GPCR ALLOSTERIC MODULATORS

3.1. Aminergic Family Receptors.

3.1.1. Serotonin 2C Receptor (5-HT$_{2C}$R).—

The serotonin (5-HT) 5-HT$_{2C}$ receptor (5-HT$_{2C}$R) is a member of the 5-hydroxytryptamine receptor family, which can activate phospholipase Cβ (PLCβ) via G$\alpha_{q/11}$ to result in production of intracellular inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) to promote intracellular calcium release (mobilization).[45] Assays described throughout this Perspective that relay information on calcium mobilization follow a similar signaling cascade, depending on the activated G protein. The 5-HT$_2$ subfamily has been of pharmacological interest for decades and is implicated in neurological, psychological, and circulatory processes.[46] It is well-known that nonselective antagonists of this subfamily can alleviate symptoms of schizophrenia and anxiety, while nonselective agonists would cause cardiac abnormalities and hallucinations, among other effects.[47] However, selective stimulation of the 5-HT$_{2C}$R is useful for treating obesity and may be useful for treating substance use disorders (SUD), depression, and other neuropsychological disorders.[48,49] Therefore, there remains a need for developing 5-HT$_{2C}$R targeted ligands that display a high degree of specificity for the 5-HT$_{2C}$R over the highly homologous 5-HT$_2$ subtypes 5-HT$_{2A}$R and 5-HT$_{2B}$R. As previously mentioned, allosteric modulation holds the potential to differentiate between receptors by targeting less conserved regions and thus provides therapeutic benefit at the 5-HT$_{2C}$R while eliminating significant CNS (5-HT$_{2A}$R) and cardiovascular (5-HT$_{2B}$R) adverse effects. Two primary groups are engaged in projects aimed at discovering PAMs of the 5-HT$_{2C}$R.[50,51]

PNU-69176E (**1**; Figure 3) was discovered by Pharmacia (later acquired by Pfizer) via screening of an internal chemical library and is the first reported 5-HT$_{2C}$R selective positive allosteric modulator.[52] In a radioligand competition binding assay of [^{3}H]mesulergine displacement by 5-HT, it was reported that a 20 μM concentration of **1** increased the affinity of 5-HT by 25-fold to the 5-HT$_{2C}$R, measured at a K_i value of 6.4 nM in the presence of **1** compared to a K_i value of 159 nM for 5-HT alone. Further, **1** displays potentiation in multiple cell lines and potentiates the effects of 5-HT at multiple receptor densities (6–45 pmol/mg of protein). Binding selectivity experiments indicate that **1** is a selective 5-HT$_{2C}$R PAM with no appreciable binding to analogous 5-HT (5-HT$_{2A}$R, 5-HT$_{2B}$R, 5-HT$_6$R, and 5-HT$_{7A}$R) and biologically relevant dopamine receptors (D_2R and D_3R). However, unlike pure PAMs, Im and colleagues reported that introduction of 10 μM concentration of **1** alone could cause IP_3 release up to 71% of the maximal response from the same concentration of 5-HT in human embryonic kidney 293 (HEK293) cells, possibly indicating a stabilization effect of the receptor active state. This intrinsic effect was conserved to various degrees while measuring both [^{35}S]GTPγS binding and [^{3}H]IP accumulation in HEK293 cells, contributing to possibility that **1** functions as an ago-PAM. Both the long alkyl chain and polar moiety (α-D-galactopyranoside) of the chemical structure are reported to be integral to

its function and may provide anchoring to the membrane and binding with the allosteric site, respectively. Interestingly, functional characterization of PAM activity of **1** via 5-HT-evoked intracellular calcium mobilization in 5-HT_{2C}R expressing Chinese hamster ovary (CHO) cells showed **1** maintained potentiation of 5-HT_{2C}R by 5-HT activation but lacked the previously reported intrinsic agonist activity. Additionally, in the same calcium mobilization assay, its diastereomer **2** (Figure 3) did not potentiate 5-HT-mediated responses nor did **2** exhibit intrinsic agonist activity.[53] Key differences between the characterization of **1** as an ago-PAM or PAM are likely borne from the selection of cell lines and corresponding 5-HT_{2C}R protein expression levels. The HEK293 cells had expression levels of ~45 pmol/mg protein, whereas the stably expressing CHO cells were characterized with physiologically relevant expression levels of ~250 fmol/mg protein for the 5-HT_{2c}R Thus, selected cell lines and receptor protein expression levels are important considerations for ligand characterization, comparison across laboratories, and the interpretation of SAR data.

Recently, our group reported CYD-1–79 (**3**), with a 4-alkylpiperidine-2-carboxamide scaffold, as a selective 5-HT_{2C}R PAM presenting a promising in vitro and in vivo profile for the treatment of cocaine use disorder (CUD).[51] Unlike **1**, **3** (Figure 3) functions as a pure PAM to potentiate 5-HT-evoked calcium mobilization in CHO cells expressing human 5-HT_{2C}R but exhibits no intrinsic activation of calcium mobilization. When investigated in preclinical pharmacokinetic (PK) and rodent efficacy studies, **3** displays measurable blood—brain barrier permeability and significantly suppresses motor impulsivity and cue reactivity assessed as lever presses for cocaine-associated cues in a rodent cocaine self-administration assay. Excitingly, elegant work in the class A GPCR crystallography field has recently produced a high-resolution crystal structure of the 5-HT_{2C}R capable of enabling molecular docking studies.[54] Shown in Figure 3, these computational studies reveal there is a predicted bridging effect formed by **3** between the extracellular loop (EL) 2 (EL2) and the transmembrane helix (TMH) VI (TMH VI) of the 5-HT_{2c}R via a bidentate, H-bonding interaction. This predicted bridging effect is mediated by a 1,2-diol moiety on **3** to the backbone carbonyl of $Leu209^{ECL2}$ residue of ECL2, and another H-bond between the ionizable N-atom of the piperidine ring and the—OH side chain of $Ser334^{6.58}$ of TMH VI (PDB code 6BQG). Significantly, these residues are not conserved in the highly homologous 5-HT_{2A}R or 5-HT_{2B}R, and these computational studies provide a possible explanation for the selective profile of **3** among closely related receptor subtypes.[51]

Lopez-Rodriguez and colleagues highlighted 5-HT_{2C}R PAMs as potential antiobesity therapeutics and recently reported the screening hit VA024 (**4**), featuring an indole scaffold, as a 5-HT_{2c}R PAM.[50] In what is only the second reported synthetic small molecule screening hit for 5-HT_{2c}R PAMs, **4** (Figure 3) was identified in a Vivia Biotech chemical library via an innovative automated flow-cytometry-based screening system, the PharmaFlow platform (previously ExviTech platform).[50,55] A minor structural modification of the pyrimidin-5-amine side chain of **4** with pyridine results in VA012 (**5**). 5-HT concentration—response curves were assessed in cells, and the introduction of **5** at a concentration of 10 μM potentiated the 5-HT E_{max} 35% greater than the efficacy of 5-HT alone. Further in vitro studies indicate **5** does not appreciably bind to important 5-HT_2 family members (5-HT_{2A}R and 5-HT_{2B}R), displays low binding competition against the

endogenous agonist (5-HT) and other orthosteric ligands (mesulergine and clozapine), and results in no significant off-target interactions as indicated in a CEREP cellular GPCR panel. Significantly, feeding models in rodents indicate **5** reduces both food intake and body weight gain without causing taste aversion when acutely administered at 2 mg/kg (ip).

As previously discussed, proteins that interact with class A GPCRs, such as G proteins, are fundamentally allosteric in their mediation of the receptor structural state and their interference or coordination in allowing other proteins to interact. Structurally, the third intracellular loop and C-terminal tail of the 5-HT_{2C}R, like most other class A GPCRs, act as scaffolds, molecular levers, and protein recruiters with multiple protein binding and phosphorylation sites for mediating receptor function.[56–59] Phosphatase and tensin homolog (PTEN) recognize key residues on the 5-HT_{2C}R intracellular loop III (ICLIII) and mediate 5-HT_{2C}R biological responses.[60] Significantly, this interaction occurs at 5-HT_{2C}R but not at the 5-HT_{2A}R. A fragment of the 5-HT_{2C}R protein, termed 3L4F (third loop, fourth fragment of the human 5-HT_{2C}R), is a peptide derived from the protein interaction site and has been shown to disrupt the 5-HT_{2C}R–PTEN complex and promote 5-HT_{2C}R mediated downstream signaling, acting as a PAM of 5-HT_{2C}R signaling.[61] Subsequent studies revealed that 3L4F-F_1 (Pro280–Arg287), a component of the first eight amino acids of the peptide 3L4F, maintains the efficacy of the full length 3L4F peptide within the picomolar range in vitro and also functions as a PAM of 5-HT_{2C}R in rats in vivo.[61] Referencing this example as a proof-of-concept, modulation of specific protein—receptor interactions at other GPCRs may be achievable to produce PAM or NAM profiles. These examples cover two unique modes for allosterically altering signaling at the 5-HT_{2C}R, where small molecules may bind to the extracellular region of the receptor and stabilize/induce an active state, or peptides and small molecules may bind intracellularly and disrupt protein–protein interactions.

3.1.2. β_2-Adrenergic Receptor (β_2AR).—

The β_2-adrenergic receptor (β_2AR) is an aminergic class A GPCR whose endogenous signaling molecule is adrenaline. The β_2AR is widely expressed in bronchial smooth muscle and plays a significant role in cardiovascular and pulmonary physiology.[18] As one of the most highly studied and characterized GPCRs, numerous studies on the β_2AR represent foundational knowledge on GPCR function, structure, and physiological importance for cell signaling.[62–67] Therapeutically, β_2AR agonists represent a large class of drugs used to treat pulmonary disorders and asthma, while β_2AR antagonists comprise selective and nonselective β-blockers (β-blockers), widely used for the treatment of hypertension, cardiac arrythmias, and other cardiovascular indications. At present, nearly all known β-adrenergic ligands act orthosterically.[68] Kobilka and colleagues have recently reported the first allosteric β-blocker, or β_2AR NAM, known as Cmpd-15 (compound-15, **6**) and, significantly, the cocrystal complex with β_2AR (Figure 4).[21,68] This recently discovered β_2AR NAM displays low micromolar affinity for β_2AR allosteric site and was identified via a DNA-encoded small-molecule library screen comprising 190 million distinct compounds.[68] SAR studies demonstrate that the formamide group in the para-formamidophenylalanine region and bromine in the meta-bromobenzyl methylbenzamide region are integral for the functional activity of **6**, and a dramatic reduction of activity was observed when removing these groups.[69] In vitro studies indicate the addition of **6** results in an inhibition of β_2AR stimulated cAMP production and fi-

arrestin recruitment. Furthermore, pharmacological studies and the β_2AR-NAM cocrystal of a polyethylene glycol—carboxylic acid derivative of **6** reveal an intracellular binding site formed by residues from helices I, II, and VI–VIII and the ICL1 of the β_2AR (PDB code 5X7D).[21] Only recently have small molecule allosteric sites been identified on the intracellular surface of GPCRs, and this finding is significant as these results could extrapolate to additional members of the class A GPCR family, opening new avenues for allosteric drug discovery.

3.1.3. Dopamine Receptors (D_1, D_2, D_3).—Dopamine receptors represent a therapeutically important subset of class A GPCRs and exist in two distinct families: (1) D_1-like family members comprise dopamine D_1 and D_5 receptors, which couple to the $G\alpha_s$ and $G\alpha_{olf}$ G proteins and stimulate cAMP production, while (2) D_2-like family members comprise D_2, D_3, and D_4 receptors, which predominantly couple to $G\alpha_{i/o}$ G proteins and attenuate cAMP production. D_2-like receptors are widely recognized as the predominant target for the treatment of schizophrenia and Parkinson's disease.[70,71] However, as a whole, dopamine receptors play a substantial role in numerous neurological and psychological disorders, including also attention deficit hyperactivity disorder (ADHD) and drug and alcohol dependence or SUD.[72] From a structural perspective of allostery, the evidence of dimerization in dopamine receptors and other GPCRs (homodimers and heterodimers) should not be overlooked, but there is still much to understand about the functional implications of GPCR dimer populations and how these populations might be therapeutically targeted.[73–79]

Due to the therapeutic potential of D_2-like receptor drugs, several allosteric modulators for D_2 and D_3 have been reported recently (Figure 5). The neuropeptide Pro-Leu-Gly-NH_2 (PLG, **7**), initially isolated from brain tissue, is an endogenous molecule that has shown potential for pharmacologically treating neurological diseases such as Parkinson's disease and tardive dyskinesia, but the peptide nature of **7** limits its development as a drug. Therefore, the rational design and modification of **7** have led to analogues containing lactam, bicyclic and spiro-bicyclic scaffolds in the search for agents with better PK properties. Subsequent studies show **7** acts as a PAM of the dopamine D_2 and D_4 receptor, and the modes of action for **7** and its peptidomimetics were validated by photoaffinity labeling peptidomimetics.[80–82] Modification of **7** in the L-proline or L-Priline and L-leucine residues led to compounds **8** and **9** displaying a similar PAM profiles as **7**, measured by increasing [^{3}H]NPA binding at concentrations between concentrations of 1pM and 1 nM in human dopamine D_2 receptors.[83] Improving upon the initial neuropeptide, analogue PAOPA (**10**) (3(*R*)-[(2(*S*)-pyroolidinylcrbony)amino]-2-oxo-1-pyrrolidine-acetamide), a dopamine D_2 recetor selective PAM, is 100- to 1000-fold more potent than **7** and significantly attenuated schizophrenia-like behavioral phenotypes in preclinical models. [84–86] Importantly, **10** displays an improved PK and toxicological profile.

Interestingly, extensive studies with the spiro-bicyclic analogues of 7 have produced both D_2 PAMS and NAMS with minor differences in the stereochemistry of the bridgehead carbon within the same series of peptidomimetics.[87]Compounds **11** and **13** are D_2 PAMS, while the corresponding distereoisomeric compounds **12** and **14,** with a difference in the 8′ a chiral

center, demonstrate D_2 NAM activity in binding experiments with the D_2 receptor agonist NPA and competitive binding with the D_2 PAM.[88] The molecular conformation is hypothesized to take either a type VI β-turn, or polyproline II helix conformation that could place the carboxamide NH_2 pharmacophore in the same topological space as that seen in the type II β-turn, which is vital for the ability to modulate dopamine receptors. Molecular modeling suggests there are two different conformations in the pucker of **14** that may result in divergent effects on the orthosteric site. The modeling results were experimentally tested by incorporating proper chemical substituents to convert the PAM to a NAM. Compounds **15** and **16** with dimethyl groups in the C2′ position of the 5.6.5 bridge were designed to display NAM activity, divergent from the parent compound PAM activity. Indeed, when compared to a control, **15** and **16** demonstrate a NAM profile, which negatively affects the binding of the dopamine receptor agonist NPA to the D_2 receptor, and the resultant shift in the EC_{50} value for [^{3}H] NPA binding to D_2 was 2.7- and 2.8-fold at concentrations of 1 μM and 10 μM, respectively.[89]

SB269652 (**17**) was found to be a negative allosteric modulator for D_2 and D_3, and the corresponding SAR shows both components of **17** possess influence to some extent on its allosteric activity (Figure 5).[90,91] The design of **17** incorporates both an orthosteric site-binding moiety and an allosteric site-binding moiety, referred to as a bitopic ligand. Modest structural modifications shifted activity in the series between competitive antagonists and compounds, such as **17**, that display allosteric pharmacology. The THIQ head group is crucial for maintaining allosteric pharmacology, while a “small” substituent in the 7-position is required, and replacement of the alkyl spacer with a linear 1,4-butylene or 1,6-hexylene spacer group conferred an increase in functional affinity. Interestingly, although the tail group is sensitive to chemical modification, an alternative 7-azaindole tail was reported and demonstrates a 30-fold increase in affinity while maintaining negative cooperativity with dopamine.[92] Further optimization of **17** led to D_3 receptor-preferring bitopic ligands, characterized by a *trans*-cyclo-propylmethyl linker replacing the *trans*-1,4-cyclohexylene linker while retaining the head and tail groups.[93] Recently, a benzothiazole scaffold compound was reported as a D_2 PAM, identified via a high-throughput screen (HTS) on 80 000 compounds, and provides another small molecule hit for the development of D_2 PAMs.[70] Although there are several identified hits coming into the arena for the allosteric modulation of D_2 and D_3, further development to provide mature clinical candidates with improved in vitro and in vivo properties, along with safer PK profiles, is still urgent.

3.1.4. Muscarinic Acetylcholine Receptors (M_1–M_5).—Muscarinic acetylcholine (ACh) receptors contain five receptor subtypes (classified as M_1–M_5) and are involved in a wide range of biological processes and diseases, including pain, Alzheimer’s disease, schizophrenia, diabetes, and obesity.[94] According to their G protein coupling preference, there are two major functional classes. The M_1, M_3, and M_5 receptors selectively couple to G proteins of the $G\alpha_{q/11}$ family, whereas the M_2 and M_4 receptors preferentially activate $G\alpha_{i/o}$ G proteins. From the perspective of biological distribution, the M_1, M_4, and M_5 receptors are predominantly expressed in the CNS, whereas the M_2 and M_3 receptor subtypes are widely distributed both in the CNS and in peripheral tissues, thus playing an important role in regulating various peripheral and central physiological functions.[95] As a

reference, a recent review by Mohr and colleagues has thoroughly summarized allosteric modulators targeting CNS muscarinic receptors.[96] Herein, we focus on the recent development of these modulators from a medicinal chemistry perspective, which is mainly reflected in M_1, M_4, and M_5 allosteric modulators.

M_1 mAChR.: Benzylquinolone carboxylic acid (BQCA, **18**), discovered through HTS efforts at Merck, is a representative scaffold for the development of highly selective M_1 mAChR PAMs and provides a basis for developing novel therapeutics to counteract the negative cognitive symptoms associated with diseases such as Alzheimer's disease and schizophrenia (Figure 6).[97] According to current ligand classifications, **18** is a PAM that lacks intrinsic activity to induce calcium mobilization at concentrations up to 10 μM but markedly increases ACh potency 129-fold at 100 μM in human M_1 mAChR expressing CHO cells. Additionally, **18** displays selectivity over the related neuronal M_2–M_5 mAChR subtypes up to >100-fold and does not modulate signaling at other examined class A GPCRs. However, **18** presents lackluster PK, resulting in high plasma protein binding and low solubility in its neutral form.

Subsequent SAR-informed optimizations to address these shortcomings were conducted. These studies suggest fluoro substituents are preferred optimizations for analogues of **18** in terms of M_1 mAChR potency in vitro,[98] which was later proven to attribute to increased intrinsic efficacy of these analogues.[99] Compounds **19** and **20** were produced based on this principle and displayed an improved inflection point (IP, also known as PAM EC_{50}) but maintained high plasma protein binding and poor brain exposure.[98,100] Amide derivative **21**, with an improved PK profile, produces higher potency binding and functional cooperativity with ACh, bearing cooperativity α and $\alpha\beta$ values of 170 and 840 in a calcium mobilization assay, respectively.[99] Modifications on the benzyl side chain and quinolin-4(1*H*)-one of **18** were examined as a means to further improve affinity, decrease plasma protein binding, and address the blood—brain barrier (BBB) permeability problem aforementioned. Aryl methyl benzoquinazolinone **22** is a resultant compound with a greater than 50-fold increase in affinity for the M_1 receptor allosteric site in comparison to **18** (K_B = 0.3 μM for **22** and 15 μM for **18**) while retaining similar positive cooperativity with ACh and relative intrinsic efficacy.[101] Mutagenesis studies and molecular modeling confirm compound **22** occupies the same allosteric binding pocket as **18**. Insights from these studies include key hydrophobic/edge-to-face π–π interactions with residues Tyr-179 in ECL2 and Trp-400$^{7.35}$ in TM7 that are critical for the increased affinity to the allosteric site and functional activity of **22**. MK-7622, a mature example in this series, advanced into a phase II clinical trial in 2013 and was terminated in 2016 for undisclosed reasons.[102]

For further optimization efforts, modifications were made to the core pharmacophore aryl ring systems. Opening of the aryl A ring of **22** furnishes 4-phenylpyridin-2-one **23**, a structure maintaining intramolecular hydrogen bonding between the carboxylic acid and ketone in **18**.[103] Compound **23** shows comparable allosteric site binding affinity to 18 with a K_B value of 43 μM in FlpIN CHO cells expressing human M_1 mAChR but interestingly improved positive cooperativity with ACh (α = 370, $\alpha\beta$ = 200), i.e., a significant 370-fold potentiation of ACh affinity, and retained high selectivity for the M_1 mAChR. Further

modification of pyridin-2(1H)-one to 6-phenylpyrimidin-4-one **24** presents an α value of 1380, a 4-fold increase in binding cooperativity with ACh, along with an 11-fold increase in intrinsic efficacy (τ_B = 2.51), suggesting further interaction with the allosteric pocket of the M_1 mAChR through the introduction of an additional tertiary nitrogen as a hydrogen bond acceptor. Ring opening of aryl B, replacing the tricyclic benzo[*h*]-quinazolin-4(3*H*)-one core with quinazolin-4(3*H*)-one, gives compound **25**.[104] Compound **25** shows improved "druglike-ness" with lower lipophilicity, topological polar surface area (tPSA), molecular weight and also reduces the toxic DNA-chelation concern for polyaromatic heterocycle scaffolds. The methyl group in the 8-position is critical to maintain affinity for the M_1 mAChR allosteric site compared to **22** with pK_B and $\alpha\beta$ values of 5.15 and 380 for **25** and 5.88 and 370 for **22**, respectively, but lower intrinsic activity (τ_B = 1.1) in radioligand binding experiments using FlpIN CHO cells. Dibenzyl-2*H*-pyrazolo[4,3-*c*]quinolin-3(5*H*)-one (**26**, DBPQ) is another example of a tricyclic scaffold similar to **22** and was discovered as a hit from a 2012 high-throughput screen of the NIH Molecular Libraries Small Molecule Repository (MLSMR).[105] A concentration—response curve of compound **26** in the presence of a submaximal concentration of ACh exhibits a PAM EC_{50} of 473 nM and limited cooperativity with ACh, resulting in a ACh maximum response of 40% measured by the calcium response assay.

Replacement of the quinolone ring system of **18** with quinolizidinone as an alternative scaffold and exchanging the side benzyl chain with a basic 4-cyanophenylpiperazine linkage led to compound **27**.[106] Compound **27** shows enhanced CNS exposure with a 13% human free fraction and improved in vivo efficacy in a mouse contextual fear conditioning model of memory, while the bioavailability is relatively low, observed as 23% in rats. Noteworthy, this modification limits the liability for efflux by the CNS efflux transporter P-glycoprotein (P-gp). Compound **28** bearing a cyano group at the 4-position of the piperidine displays increased PAM potency (M_1 mAChR EC_{50} = 135 nM) in human M_1 mAChR expressing CHO cells determined in the presence of an EC_{20} concentration of ACh by a calcium mobilization readout on a fluorometric imaging plate reader (FLIPR384).[107] The plasma free fraction value is an acceptable 30%, and oral bioavailability is greatly improved to 68% in rats with a 1.7 h half-life. Further modification by replacing the carboxylic acid with cyclic amide substituents to produce **29** significantly improved the PAM potency in functional assays (M_1 mAChR EC_{50} = 31 nM); however this led to P-gp efflux liability.[108] A replacement of the core to methoxynaphthalene led to compound **30** with a M_1 mAChR PAM EC_{50} value of 17 nM.[109] Although **30** addressed the P-gp substrate problem, **30** results in high protein binding as observed at 99.9% in rat.

VU0108370 (**31**), a hit discovered via a functional HTS of the NIH's Molecular Libraries Probe Production Centers Network (MLPCN) library, represents a second unique chemotype based on an indole core (Figure 7).[110] Compound **31** shows relatively low PAM potency for ACh at the M_1 mAChR with an EC_{50} value of 9.71 μM. Structural optimization around **31** afforded ML169 (**32**) as a selective M_1 mAChR PAM MLPCN probe, presenting a moderate yet improved potency (PAM EC_{50} = 1.38 μM). Difluoro substitution of the indole core and a pyrazine in the benzyl side chain results in VU0456940 (**33**) and further improves the PAM EC_{50} to 310 nM.[111] Azaindole (**34**) results from the replacement of the quinolone ring of **18**

with an azaindole core producing an EC_{50} value of 1.8 μM.[112–114] Modification of the substitution on a benzyl with pyrazole furnishes PF-06764427 (**35**) as a highly selective M_1 mAChR PAM and demonstrates excellent cooperativity with a 30-fold potency improvement and favorable in vivo efficacy in an amphetamine-stimulated locomotor activity model in rodents.[115] Difluoro substitution on **35**, resulting in VU6004256 (**36**), improves its safety profile and was reported as lacking severe adverse effects, such as behavioral convulsions and peripheral cholinergic adverse effects in mice, although no improvements to its activity were observed.[116]

A third unique scaffold for M_1 mAChR PAMs consists of an isatin core. VU0119498 (**37**) is a pan-M_1,M_3,M_5-PAM discovered through a HTS campaign, and subsequent SAR enabled the rational design of subtype selective PAMs.[117] Chemical optimizations on **37** led to selective M_1 mAChR PAMs ML137 (VU0366369, 38) and VU0448350 (**39**) with EC_{50} values of 0.83 μM and 2.4 μM, respectively.[117,118] Further modification on this scaffold yields VU0453595 (**40**), a highly selective M_1 mAChR PAM with improved brain exposure in mice after systemic administration.[119] Recent modification around isoindolin-1-one produces compound **41**, which displays a significantly improved PAM EC_{50} value of 47 nM, minimal intrinsic agonist activity, and oral bioavailability of 91% in rodents and 65% in canines.[120] Other work in this arena includes the benzodiazepine derivative **42**, which was recently reported by a team at Roche as a selective M_1 mAChR PAM with an PAM EC_{50} value of 80 nM in human M_1 mAChR expressing cells.[121] Overall, there are variable improvements in brain exposure and in vivo efficacy for selective M_1 mAChR allosteric modulators and development continues for multiple scaffolds presented herein.

M_5 mAChR.: The M_5 mAChR has historically been the least studied receptor of the mAChR family, primarily due to low endogenous expression levels; however there is a current upsurge in interest from drug discovery groups to identify selective PAMs that are efficacious in relevant disease models.[122] Physiological studies indicate the M_5 mAChR is enriched in the cerebrovascular system and present a potential target for the treatment of numerous CNS disorders including schizophrenia, Alzheimer's disease, ischemia, and migraine.[122] As previously highlighted, the small molecule series with an isatin core display PAM activity at the M_5 mAChR in addition to other mAChR subtypes, and thus medicinal chemistry efforts have partly focused on identifying molecular switches to convey subtype specificity in this series. VU0119498 (**43**) is the first reported hit discovered via a HTS and displays micromolar potencies as a pan-PAM for the potentiation of ACh at M_1, M_3, and M_5 mAChR subtypes in cell-based calcium mobilization assays (Figure 8).[123] Subsequent optimization led to the discovery of ML129 (VU0238429, **44**), containing a 5-trifluoromethoxy isatin scaffold, as a selective M_5 mAChR PAM with an EC_{50} of approximately 1.16 μM.[124] Further modifications included substitutions on the benzyl chain where it was shown that VU0365114 (**45**) and ML172 (VU0400265,**46**) could maintain potency, while the addition of a phenoxyethyl substituent in ML326 (**47**) achieved submicromolar level potency in a human M_5 mAChR expressing cell line (EC_{50} = 410 nM, ACh fold-shift = 20, Figure 9).[122,125,126] However, the suboptimal ionization properties of **47** preclude its utility for in vivo assessment due to low CNS exposure.[127] ML380 (**48**), a promising and divergent scaffold lacking the common isatin core, was discovered during a

HTS of the MLPCN screening deck of ~360 000 compounds against M_1, M_4, and M_5 mAChR subtypes. Compound **48** displayed selective M_5 mAChR PAM activity with submicromolar potency and markedly improved CNS penetration (EC_{50} = 190 nM, ACh fold-shift = 9.3).[127]

In regard to NAM discovery, ML375 (**49**) is the first selective M_5 mAChR NAM with submicromolar allosteric site potency, and characterization reveals an IC_{50} of300 nM at the human M_5 mAChR and an IC_{50} of 790 nM for rat M_5 mAChR (Figure 9).[128] Compound **49** was also found to exhibit favorable CNS exposure (brain/plasma K_p = 1.8).[128] However, high protein binding in both blood and brain tissue (rat f_u = 0.029, rat brain f_u = 0.003) has limited its utility as an in vivo tool compound to date.[129] Through a combination of matrix libraries and iterative parallel synthesis, optimization of **49** led to VU6000181 (**50**), which maintains potency levels but also retains an unfavorable PK profile.[129]

M_4 mAChR.: Interestingly, the analytical specialty chemical thiochrome was one of the first reported selective M_4 mAChR PAMs but was observed to exhibit low affinity for the receptor.[130] Heightened activity in this arena occurred with the discovery of LY2033298 (**51**) as a benchmark example of selective M_4 mAChR PAMs (Figure 10).[131] Compound **51** contains a 5-aminothieno[2,3-*c*]pyridine scaffold and has a measured K_B value of 200 nM at the allosteric site on the unoccupied human M_4 mAChR.[131] Chemical modification of **51** on the substitutions in the thieno[2,3-*c*]pyridazine and side chain resulted in the discovery ofVU10010 (**52**).[132] Compound **52** has a high potency with an EC_{50} value of400 nM and elicits a 47-fold leftward shift of an ACh concentration—response curve in a calcium-mobilization assay in rat M_4 mAChR expressing cells. According to follow-up manuscripts focusing on the optimization of **52**, it was stated that **52** was not centrally active in vivo and was considered to be a substrate for the P-gp efflux pump.[133] However, upon work to the scaffold, the authors concluded lack of in vivo activity of **52** was due to physicochemical properties (e.g., log P = 4.5) that led to a poor pharmacokinetic profile. Optimization of **52** led to ML108 (**53**) and VU0152099 (**54**), which present nearly equivalent potency while improving CNS exposure as evidenced by peak brain concentrations ranging from 3 to 5 μg/mL after 56.6 mg/ kg ip administration in rodents.[133] However, the metabolic stability of this scaffold is poor due to the hydroxylation of the 6-methyl group on the pyridine ring, resulting in less than 10% parent compound remaining after 90 min. Noteworthy, the replacement of the metabolically labile 6-methyl group with an ether linked substituent led to ML173 (**55**) and a significantly improved microsomal stability profile with greater than 90% parent remaining after 90 min, in both rat and human microsomal assays.[134] Although in vitro potency in human M_4 mAChR expressing cells was measured at an EC_{50} of 95 nM, subsequent preclinical in vivo studies demonstrated limited efficacy for **55** in the reversal of amphetamine-induced hyperlocomotion in a rat behavioral model. This result necessitates highlighting an important phenomenon that can be encountered during the preclinical phases of compound optimization: species bias. Additional data indicate that although high potency was observed at the human M_4 mAChR, **55** displays a strong species bias with an EC_{50} of only 2.4 μM for the rat M_4 mAChR, which leads to exceedingly difficult optimization and ultimately precludes preclinical development of this compound.[134] In an expert review,

Conn, Lindsley, Meiler, and Niswender advise identifying and avoiding compounds with an intractable species bias from experience in this series and others.[22]

ML293 (**56**) is characterized by a unique scaffold with a benzothiazole core and was developed through an iterative optimization effort at the Vanderbilt Center for Neuroscience Drug Discovery (Figure 10).[135] Although **56** displayed low micromolar potency at the human M_4 mAChR, the promising in vivo PK properties warrant attention with a lower iv clearance rate (11.5 mL min^{-1} kg^{-1}) than previous series and excellent brain exposure when orally administered to rats and measured as a brain to plasma ratio (10 mg/kg at 1 h, [brain] = 10 μM, *B:P* = 0.85).[135] The modest potency and half-life (EC_{50} = 1.3 μM, $t_{½}$ = 57 min) necessitates further optimization of this series. Recently reported, modification of the previously described 3-aminothieno[2,3-*b*]pyridine scaffold (as in **53**) by replacing the pyridine with a pyridazine ring results in VU0464090 (**57**).[136] Compound **57** displays a 3-fold potency improvement with an EC_{50} value of 150 nM in human M_4 mAChR expressing cells and a significant 9-fold improvement in free fraction values compared to **53** (f_u rat, human of 0.022, 0.035 for **57** and 0.015, 0.004 for **53**).[136] This relatively minor chemical modification results in large electronic changes due to the high dielectric constant of the pyridazine ring and likely led to the observed improvement in PK properties. However, the *p*-methoxybenzylamide in **57** proved to be metabolically labile via cytochrome P450 (CYP)-mediated oxidative demethylation, and alternative amides were explored. Concentrating on sulfur-containing amide moieties, Lindsley and colleagues discovered VU0467154 (**58**) through an iterative optimization campaign.[136] Although the potency of **58** for human M_4 mAChR is modest with an EC_{50} value of631 nM, **58** has proven to be an exemplary rodent M_4 mAChR PAM tool compound, due to its reported minimal off-target interactions, excellent subtype selectivity, and good PK profile.[136,137] The following optimizations to the 5-aminothieno[2,3-*c*]pyridazine class ofM_4 mAChR PAMs were recently reported and feature an azetidine substituted side chain.[138] Out of a series of examined cyclic amines, azetidine based linkers in the side chain retained activity. Chemically, compound **59** in this series maintains an amide; however utilization of the azetidine results in a rigid amide lacking H-bonding ability (Figure 10). On the basis of an interesting analysis of X-ray crystallographic data from compound **59**, the removal of the amide N–H and the introduction of conformational restraint in the side chain were hypothesized to limit intermolecular interactions between adjacent molecules due to π-stacking and H-bonding, ultimately improving upon the poor solubility of previous derivatives. Indeed, **59** maintains high potency with a human M_4 mAChR EC_{50} of 72 nM and markedly improved CNS exposure in rat (K_p = 2.6, $K_{p,uu}$ = 2.1).[138] In vivo, **59** was examined in an established rat amphetamine-induced hyperlocomotion assay to determine antipsychotic efficacy and at 30 mg/kg (oral) **59** can attenuate hyperlocomotion by up to 32%. However, the azetidine amide linker in this scaffold is a metabolic weakness with high predicted clearance resulting from both rat and human microsomal preparations (CL_{hep} = 64 (r), 20 (h) mL min^{-1} kg^{-1}). Subsequent developments detail a replacement by a 3-aminoazetidine moiety, resulting in VU6000918 (**60**).[139] Compound **60** is characterized by improved in vitro human M_4 mAChR potency (EC_{50} = 30 nM), a relatively short half-life (1–2h), and suboptimal oral bioavailability, as measured at 11% in canine (2 mg/kg). At present, this extended series of compounds has demonstrated excellent in vitro activity and preferred species selectivity;

however achieving an optimal combination of distribution, metabolism, and pharmacokinetic (DMPK) properties such as bioavailability, CNS exposure, P-gp efflux liability and metabolic stability has proven to be challenging.

M_2 mAChR.: The M_2 mAChR is distributed in both the CNS and periphery and has been predominately studied for its role in regulating parasympathetic cardiac function. In this capacity, the M_2 mAChR modulates potassium channels and its activation leads to the closing of calcium channels, necessary for heart rate reduction.[140] Early examples of M_2 mAChR allosteric modulators, discovered by Mohr and colleagues, include alkane-bisammonio-type ligands.[141] This chemotype was subjected to subsequent rounds of chemical modifications, where a switch from negative to positive cooperativity was observed and conjugation to orthosteric ligands resulted in bitopic molecules of interest.[142] The importance of the M_2 mAChR lies not in its therapeutic utility as the general consensus is that M_2 mAChR activation leads to undesirable off-target effects for mAChR modulators.[143] However, from a structural perspective, elegant work on the M_2 mAChR and its PAM LY2119620 (**61**) has enabled major leaps in the understanding of mAChR, and GPCR, allosteric modulation.[19,144] Compound **61** is reported as a high-affinity PAM of both the M_4 and M_2 mAChR, and it potentiates the activity of agonist iperoxo. The pioneering cocrystal structure of **61** bound to the M_2 mAChR shows **61** occupying a site above the orthosteric iperoxo binding site on the extracellular side ofhelices II, VI, and VII and forming extensive contacts with ECL2. and ECL3 (PDB code 4MQT, Figure 11).[19] These contacts include a charge—charge interaction between the piperidine group and residue Glu172 in ECL2, hydrogen bonds between both the amide oxygen and N–H with the side chains of Tyr80$^{2.61}$ and Asn419 in ECL3, and additional hydrophobic interactions with aromatic residues in ECL2. This structural analysis provides insight into one way a PAM can impact agonist binding and resultant receptor activity.

Further contributions from the structure of the M_2 mAChR include a recent discovery effort aimed at finding highly selective PAMs that potentiate binding of nonselective antagonists, whereby the antagonist is essentially turned selective due to PAM induced cooperativity.[145,146] These studies utilized extensive molecular libraries for ensemble docking at the M_2 mAChR and realize the potential of allosteric modulators to improve orthosteric ligand selectivity. The resultant compound ‘628 (**62**) features a unique triazoloquinazolinone and could enhance binding of the M_2 mAChR antagonist *N*-methyl scopolamine (NMS) with a cooperativity factor (α) of 5.5. Interestingly, **62** can markedly slow the dissociation rate of NMS from the M_2 mAChR by 50-fold. The specific PAM effect of **62** on NMS antagonism was further validated in cell-based functional assays, and the observations translated to membranes from adult rat hypothalamus and to neonatal rat cardiomyocytes.[146]

3.2. Lipid Family Receptors.

3.2.1. Cannabinoid Receptors (CB_1 and CB_2).—

The CB_1 receptor and the CB_2 receptor are key mediators of the endocannabinoid system. The cannabinoid CB_1 receptor is widely distributed throughout the CNS and endogenous agonists of the CB_1 receptor include anandamide (AEA) and 2-arachidonylglycerol (2-AG), which regulate many physiological processes related to pain, metabolism, nociception, and neurotransmission.[147] To date, CB_1

receptor orthosteric agonists and antagonists have not realized therapeutic expectations largely due to adverse effects, for example, the orthosteric antiobesity antagonist rimonabant was withdrawn from the market owing to neuropsychiatric adverse effects.[148] Alternatively, development of both NAMs and PAMs of the CB_1 receptor has been of high interest in recent years, encompassing structurally distinct synthetic, plant-derived and endogenous allosteric ligands. NAMs of the CB_1 receptor were reported first, mainly comprising two scaffolds that have been extensively characterized: the 1*H*-indole-2-carboxamide and the diarylurea analogues. The CB_2 receptor, another important member of the endocannabinoid system, is highly expressed in the periphery, especially in blood cells, and in blood-cell producing organs.[149] Additionally, recent work has highlighted the expression of CB_2 receptors in the CNS and, functionally, CB_2 receptor modulation of dopamine-mediated neural activity and cocaine self-administration in rodents.[150,151] Despite recent pharmacological advances in the characterization of cannabidiol as an allosteric modulator of the CB_2 receptor,[152] the therapeutic potential of PAMs and NAMs targeting the CB_2 receptor requires additional investigation, and thus we focus on CB_1 receptor ligand discovery herein.

1H-Indole-2-carboxamide CB_1, Receptor NAMs.: A major advance was made with the discovery of 1*H*-indole-2-carboxamide analogues as CB_1 receptor NAMs. Org27569, Org29647, and Org27759 (**63–65**, Figure 12) displayed an interesting pharmacological profile by enhancing the affinity yet reducing the efficacy of CB_1 receptor agonists and suggests the existence of an allosteric binding site at the CB_1 receptor. These allosteric ligands have proven to be an excellent series of tool compounds and provide a basis for medicinal chemistry development. When examined in a binding assay, **63–65** augment specific binding of the CB_1 receptor agonist 2-[(1*R*,2*R*,5*R*)-5-hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol, [^{3}H]-CP55,940 in membranes from cells expressing the CB_1 receptor. However, in a luciferase reporter gene assay, guanosine 5′-O-(3-[^{35}S]thio)triphosphate binding assay and mouse vas deferens assay, **63–65** elicit a significant reduction in the E_{max} for CB_1 receptor agonists, showing a resultant CB_1 receptor NAM profile.[153]

Piscitelli et al. examined a number of 4-substitutions on the phenyl B ring and discovered that piperidinyl or dimethylamino groups at the 4-position of the phenyl ring are preferential for CB_1 receptor NAM activity (Figure 12).[154] Compound **66**, with hydrogen in the C3 position of the indole, and compound **67**, with a dimethylamino substituent in the 4-position of phenyl, show potent NAM activity with IC_{50} values of 90 nM and 50 nM, respectively. The analyses of these compounds also provide evidence that the carboxamide functionality is required, and when replaced by an ester, potency is greatly reduced. Lu et al. subsequently conducted a series of SAR studies around the 1*H*-indole-2-carboxamide scaffold by measuring two essential parameters: K_B, which reflects the binding affinity of the allosteric ligand, and α, which measures the allosterically induced effects between the orthosteric and allosteric ligands when both are bound to the receptor. These SAR studies report the indole core is essential for the binding affinity (K_B) but not for generating allostery (α) on the orthosteric site, and the C3 substituents of the indole-2-carboxamides significantly impact the cooperativity. Replacing the indole with benzofuran ring (**68**) led to a 10-fold increase in

the K_B value (2594 nM for **68** vs 217.3 nM for **63**), and masking the indole nitrogen with a methyl group as in **69** led to a significant 27-fold decrease. Substitution of the C3 position with a linear *n*-pentyl group, resulting in **70**, displayed a noteworthy cooperativity enhancement (α = 17.6 for **70** vs a = 11.9 for **69**) while producing only a moderate binding affinity of 496.9 nM. Additionally, replacing the phenyl B ring piperidinyl substituent in **70** with a dimethylamino moiety yields **71** with improvement to the NAM binding affinity (K_B = 167.3 nM) and maintenance of the cooperativity (α = 16.55), while functional assays displayed a potent inhibition of GTPγS binding.[155,156] Further investigations modifying the C3 position with variations of the linear alkyl moieties, such as *n*-propyl and *n*-hexyl, yield compounds **72** and **73**, with **72** displaying the highest allosteric ligand affinity observed (K_B = 89.1 nM) and a reduction in cooperativity (α = 5.1), while **73** was characterized by modest affinity (K_B = 217.3 nM) and a significant enhancement of binding cooperativity (α = 24.5).[157] In addition, modification to the substitution on the A ring shows that a 5-halogen substituent is vital to maintain allosteric activity. Functional assays on **72** and **73** demonstrate an interesting effect on CB_1 receptor signaling pathways with a NAM concentration-dependent inhibition of agonist-induced GTPγS binding, yet they have a PAM effect in a β-arrestin mediated extracellular signal-regulated kinases ½ (ERK½) phosphorylation assay. Aside from alkyl chain substituents on the C3 position, less bulky substituents such as methyl and hydrogen on the C3 position and a fluoro or chloro substitution at the C5 position yielded analogues **74** and **75**, which possess improved CB_1 receptor NAM activity in a calcium-mobilization assay with an IC_{50} of 151 nM and 79 nM, respectively, which is approximately 5- and 10-fold more potent than the parent compound **71**.[158]

Ligand-assisted protein structure (LAPS) is an approach for elucidating structure—function correlates of ligand binding to GPCRs in functionally and physiologically relevant conditions, which is useful for obtaining structural information about the allosteric site since the structures of some receptors are obscure.[159] In this approach, noncovalent, pharmacologically active ligands can be designed and synthesized to accommodate chemically reactive moieties, such as electrophilic groups (e.g., isothiocyanate, benzophenone, etc.) and photoactivatable groups (trifluoromethyl diazirine, aliphatic/aromatic azide, etc.). Thus, upon ligand binding, the reactive group forms a covalent bond at an actionable amino acid residue providing spatial resolution of the allosteric site, in this case. Modification at the 5-position of **63** with the chemically reactive electrophilic group isothiocyanate (NCS) generates CB_1 receptor NAM covalent ligand **76** that retains the affinity—efficacy profile of **63** but displays reduced inverse agonist activity.[159] Functional assays show a signaling bias toward β-arrestin with an EC_{50} of 2 nM, an 87-fold difference over cAMP-dependent signaling. To validate the ability of **76** to covalently label human CB_1 receptor, time-course experiments were executed, which showed the binding of [^{3}H]-CP55,940 to human CB_1 receptor increases in a time-dependent manner, reaching a maximum by 60 min preincubation time with **76**. Compounds **77–80** are another series of photoactivatable analogues developed by Lu et al. as allosteric modulators for the CB_1 receptor.[160] These compounds preserved the pharmacological properties of **63** negatively modulating the CB_1 receptor agonist CP55,940-induced G protein coupling in a concentration dependent manner with a complete functional inhibition at 10 μM in their

assay system. These results show that the *N*-phenylethyl-1*H*-indole-2-carboxamide scaffold is a potent representative for CB_1 receptor allosteric modulation.

Diarylurea Analogues as CB_1 Receptor NAMs.: A second substantial body of work describes the discovery and interrogation of the diarylurea scaffold as a CB_1 receptor NAM. Numerous studies have now reported chemical leads bearing this scaffold, along with thorough SAR. 1-(4-Chlorophenyl)-3-(3-(6-(pyrrolidin-1-yl)pyridine-2-yl)phenyl)urea PSNCBAM-1 (**81**, Figure 13), discovered via HTS, displays PAM-like positive cooperativity in agonist binding affinity but decreases functional response in cellular assays. As an excellent case for a thorough pharmacological workup, **81** enhanced radioligand [^{3}H]-CP55940 binding levels but decreased functional responses stimulated by orthosteric agonists in numerous assays, including intracellular calcium mobilization, [^{35}S]GTP-γ-S binding, cAMP accumulation, and β-arrestin recruitment.[161,162] Interestingly, this profile is similar to *N*-phenylethyl-1*H*-indole-2-carboxamide Org27569 (**63**) with the notable exception of intrinsic efficacy as an agonist in some assays such as ERK½ phosphorylation; **81** has not displayed marked agonist activity in similar assays thus far.[163,164]

Detailed SAR studies on **81** have been reported, focusing on various substitutions on the diphenyl rings of the urea. Zhang et al. first reported chemical modifications to the 4-chlorophenyl at the A ring and the alkyl substitution at the 6-aminopyridinyl rings of the B ring. The smaller *N,N*-dimethylamino analog **82** displays comparable potency to **81** (27.4 nM vs 32.5 nM), suggesting the pyrrolidinyl ring is not required for this parameter. Further investigation on the A ring uncovered that the key property correspondent to activity was electron density. For example, electron withdrawing groups at the 4-position provided good potency, with the fluoro (**83**, IC_{50} = 32 nM) and the cyano analogues (**84**, IC_{50} = 33 nM). Among them, the cyano analogue possesses much better potency in CB_1 receptor calcium mobilization and radioligand [^{3}H]-CP55940 binding assays with an EC_{50} of 55.2 nM. Subsequent replacement of the pyridine ring by a pyrimidine ring, resulting in **85** and **86** (Figure 13), presents comparable activity to **81** in binding assays. Functional studies suggest that these compounds now display signaling biased PAM activity, promoting the β-arrestin-1 pathway toward ERK½ phosphorylation at 10 μM without the G_i-mediated signaling activity typically displayed by the agonist CP55940.[165] Further replacing the pyridinyl group with lipophilic aromatic rings (**87** and **88**) or introducing a spacer (N–H group) between the pyridine ring and the core phenyl (**89**) maintained activity at the CB_1 receptor and agreed with other work showing the exact properties lent by the pyridinyl and the pyrrolidinyl ring were not necessary for CB_1 receptor activity.[166,167] Among this series, RTICBM-74 (**87**) displayed comparable potency to **81** with an IC_{50} of 23 nM (33 nM for **81**) in a calcium mobilization assay; however, it was not as effective in antagonizing agonist-stimulated [^{35}S]GTP-γ-S binding to mouse CB_1 receptor in mouse cerebellar membranes (**81**, IC_{50} = 89 nM; **87**, IC_{50} = 153 nM). A significant metabolic and PK improvement was afforded by the lipophilic phenyl substituent in **87**, as examined in rat liver microsomes showing a more than 22-fold improved half-life ($t_{½}$ > 300 min) and clearance (CL = 4.6 μL min^{-1} mg^{-1}). Possibly due to the improved metabolic and PK profile, **87** is more effective than **82** in vivo in attenuating the reinstatement of extinguished cocaine-seeking behavior in rats, producing an effect at 10 mg/kg equal to that of 30 mg/kg of **81**.

Finally, some noteworthy additional scaffolds have been reported as CB_1 NAMs recently. Fenofibrate (**90**), a PPARα agonist, and cannabidiol (**91**), a nonpsychoactive phytocannabinoid with therapeutic utility in numerous disorders, have both been shown to act at the CB_1 receptor as NAMs (Figure 13).[168,169] The steroid pregnenolone (**92**) is also reported acting as a NAM of CB_1 receptor-mediated ERK½ phosphorylation, devoid of effects on orthosteric agonist binding affinity or cAMP-mediated signaling.[170] Lastly, there have been increased investigations on peptide endocannabinoids (Pepcans) and their ability to allosterically modulate cannabinoid receptor signaling, where some have shown CB_1 receptor NAM activity.[171]

CB_1 Receptor PAMs.: With a greater understanding of CB_1 receptor biology and the possible therapeutic benefit of agonism, the discovery and development of CB_1 receptor PAMs is a current and evident trend. RTI-371 (**93**), a dopamine transport inhibitor with a 3-phenyltropane backbone, was first found to be a CB_1 receptor PAM via an initial functional assay screen. Compound **93** (Figure 14) at 10 mM demonstrates potentiation of the efficacy of agonist CP55940 with an E_{max} value of 36% in a human CB_1 receptor cell-based calcium mobilization assay.[172] Next, a study found the endogenous anti-inflammatory mediator, lipoxin A4 (**94**), demonstrated allosteric enhancement of CB_1 receptor signaling.[173] Interestingly, this endogenous molecule could enhance the affinity of the assayed ligands to the CB_1 receptor with 100% enhancement of [^{3}H]-CP55940 binding and nearly 30% of [^{3}H]-WIN55212–2 binding while increasing the potency of AEA in decreasing forskolin (FSK)-induced cAMP levels by 386 times at a concentration of 100 nM in HEK-CB_1 cells. When studied in vivo and administered via an intracerebroventricular route (1 pmol/2 μL), **94** could promote neuroprotection against β-amyloid (1–40) (400 pmol/2 μL, icv)-induced performance deficits in the Morris water maze assay in mice. A novel synthetic small molecule GAT211 (**95**), with a 2-phenyl-1*H*-indole scaffold, was first reported as a CB_1 receptor PAM in a patent filed by Northeastern University, and recently Thakur et al. described the synthesis and in vitro and ex vivo pharmacology of **95** (racemic) and its resolved enantiomers, GAT228 (**96**, *R*) and GAT229 (**97**, *S*).[171,174] In membranes taken from CHO cells expressing human CB_1 receptor, **95** enhances the binding of the agonist [^{3}H]-CP55,490 at 100 nM and 1 μM and maintains binding enhancement from 1 nM to 10 μM in ex vivo mouse brain membranes while markedly reducing the binding of the antagonist/inverse agonist [^{3}H]SR141716A from 1 μM to 10 μM in ex vivo mouse brain membranes. Compound **95** (1 μM) displays both PAM and intrinsic agonist activity in human CB_1 receptor expressing HEK293A and Neuro2a cells and in mouse brain membranes rich in native CB_1 receptor. However, **95** also exhibits strong PAM activity at a concentration of 1 μM in isolated mouse vas deferens endogenously expressing CB_1 receptor without displaying intrinsic activity. Upon further investigation, the R-(+)-enantiomer (**96**) is the contributing factor for the Ago-PAM property of **95** and displays allosteric agonist activity when tested alone, whereas the *S*-(—)-enan-tiomer (**97**) contributes to the PAM activity of **95**, lacking intrinsic efficacy when isolated and tested. This example highlights the importance of interrogating the activities of racemates independently of the racemic allosteric modulator hit and provides evidence that minor stereochemical differences contribute divergent activities. Also, excellently shown in this case is that the ability to detect allosteric agonism (e.g., ago-PAMs) is dependent on the receptor expression levels in

a given cell-type and sensitivity of detection may differ between in vitro and ex vivo assays. Thus, these factors should be accommodated in programs screening racemic allosteric modulators in highexpressing cell lines that are not representative of in vivo receptor expression levels.

Subsequently, a report on the 2-phenyl-1*H*-indole analogue ZCZ011 (**98**) revealed in vitro and in vivo evidence of PAM activity. In vitro, **98** (Figure 14) increases the CB_1 receptor agonists [^{3}H]-CP55,940 and [^{3}H]-WIN55212 binding affinity and results in an enhanced functional output of E_{max} value of 207% and 225%, respectively (normalized to each agonist functional response at E_{max} 100%). Compound **98** (10 nM) displays enhanced AEA-stimulated signaling via [^{35}S]GTPγS binding with a 40% increase over AEA alone in mouse brain membranes. When additional signaling pathways were investigated, **98** displays a concentration-dependent enhancement of AEA-stimulated β-arrestin recruitment with an E_{max} value of 195% at 1 μM and shows an increase in agonist (AEA and CP55,940) potency by ERK½ phosphorylation assays in human CB_1 receptor expressing cells. In vivo, **98** (40 mg/kg, ip) is brain penetrant and increases the potency of administered orthosteric agonists when examined in cannabimimetic activity behavioral assays in rodents. Therefore, due to broad PAM activity across signaling pathways and multiple agonists, **98** may be useful as a pharmacological tool for mechanistic studies as well as for exploring proof-of-concept studies and potential therapeutic applications of CB_1 receptor PAMs.[175]

Straiker and colleagues recently report a physiologically relevant neuronal model of endogenous cannabinoid signaling as an assay to test CB_1 receptor allosteric modulators. In this model, CB_1 receptor ligands are applied to cultured autaptic hippocampal neurons that exhibit depolarization-induced suppression of excitation (DSE), a form of synaptic plasticity that is mediated endogenously by the CB_1 receptor and 2-arachidonoyl glycerol (2-AG).[176,177] The aforementioned NAMs **63**, **81**, and Pepcan12 attenuate DSE and do not directly inhibit CB_1 receptors, while NAMs **92** and hemopressin as well as the PAM **94** are without effect in this model. Compounds **95** and **98** each show PAM-like responses in autaptic hippocampal neurons, representing the first PAMs to display efficacy via the 2-AG-utilizing neuronal model system. In context of the abovementioned 2-phenyl-1*H*-indole **95**, further examination of its enantiomers **96** and **97** shows that the (*S*) enantiomer **97** exhibits pure PAM-like behavior and the (*R*) enantiomer **96** appears to directly activate the CB_1 receptor as an allosteric agonist, which is in accordance with the previous report.[171]

Molecular dynamics (MD) simulations of GPCR-ligand binding can provide a unique view into the subtleties ofreceptor activation and modulation and, importantly, illuminate ligand interactions for complexes that have proven difficult to crystallize thus far. A recent MD study by Tautermann et al. proposes a mechanism of interaction for certain CB_1 receptor ago-PAMs with two points. First, the agonism may result from the ligand binding to the orthosteric binding site, and the PAM effect is the result of the ligand interacting with an adjunct, deeper binding site in the receptor. Second, the pockets may overlap, resulting in the interaction with residues in both sites simultaneously as one unified pocket and producing the activation and/or modulation effects. Interestingly, this mechanism may explain the observation that Ago-PAM **97** displays competitive binding with CP 55,940 at high concentrations. This interaction mechanism is experimentally validated by showing that

multiple binding sites of **98**, another ago-PAM, contribute to its activity, where positive modulation of the orthosteric agonist is observed until the concentration of **98** is increased above its PAM EC_{50} value and it begins to compete with CP 55,940, owing to its additional affinity for the agonist binding site.[147]

The past decade has seen the identification and characterization ofmultiple promising compounds as NAMs targeting the CB_1 receptor; however these purported NAMs are beset by moderate efficacy and some CB_1 receptor inverse agonist activity that may hinder their future development. Additionally, in vivo studies on CB_1 receptor NAMs are thus far limited and will need to progress toward proof-of-concept studies to show therapeutic utility. As for CB_1 receptor PAMs, the reported small molecules trend toward multifaceted and complicated pharmacology that is sensitive to small molecular modifications, as seen from PAMs of other targets in this Perspective. Additionally, the structural diversity remains relatively small and novel scaffold discovery may open opportunities for CB_1 receptor PAMs with tractable pharmacology. Thus, due to the physiological importance and biological abundance of the CB_1 receptor, innovative medicinal chemistry efforts are necessary to further discovery and development of chemical probes and drug-candidates with improved DMPK characteristics.

3.2.2. Free Fatty Acid Receptors (FFA1—FFA3).—

Free fatty acid receptors (FFARs) are a recently “deorphanized” family of receptors that are activated by nonesterified, or free, fatty acids (FFAs), which comprise a carboxylic acid linked to an aliphatic chain ofvarying length. The receptors are classified based on the chain length of the endogenous agonist, which are termed short chain fatty acids (SCFAs), medium chain fatty acids (MCFAs), or long chain fatty acids (LCFAs). For example, the FFA1 receptor (also GPR40) and FFA4 receptor (also GPR120) are activated by MCFAs and LCFAs, while the FFA2 (also GPR43) and FFA3 (also GPR41) receptors are activated by SCFAs. The change in name designation, e.g., GPR40 to FFA1, aligns with the discovery of endogenous signaling ligands. As key sensors for dietary and other signaling FFAs, this family of receptors has attracted elevated interest for their role in regulating metabolic and inflammatory processes and has recently been implicated as targets in metabolic disorders and type 2 diabetes. Milligan et al. recently published an extensive review of this receptor family, including FFAR biological importance and druggability.[178]

FFA1 Receptor (GPR40).: The FFA1 receptor, which has been previously designated GPR40, is predominately expressed in pancreatic β cells and intestinal enteroendocrine cells and has been validated as a potential target for the treatment of type 2 diabetes.[179] Allosteric modulator discovery for this target has produced a rich collection of pharmacologically diverse ligands, and elegant structural work has identified multiple allosteric binding sites.[180,181] In recent years, numerous full and partial allosteric agonists of FFA1 have been discovered and are described as binding to a select number of distinct allosteric sites, such as TAK 875 (**99**), AM 8182 (**100**), AM 1638 (**101**), AMG 837(**102**), MK8666 (**103**), and AP8 (**104**). Additionally, recent work in FFAR structural biology and biochemistry describes and validates this multiple-site postulation.[18,30,20]

The FFA1 partial agonist TAK-875 (**99**) was discovered as an ago-PAM, binding to a distinct allosteric site and characterized as enhancing the activity of endogenous FFAs.[182] Importantly, **99** (Figure 15) was progressed into phase III clinical trials for the treatment of type 2 diabetes mellitus, but the trial underwent early termination due to toxicity. The cocrystal complex of **99** with FFA1 reveals that the binding site for **99** is formed by helices III–V and the ECL2 and is adjacent to the exterior lipid membrane surface (PDB code 4PHU).[30] The allosteric partial agonist MK-8666 (**103**) has recently been approved to advance into a phase I clinical trial for the treatment of type 2 diabetes mellitus. Interestingly, the cocrystal complex of **103** bound to FFA1 demonstrates a binding site adjacent to the lipid membrane, similar to **99** (PDB code 5TZR).[20] Identification of membrane-adjacent binding sites for allosteric modulators is thus far uncommon; however due to the allosteric nature of GPCR interactions with membrane lipids and cholesterol, this site of action may be more common than currently appreciated or may be yet unexploited at additional receptors. A novel ago-PAM, AP8 (**104**), was subsequently discovered and displays a far higher potency for potentiating endogenous FFAs than **99** or **103**.[180] The ternary complex structure of FFA1-**103**-**104** reveals that **104** binds to a lipid-facing pocket formed by helices II–V and ICL2, which is outside the intracellular halves of the TM helical bundle, and this site is completely distinct from the allosteric binding site of **103** (PDB code 5TZY).[20] Further validation of the allosteric mechanism and signaling bias of allosteric modulators for FFA1 are still progressing.[181]

FFA2 Receptor.: FFA2 and FFA3 receptors are predominately expressed in the gut enteroendocrine cells, pancreatic β cells, and adipose tissue and have been found expressed in various cancer cells, including breast, colon, and liver.[181] The phenyl-acetamide scaffold series of FFA2 receptor allosteric modulators remain the most studied and characterized allosteric ligands for this target. 4-CMTB (4-chloro-α-(1-methylethyl)-*N*-2-thiazolylbenzeneacetamide, **105**) and its analogue **106** (Figure 16) were identified via high-throughput screening and represent the first series of synthetic small molecules that display allosteric agonism and PAM-like effects at the FFA2 receptor.[183,184] Initial characterization shows that **105** can stimulate signaling via both $G\alpha_{i/o}$ and $G\alpha_{q/11}$ promoted pathways. However, subsequent chemical modifications based on SAR around 4-CMTB result in limited improvements, and poor PK properties in male Sprague-Dawley rats have limited its further development as a preclinical candidate.[185,186] As an alternative, phenylacetamide **107** was used for in vivo proof-of-concept studies to demonstrate an FFA2-mediated reduction in plasma nonesterified fatty acids in wild-type mice.[186] Recently, AZ1729 (**108**) was discovered by introducing a phenyl linkage between the amide and thiazole,and **108** displays an interesting G_i-biased profile as a FFA2 receptor allosteric agonist and can potentiate agonist signaling as a PAM.[187]

FFA3 Receptor.: Hexahydroquinolone-3-carboxamides and derivatives thereof are the predominantly reported allosteric FFA3 receptor ligands and derive from a patent by Arena Pharmaceuticals.[188] Compound **109** (Figure 17) displays intrinsic efficacy as well as orthosteric agonist potentiation as an ago-PAM of the human FFA3 receptor with modest potency and is without activity at the FFA2 receptor.[189] Interestingly, modification of the hexahydroquinolone results in molecular switches that significantly alter the activity profile.

For example, when replaced by a 2-bromophenyl group, the modification yields **110** demonstrating a pure PAM profile for the FFA3 receptor without intrinsic agonism, while modification of the phenyl to 3-phenoxyl yields **111** demonstrating a inhibition of the agonist maximal response and an increase binding cooperativity.[189] The authors continue to describe a unique case for **111**, where there was a negative influence on signaling (log β = −21.92 ± 0.20) but a positive impact on binding cooperativity (log α = 1.20 ± 0.39), leaving the conclusion that **111** may be appropriately described as a PAM-antagonist. At present, although FFARs are considered to be an important target for drug discovery and two allosteric ligands have progressed into clinical trials, the overall body of literature remains relatively small, especially for FFA2-FFA4 receptors. Pharmacological tool compounds characterized by high selectivity for a single FFAR and high potency are needed for further evaluation and validation of mechanisms, binding sites, and in vivo tolerability. Thus, there remains high value in medicinal chemistry around these receptors and the evaluation of unique allosteric binding sites may provide insight for targeting FFAR family members as well as other GPCRs.

3.3. Nucleotide Family Receptors.

3.3.1. Adenosine Receptors (A_1R–A_3R).—

Adenosine receptors (ARs), classified as A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R, have been involved in the treatment of diseases that span cardiovascular disease, CNS disorders, inflammatory and allergic disorders, and cancer.[190] Historically, both agonists and antagonists have been used to indiscriminately modulate ARs, the most well-known being the endogenous agonist adenosine and the common antagonist caffeine. Recent work has begun to identify subtype selective orthosteric ligands; however allosteric modulation may provide multiple benefits for therapeutically targeting ARs.[191] Allosteric modulators of ARs are increasingly pursued to avoid side effects caused by agonists acting through indiscriminate AR activation and the propensity for orthosteric agonists to cause receptor desensitization upon prolonged exposure. ARs interact with multiple and divergent second messenger systems, as the A_1R and A_3R reduce the production of cAMP by coupling to $G\alpha_i$ protein resulting in adenylate cyclase inhibition, while the A_{2A} and A_{2B} subtypes stimulate the production of cAMP via coupling to $G\alpha_s$ or $G\alpha_{olf}$ protein.

A_1R.: Evaluation of the A_1R allosteric modulators reviewed herein have been predominately performed in two in vitro functional assays; the first evaluates the inhibitory activity of forskolin-stimulated cAMP accumulation in CHO cells stably expressing the human A_1R, while the second measures phosphorylation of ERK½ in the same cell type. Additionally, groups have reported results on A_1Rbinding parameters (affinity K_D and density B_{max}) and radioligand binding assays that provide association and dissociation kinetics to assess the allosteric modulation of the orthosteric agonist—receptor—G-protein ternary complex. Finally, some observations of antagonist competition binding assays are reported.

In pioneering work, Bruns et al. in 1990 developed a novel series of PAMs (designated allosteric enhancers) known as the “PD” series [PD 81,723 (**112**), PD 71,605 (**113**), PD 117,975 (**114**)], and they were characterized by a 2-amino-3-benzoyl-thiophene (2A3BT) scaffold, selectively enhancing the binding of N^6-cyclopentyladenosine (CPA) to the A_1R

(Figure 18).[192–194] Analogues of PD 71,605 (**113**) led to the discovery of T-62 (**116**) and LUF 5484 (**117**) with markedly improved potency over **112**.[192,195] Among them, **116** (2-amino-4,5,6,7-tetrahydrobenzo [*b*]thiophen-3-yl-(4-chlorophenyl)-methanone) was developed by King Pharmaceuticals and advanced into clinical trials for the potential treatment of neuropathic pain associated with hyperalgesia and allodynia.[194] However, the program was terminated after failure to meet the end point for efficacy in phase IIB.[196,197] The 2-aminothiophene series of PAMs has been recognized as a representative core scaffold for the A_1R, but subsequent observations of intrinsic antagonist activity at high concentrations and moderate efficacy at lower concentrations necessitated further chemical modifications and optimization.[198]

The early SAR studies around the 2-amino-3-benzoyl thiophene core generated a preliminary principle that the 2-amino group, 3-benzoyl, and the corresponding hydrophobic *para*- and *meta*-substituents on the phenyl in the C3-position are critical to the PAM activity of these analogues.[199] Large hydrophobic groups at the 4-position of the thiophene ring and small substituents (H and CH_3) at the 5-position could improve the PAM activity, while bulky substituents at the C5-position resulted in allosteric enhancer activity with an apparent intrinsic antagonist profile.[200] The proper combination of these modifications remains a challenging task and requires achieving a high PAM potency with as minimal as possible antagonist activity. On the basis of this SAR, two functionally divergent binding pockets within the larger allosteric site are proposed, with one interacting with the 2A3BT core and a second possible lipophilic domain to accommodate the C4-/C5-substituents on the thiophene ring.[201,202]

Subsequently, detailed SAR studies on the 4- and 5-substitution of 2-amino-3-benzoyl thiophene was pursued. Modifying the C4-/C5-substituents with a fused cycloalkyl ring increased lipophilicity and led to **118**, which displays allosteric enhancer and partial agonist characteristics with >50% intrinsic activity and 98% of the maximum agonist R-PIA response at 10 μM on A_1R-mediated stimulation of ERK½ phosphorylation in vitro. However, increasing the ring size from a cyclopentyl moiety gradually to a cycloheptyl substituent in efforts to improve allosteric enhancement results in the loss of activity. Opening the C4/C5 ring furnishes a series of compounds with improved PAM potency. Among the different substitutions on C4/C5, **119** with a bromide in C5 displays higher potency than compound **112** in the kinetic binding assay (2-fold greater affinity than **112** and 3-fold less inhibition of antagonist [^{3}H]-CPXbinding).[203] Compound **120** contains a 5-phenyl substituent that increases potency (EC_{50}) 6-fold over **112**, but when no substituent is present in the 5-position (**121**), allosteric enhancement efficacy is improved (77% vs 28%) along with greater antagonist activity compared to **112**.[204] Additional modifications and SAR provided support for these results, and it was concluded that alkyl and aryl groups at the C4-position are favored for allosteric enhancer activity.[205]

Romagnoli and colleagues further modify the C4-substitution to compound **122** (Figure 18), characterized by aryl piperazine moieties linked to a methylene at the 4-position of the thiophene ring, and evaluate **122** with a cAMP functional assay in human A_1R expressing CHO cells. A maximal 87% attenuation of cAMP production is observed at 10 μM without antagonist activity, observed by negligible binding inhibition activity to displace the binding

of selective agonists to A_1R, $A_{2A}R$, and A_3R Saturation binding experiments show **122** produces a A_1R density (B_{max}) shift of [^{3}H]-CCPA binding 7.7-fold to A_1R in CHO cells and enhances the apparent affinity of CCPA approximately 6.3-fold in the A_1R CHO cell membranes by titrating the radioligand [^{3}H]-DPCPX at 10 μM concentration. The number and position of electron-withdrawing or electron-releasing groups on the phenyl attached to the piperazine moiety were determined as highly influential for the overall allosteric enhancement activity. Among them, **123–126** (Figure 18) possess 4-chloro, 3,4-difluoro, 3-chloro-4-fluoro, and 4-trifluor-omethoxy derivatives, and each has been reported to maintain improved potency in the binding (saturation and competition) and functional cAMP studies.[200,206] Subsequently, an aryl substitution at the 5-position was discovered to have a fundamental effect by contributing additively to the allosteric enhancer activity. Compounds **127–131** with a 5-aryl substituent have substantially higher activity than **112** without significantly inhibiting antagonist binding at the A_1R, A_2R, or A_3R. Saturation and competition experiments have also shown that this series of 5-aryl-substituted thiophene derivatives were more active than the corresponding 5-unsubstituted analogues with a highest 13.3-fold decreased CCPA K_i value at 10 μM in competition binding experiments (compound **128**).[207] Encouraging results are also reported on **132–136**, which possess a neopentyl and an aryl moiety at the 4- and 5-positions. Moderate to good enhancing activity of cAMP attenuation is observed (up to 64% inhibition at 10 μM) without significant inhibition of antagonist binding across AR subtypes.[208]

Further modification around this series obtained compound **137**, characterized by a common 2-amino-3-(*p*-chlorobenzoyl)-thiophene core with neopentyl substituent at the C-4 position and benzyl acetylene at the C-5 position of the thiophene ring. Attenuation of cAMP production by **137** shows a 75% inhibition in the presence of 1 pM of orthosteric agonist CCPA, which is almost 4-fold greater than the former allosteric enhancer **112** (19%).[199] Interestingly, in [^{3}H]CCPA saturation binding experiments there was no change observed in the affinity (K_D) of [^{3}H]CCPA with the inclusion of modulator **137**. However, when using [^{3}H]DPCPX as a radioligand in competition binding studies of CCPA with and without **137**, the derived apparent affinity (K_i) value for CCPA was decreased by a 10-fold shift with the inclusion of **137**. Thus, it was concluded that **137** was unable to increase the affinity (K_D) of CCPA when bound to the A_1R high affinity site but could shift the population of A_1Rs toward a high affinity state as evidenced by the increased apparent affinity of CCPA to A_1R in competition binding studies. Compound **137** also displayed a slowing of the dissociation rate of the radioagonist [^{3}H]-NECA by 2.1-fold with a corresponding 1.9-fold increase of apparent affinity. Compound **138** with *p*-chlorobenzyl at the C4-position of thiophene and *p*-chlorophenyl substituent at the C5-position showed a 4-fold increase in cAMP production attenuation compared to **112** in a functional assay (84% vs 21%) and delayed the dissociation rate constant of agonist [^{3}H]-NECA by 2.5-fold.[209] No significant binding inhibition was noted for **138** for antagonists of the A_1R, A_2R, and A_3R subtypes in competition binding assays. Additionally, significant antinociceptive effects were observed in mice at doses of 0.3 and 3 mg/kg of **138**, compared to vehicle-treated mice.

Scammells et al. reported studies interrogating the stimulus bias between cAMP and ERK½ associated pathways (i.e., biased signaling, functional selectivity) of their AR molecules,

which may provide a strategy to achieve selectivity ofsignaling at GPCRs associated with ligand directed signaling outcomes manifested as changes in rank orders ofpotency and or maximal effects relative to a reference (e.g., the endogenous) agonist.[202] This highlights how a GPCR bound with both an allosteric modulator and an orthosteric agonist should be viewed as a unique protein state that differs from those promoted by either orthosteric or allosteric agonist alone. Two novel 2A3BT derivatives **139** and **140**, differing by the absence or presence of a halogen atom in the 4-position of the benzoylthiophene ring, induce functionally biased states of the A_1R. In comparison to the orthosteric agonist response from *R*-PIA, **139** alone is biased as an allosteric agonist toward cAMP accumulation over ERK½ phosphorylation with a 45-fold bias factor. Compound **139** also allosterically shifted the biased signaling of the agonist *R*-PIA (strongly biased toward the pERK½ pathway) toward activation of the two pathways in a nonbiased manner. Conversely, **140** shows minimal bias as an allosteric agonist (with bias factor 3.5) but demonstrates a pathway-biased allosteric modulation when combined with *R*-PIA. Additionally, they also found that the well-characterized 2A3BT, T62, as well as VCP520 (**141**) and VCP333 (**142**), exhibited stimulus bias toward cAMP inhibition compared to pERK½. Finally, SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5-(2*H*)-ylidene)-methanamine, **143**) has also been reported as an allosteric modulator of ARs. **143** could not only selectively slow the agonist dissociation at A_1R but also accelerate agonist dissociation at A_3R and antagonist competitive dissociation of adenosine A_{2A}R at a concentration of 10 μM.[210]

The understanding of A_1R pharmacology has greatly benefited from the recent reports of solved structures of both the active and inactive state A_1R.[211,212] In the study by Glukhova et al., the 3.2 Å resolution crystal structure of A_1R bound to the covalent antagonist DU172 was presented along with structural insights that potentially explain the interactions of A_1R allosteric modulators such as **121**, which was used in docking studies with the inactive A_1R structure. As shown previously, some allosteric modulators of the A_1R have displayed a complex pharmacological profile that results in PAM activity toward orthosteric agonists while inhibiting the effect of orthosteric antagonists. Although a large, extended orthosteric pocket is present, docking studies of **121** suggest that it preferentially binds to the orthosteric pocket of the inactive state A_1R.[212] The cryoelectron microscopy active state A_1R has also been reported and provides additional insight for allosteric modulator binding when considered in context ofprevious mutagenesis studies that identify ECL2 as a critical domain for PAM activity.[211,213] Taken together, the structural information shows that ECL2 maintains a similar position between the active and inactive states leading to the suggestion that A_1R PAM activity is governed by the availability of active state receptors that provide a preferential PAM binding site upon A_1R activation and subsequent collapsing inward of the TM domains.

A_{2A}R.: Amiloride (**144**) and analogue HMA (2,5-(*N,N*-hexamethylene)amiloride, **145**) are reported to bind to the sodium ion site of adenosine receptors.[214,215] Compounds **146–149** are derivatives of **144** and **145** (Figure 19) via employing varied 5′-substitutions on amiloride, showing consistent potency as A_{2A}R allosteric antagonists by displacing orthosteric radioligand [^{3}H]-ZM-241,385 from the wild-type human A_{2A}R (59–73%) and displaying even greater potency in the W246A sodium ion site mutant human A_{2A}R

(94.6%–100%).[216] Docking studies on the high resolution agonist-bound $A_{2A}R$ show that these analogues conform to similar binding poses to that of amiloride and HMA observed in previous docking studies, with hydrogen bonding and salt bridge interactions with $Asp52^{2.50}$ and $Thr88^{3.36}$ and occupation of the $Trp246^{6.48}$ position.[217] Noteworthy, the interactions with the $Trp246^{6.48}$ are predicted to be π–π stacking between $Trp246^{6.48}$ and the phenyl group of most analogues, which is not present in **145**. The phenethyl moieties and the substituents attached on the phenyl groups are predicted to reach into a part of the orthosteric binding site surrounded by hydrophobic residues of $Phe168^{EL2}$, $Met177^{5.38}$, $Leu249^{6.51}$, $Asn253^{6.55}$, and $Ile274^{7.39}$, suggesting that **146–149** can intrude the orthosteric site from the allosteric site and displace orthosteric ligand ZM-241,385 in a direct, competitive manner.

$A_{2B}R$.: The $A_{2B}R$ is the least characterized subtype in the AR family. Among ARs, the $A_{2B}R$ subtype exhibits low affinity for the endogenous agonist adenosine compared to the A_1R, $A_{2A}R$, and A_3R subtypes and is therefore suggested to be activated when local concentrations ofadenosine increase to a large extent following tissue damage. Compounds **150–156**, with small differences in the side chain of the 1-benzyl-3-ketoindole scaffold, are the only reported allosteric modulators for the $A_{2B}R$ (Figure 20) and have been characterized through binding and functional assays, including cAMP functional assays, dissociation kinetic assays, equilibrium binding assays, and [^{35}S]GTPγS binding assays in CHO cells expressing human A_1R, $A_{2A}R$, $A_{2B}R$, and A_3Rs.[218,219] The PAMs **150–152** potentiate agonist efficacy but not agonist potency (similar submicromolar potencies at $A_{2B}R$) with PAM EC_{50} values between 250 nM and 446 nM. PAM **151** demonstrates a significant reduction in the radioligand [^{3}H]NECA dissociation constant from 0.0162 min^{-1} to 0.0086 min^{-1} and increases the efficacy of agonist BAY 60–6583 to stimulate guanine nucleotide exchange with E_{max} values from 155.2% to 175.0% at 1 μM in [^{35}S]GTPγS binding assays. Slight alterations to the side chain are discovered as chemical switches and yield NAMs **153–156** that reduce agonist potency and efficacy. Compound **156** significantly increases the dissociation rate of [^{3}H]NECA from $A_{2B}R$ with K_{off} value 0.0481 and results in a pronounced attenuation of the orthosteric ligand BAY 606583 mediated stimulation of guanine nucleotide exchange. None of the compounds reported (**150–156**) display appreciable affinity for the A_1R, $A_{2A}R$, and A_3R except for **152** and **153**, which display submicromolar affinity for the A_1R(**152** K_i = 161.5 nM, **153** K_i = 343.0 nM). More work will certainly be done to elucidate the therapeutic potential of the $A_{2B}R$, especially in inflammation and injury, and there remains great potential for further interrogating allosteric modulators of the $A_{2B}R$ through medicinal chemistry. However, these current allosteric modulators comprise both PAMs and NAMs and may provide useful chemical probes to explore the biology and therapeutic potential of $A_{2B}R$ allosteric modulators.

A_3R.: The A_3R is widely expressed and displays tissue specific regulation regarding cellular energy consumption and energy deficits. Agonists and antagonists have recently been studied, and antagonists have been prime candidates for rheumatoid arthritis, glaucoma, psoriasis, and hepatocellular carcinoma, as the A_3Ris found to be overexpressed in cancer cells.[220] Allosteric modulators of the A_3R are a recent development and may provide a unique therapeutic approach toward these disorders and others. Relative to the other

members of the AR family, modest numbers of selective allosteric compounds have been reported for the A_3R The core scaffolds of A_3R allosteric modulators predominately comprise 1H-imidazo[4,5-*c*]-quinolin-4-amine and 2,4-disubstituted quinoline analogues. Reported characterizations of A_3R allosteric ligands are mainly based on the results of radioligand displacement, kinetic dissociation experiments, and functional (cAMP-based) assays. LUF6000 (**157**) with a 1*H*-imidazo[4,5-*c*]quinolin-4-amine scaffold was discovered (Figure 21) and prioritized for optimization due to significant potentiation of agonist efficacy compared to the former discovered A_3R PAMs. However, like $A_{2B}R$ PAMs, it shows no enhancing effect at 10 μM on agonist potency for human A_3R expressed in CHO cells, observed via cAMP functional readouts.[221,222] Ring opening around **157** to LUF6096 (**158**), also bearing a 2,4-disubstituted quinoline core, is another example of A_3R PAMs and equally potentiates orthosteric agonist efficacy. Interestingly, **158** also displays allosteric effects on the agonist potency and could produce a shift in the EC_{50} value of the agonist Cl-IBMECA from 31 nM alone to 9 nM with PAM, a ~3-fold shift.[223]

3.3.2. P2Y Receptors ($P2Y_1$ and $P2Y_2$ Receptors).—

Purine and pyrimidine receptors exist in two families: P1 receptors (adenosine receptors) activated by adenosine, discussed above, and P2 receptors activated by adenosine 5′-tri- or diphosphate (ATP or ADP) and/or uridine 5′-tri- or diphosphate (UTP or UDP). P2 receptors are further divided as P2X and P2Y receptors, which are ligand gated ion channels and GPCRs, respectively.[224] The human purinergic GPCRs (P2Y) are divided into two subfamilies based on their coupling to specific G-proteins, $G\alpha_{q/11}$ coupled P2Yrlike receptors and $G\alpha_{i/o}$ coupled $P2Y_{12}$-like receptors. They are activated by ADP to trigger glutamate release, facilitating thrombus formation and are essential for platelet aggregation and thus considered promising new drug targets.[225]

$P2Y_1$ Receptor.: The $P2Y_1$ receptor is a promising therapeutic target due to its critical role in ADP-induced platelet aggregation and the potential for an improved safety profile over $P2Y_{12}$ receptor inhibitors regarding bleeding liability.[225] Early efforts in this arena essentially focused on nucleotide derivative orthosteric antagonists. However, the discovery of BPTU (**159**) by Bristol-Myers Squibb (Figure 22), a hydrophobic diarylurea derivative, as a non-nucleotide allosteric antagonist of the $P2Y_1$ receptor has provided the foundation for allosteric modulators to be considered as potential therapeutic agents of this receptor.[226] Thus, there have been recent innovative approaches to design non-nucleotide, diarylurea scaffold allosteric antagonists as antithrombotic agents with improved safety profiles.[227,228] As a relatively new target and mode of antagonism, the most extensive characterizations have been performed on **159**, which demonstrates a 68 ± 7% thrombus weight reduction in a rat arterial thrombosis model (10 mg/kg **159**, 10 mg kg^{-1} h^{-1} rate) with minor effects on overall bleeding in provoked rat bleeding time models. Important structural and pharmacological studies by Jacobson and colleagues have recently begun to illuminate the complex allosteric mechanisms of **159** and its effect on both multiple downstream signaling pathways as well as multiple agonists.[31,229] In the recent cocrystal complex, **159** is the first $P2Y_1$ receptor antagonist shown to bind to an allosteric site entirely outside of the helical bundle, not only outside of the orthosteric site. The allosteric binding site of **159** is situated on the outside of the TM domain bundle adjacent to the lipid membrane and engages **159** by

mostly hydrophobic and aromatic residues located in the TM helices I–III as well as minor involvement of ECL1. The two nitrogen atoms of the urea group in **159** promote two bidentate hydrogen bonds with the backbone carbonyl of Leu102$^{2.55}$, which result in the only polar interactions present. The pyridyl group, the benzene ring of the phenoxy group tethered to pyridine, and the *tert*-butyl substituent on the phenoxy group are responsible for forming the main hydrophobic interactions with the $P2Y_1$ receptor.[225] Insights from the cocrystal complex indicate that this interaction may reasonably stabilize the extracellular helical bundles and restrain the receptor in an inactive state. Thorough pharmacological studies of **159** $P2Y_1$ receptor allosteric antagonism provide a more nuanced view of its mode of action, including a description of probe dependence and signaling bias.[229] When provoked by structurally diverse agonists, **159** displayed varying degrees of antagonism across multiple signaling pathways. For example, allosteric antagonism of the agonists 2MeSADP and MRS2365 resulted in decreased potency for ERK½ stimulation with no effect on maximal response (E_{max}); however in [^{35}S]GTPγS binding assays and β-arrestin2 recruitment, **159** was able to significantly suppress the respective agonist-mediated responses. Antagonism of the agonist Ap4A resulted in insurmountable suppression of the maximal response across all assays tested. These studies highlight the high level of complexity for allosteric GPCR modulation but also call to attention the high degree of specificity that can be achieved if probe dependence and signaling bias are therapeutically desired outcomes based upon biological understanding. The FDA approval of ticagrelor (AZD6140), a $P2Y_{12}$ receptor allosteric antagonist discovered by AstraZeneca, has paved the way for antithrombotic drugs in this class with safer bleeding profiles to emerge as therapeutics.[230]

$P2Y_2$ Receptor.: The $P2Y_2$ receptor couples primarily to $G\alpha_{q/11}$ to activate PLC-β and has been implicated in diverse physiological processes, including platelet aggregation, immunity, lipid metabolism, gastrointestinal functions, and bone homeostasis.[231] Allosteric agonists for the $P2Y_2$ receptor have been recently reported.[224,232] Of these, **160** is characterized as a partial allosteric agonist and was discovered by the modification of 5′-methylenephosphonate, a derivative of UTP (uridine 5′-triphosphate).[224] Additionally, compound **89** (**161**), with a novel 4(1*H*)-quinolinone scaffold, is among the first non-nucleotide $P2Y_2$ receptor allosteric agonists and is selective over closely related subtypes. Initial characterizations displayed activity in calcium mobilization assays in the 1321N1 human astrocytoma cell line, induction of nuclear receptor 4A (NR4A) in a gene reporter assay, and the attenuation of isoproterenol-induced cardiac hypertrophy in neonatal rat cardiomyocytes (NRCMs).[232] These studies relay **161** (Figure 22) as a validated chemical tool compound for utilization in further proof-of-concept studies to investigate the therapeutic potential of $P2Y_2$ receptor allosteric agonists for the treatment of cardiovascular disorders. Interestingly, the $P2Y_2$ receptor serves as an attractive drug target for dry eye disease (DED) and nucleotide-derived agonists have been approved for the treatment of DED in Japan and Korea.[233] Further development of **161** and other allosteric agonists may prove advantageous for DED, cardiovascular indications, and others.

3.4. Peptide and Protein Family Receptors.

3.4.1. Chemokine Receptors (CCR5, CCR9, CXCR1, CXCR2, CXCR4).—

Chemokine GPCRs contain four families as CCR, CXCR, CX3CR, and XCR based on the relative positioning of conserved cysteine residues in the N-terminal domain of their mature ligands. At present, there are roughly 50 chemokines and at least 18 chemokine GPCRs have been identified in humans.[234,235] The development of allosteric modulators for chemokine receptors (Figure 23) represents a profound advance for allosteric modulators of class A GPCRs with marketed drugs (maraviroc, NAM of CCR5; plerixafor, NAM of CXCR4), clinical candidates (reparixin, NAM of CXCR1; ladarixin, NAM of CXCR1/CXCR2; vercirnon, allosteric antagonist of CCR9), and structural studies of allosteric modulators binding to the receptors in high resolution (for CCR2, CCR5, and CCR9).[235,18] Besides synthetic drugs, chemokine receptor allosteric sites have been shown to also bind endogenous mineral cations such as sodium, calcium, zinc, and magnesium. These studies included CCR1, CCR4, CCR5, and CCR8, and additional work has shown the metal ion Zn(II) or Cu(II) complex to be an allosteric enhancer of CCL3.[236] Herein, we describe representative allosteric modulators for seven chemokine receptors and the structural and chemical knowledge relating to their discovery.

CCR5.: The chemokine receptor CCR5 is widely implicated for its role in the process ofhuman immunodeficiency virus type 1 (HIV-1) infection. Mechanistically, CCR5 forms a co-receptor with the viral envelope glycoprotein gp120, which is required for HIV-1 cell recognition and entry leading to infection.[237,238] Maraviroc (**162**) is a marketed allosteric drug for anti-HIV (Figure 23), stabilizing CCR5 in an inactive conformation that blocks CCR5-gp120 interaction by allosterically binding to CCR5.[239,240] The cocrystal complex of CCR5 and maraviroc demonstrate that maraviroc occupies an extracellular site of the 7TM helical bundle.[27] The protonated nitrogen of the tropane group forms a salt bridge with Glu283$^{7.39}$. The carboxamide nitrogen and the amine of the triazole group of the ligand form hydrogen bonds with Tyr251$^{6.51}$ and Tyr37$^{1.39}$, and the phenyl, triazole, and cyclohexane ring are responsible for the formation of hydrophobic interaction (PDB code 4MBS). Additionally, CCR5 and the highly homologous CCR2 are promising targets for immunologic and cardiovascular diseases due to their important functions in macrophages, T-lymphocytes, and natural killer cells. Chemokine receptors are activated by more than 50 chemokine ligands in a concerted manner in response to various immunologic or inflammatory events. Thus, probe dependence may be a primary advantage for allosteric modulators of chemokine receptors. In a recent study, Wünsch and colleagues discovered the first probe dependent CCR5 PAM.[241] Through an innovative bioluminescence resonance energy transfer (BRET)-cAMP assay, the endogenous agonists CCL4 and CCL5 were screened at CCR2 and CCR5. Chemical modifications and resulting SAR were performed on a 2-benzazapine scaffold showing a sensitive 7-position where the addition of *p*-tolyl moiety led to a chemical switch toward CCR2 modulation without CCR5 activity. The parent compound displayed PAM activity at CCR5, with no activity at CCR2, and selectively modulated CCL4 versus CCL5.[241] Bipyridine and terpyridine, small molecule metal chelators, have also been shown to modulate CCR5 with prode dependency. Biochemical studies indicate that bipyridine and terpyridine are PAMs of CCL3, weakly potentiate CCL4, and compete with CCL5 binding to CCR5.[242] CCR5 remains an active

target for PAM and NAM discovery, and the identification of probe dependent ligands will broaden the biological knowledge of chemokine signaling and the therapeutic relevance.

CXCR1.: CXCR1 and CXCR2 are largely expressed on T lymphocytes and natural killer cells, playing a key role in acute and chronic inflammatory conditions.[235] Reparixin (**163**) is a noncompetitive NAM for CXCR1 (Figure 23) presenting a 400-fold higher efficacy in inhibiting CXCR1 activity versus CXCR2.[243] Compound **163** inhibits the signaling triggered by chemokine CXC ligand 8 (CXCL8) and binds CXCR1 at an allosteric site between TM I, III, and VI and has been advanced into a phase III clinical trial for pancreatic islet autotransplantaion.[243] Ladarixin (DF 2156A, **164**) is the second representative example of this series as a highly potent allosteric inhibitor of CXCR1/CXCR2 with an IC_{50} value 0.1 nM and has been advanced into clinical trials for type 1 diabetes.[235]

CXCR4.: CXCR4 is expressed by hematopoietic stem cells and progeny, as well as by over 48 different cancers types, and is essential for hematopoietic stem cell colonization of fetal bone marrow during development.[244] Interestingly, plerixafor (**165**), a NAM of CXCR4 with tetraazacyclotetradecane scaffold (Figure 23), was initially in development as an anti-HIV drug but has been repurposed and is now marketed for an indication of bone marrow transplantation for patients with non-Hodgkin's lymphoma or multiple myeloma, where upon administration **165** is efficacious in mobilizing stem cells into the peripheral blood for collection.[235]

CCR9.: CCR9 is another member of the CC chemokine receptor subfamily implicated in inflammatory bowel disease. Vercirnon (**166**) is a selective allosteric antagonist of CCR9 (Figure 23) that has entered phase III clinical trials for the treatment of Crohn's disease.[245] The cocrystal structure of CCR9 with **166** shows that **166** binds to the intracellular side of the CCR9, which is similar to that of CCR2-RA-[*R*] bound to CCR2. The sulfone group, ketone group, and pyridine-*N*-oxide group of **166** could contribute to forming multiple hydrogen bonds with intracellular side of the CCR9. The *tert*-butylphenyl and chlorophenyl group are responsible for the hydrophobic interactions with the hydrophobic cleft (PDB code 5LWE).[29] Allosteric modulation via binding intracellular allosteric sites is still uncommon for class A GPCRs; however this mode of action may have important therapeutic implications, especially for peptide and protein receptors such as chemokine receptors.

CCR2.: CCR2 is implicated in numerous inflammatory and neurodegenerative diseases.[246] CCR2-RA-[*R*] (**167**) is a NAM of CCR2 (Figure 23) with good selectivity against CCR1 and CCR5, in vitro activity characterized by an IC_{50} of 0.17 μM, and also an excellent DMPK profile.[18] The cocrystal structure of CCR2 in a ternary complex with **167** and orthosteric BMS-681 antagonist demonstrates that **167** occupies an intracellular allosteric binding site, as seen in other chemokine receptors. The pyrrolone structure is very important for forming hydrogen bonds between hydroxyl group and Glu3 1 $0^{8.48}$ and $Lys311^{8.49}$, and carbonyl group with the backbone amide of$Phe312^{8.50}$. The existence of the phenyl group is vital for hydrophobic interactions with various amino residues.[28]

CCR4.: Chemokine receptor 4 (CCR4) is mainly expressed in T helper 2 (Th2) cells and contributes to the pathogenesis of allergic diseases in inflamed tissues. Endogenous agonists chemokine ligand 17 (CCL17) and chemokine ligand 22 (CCL22) are two signaling proteins that bind the orthosteric site of CCR4 and are crucial for recruiting T cells during the inflammatory response upon exposure to allergens.[247,248] Interestingly, a functional interrogation of these signaling ligands shows that CCL22 activated CCR4 was able to couple efficiently to β-arrestin and stimulate GTPγS binding, while CCL17 activated CCR4 did not couple to β-arrestin and only partially stimulated GTPγS binding.[248] Thus, the physiological conditions under which some chemokines are released and activate their respective receptors remain an active area of research. The CCR4 has been a target for the discovery of small molecule therapeutics for many due to its central role in pathogenesis such as asthma, atopic dermatitis, cancer, and mosquito-borne tropical diseases.[247]

Indazole sulfonamide series were recent synthesized and examined as human CCR4 antagonists, and SAR studies around the C4, C5, C6, C7 and N1, N3 positions were conducted to provide compounds with a better in vivo profile. Among them, **168** with a methoxy group as C4 substituents, 5-chlorothio-phene-2-sulfonamide at N3, and a meta-substituted benzyl group possessing an α-amino-3-[(methylamino)acyl] group at N1 was the most potent, presenting a pIC_{50} of 7.4 for CCR4. Compound **168** also demonstrates a good PK profile in three species (rat, dog, and human) and was selected for further development.[247] Subsequent studies on CCL17- and CCL22-induced responses of human CCR4 expressing T cells suggest there are two additional allosteric sites to which small molecules bind. Compound **168** and its analogues bind to one of them, the intracellular allosteric binding site. Lipophilic heteroarenes possessing basic amino groups have been shown to bind to another site. Additionally, a heteroarylpyrazole arylsulfonamide scaffold was also reported as a potent lead for further development.[249]

CXCR3.: The chemokine receptor CXCR3 is mainly activated by γ-inducible chemokines CXCL11, CXCL10, and CXCL9, directing activated T cells to the sites of inflammation, and is implicated to play a role in a myriad of inflammatory diseases, such as rheumatoid arthritis, multiple sclerosis, cancer, atherosclerosis, and allograft rejection; thus, CXCR3 is viewed as a promising drug target.[250–252] 8-Azaquinazolinone derivatives (**169–173**) were characterized as promising allosteric modulators of the chemokine receptor CXCR3 and commonly demonstrate properties of signaling bias and probe-dependence.[252] Among them, **172** can inhibit CXC chemokine 11 (CXCLll)-dependent G protein activation over β-arrestin recruitment with 187-fold selectivity, and it inhibits CXCL11-over CXCL10-mediated G protein activation with 12-fold selectivity.[252]

Structure-based drug discovery (SBDD) is still in the early phases for receptors such as CXCR3 due to limited structural information and complicated interactions between receptors and chemokine signaling proteins. As an alternative, photoaffinity labeling is an effective biochemical tool to elucidate the binding pocket at the CXCR3 receptor. By this principle, photoactivatable **174** with a nanomolar affinity was synthesized based on the 2,3-disubstituted-8-azaquinazolinone scaffold. Notably, **174** could attenuate radioligand binding by 80% in [^{3}H]-RAMX3 radioligand displacement assay and proved to be a promising chemical tool for further exploration of the allosteric binding site of CXCR3.[251] Aside from

photoaffinity labeling, site-directed mutagenesis is another approach to reveal information about ligand—receptor interactions through the mutations of amino acid residues and detection of their influence on modulator binding, signaling and transmission of cooperativity. Compounds **171** and **173** are two biased NAMs and have been shown to exhibit probe-dependent inhibition of CXCR3 signaling. Homology modeling and docking provided direction for site-directed mutagenesis, and functional outcomes of the mutations were measured by a BRET-cAMP and β-arrestin recruitment assay. These studies indicated that F131$^{3.32}$, S304$^{7.39}$, and Y308$^{7.43}$ act as key residues for the compounds to modulate the chemokine response, and notably, mutations of D186$^{4.60}$, W268$^{6.48}$, and S304$^{7.39}$ led to a G-protein-active rather than β-arrestin-inactive conformation.[252]

3.4.2. Opioid Receptors (δ-OR, κ-OR, μ-OR).—Opioid receptors (ORs), specifically the μ-opioid receptor (MOR), is the therapeutic target for numerous clinically used medications, predominately analgesics. Although MOR activation produces profound analgesia, tolerance develops for opioid drugs and addiction can be severely problematic.[253,254] Additionally, side effects such as respiratory depression, nausea, constipation, and others have highlighted the urgent need for better therapeutic agents targeting the MOR. Allosteric modulators may be suitable in this scenario, as receptor desensitization is theoretically less likely and allosteric modulators have a generally safer profile, owing to the ceiling effect.[255] Both NAMs and PAMs have been explored for opioid receptors, and while most work on allosteric modulation has been directed toward the MOR, a few ligands have been reported as hits for the δ-opioid receptors (DOR).[255]

Cannabidiol and salvinorin-A are among the earliest identified NAMs for the MOR and DOR.[256,257] Recently, BMS-986121 (**175**) and BMS-986122 (**176**) are reported as the first series of selective PAMs for the MOR (Figure 24) and were identified via a high-throughput screen.[258,259] The PAM activity is further characterized by three functional assays, including β-arrestin recruitment, inhibition of adenylyl cyclase activity, and G protein activation via [^{35}S]GTPγS binding. Compound **176** was shown to shift leftward the concentration—response curve of endomorphin-I (α = 7) in β-arrestin recruitment assays, and in G protein activation assays it was revealed that **176** displayed low levels of intrinsic agonism at concentrations above those required for endomorphin-1 potentiation. Intrinsic agonist activity of **176** at high doses was possibly explained by the lack of reciprocal affinity modulation from the orthosteric agonist. Subsequent chemical SAR study of **176** around the substituents on the side phenyl led to the discovery of BMS-986124 (**177**) as a neutral allosteric ligand or NAL.[258,259] Additionally, diterpene alkaloid ignavine (**178**) demonstrates positive modulatory activity for MOR agonists DAMGO, endomorphin-1, and morphine in a cAMP assay with an analgesic effect in vivo.[260] BMS-986187 (**179**), with a chemically novel core compared to previous BMS series, was discovered as an effective PAM at the DOR and at the κ-opioid receptor (KOR) rather than the MOR with an approximately 20-to 30-fold higher affinity in the allosteric ternary complex model.[261] Recently, significant attention has been directed toward the misuse and fatalities associated with prescription OR agonists, as opioid use disorder rises to epidemic proportions. However, the MOR remains a highly effective and validated target for analgesia and OR ligands are essential therapeutics. Thus, there is great interest in the discovery and development of novel OR therapeutics that

have a safer profile without the loss of efficacious analgesia. Allosteric modulation of ORs holds promise of delivering safer analgesics and other therapeutics to the clinic; however significant optimization and development are still needed.

3.4.3. Other Peptide and Protein Family Receptors.

Melanin-Concentrating Hormone Receptor 1 (MCH_1R).: The MCH_1 receptor is activated by melanin-concentrating hormone (MCH) and is widely expressed in the human CNS and to a lesser degree in the periphery. Due to its specific expression patterns in the CNS, the MCH_1 has been postulated to be drug target in anxiety, depression, and obesity disorders.[262] Predominately explored as an antiobesity target, the MCH_1 receptor is reported to be allosterically inhibited by the small molecule MQ1 (**180**, Figure 25) in multiple signaling pathways for $G\alpha_{i/o}$, $G\alpha_{q/11}$, and β-arrestin. MQ1 has been shown to be a slowly dissociating reversible MCH1 receptor blocker in washout experiments as well as affinity selection-mass spectrometry.[263] Increased efforts in this area should provide important in vivo tool compounds to validate the therapeutic potential of this attractive target.

Neuropeptide Y Receptors (Y_1R-Y_5R).: The human neuropeptide Y receptors comprised Y_1R, Y_2R, Y_4R, and Y_5R and are activated by a set of three known endogenous peptides, neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP). Each of these receptors is characterized by varying affinity for the endogenous peptides as well as varying physiological distribution. While each member of this receptor family has been identified as a probable therapeutic target for diseases such as obesity, cancer, or other metabolic disorders, most studies have focused on the Y_4R. Importantly, the Y_4R has higher affinity for the endogenous ligand PP, which is secreted by pancreatic cells in proportion to caloric content intake and is thought to modulate satiety in feeding, food intake, energy homeostasis, and colon transit.[264] Niclosamide (**181**) and structurally related compounds are revealed as nonselective small molecule PAM ligands for Y_4R versus Y_1R, Y_2R, and Y_5R via HTS.[265] Further efforts yielded the small molecule *tert*-butylphenoxycyclohexanol (tBPC, **182**), a purely efficacy-driven selective Y_4R PAM, and is reported to potentiate Y_4R activation in G-protein signaling and arrestin recruitment experiments.[266] Thus, the early efforts toward this target are promising and may yield important clinical candidates for obesity in the future.

Proteinase-Activated Receptors (PAR2).: PAR2 has recently been recognized as an important modulator of coagulation and inflammatory responses.[267] PAR2 is activated upon cleavage of the extracellular amino terminus by proteinases. This cleavage results in a new amino terminus that subsequently acts as a “tethered” agonist for receptor activation.[268] Thus, allosteric modulation of this receptor may be an attractive strategy for retention of this unique mechanism of activation. AZ3451 (**183**) is an allosteric antagonist binding to a remote, lipid-facing allosteric site of PAR2, and the solved cocrystal structure represents increased success in the structural elucidation for allosteric modulators of class A GPCRs.[32]

Tachykinin Receptors (NK2).: The NK2 receptor is widely expressed throughout the human gastrointestinal system and has been shown to be a key player in intestinal motility and the high degree of motility observed upon intestinal inflammatory responses. Thus,

efforts toward discovering small molecule agents for this target have focused on antagonists and NAMs to block inflammation and motility for indications such as irritable bowel syndrome.[269] Compound **184** was discovered as a NAM for the NK2 receptor in a FRET-based binding assay. Interestingly, the NK2 receptor has been shown to be active in two distinct activation conformations, which correspond to downstream signaling biases. Compound **184** can shift the active conformations away from a cAMP-producing conformation, suppressing agonist-induced cAMP production by 30% in the presence of 10 μM of **184**.[270] This shift in conformation populations results in a slight potentiation of agonist-induced calcium response, indicating a possible increase of this particular conformation. Further modification on **184** to **185** with a butyronitrile side chain yielded a compound that retains suppression of cAMP production while increasing potentiation of agonist-induced calcium mobilization E_{max} (50% to 68%).[271] Additional efforts toward NK2 NAMs may elucidate the discrepancies between active conformations and provide evidence for NAMs as therapeutic candidates for gastrointestinal-related diseases.

4. CHALLENGES AND EMERGING CONCEPTS IN CLASS A GPCR DRUG DISCOVERY

4.1. Complexities and Nuance Observed in Screening, Optimizing, and Advancing Class A GPCR Allosteric Modulators.

The discovery of allosteric modulators for class A GPCRs has been advanced by numerous academic and industry groups over the past decade and has provided a framework for optimization and development of such ligands. This framework includes understanding the multiple facets of allosteric modulator influences on the receptor complex with orthosteric agonists and antagonists, as well as the influence on coupling to effector molecules. As previously suggested, an allosteric modulator—receptor complex can be described as a "new receptor" that results in new or differential biology compared to the native receptor.[5] Thus, it is important to quantify the allosteric modulator's effect on the receptor, orthosteric signaling molecule, and the downstream effectors. The quantification metrics (cooperativity, intrinsic efficacy, etc.) used throughout hit optimization have been shown to sometimes improve (or decrease) in tandem; however, these measures have often been shown to "uncouple" such that orthosteric agonist affinity may improve, but there may be no impact on other metrics such as agonist efficacy. Additionally, this trend is observed in which, upon chemical modification, allosteric modulators may acquire intrinsic efficacy as agonists or serve as antagonists. While these outcomes may be advantageous for a particular target, the trend likely will not translate to other orthosteric ligands of the receptor (probe dependence). This can be problematic when the endogenous signaling ligand is not amenable to screening in functional assays (peptides, proteins, etc.) and should be approached with caution. Importantly, GPCRs exist in an ensemble of states and can be stabilized by high-affinity allosteric modulators in a variety of functionally relevant states, such as active states, pathway-specific active states, or inactive states.[40] The induced receptor conformation may then be more likely to couple with some effectors (e.g., β-arrestins) than others (e.g., G proteins), leading to signaling bias. These complexities may yield therapeutically important results; however, the team must obtain a thorough characterization and SARs of the candidate molecule to effectively move through optimization and development. Allosteric

modulators exert their effects on receptor activation in numerous ways, which contribute to the nuances described above. As seen throughout this Perspective, drug discovery teams utilize various assays to quantify the effects of allosteric modulators, but the assays and assay conditions employed are likely to diverge between groups. Additionally, experts have warned against characterizing allosteric modulators with singular assays, such as calcium mobilization, to determine potency estimates that solely drive SAR.[26] Robust in vitro characterizations that lead to results that are readily compared across drug discovery groups is a continuing challenge for the field.

Advancing allosteric modulators from in vitro to in vivo is beset with challenges as well. For instance, dissection of signaling bias in animal models and behavioral paradigms is difficult to achieve and may be highly sensitive to probe dependence, especially if the endogenous signaling agonist could not be used for iterative screening and SAR.[22] Additionally, allosteric sites can display a greater divergence between species (e.g., rat vs human) in comparison to orthosteric sites, complicating interpretations of drug effect across species.[134] One possible explanation for this observation is that allosteric sites are less homologous due to decreased evolutionary pressure, a feature exploited for subtype selectivity, and may be more pronounced between species. Thus, screening preclinical candidate allosteric modulators at both human and rat GPCRs will help to alleviate this unknown when advancing compounds. Also relevant to in vivo characterization is the presence of “chemical switches” (slight chemical changes that significantly alter or reverse activity) that become apparent during chemical optimization.[272] Although standard chemical switches are likely to be addressed at an early stage, recent work has highlighted the presence of metabolic chemical switches that lead to major metabolites displaying a different or opposite activity profile.[273] Chemical switches may be identified and addressed through early core optimization and the use of the “fluorine walk” to determine scaffold positions amenable to modification, and some success has been reported in the use of halogens or deuterium to dissuade metabolism of some scaffolds.[22] Species bias and metabolic switches may complicate preclinical development and should be a key, deciding factor for the abandonment or development of select scaffold.

4.2. Emerging Strategies for Class A GPCR Small Molecule Allosteric Modulator Discovery and Development.

Structure-based drug design (SBDD) is emerging in the discovery and optimization of allosteric modulators for class A GPCRs.[274] The growing number of crystal structures available and the structural studies on allosteric modulator mechanisms are providing the material and insight into engage in SBDD for allosteric modulators.[275,276] Importantly, the resolution at allosteric “hot spots”, such as ECLs, has greatly improved in recent reported crystals and is suitable for docking studies or molecular dynamics simulations. It should also be noted from a receptor structure perspective, that there is an ever-growing number of class A GPCR structures being reported via electron cryomicroscopy (cryo-EM) techniques. The resolution of these structures has significantly improved in recent years, and thus, cryo-EM structures will likely play a pivotal role in understanding receptor complexes and informing SBDD.[277] From a ligand optimization perspective, these structural studies can be combined with functional assays and site-directed mutagenesis to provide greater clarity on the

allosteric mechanism of action at the receptor. Compounds displaying chemical switches may be used in simulations and further inform the structural biology of GPCR activation and signaling enhancement. From a discovery perspective, exciting studies are emerging with de novo allosteric modulators discovered via virtual screening oflarge libraries. For example, Valant and colleagues recently published the results of an iterative molecular docking and screening project where two subtype selective M_2 mAChR NAMs and one PAM were discovered from the National Cancer Institute (NCI) compound library.[278] The success of molecular docking approaches in this example, in which validated and chemically diverse PAMs and NAMs were discovered, is encouraging, and this arena is projected to be highly important in the future. Additionally, fragment-based drug design (FBDD) may be an important tool for the discovery of novel allosteric modulator chemotypes and becomes a reality with improved crystal structures.[279,280] An in silico approach has been shown by Bian et al. for the class C GPCR metabotropic glutamate receptor 5.[281] Additionally, a more traditional approach without structural information has been shown for the class C GPCR metabotropic glutamate receptor 2 by Szabo et al.[282] Considerations of receptor activation state and the ability to predict allosteric modulation by docking to unknown sites remain to be addressed for different members ofclass A GPCRs.

Another emergent strategy is the utilization of covalent allosteric probes to identify and define the allosteric binding site. Chemically reactive groups can be adapted to allosteric modulators to afford covalent binding to the allosteric site, and subsequent peptide mass spectrometry can yield surrounding residues. Followed by site-directed mutagenesis and informed by known structural information, this may be a powerful tool for structurally informed rational design. This strategy was successfully implemented by Thakur and colleagues to map the CB_1 receptor allosteric binding site.[159] Electrophilic and photoactivatable moieties were added to CB1 receptor NAMs, which retained their activity and provided useful chemical tool compounds. Of note, the authors engaged in iterative rational design of numerous covalent derivatives based on previous knowledge of the NAM SAR and discussed modifications that abolished activity. Thus, this powerful strategy may not be suitable for scaffolds prone to chemical switches or shallow SAR.

4.3. New Concepts in Pharmacology for Class A GPCRs and Implications for Allosteric Modulator Agents.

As studies continue to shed light on the intricate pharmacology of class A GPCRs, new paradigms in drug discovery will emerge. The initial concept of allosteric modulation was developed based on the understanding that allosteric regulation was a ubiquitous and essential element for functional proteins throughout biology.[9] Likewise, biological and pharmacological studies will elucidate new mechanisms for GPCR regulation, expression, function, and modulation. There are emerging studies regarding class A GPCR dimerization/oligomerization, subcellular location of GPCRs, and temporal regulation of GPCRs that pose interesting paradigms for GPCR modulation. The dimerization, whether homodimers, heterodimers, or higher order oligomers, of class A GPCRs has been a thoroughly discussed topic in relation to its biological relevance.[283–285] A noteworthy reminder, class C GPCRs are known to form obligatory dimers; thus the discussion is centered on class A GPCRs.[286] A recent review by Gurevich and Gurevich addresses dimerization from a signaling

perspective and discusses the stoichiometry observed between class A GPCRs and their effectors: G proteins, β-arrestins, and GRKs.[287] The conclusions state that a single monomeric class A GPCR is sufficient for effector coupling and downstream signaling through multiple pathways, and this is supported by biochemical and functional assays. It is also known that class A GPCRs do indeed interact as dimers during their "life cycle" and that these interactions may be important regulators for expression, localization, and trafficking. Whether or not functional signaling dimers exist, there is evidence for receptor crosstalk that can be modulated, and allosteric modulators can play an important role in regulating the monomer—dimer equilibrium and may impart therapeutic effects in this manner. Indeed, a recent report shows how the CCR5 receptor allosteric modulator maraviroc (**162**) can influence the dimer population by inducing a third inactive dimer conformation.[16] Dimerization of CCR5 is necessary for translocation to the membrane, and the dimer induced by **162** may contribute to its efficacy in blocking HIV entry in to the cell. Much more information is needed on these facets of GPCR pharmacology, but novel chemical probes and tool compounds can provide new ways of investigating dimers and the future may hold targeted therapies for such complexes.

Additionally, as class A GPCR dimers have been visualized in the cell, so have functional intracellular class A GPCRs. Opioid receptors (ORs) were highlighted in a recent report that identified differential signaling patterns between endogenous peptide-bound ORs at the cell surface and opioid drug-bound ORs in the Golgi membrane within the cell.[288] The authors argue that this "distortion" of typical endogenous peptide activation may drive neuronal toxicity and adverse effects from OR-targeted therapeutics. This effect has been termed location bias for GPCR activation. In a review by Grundmann and Kostenis, the recent consensus on time-encoded GPCR signaling ("temporal bias") is presented, as are other important kinetic parameters for class A GPCR signaling.[289] Additional indepth reviews have highlighted signaling and ligand kinetics as an important consideration for interpreting results of receptor activation, especially in the context of allosteric ligands.[290] Current pharmacological studies have identified signaling bias and probe dependence as emerging therapeutic strategies, and the future will likely see dimer equilibrium, location bias, and temporal bias become topics of discussion in class A GPCR allosteric modulator drug discovery.

5. CONCLUSIONS AND FUTURE DIRECTIONS

Class A GPCRs hold significant clinical importance and are targeted by a high percentage of currently marketed drugs. Utilizing allosteric modulation to precisely alter the function of these receptors may enable the therapeutic targeting of previously intractable GPCRs or provide safer therapeutics for currently targeted receptors. The ability to modulate signaling in a spatial and temporal dependent manner, as well as the potential to therapeutically exploit probe dependence, moves beyond achieving subtype selectivity and toward a remarkably precise therapeutic paradigm. However, the complexities that confer these advantages also must be addressed during allosteric modulator discovery, optimization, development, and advancement into preclinical/clinical assessments. Multiple signaling pathways downstream of effectors, such as G proteins and β-arrestins, should be examined to provide informed SAR of the molecule series. Additionally, the endogenous agonist

should be assayed, when appropriate, to ensure the translation of the allosteric modulation in vivo and avoid unforeseen probe dependence. There are cases, especially in the chemokine receptor family, where probe dependence will be a desirable outcome and should be addressed early in scaffold optimization. Other in vivo considerations arise from species bias, which has been observed in mAChRPAMs and NAMs, where activity does not translate from in vitro assays employing human mAChRs to rat in vivo assays. Thus, early examination of rat and human class A GPCRs in vitro may help avoid this situation. The discovery and development of class A GPCR allosteric modulators have progressed tremendously in recent years and have provided a framework for overcoming challenges and maturing clinical candidates.

The chemical diversity of class A GPCR allosteric modulators has grown along with diversity of allosteric binding sites. Allosteric binding sites have been shown to exist in extracellular regions, transmembrane regions, and intracellular regions of receptors, all contributing unique mechanisms for modulation. Most ligand interactions with these sites are classified as hydrophobic interactions, while aromatic π–π interactions are also common. Core scaffolds for class A GPCR allosteric ligands commonly contain a nitrogen amenable to H-bond polar interactions. Druglikeness has improved and is attainable in situations where there are large amounts of SAR to dictate sites available for modification. Chemically successive, iterative ligand design and synthesis should be performed to enable informed SAR and can be aided by strategies such as the “fluorine walk”. Importantly, emergent structural information may provide information toward chemical modifications that lead to high affinity allosteric ligands and should be utilized where available.

Finally, new conceptual frames for pharmacology may direct allosteric modulator discovery toward modes of action other than simple potentiation of activation. As seen in CCR5 dimerization, maraviroc may alter dimer populations to provide antiviral efficacy. Dimer stabilization/destabilization, location bias, and temporal bias may become considerations for allosteric mechanism of action. Probe dependence can hinder development; however, it may also be used to selectively potentiate (or diminish) marketed orthosteric drugs. In this way, a promiscuous orthosteric drug can have improved selectivity at its site of action or be altered for a higher affinity at an additional site. Thus, allosteric modulators may improve marketed drugs to provide greater selectivity, or if polypharmacology is desired, as in difficult psychiatric conditions, allosteric modulators could enhance activity at other receptors for a given marketed drug that displays moderate affinity for these targets. Allosteric modulation is a fundamental mechanism in biology, and exploitation of this paradigm has delivered FDA-approved therapies, multiple drug-candidates in the pipeline and promises to provide more precise and safer small molecule therapeutics in the future.

ACKNOWLEDGMENTS

This work was supported by Grants R21 MH093844 (J.Z., K.A.C.), R01 DA038446 (J.Z., K.A.C.), K05 DA020087 (K.A.C.), P30 DA28821 (K.A.C.), T32 DA07287 (E.A.W.), and F31 DA045511 (E.A.W.) from the National Institutes of Health, R. A. Welch Foundation Chemistry and Biology Collaborative Grant from the Gulf Coast Consortia (GCC), John Sealy Memorial Endowment Fund, and the Center for Addiction Research (CAR) at UTMB.

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ABBREVIATIONS USED

GPCR	G-protein-coupled receptor
oGPCR	orphan G-protein-coupled receptor
CNS	central nervous system

PAM	positive allosteric modulator
NAM	negative allosteric modulator
SAL	silent allosteric ligand
TM	transmembrane
SAR	structure–activity relationship
5-HT	5-hydroxytryptamine
5-HT$_{2C}$R	serotonin 5-HT$_{2C}$ receptor
5-HT$_{2A}$R	serotonin 5-HT$_{2A}$ receptor
5-HT$_{2B}$R	serotonin 5-HT$_{2B}$ receptor
5-HT$_{6}$R	serotonin 5-HT$_{6}$ receptor
5-HT$_{7A}$R	serotonin 5-HT$_{7A}$ receptor
IP$_3$	inositol 1,4,5-triphosphate
SUD	substance use disorder
CHO	Chinese hamster ovary
CUD	cocaine use disorder
PK	pharmacokinetics
EL	extracellular loop
TMH	transmembrane helix
PTEN	phosphatase and tensin homolog
ICL	intracellular loop
β_2AR	β_2-adrenergic receptor
cAMP	cyclic adenosine monophosphate
DR	dopamine receptor
ADHD	attention deficit hyperactivity disorder
PLG	Pro-Leu-Gly-NH$_2$
HTS	high-throughput screen
mACh	muscarinic acetylcholine
BQCA	benzylquinolone carboxylic acid
BBB	brain–blood barrier

IP	inflection point
tPSA	topological polar surface area
P-gp	P-glycoprotein
CB	cannabinoid
GTPγS	guanosine 5′-*O*-[γ-thio]triphosphate
ERK½	extracellular signal-regulated kinase ½
FSK	forskolin
LAPS	ligand-assisted protein structure
DSE	depolarization-induced suppression of excitation
2-AG	2-arachidonoyl glycerol
MD	molecular dynamics
FFAR	free fatty acid receptor
AR	adenosine receptor
AE	allosteric enhancer
AM	allosteric modulator
UTP	uridine 5′-triphosphate
NR4A	nuclear receptor 4A
NRCM	neonatal rat cardiomyocyte
DED	dry eye disease
CCR	chemokine receptor
HIV-1	human immunodeficiency virus type 1
BRET	bioluminescence resonance energy transfer
SBDD	structure-based drug design
FBDD	fragment-based drug design
OR	opioid receptor
MOR	μ-opioid receptor
DOR	δ-opioid receptors
KOR	κ-opioid receptor
PP	pancreatic polypeptide

PAR	proteinase-activated receptor
MCH1R	melanin-concentrating hormone receptor 1
MCH	melanin-concentrating hormone

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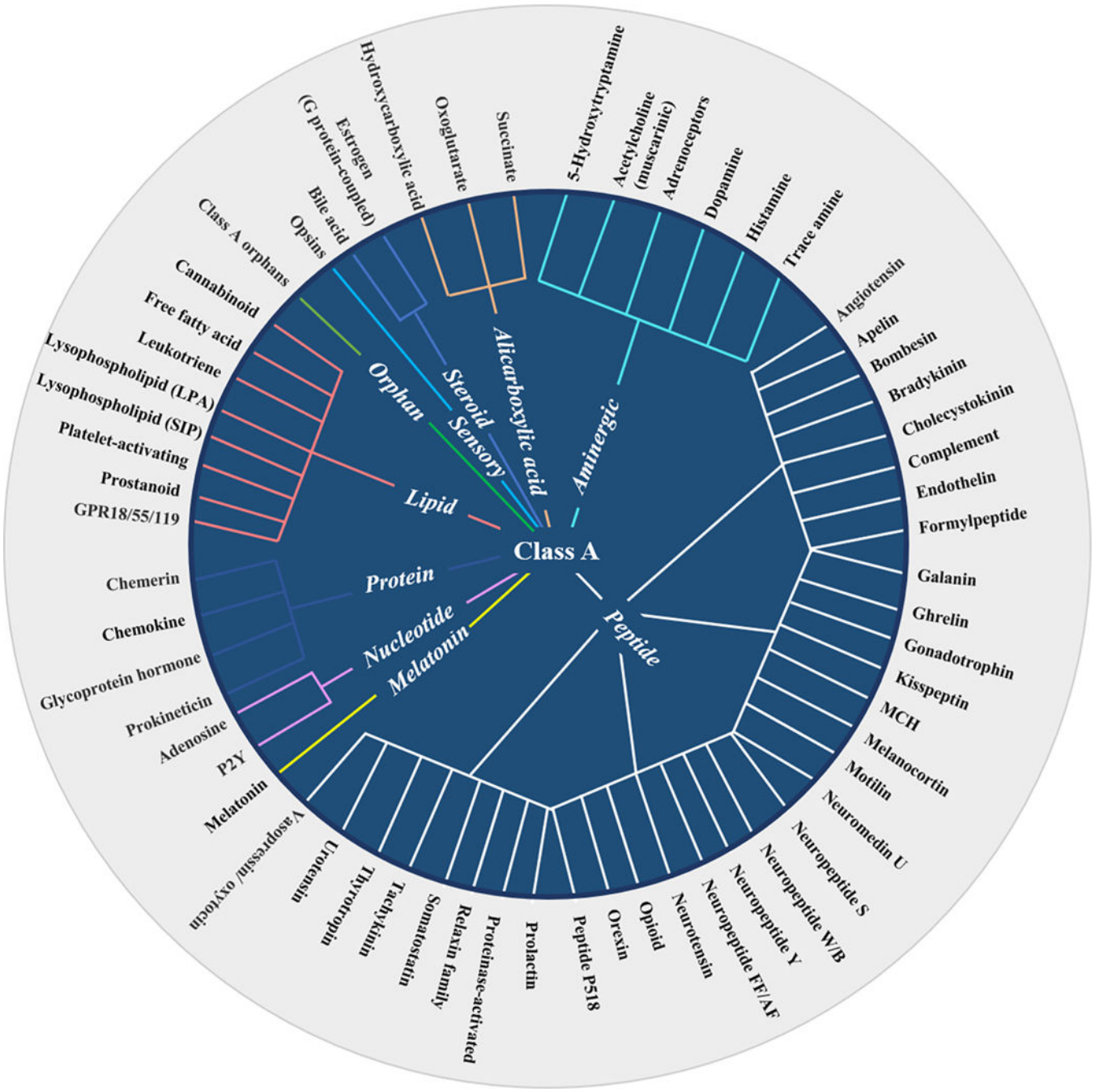


Figure 1.

Signaling molecule diversity and classification for class A GPCRs. The inner blue region highlights the diverse endogenous signaling molecules that activate class A GPCRs. Data were retrieved from Web site http://www.gpcrdb.org/drugs/drugbrowser.

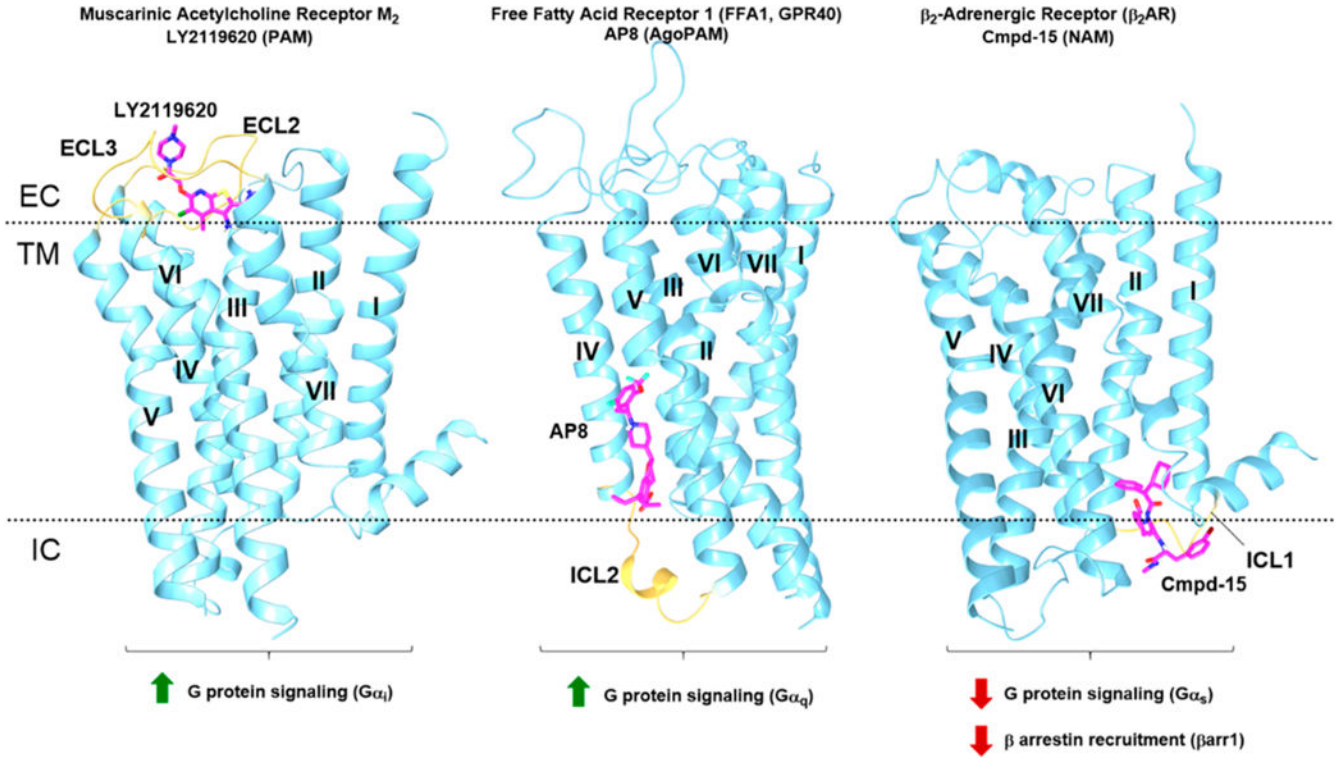


Figure 2.

Diverse allosteric binding sites reported across class A GPCRs. The representative class A allosteric modulators (stick representation,magenta) are shown bound in the corresponding cocrystal structure at distinct and diverse sites. The M2 mAChR-shows an extracellular (EC) site LY2119620 (left, PDB code 4MQT);19 FFA1-AP8 cocrystal shows AP8 in the transmembrane (TM) region adjacent to the lipid membrane (center, PDB: 5TZY);20 β_2ARCmpd-15 cocrystal shows an intracellular (IC) binding site (right, PDB code 5X7D)21 Bottom: the diverse signaling outcomes on orthosteric agonism by class A GPCR allosteric modulators.

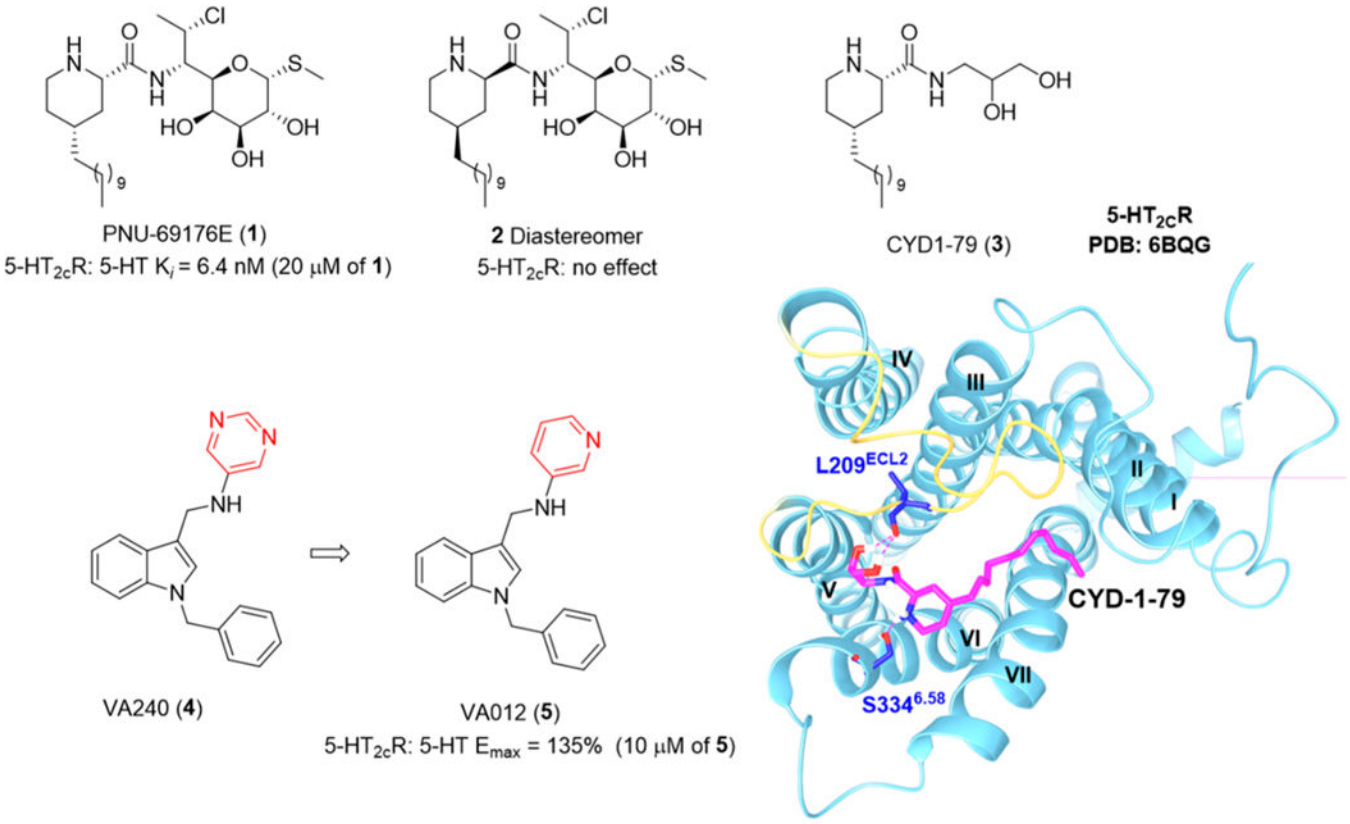


Figure 3.

Representative 5-HT_{2C}R PAMs **1–5**. Right: CYD-1–79 (stick representation, magenta, **3**) predicted binding pose from molecular docking on the recently solved 5-HT2CR crystal structure (PDB code 6BQG) interacting with L209ECL2 and $S334^{6.58}$.[51,54]

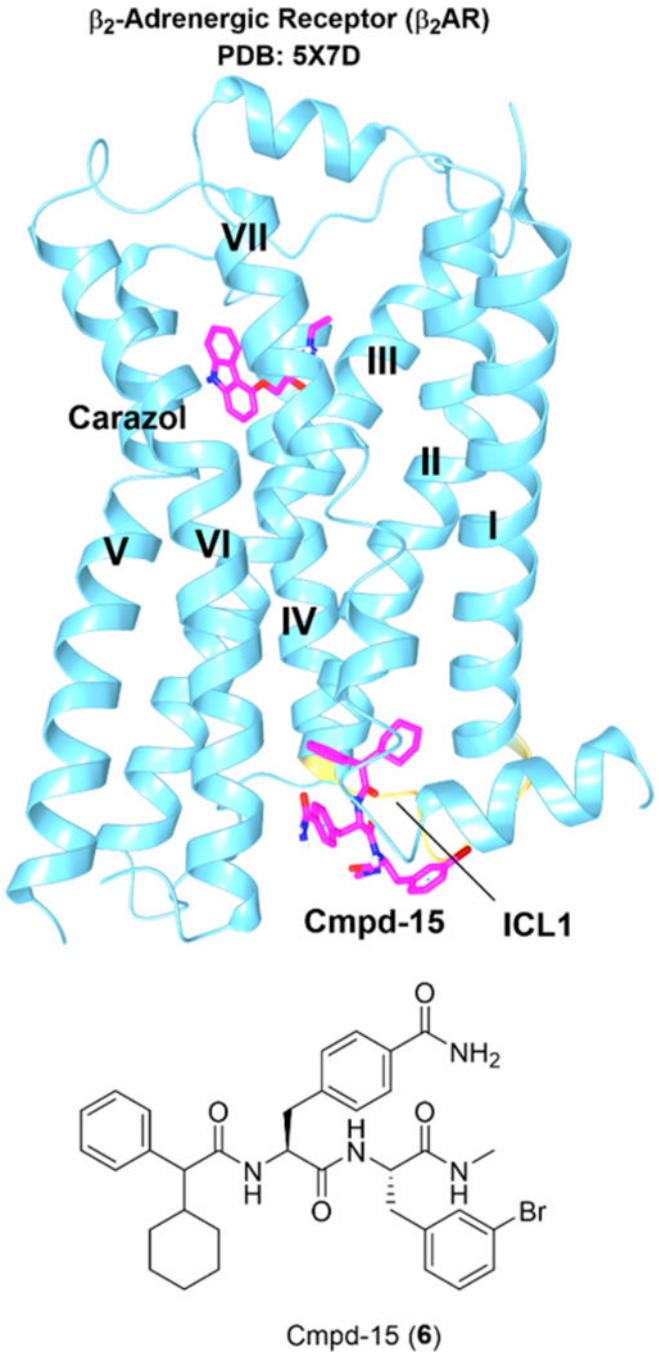


Figure 4.

Top: β_2AR cocrystal structure with Cmpd-15 (**6**) and antagonist carazol (PDB code 6X7D)[21] Bottom: structure of β_2AR NAM Cmpd-15 (**6**).

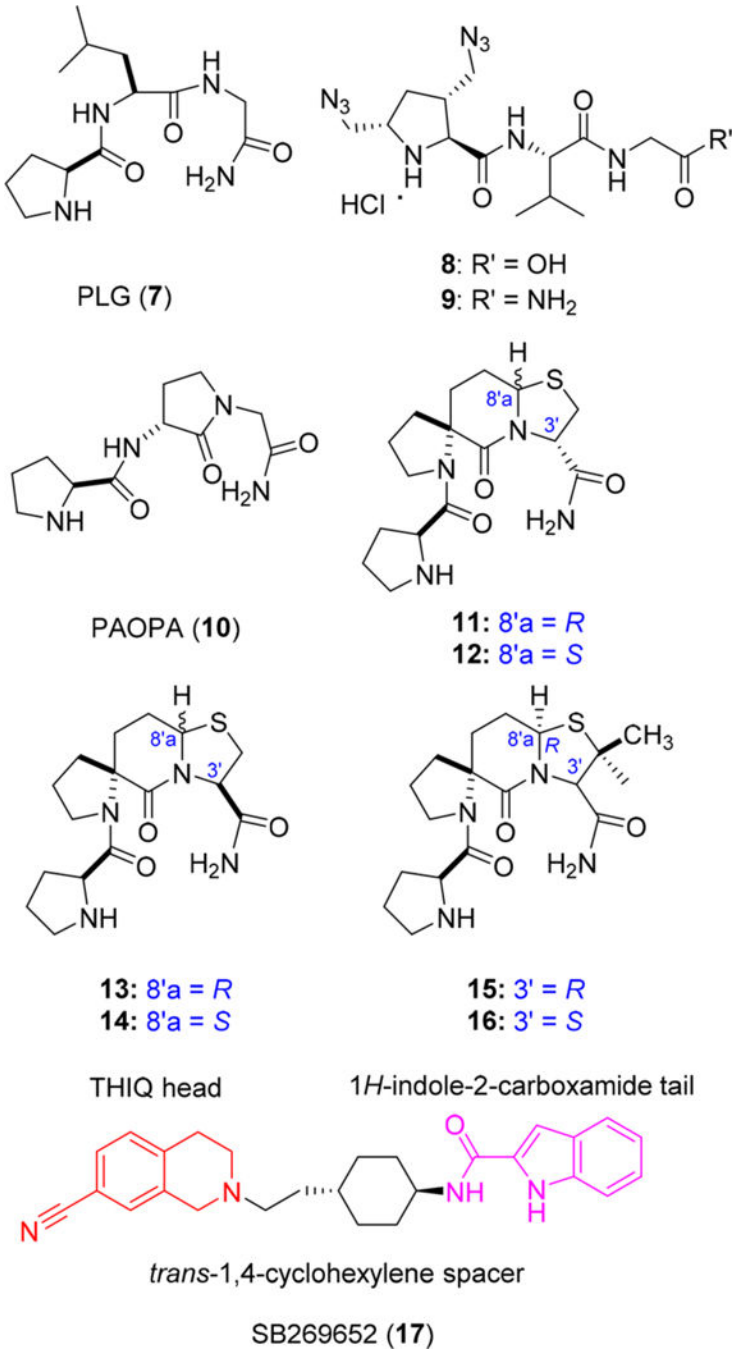


Figure 5.

Representative allosteric modulators of the dopamine D_2 and D_3 receptors with chemical modifications at selected positions.

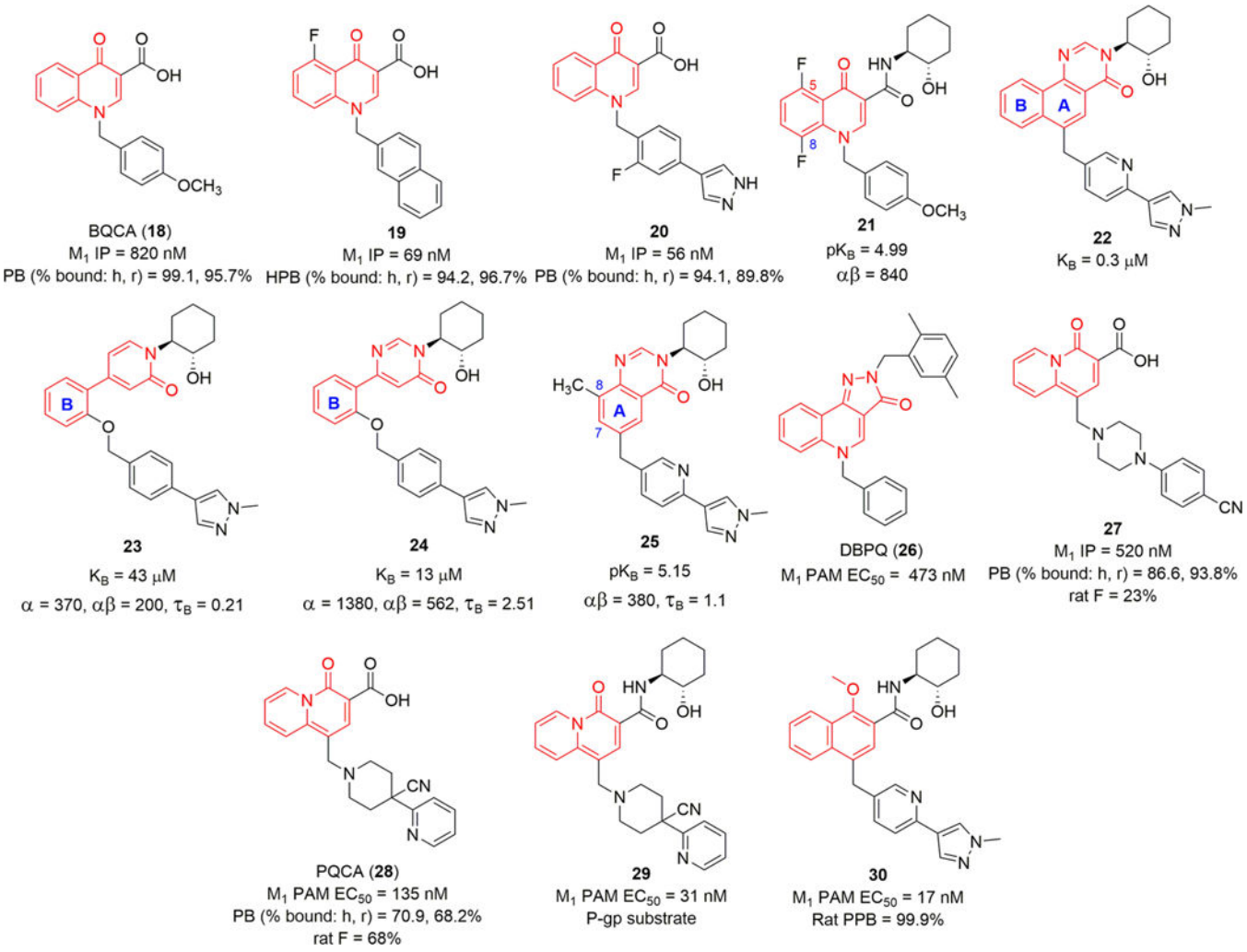


Figure 6.

M_1 mAChR PAM BQCA (**18**) and derivatives (**19–30**) with corresponding SARs.

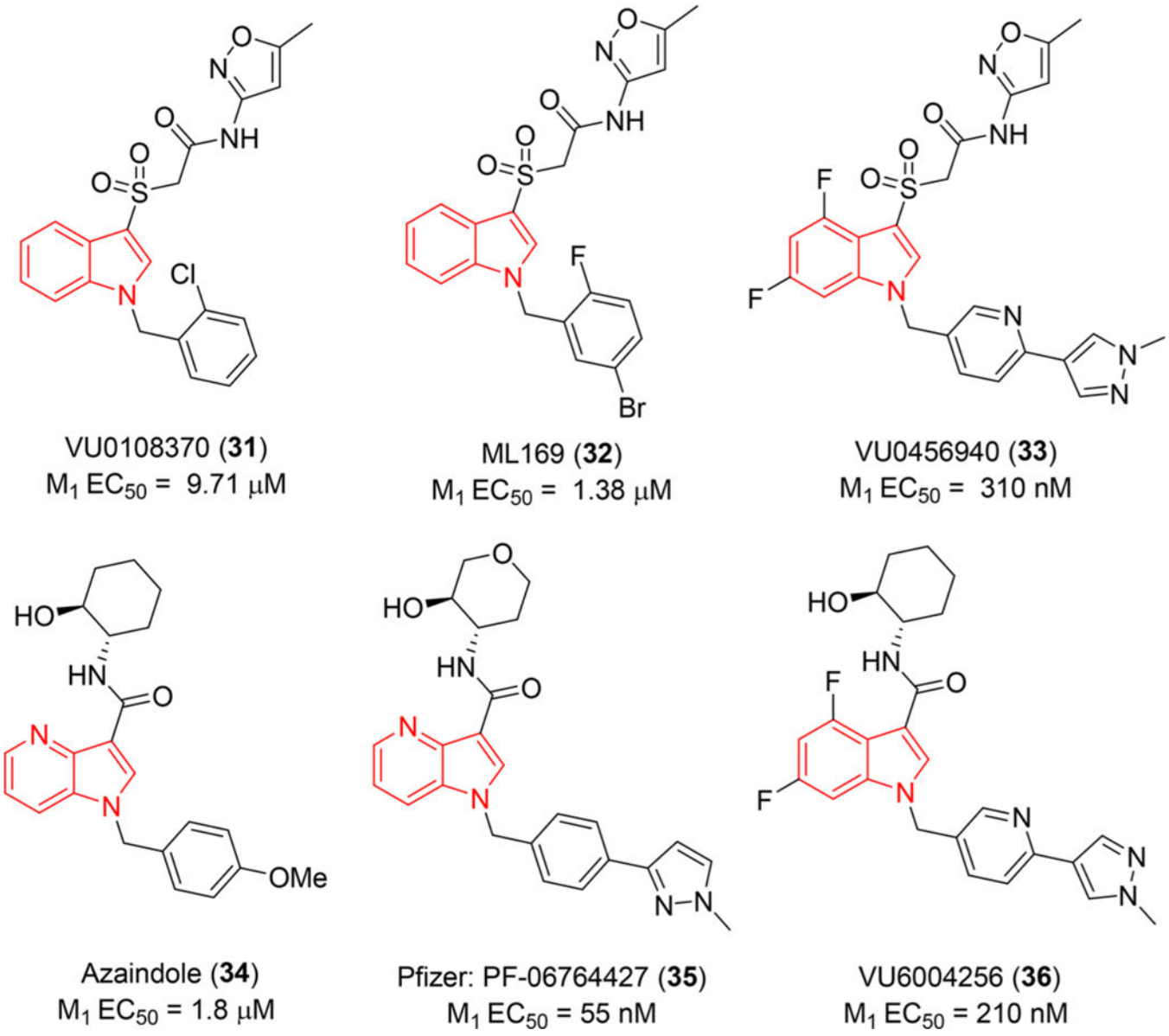


Figure 7.
Representative M_1 mAChR PAMs with an indole core (red) and SAR (compounds **31–36**).

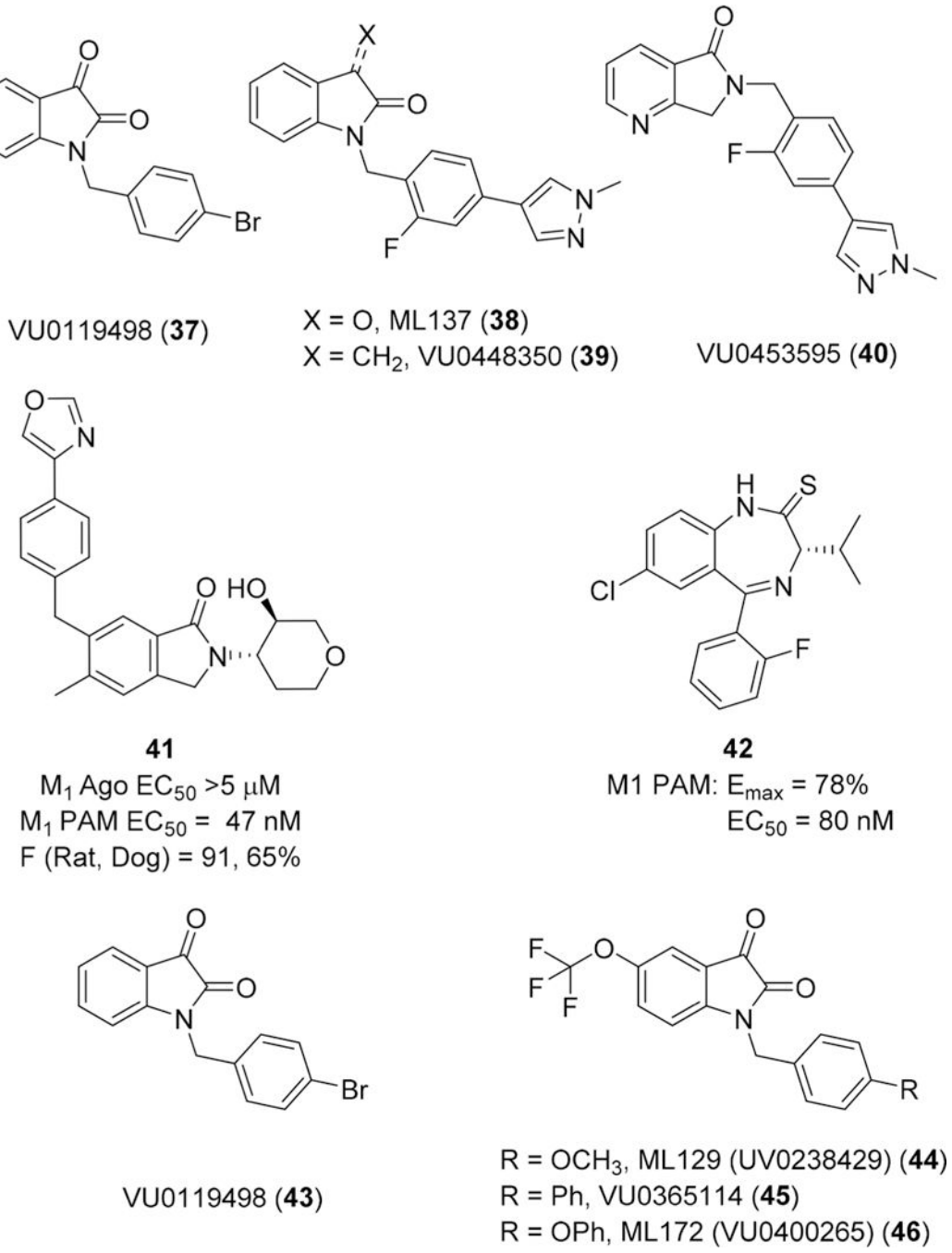


Figure 8.
Chemically diverse M_1 mAChR PAMs with key modifications (compounds **37–46**).

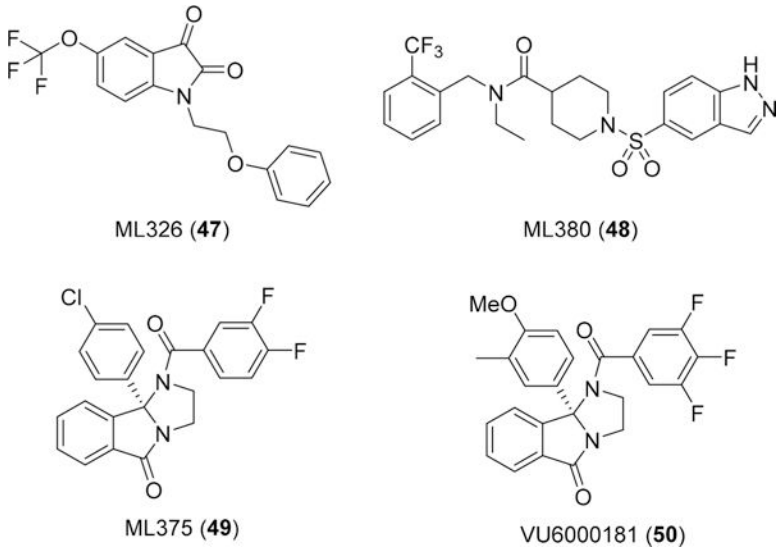


Figure 9.
Representative M_5 mAChR PAMs (**47, 48**) and NAMs (**49, 50**).

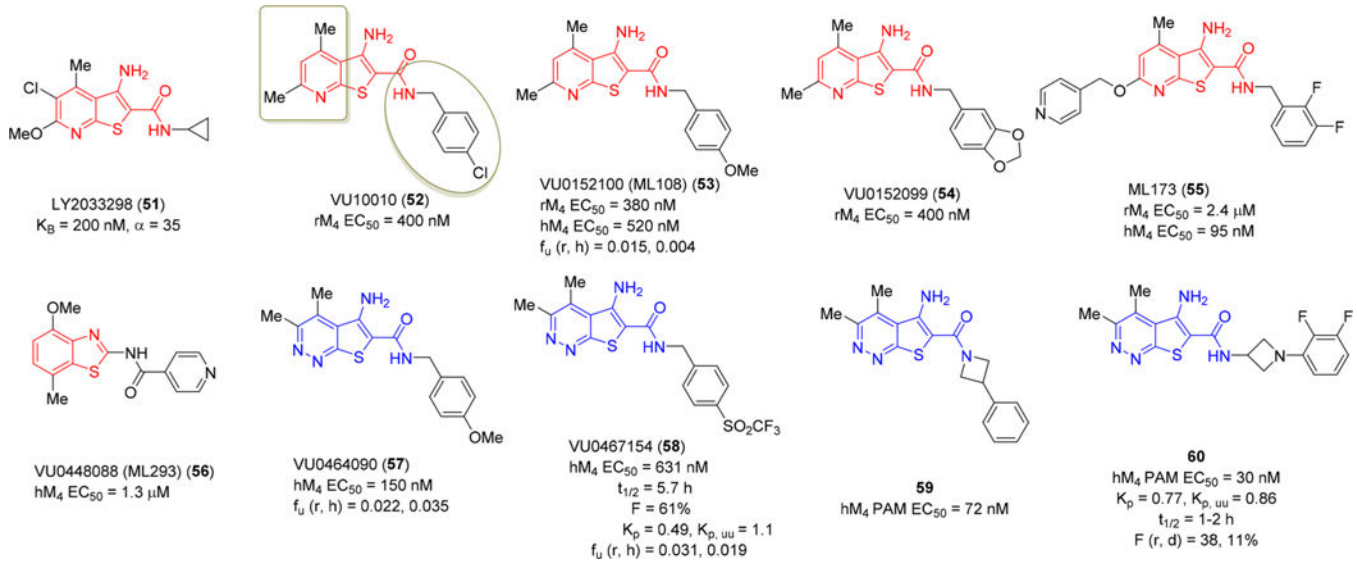


Figure 10.

M_4 mAChR PAM derivatives around the 5-aminothieno[2,3-*c*]pyridine scaffold and corresponding SARs (**51–60**).

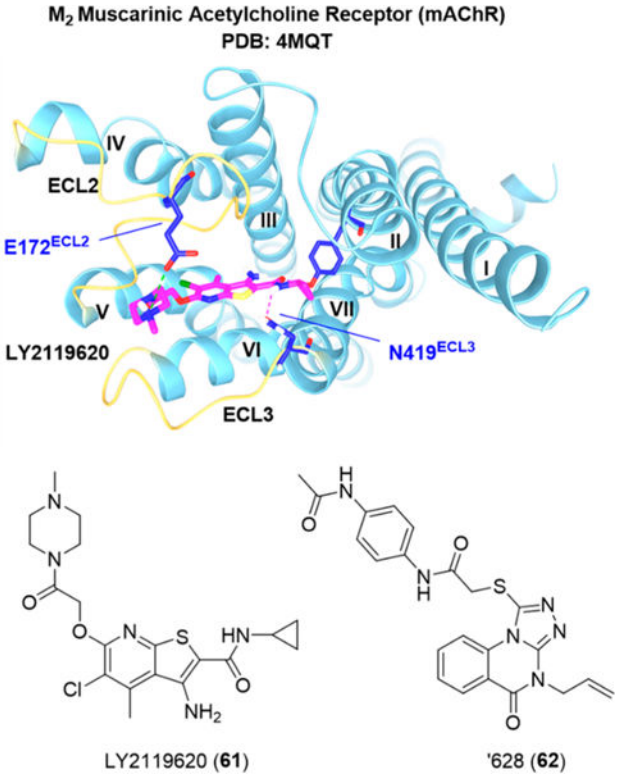


Figure 11.

Top: M_2 mAChR-LY2119620 (stick representation, magenta, **61**) cocrystal extracellular view highlighting interactions with ECL residues $E172^{ECL2}$ and $N419^{ECL3}$ (PDB code 4MQT).[19] Bottom: representative M_2 mAChR PAMs (**61, 62**).

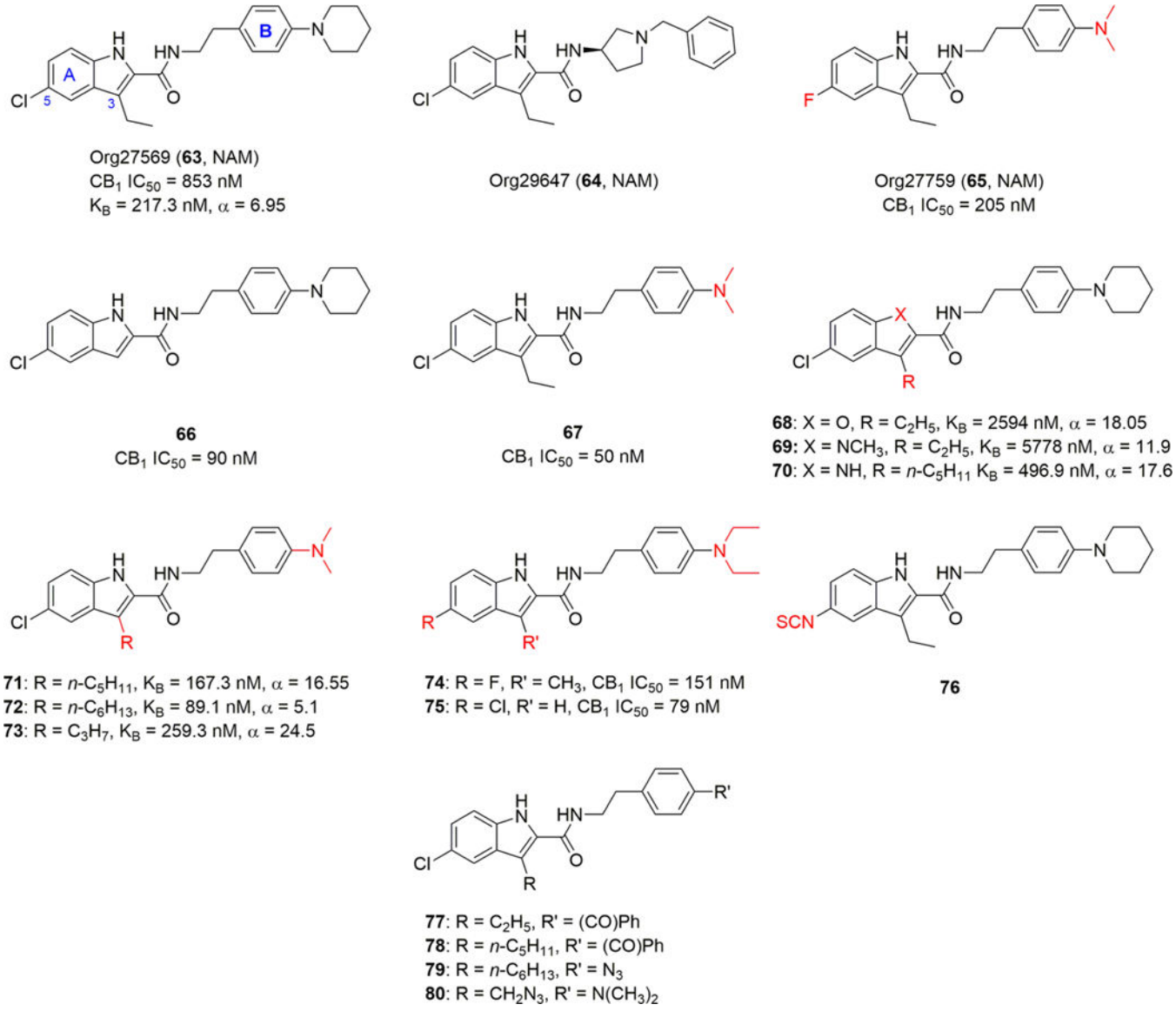


Figure 12.

CB_1 receptor NAMs (**63–80**) based on the 1*H*-indole-2-carboxamide scaffold and corresponding SAR.

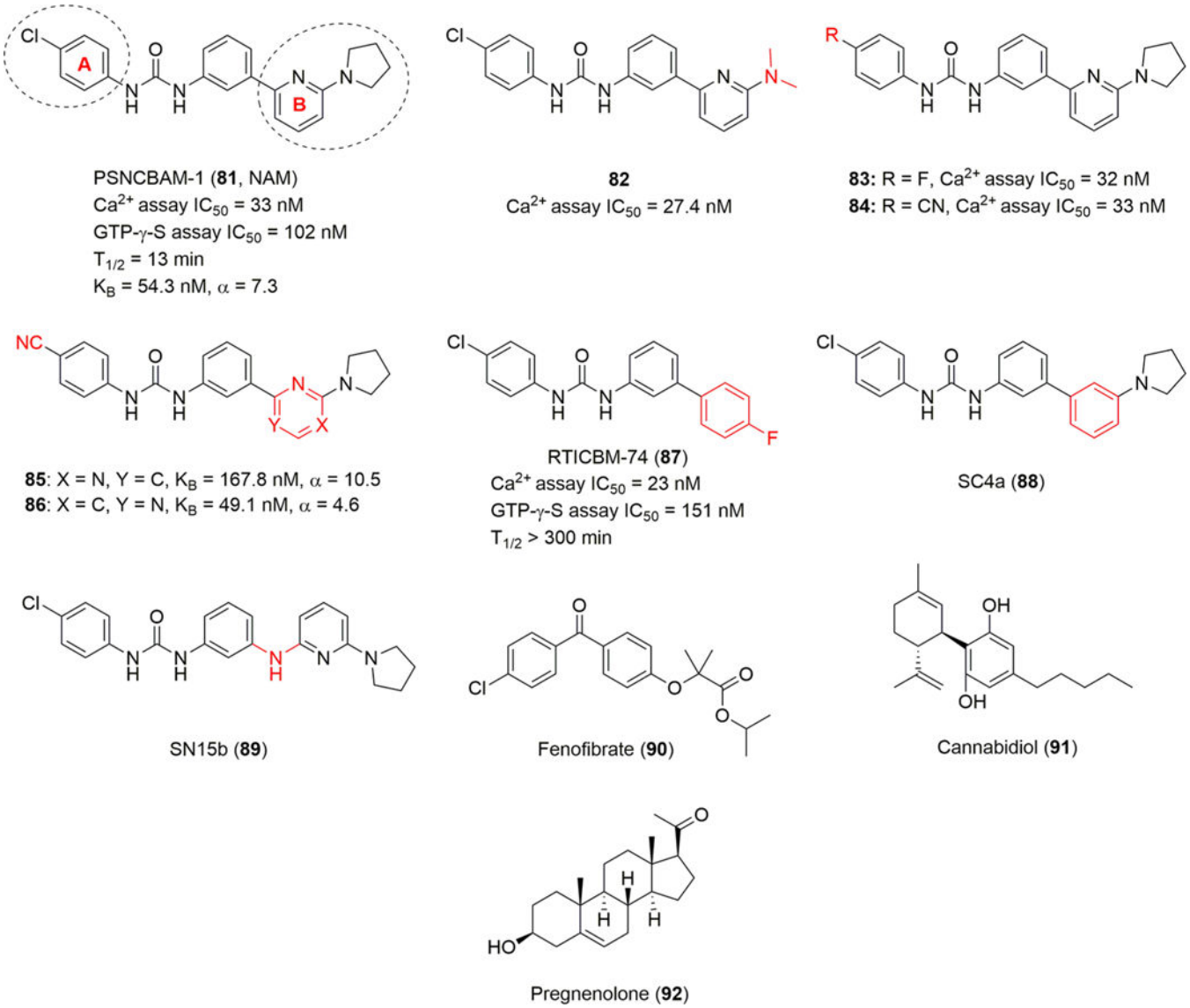


Figure 13.

CB_1 receptor NAMs based on the diarylurea scaffold (**81–89**) and corresponding SAR; additional CB_1 receptor NAMs (**90–92**).

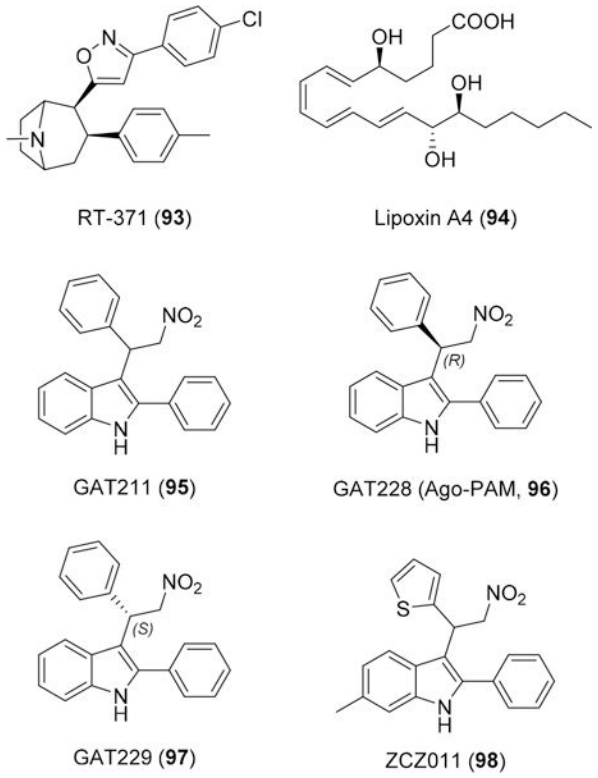


Figure 14.

Representative CB_1 receptor PAMs (**93, 94**) and 2-phenyl-1*H*-indole scaffold derivative CB_1 receptor PAMs (**95–98**).

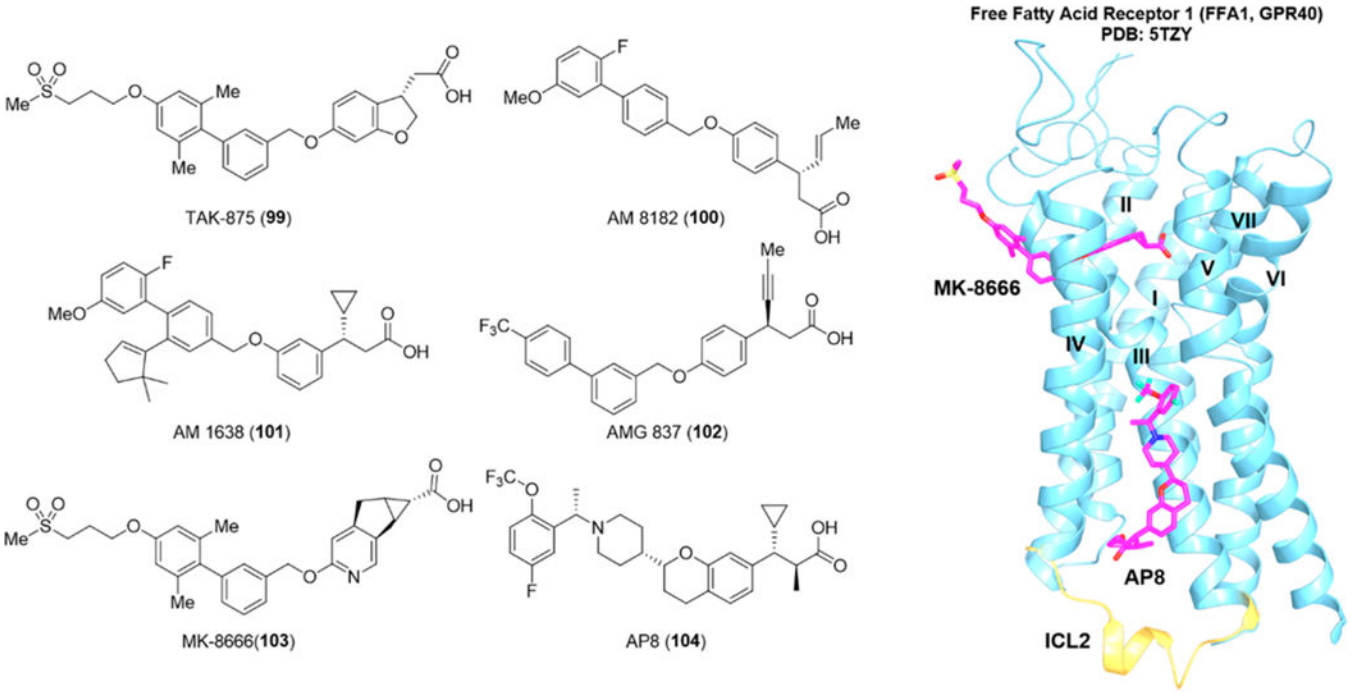


Figure 15.

Left: representative FFA1 receptor PAMs (**99–104**). Right: cocrystal complex of FFA1-MK-8666-AP8 showing distinct allosteric sites for both PAMs (PDB code 5TZY).[20]

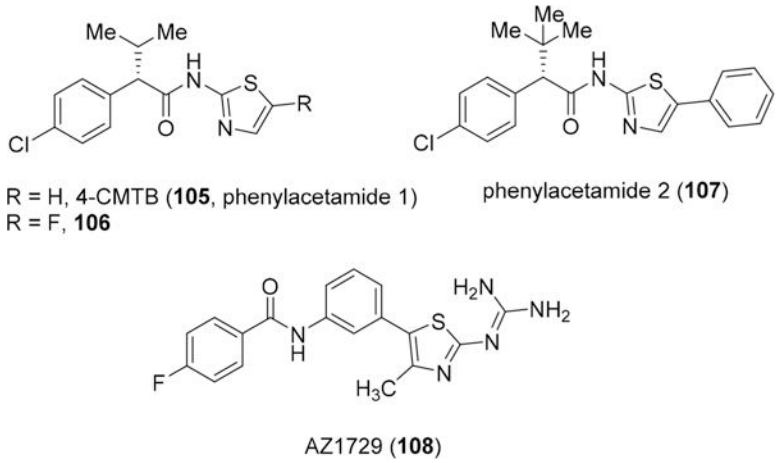
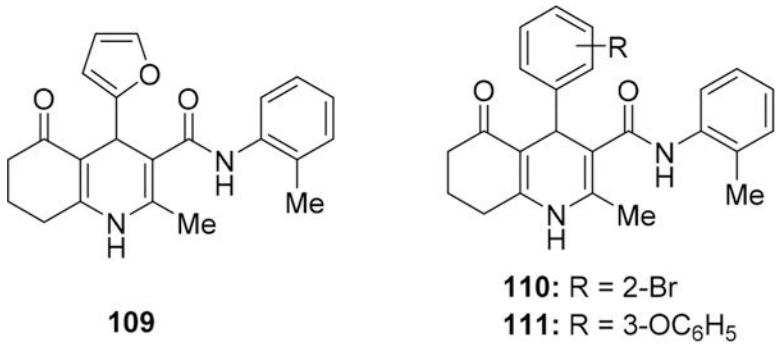


Figure 16.
Representative FFA2 receptor PAMs (**105–108**).



109

110: R = 2-Br
111: R = 3-OC_6H_5

Figure 17.
Representative FFA3 receptor PAMs (**109–111**).

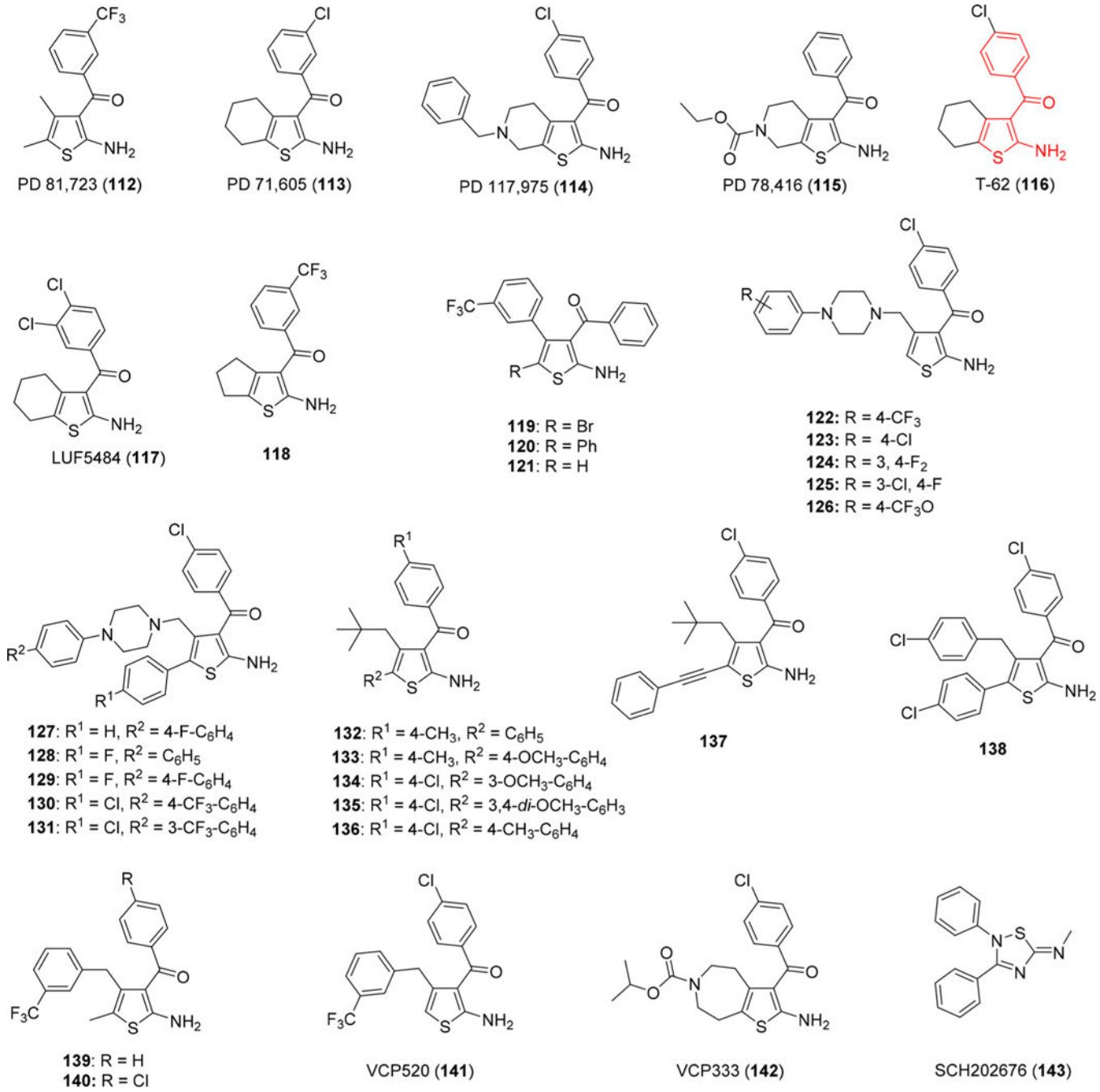


Figure 18.

A_1RPAM derivatives designed around the 2-amino-3-benzoylthiophene scaffold (**112–143**).

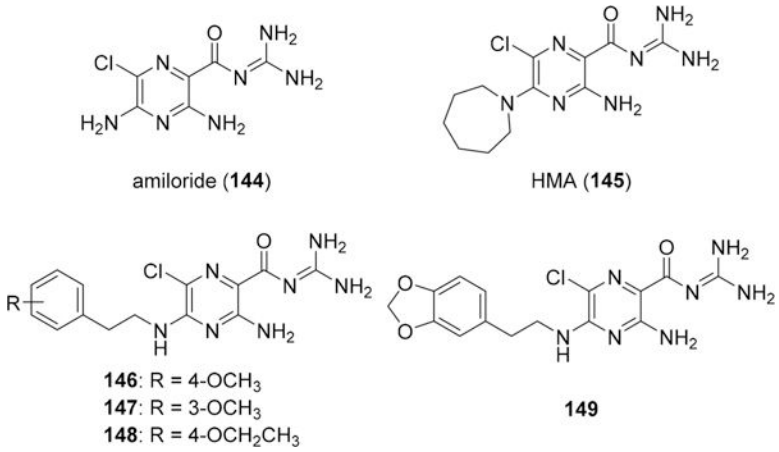


Figure 19.

Representative $A_{2A}R$ PAMs with centric pyrazine core (**144–149**).

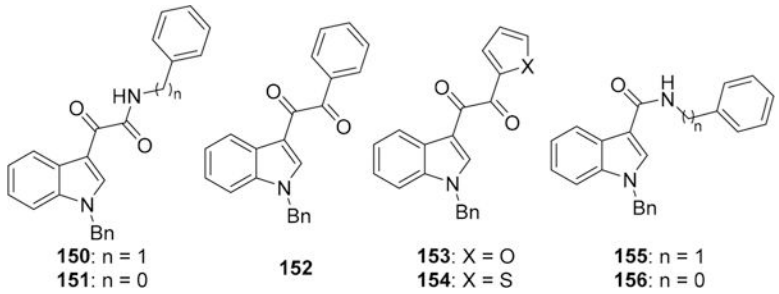


Figure 20.

$A_{2B}R$ PAM derivatives on the 1-benzyl-3-ketoindole scaffold side chain (**150–156**).

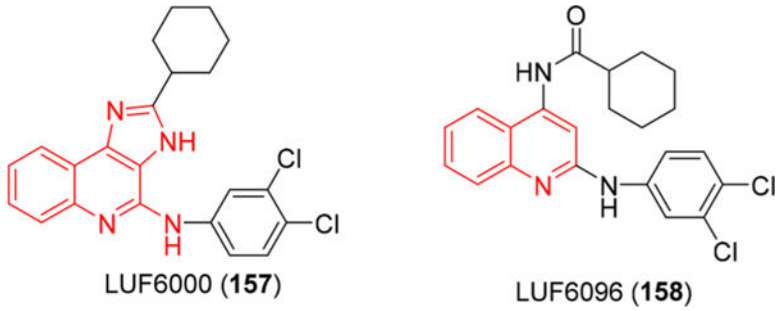


Figure 21.

Representative A_3R PAMs displaying a ring opening on the 1*H*-imidazo[4,5-*c*]quinolin-4-amine scaffold (**157, 158**).

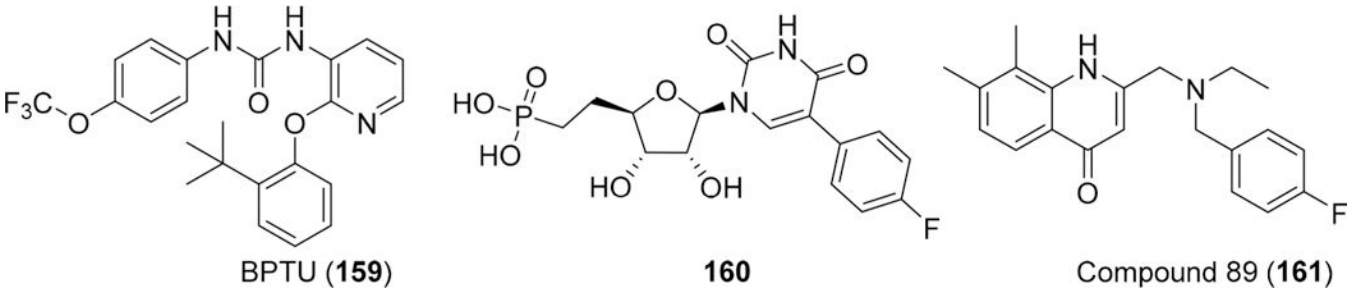


Figure 22.

$P2Y_1$ receptor allosteric antagonist BPTU (**159**) and $P2Y_2$ receptor allosteric agonists (**160, 161**).

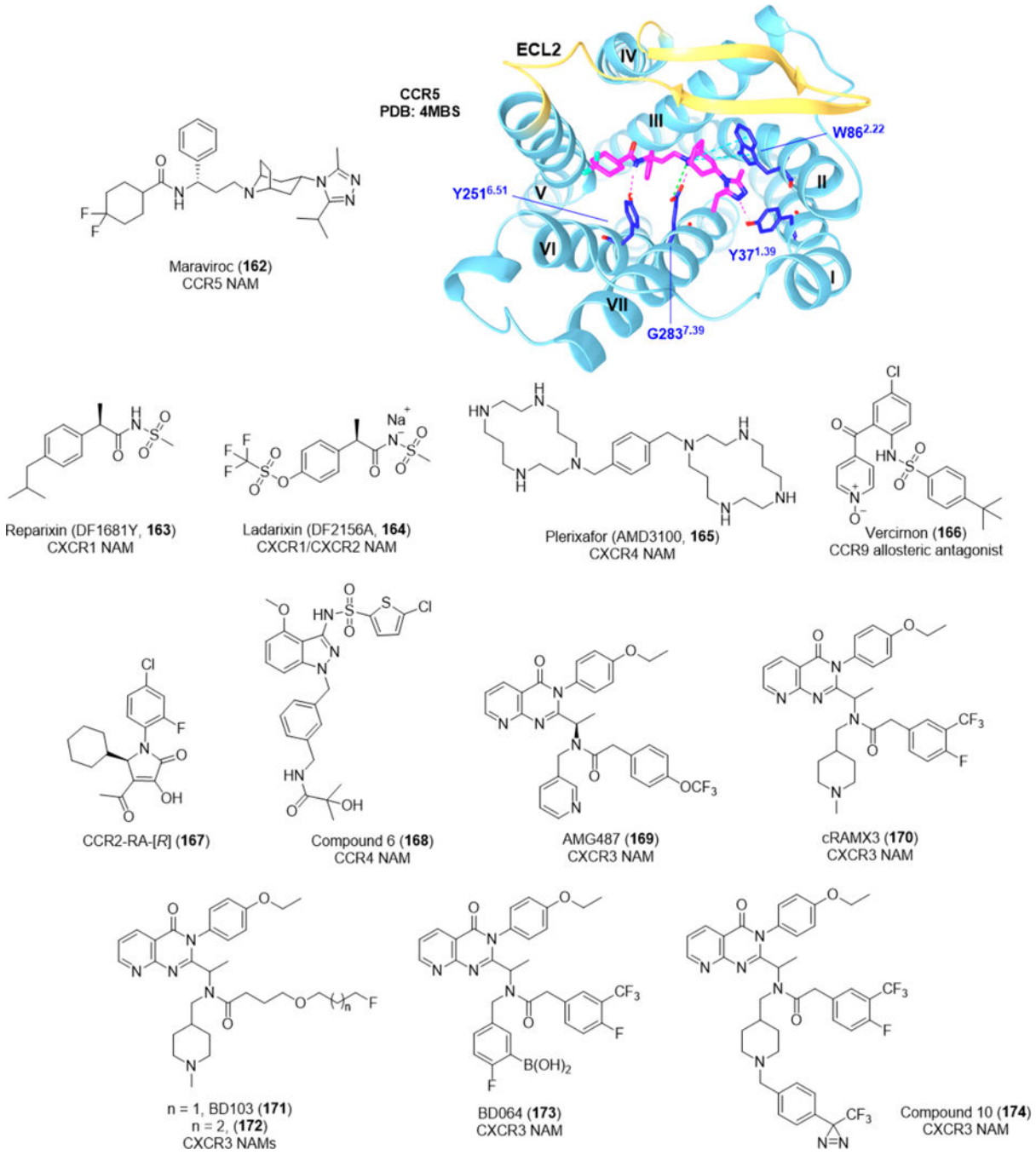


Figure 23.

Top: chemical structure of maraviroc (**162**), an FDA-approved CCR5 NAM with the CCR5-maraviroc cocrystal displaying numerous interactions with residues in the TM bundle (PDB code 4MBS).[27] Bottom: representative allosteric antagonists and NAMs discovered for chemokine receptors (**163–174**).

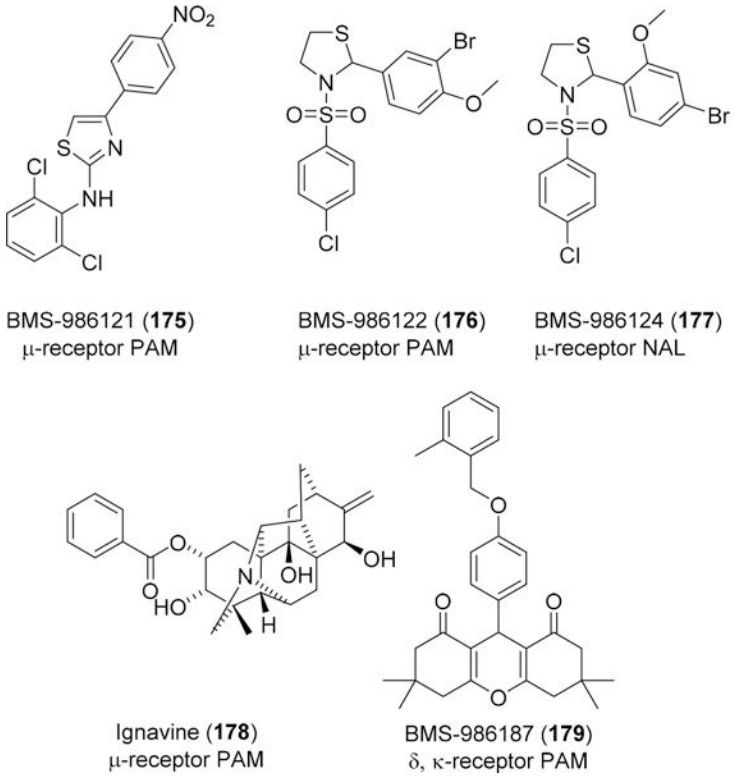


Figure 24.
Chemically diverse allosteric modulators for ORs (**175–179**).

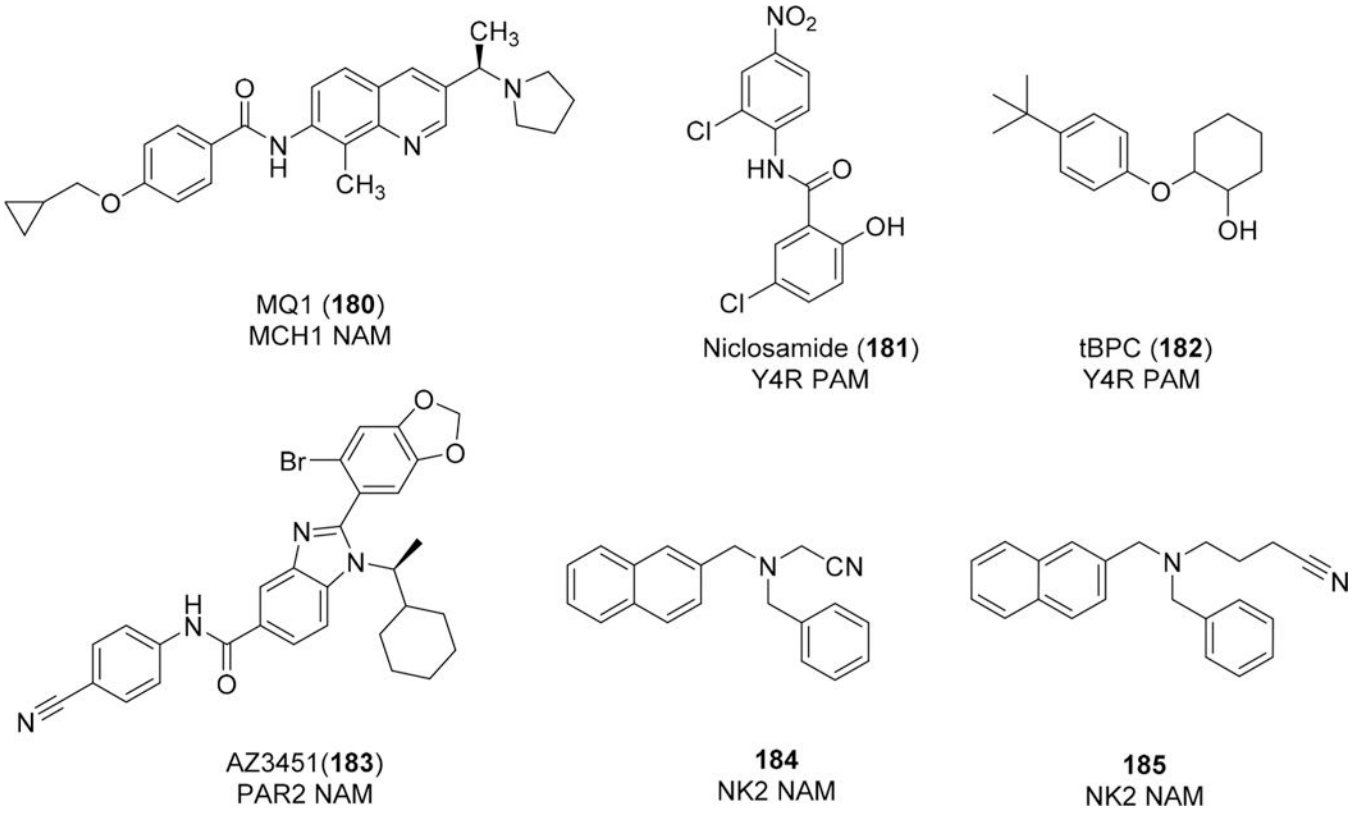


Figure 25.

NAMs and PAMs for additional peptide and protein family receptors, as described (**180–185**).

Table 1.

Selected Allosteric Modulators of Class A GPCRs Currently in Clinical Trials or Approved for Clinical Use[a]

family	target	name	indication	mechanism of action	phase
acetylcholine receptors	Ml	VU319	cognitive impairment	PAM	phase I
free fatty acid receptors	FFAR1	MK-8666	type 2 diabetes mellitus	partial allosteric agonists	phase I
chemokine receptors	CXCR1, CXCR2	ladarixin (DF2156A)	onset type 1 diabetes	NAM	phase II
free fatty acid receptors	FFAR1	TAK-875	type 2 diabetes mellitus	partial allosteric agonists	phase III
chemokine receptors	CCR9	vercirnon	Crohn's disease	NAM	phase III
chemokine receptors	CXCR1	reparixin (DF1681Y)	β-cell transplantation	NAM	phase III
P2Y receptors	$P2Y_{12}$	ticagrelor	anti-thrombosis	allosteric antagonist	approved
chemokine receptors	CCR5	maraviroc	HIV infection	NAM	approved
chemokine receptors	CXCR4	plerixafor	bone marrow transplantation	NAM	approved

[a]Data retrieved from (a) Cortellis database, https://www.cortellis.com/intelligence/login.do, (b) ref 3, (c) http://www.gpcrdb.org/drugs/ drugbrowser, and d) https://www.drugbank.ca.

Table 2.

Allosteric Modulator and Class A GPCR Cocrystal Structures[a]

GPCR	allosteric modulator	mechanism of action	allosteric site distribution	PDB code	ref
CCR5	maraviroc	NAM	extracellular side	4MBS	27
CCR2	CCR2-RA-[*R*]	NAM	intracellular side	5T1A	28
CCR9	vercirnon	allosteric antagonist	intracellular side	5LWE	29
M_2	LY2119620	PAM	extracellular side	4MQT	19
FFA1 (GPR40)	TAK-875	partial allosteric agonist	extracellular side	4PHU	30
FFA1 (GPR40)	MK-8666	partial allosteric agonist	extracellular side	5TZR	20
FFA1 (GPR40)	MK-8666, AP8	Ago-PAM	intracellular side	5TZY	20
$P2Y_1$	BPTU	allosteric antagonist	intracellular side	4XNY	31
β_2AR	Cmpd-15PA	NAM	intracellular side	5X7D	21
PAR2	AZ3451	allosteric antagonist	extracellular side	5NDZ	32
C5aR1 (CD88)	NDT9513727	allosteric antagonist	transmembrane	6C1Q	33
C5aR1 (CD88)	NDT9513727	allosteric antagonist	transmembrane	6C1R	34
$P2Y_{12}$	AZD1283	allosteric antagonist	extracellular side	4NTJ	35
$A_{2A}R$	Na^+	NAM	sodium ion site	5NLX	36
DOR	Na^+	NAM	sodium ion site	4N6H	37

[a]Data as of July 31, 2D18.