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Functionalized Congener Approach to the Design of Ligands for G Protein–Coupled Receptors (GPCRs)

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Abstract

Functionalized congeners, in which a chemically functionalized chain is incorporated at an insensitive site on a pharmacophore, have been designed from the agonist and antagonist ligands of various G protein–coupled receptors (GPCRs). These chain extensions enable a conjugation strategy for detecting and characterizing GPCR structure and function and pharmacological modulation. The focus in many studies of functionalized congeners has been on two families of GPCRs: those responding to extracellular purines and pyrimidines—i.e., adenosine receptors (ARs) and P2Y nucleotide receptors. Functionalized congeners of small-molecule as ligands for other GPCRs and non-G protein coupled receptors have also been designed. For example, among biogenic amine neurotransmitter receptors, muscarinic acetylcholine receptor antagonists and adrenergic receptor ligands have been studied with a functionalized congener approach. Adenosine A_1, A_{2A}, and A_3 receptor functionalized congeners have yielded macromolecular conjugates, irreversibly binding AR ligands for receptor inactivation and crosslinking, radioactive probes that use prosthetic groups, immobilized ligands for affinity chromatography, and dual-acting ligands that function as binary drugs. Poly(amidoamine) dendrimers have served as nanocarriers for covalently conjugated AR functionalized congeners. Rational methods of ligand design derived from molecular modeling and templates have been included in these studies. Thus, the design of novel ligands, both small molecules and macromolecular conjugates, for studying the chemical and biological properties of GPCRs have been developed with this approach, has provided researchers with a strategy that is more versatile than the classical medicinal chemical approaches.

Keywords

purines; adenosine receptor; P2Y nucleotide receptor; carrier-bound drugs; muscarinic receptor; xanthines

Introduction

G protein–coupled receptors (GPCRs) serve as the mechanistic basis for a large fraction (roughly half) of the pharmaceuticals currently in clinical use. These receptors may serve as the direct target of an agonist or antagonist drug, or the activity of a given receptor induced by its naturally occurring extracellular transmitter may be modulated indirectly (through an allosteric mechanism or by alteration of uptake or metabolism) [1]. GPCRs have an overall structural motif of seven transmembrane helical domains (TMs) that are embedded in and

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traverse the plasma membrane. Such heptahelical proteins constitute ~4% of the human genome.

The functionalized congener approach (Figure 1) is a widely applicable conceptual framework for the design of small, biologically active molecules that bind to biopolymers, such as receptor proteins on the cell surface. Beginning in the early 1980s, this approach was found to be particularly useful in the design of certain Family A GPCR ligands [2,3], and since that time it has been applied to a wide range of GPCRs. This strategy allows the affinity or selectivity of a nonpeptide drug for its receptor to be greatly influenced by functions introduced at the terminus of an attached chain at a permissive site on the drug molecule. Ideally, the steric constraints of the pharmacophore-binding site are relaxed at the distal region of this chain. Figure 1A details this approach schematically, as it was conceived in 1987 [4] and applied to the adenosine receptors (ARs), long before knowledge of the structure of these receptors was available.

A further benefit of this approach is the ability to couple a biologically active ligand to a carrier such as a peptide and maintain or enhance its pharmacological profile [5,6]. A distal moiety of an appended chemically functionalized chain may provide secondary favorable interactions with the receptor (Figure 1A) to enhance ligand affinity and selectivity. This makes possible the design of spectroscopic probes of high receptor affinity, dual-acting drugs, carrier-bound drugs, and so forth (Figure 1B) [4,7–11]. Thus, prosthetic groups to provide various chemical or physical properties may be covalently coupled to these small molecules through an appropriately designed functionalized chain.

Beginning in 1984, a research group that later became the Molecular Recognition Section (MRS) of the National Institute of Diabetes and Digestive and Kidney Diseases sought to expand this design approach to a relatively unexplored GPCR as a test system and selected the ARs for this purpose. At the time, the newly discovered ARs were appealing because there were few potent ligands with which to probe these receptors pharmacologically [12]. The widely used antiasthmatic drug theophylline (1,3-dimethylxanthine) had recently been identified as a prototypical adenosine antagonist [13,14], and institute scientists delineated properties that distinguished an AR that stimulates adenylate cyclase from one that inhibits [15]. Today, the ARs are considered one of the more advanced examples of GPCRs used in medicinal chemical development, with highly selective agonists and antagonists of nanomolar affinity at each of the four AR subtypes [16].

Background: probing GPCR structure and focus on receptors for extracellular purines and pyrimidines

GPCRs sense chemical signals from the exofacial side of a cell membrane and transmit the activation signal via both signaling cascades that are G protein dependent and those that are not, such as arrestin [17]. The ligand-binding function of Family A GPCRs occurs at the extracellular side and/or within the TM cleft of the GPCR, and signaling propagation depends on cytosolic regions of the receptors. Nonpeptide ligands tend to bind deeper in the TM domain than do peptide ligands, which typically associate largely with the extracellular domains of the receptor [18]. The binding of an agonist ligand induces a conformational change of the receptor that enables the second and third intracellular loops to contact the G protein(s) [19].

The receptor concept remained a theoretical construct for decades, until the cloning of the β2-adrenergic receptor by Lefkowitz and coworkers in 1986 [20]. Because drug-relevant GPCRs are membrane-bound proteins, directly determined structural information was unavailable until the recent X-ray structural determination of the adrenergic receptors [21].

The X-ray structures of other GPCRs, including the A_{2A} AR (Figure 1C), followed closely on the heels of this initial breakthrough [22]. Before this development, rhodopsin-based homology modeling was the only means of looking into the three-dimensional structure of the GPCRs, and this approach was used successfully in conjunction with site-directed mutagenesis and ligand docking [23–25]. Often, it has been possible to correlate the structure-activity relationship (SAR) studies of ligands with structural insights gained from molecular modeling hypotheses. Hypotheses about ligand binding and receptor activation gleaned from molecular modeling have been supported by studies involving reengineered GPCRs and chemical cross-linking of strategically placed Cys residues in the TM regions [19,26].

When our lab began medicinal chemical studies of GPCRs, we recognized the potential value for drug design of structural exploration of what were then elusive proteins. Knowledge of the three-dimensional structure of GPCRs (gained by indirect methods) could contribute to the design of novel agonists and antagonists. We began our testing with purine and pyrimidine receptors. With the synthesis of chemically related agonist and antagonist classes for a given GPCR, recognition elements that distinguish these pharmacological actions can be pinpointed, thereby providing insights into the activation mechanisms [27,28]. Subtle structural differences within the same class of GPCR ligands for a given GPCR can alter the spectrum of activation of different signaling pathways. Elucidating how these GPCRs function in ligand recognition and activation as “nanomachines” is important because of the therapeutic potential of modulation of this large family of nucleoside and nucleotide receptors. If general phenomena can be identified, the analysis can be extended to other members of the GPCR superfamily.

Many studies of functionalized congeners have focused on GPCRs that respond to extracellular purines and pyrimidines, i.e., ARs for purine nucleosides and P2Y receptors for nucleotides. Extracellular adenosine **1** (Figure 2) acts as a depressant neuromodulator, and the alkylxanthine stimulants such as caffeine **2** and theophylline **3** competitively antagonize the actions of adenosine [16]. Purine and pyrimidine nucleotides (such as ATP **4**, ADP **5**, UTP **6**, UDP **7**, and UDP-glucose **8**) act as neurotransmitter/neuromodulators at their own GPCRs [29]. These ubiquitous signaling molecules modulate the function of diverse mammalian cell types and tissues under both normal and pathophysiological conditions. The receptors for extracellular nucleosides and nucleotides have been characterized through medicinal chemical, molecular biological, and pharmacological approaches. The nucleotide receptors (denoted P2) include both ligand-gated ion channels, termed P2X, and GPCRs, termed P2Y [30]. The P2Y receptors are distinct from the ARs, for which four subtypes (A_1, A_{2A}, A_{2B}, and A_3, all GPCRs) have been defined [16].

In collaboration with Ad IJzerman, we initiated molecular modeling of the A_1 and A_{2A} ARs soon after their cloning in 1989 [31] and developed the first computer models of the putative binding sites of adenosine agonists and antagonists with the TM domain [32]. The first model of a P2Y receptor (chick $P2Y_1$) was published in 1994 and was one of the first models based on rhodopsin [33]. The initial purine receptor models, before 2000, were based on the structures of bacteriorhodopsin and earlier poorly resolved or incomplete structures of rhodopsin; yet even these early models suggested some features of ARs and the $P2Y_1$ receptor that were substantiated by more sophisticated modeling derived from the high-resolution rhodopsin structure. This computational probing of the architecture of both purine and pyrimidine receptors has been supported by our site-directed mutagenesis and SAR studies [34,35].

These structural insights have successfully guided the design of new ligands for these GPCRs. In the course of the modeling studies, our laboratory discovered the importance of

"meta" binding sites on the extracellular loops, particularly the EL2, in directing small-molecule ligands, such as ATP, to their principal binding sites in the transmembrane cleft [36]. Later, when a high-resolution structure of rhodopsin was determined by Palczewski et al. [37], the conformation of the TM domains of a purine receptor almost exactly overlayed the X-ray structure.

Thus, these important GPCRs have been studied from the convergent perspectives of SARs of ligands and of structural probing of the receptor protein targets through mutagenesis and computational modeling. The protein structure of the first AR determined by X-ray crystallography also was closely estimated by the three-dimensional model predicted through homology modeling, including side chain orientations in the ligand-binding region [38].

Biogenic amine receptor ligands and origins of the functionalized congener approach

In the late 1970s, Murray Goodman and coworkers at the University of California, San Diego derivatized β-adrenergic receptor agonists and other biogenic amines for attachment to peptides as drug carriers [3,39]. Isoproterenol **10** (Figure 2) was the first analogue of a biogenic amine (norepinephrine **9**) successfully derivatized this way. A functionalized chain terminating in a carboxylic acid **11** was created as an extension of the *N*-isopropyl group of **10**. Unfortunately, the early experiments lacked a stereochemical control at the secondary carbon of the alkyl chain, which can have pharmacological consequences [40]. These studies, a collaboration with Kenneth Melman at Stanford University, aimed to design drugs that could be attached to polypeptides for *in vivo* targeting [40,41]. The project grew out of a collaboration between Goodman and Nathan Kaplan at the University of California, San Diego and his graduate student Craig Venter on immobilized drugs, which were later shown to be chemically heterogeneous [41].

While scrutinizing and modeling the chemistry of linkage of the catecholamines to peptides by means of small-molecule derivatives with elongated chains, Goodman and colleagues found β2-adrenergic agonists that were superpotent. A *p*-trifluoromethylanilide derivative **12** (which was later resolved into four pure stereoisomers [40]) and a dipeptide conjugate **13** were significantly more potent than **10** at the β2-adrenergic receptor. This potency enhancement was accomplished by manipulating the molecules at points distal from the pharmacophore (the business end). The pharmacological profile of the substances could be tuned without altering the pharmacophore moiety. The strategy of catecholamine derivatization depended on treating the hypothetical construct of β-adrenergic receptors as distinct molecular entities on the cell surface, which indeed they were shown to be, nearly a decade later [20].

Adenosine and P2Y receptor functionalized congeners

Extracellular adenosine **1** is involved in many of the body's cytoprotective functions through its actions at the four subtypes of ARs. For example, adenosine serves to precondition heart muscle against ischemia and toxicity by activation of the A_1 and A_3 ARs on the surface of cardiac myocytes [42]. We showed that activation of the low-affinity A_{2B} AR by high concentrations of adenosine in the heart protects the muscle in the postreperfusion period [43]. Adenosine counteracts the damaging effects of excitotoxicity and seizure activity in the central nervous system by activation of neuonal A_1 ARs, and it suppresses excessive immune and inflammatory responses by activation of the A_{2A} AR on immune cells. Selective agonist and antagonist ligands for all four of the AR subtypes are used as essential pharmacological probes. Selective ligands for the A_3 AR have therapeutic potential as anti-

inflammatory [44], cardioprotective [45,46], cerebroprotective [47], anticancer [48], and antiglaucoma [49] agents.

Functionalized congeners of ligands of the A_1 AR were first introduced in our laboratory in collaboration with John W. Daly (all of the National Institute of Diabetes & Digestive & Kidney Diseases), who was already exploring SAR at this receptor of simple derivatives of the agonist adenosine **1** and antagonist theophylline **3** [2,12]. In the agonist series, we hypothesized that the N^6-substituent had the greatest freedom of substitution; aryl and arylalkyl were the favored substitutions [50]. Therefore, we incorporated functionalized chemical chains on an N^6-phenyl substituent in **14** and extended them in a stepwise fashion, optimizing affinity in binding to the rat brain A_1 AR at each stage (Figure 3).

It was evident that the substitution of the phenyl ring with electron-donating groups was conducive to high affinity. However, it was not known whether the binding-site geometry within the receptor would accommodate a long extension of the chain. To answer this question, we expanded a *p*-methyl group in **15** into a carboxymethyl group to form **16** for attachment to larger moieties. Not only did amide derivatives at this position still bind to the A_1 AR, but aromatic amides (e.g., **18**) were clearly favored over aliphatic amides (e.g., **17**), suggesting that a distal region of the receptor-binding site was not sterically constrained and was hydrophobic and/or aromatic. The motif of *p*-substitution was continued with the attachment of an additional *p*-aminophenyl acetyl moiety, e.g., **19**.

The preferred terminal group at this stage of the chain extension was found to be a 2-aminoethylamido group in **20**, which was considerably more potent than a neutral amide in A_1 AR binding. This suggested that a distal negatively charged region exists on the receptor or in its vicinity. Thus, this prototypical adenosine amine congener **20** (ADAC) was used for additional derivatization, which indicated an essentially unlimited ability to elongate the carrier moiety [2]. This suggested that the primary amino group of ADAC **20** protrudes out of the binding region for the adenosine pharmacophore, toward or into the extracellular medium, as shown in Figure 1A. Later, it was found that the charged residues in the extracellular regions of the ARs are predominantly anionic, which explained the observed affinity enhancement in positively charged functionalized congeners [31,32]. Acylation of **20** to form the neutral compound **21** somewhat reduced the potency of binding to the A_1 AR. Because an amino group in the chain enhances affinity at the ARs, we introduced a new functionalized congener **25**, which is related to ADAC but containing an extra amino group in the chain and also has favorable affinity at the human A_1 AR (Table 1). This secondary amine would remain free following coupling of the terminal primary amine to a carrier [51]. Certain conjugates of ADAC with long chain fatty acids and other lipid derivatives displayed unusuallly high binding affinity at the A_1 AR, suggestive of distal anchoring of the hydrophobic chain in a lipid-rich environment surrounding the receptor [140].

Although the potency the rat A_1 AR was enhanced in this agonist series by the stepwise chain derivatization, the selectivity in comparison to the rat A_{2A} AR was not increased. The selectivities of N^6-phenyladenosine **14** and ADAC **20** were 200 and 250-fold, respectively.

A similar derivatization scheme was applied to xanthines, e.g., **26** and **27**, as AR antagonists (Table 1) [52]. Curiously, as with adenosine, an attachment of a phenyl ring at the C8 position was highly favored in A_1 AR binding over aliphatic groups at that site. Chain extension with simple functionalized 8-alkyl chains greatly reduced AR-binding affinity. Thus, the 8-aryl ring is a specific recognition element for receptor binding and was therefore included in the functionalized congener series. The 1,3-dipropyl substitution of the methyl groups present in theophylline **3** was also included because it is known to enhance the A_1 AR affinity by 30- to 40-fold. Also, as in the N^6 position in the agonist series, the

substitution of the 8-phenyl ring in the xanthine series with an electron-donating group was conducive to high affinity [6,53]. Thus, a methoxy substituent was elaborated into a carboxymethyloxy group for attachment to larger moieties through an amide linkage. This xanthine carboxylic congener **26** (XCC) and the elongated 2-aminoethylamide (xanthine amine congener, XAC **27**) overcame the biologically limiting low water solubility of the 8-arylxanthines noted previously [12].

As in the agonist series (Figure 3), the A_1 AR-preferred terminal group is an amine as in **27**, again suggesting that a distal negatively charged region exists on or near the receptor. As a systematic means of probing the effects of varied functionality at the distal end of the xanthine chain, amino acid conjugates of XAC such as **34** were prepared and analyzed in binding and functional assays [6]. In general, a free amino group on a wide variety of amino acid conjugates, such as the diamine **34**, favored higher binding affinity at the A_1 AR, suggesting an electrostatic cause. The D-Lys conjugate **34** was intended for enhanced *in vivo* stability toward in the presence of peptidases through the pendant unnatural D-amino acid.

After the successes with the A_1 AR, we explored functionalized congeners of ligands of the A_{2A} AR, in collaboration with Gary L. Stiles (Figure 4) [54]. In 1989, Michael Jarvis, Michael Williams, and coworkers reported the first potent A_{2A} AR–selective agonists [55]. This group, which first reported CGS21680 **37** as an A_{2A} AR agonist, partnered with our lab to see if the functionalized congener approach could be extended to the A_{2A} AR. This resulted in a series of long-chain functionalized congeners of adenosine (more precisely, of its 5′-ethyluronamide derivative, NECA **36**) derivatized at the C2 position instead of the N^6 position. As with the A_1 AR, the chain was optimized in receptor binding at each stage of elongation. Consistent with the previous pattern, an amino terminal group was found to be beneficial for affinity at this receptor subtype.

The potent A_{2A} AR agonist CGS21680 **37** was condensed with an ethylenediamine unit to form APEC **39** (2-[[2-[4-[2-(2-aminoethyl)aminocarbonyl]ethyl]-phenyl]ethylamino]-5′-N-ethyl-carboxamidoadenosine), which became a prototypical amine functionalized congener with selectivity and higher affinity for the rat A_{2A} AR than for the rat A_1 AR. In Figure 1C, APEC is shown in its docked complex with the human A_{2A} AR, and its functionalized chain reaches the extracellular regions of the receptor. The A_{2A} AR, which induces vasodilitation to lower blood pressure, has been the subject of numerous biological investigations over several decades. It was not until a photoaffinity label selective for the A_{2A} AR derived from APEC was introduced that the molecular form of this receptor was proven to be distinct from the A_1 subtype [56]. Compound **40**, which contains a *p*-aminophenylacetyl (PAPA) prosthetic group, could be radioiodinated on the aryl amine and then crosslinked to the receptor, either by conversion to the 4-azide or by chemical cross-linking with *N*-succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate (SANPAH). APEC itself is also useful for the study of behavioral effects, because it penetrates into brain better than its precursor carboxylic acid derivative [57].

8-Styrylxanthine derivatives that act as selective antagonists for the A_{2A} AR were also designed as functionalized congeners, e.g. the carboxylic acid derivative **44** [58]. These 8-styrylxanthine derivatives include a 7-methyl group and could therefore be considered analogues of caffeine. Chain extension at the 7-alkyl position was not tolerated in receptor binding. Careful probing of the SAR identified the aryl ring of the styryl group as the most insenstitive site for functionalization in this series.

Selective ligands of the A_{2B} AR derived from functionalized congeners were recently reported. The same group that was used for xanthine antagonists of the A_{2B} AR (C8

substituted chain containing a 4-carbonylmethyloxy phenyl group) was introduced at the N^6 position in the agonist series [59,60] resulting in A_{2B} AR selectivity.

Functionalized congeners of selective A_3 AR agonists were first studied in the ribose series, in which a functionalized chain extended from the 3 position of an N^6–benzyl group, e.g., **45**, where tolerance for steric bulk was discovered and quantified in a CoMFA (Comparative Molecular Field Analysis) model [61]. Simple amino acid moieties could be coupled through a 3-aminobenzyl group with retention of moderate affinity. However, even at this position, which was somewhat removed from the nucleoside pharmacophore, we observed constraints on the amount of steric bulk tolerated in A_3 receptor binding. Only a β-alanyl conjugate **45** was relatively unimpaired in binding. Later, we modified the linking chemistry for similar A_3 AR agonists to include a 3-alkynylbenzyl group of linear geometry, e.g., **46**, which better preserved nanomolar affinity [46].

Functionalized congeners were also reported for a series of selective A_3 AR agonists consisting of 5′-*N*-methylcarboxamidoadenosine analogues containing the (N)-methanocarba (bicyclo[3.1.0]hexane) ring system as a ribose substitute, e.g., **47** amd **48** [62]. The rigid bicyclic ring system in this class of agonists provided a steric constraint of the riboselike moiety to enhance A3 AR selectivity. Functionalized ethynyl chains terminating in amino or carboxylate groups were incorporated at both the N^6 and C2 positions and effects on affinity compared. Substitution at the C2 position of the purine moiety in the (N)-methanocarba series, e.g., **48**, preserved A_3 AR selectivity more effectively than similar chains attached at the 3 position of the N^6-benzyl group, e.g., **47**.

We also applied the functionalized congener approach to antagonists of the A_3 AR [63]. 1,4-Dihydropyridine (DHP) derivatives containing 4-phenylethynyl and 6-phenyl groups are high-affinity antagonists at this receptor. We probed various sites of chain substitution on these DHPs to identify the least-sensitive position in receptor binding. Such functionalization of the 4-arylalkynyl moiety was not tolerated in A_3 AR binding. A para-substituted *O*-benzyl ester group at either the 3 or 5 position of the DHP was suitable for the attachment of a functionalized chain, which could then be linked to prosthetic groups. An amine derivative of a 3-benzyl ester **49** was conjugated to biotin to provide **50**.

Macromolecular conjugates of AR ligands

At the time ADAC **20** and XAC **27** were first reported, little was known of the physical or chemical nature of the A_1 AR or of any GPCR. The structural similarity to rhodopsin was not yet evident. Thus, the functionalized congener approach served as a means of “blindly” exploring the solvent accessibility and environment of the hypothesized binding site for the pharmacophore. An early study by Ray Olsson and coworkers, in which adenosine agonists were coupled to polymeric carbohydrates (oxidized stachyose) with the retention of vasodilatory activity, led to the conclusion that the AR was located on the cell surface [64]. More chemically well-defined, biologically active macromolecular conjugates were needed, and the functionalized congener approach proved useful for this purpose. The types of conjugates used in this effort are now more sophisticated. For example, dendrimers have recently been used as macromolecular carriers of AR functionalized congeners.

The ability to substitute ADAC **20** with a macromolecular carrier was first demonstrated through the biotin-avidin system [65–67]. Acylation of **20** with biotin or with various chain-elongated derivatives of biotin provided conjugates **54** and **55**, which retained a relatively high affinity at the A_1 AR (Figure 5). Precomplexation of these ADAC-biotin conjugates with the tetrameric protein avidin of molecular weight ~68,000 D only slightly reduced the receptor affinity. This clearly indicated that the steric constraints of the pharmacophore-binding site were entirely overcome. Thus, this functionalized chain must protrude toward

the exofacial side of the receptor. The sequential elongation of the chain connecting biotin to the adenosine moiety allowed a crude estimation of the depth of the binding site, i.e., a "dipstick" approach to exploring the binding site. This calculation was based on the extended conformation of the shortest chain to be unimpaired in its ability to bridge the pharmacophore and the macromolecular carrier, i.e., avidin. By this method, the depth of the binding site of adenosine was estimated to be ≤12 Å. Later, molecular modeling of the receptor confirmed that this was a reasonable approximation. We used a similar method to conjugate the A_{2A} AR agonist APEC to biotin to provide **56**. Xanthine biotin conjugates **57–60**, when complexed to avidin, required a longer chain length to achieve moderate A_1 AR-binding affinity.

Dual-acting ligands: binary drugs

We designed ligands to interact equipotently with multiple subtypes of GPCRs, whether by tethering two components or by either fusing or merging favorable structures to create a dual selectivity in a single functional unit [68]. Successes in the design of multiple ligands were recently reviewed by Morphy and Rankovic [9]. One of the first examples of tethering two pharmacophoric units was reported by Portoghese and coworkers; this was achieved with the opioid system, in which ligands for either the same or different GPCRs were covalently coupled through flexible chains [8]. The objective of tethering two GPCR ligands is to achieve a synergistic, net biological effect of activation or antagonism of multiple pathways [46] and potentially to bridge GPCR dimers (Figure 2C) [8,69,70]. An approach to designing dual-acting ligands (termed binary drugs) derived from functionalized congeners was reported in 1987 (Figure 6) [71].

A functionalized congener antagonist of the A_1 AR, the 1,3-dialkylxanthine derivative XAC **27** (Table 1), was attached through an L-Lys linker to a segment derived from the neurotransmitter peptide substance P (SP) to form binary drug **61** (Figure 6A). The Lys linker preserved a free amino group in the spacer chain, which increased A_1 AR affinity and solubility. This binary conjugate bound to the rat A_1 receptor with a K_i value of 35 nM and to the NK1 (neurokinin type 1) receptor with a K_i value of 300 nM [71]. Coupling of the functionalized adenosine agonist N^6 -[4-(carboxymethyl)phenyl]adenosine **16** (Figure 2A) to an SP C-terminal peptide also resulted in a binary drug **62** that bound to both receptors. The demonstration that the biochemical properties of two unrelated drugs, both of which act through binding at extracellular receptors, may be combined in the same molecule suggested a novel strategy for drug design. In principle, two different substances that produce the same final effect (e.g., hypotension by adenosine agonists and by SP analogues) should produce a combined effect. Adenosine analogues have analgesic properties, and the binary drugs derived from SP and adenosine agonists or antagonists were intended to serve as ligand tools for probing interrelationships of NK pathways and sites for the antinociceptive action of adenosine.

Considering the antinociceptive properties of AR ligands, we also sought to combine such ligands with opioid receptor agonists. Thus, nonpeptide ligands of the opioid receptors were conjugated to AR ligands, resulting in conjugates that retain affinity for both the A_1 AR and the μ-opioid receptor (Figure 6B) [72]. We probed three structural classes of opioids, derivatives of tetrahydrooripavine, etonitazine, and fentanyl, for sites of functionalization for chain attachment. An amino group on the 7-a position of 6,14-endoethenotetrahydrooripavine was suitable for derivatization with long-chain substituents, such as Cbz-glycyl-glycine, without loss of affinity for the μ-opioid receptor. A binary conjugate of this tetrahydrooripavine with an alkylxanthine moiety **63** had morphinelike activity in the paraphenylquinone *in vivo* assay. Thiourea-linked conjugates of a benzimidazole derivative that was a potent opioid ligand and an AR functionalized

congener, either ADAC **20** or XAC **27**, bound effectively to both receptors. For example, the ADAC conjugate **64** bound to the rat A_1 receptor with a K_i value of 31 nM and to the rat μ-opioid receptor with a K_i value of 150 nM.

Binary drugs that activate two subtypes of ARs have been proposed for cardioprotection (Figure 6C) [73]. Adenosine released during cardiac ischemia exerts a potent, protective effect in the heart via activation of A_1 and A_3 receptors [42,45,74], which activate separate protective signaling cascades. To explore the interaction between these two cardioprotective ARs and the question of which receptor is the more important anti-ischemic receptor, we designed specialized binary ligands by tethering functionalized congeners. One objective was to use this ligand tool to test the hypothesis that activation of both receptors exerts a cardioprotective effect significantly greater than activation of either receptor individually. We used a novel design in which new binary conjugates of adenosine functionalized congeners that were pharmacologically complementary were synthesized and tested in a novel cardiac myocyte model of adenosine-elicited cardioprotection.

Binary drugs with mixed selectivity for both A_1 and A_3 ARs were created through the covalent linking of functionalized congeners of adenosine agonists, each of which is selective for either the A_1 or the A_3 AR subtype. MRS 1740 **65** and MRS 1741 **66**, thiourea-linked regioisomers of a binary conjugate, were highly potent and selective in radioligand-binding assays for the A_1 and A_3 ARs (K_i values of 0.7–3.5 nM) compared with the A_{2A} AR. The myocyte models used cultured myocardial muscle cells from chick embryo, either ventricular cells expressing native adenosine A_1 and A_3 ARs or engineered atrial cells, expressing either human A_3 receptors alone or both human A_1 and A_3 receptors. The binary agonist **66** activated both A_1 and A_3 receptors simultaneously, with full cardioprotection (EC_{50} ~0.1 nM) dependent on the expression of both receptors. Thus, coactivation of both adenosine A_1 and A_3 receptors by the binary A_1/A_3 agonists represents a novel general cardioprotective approach for the treatment of myocardial ischemia.

Scammells et al. recently reported bivalent conjugates of ligands of β2-adrenergic and adenosine A_1 receptors that activated both receptors and exploited crosstalk between these receptors [75]. The N^6 position of adenosine was used as the site for chain derivatization. Hexyl and butyllinked variations in the connecting chain were compared. The hexyl-linked conjugate displayed K_i values of 436 and 311 nM at A_1 and β2 receptors, respectively, and was even more potent in activation of the β2-adrenergic receptor with an EC_{50} of 6 nM.

Irreversibly binding AR ligands for receptor inactivation and crosslinking

In collaboration with Gary Stiles and Mark Olah, then at Duke University, we coupled AR functionalized congeners in both the A_1 agonist and A_1 antagonist series to a variety of bifunctional crosslinking reagents present in excess to achieve a 1:1 stoichiometry in the product, thus leaving one chemically reactive group [76,83]. These crosslinking reagents were typically symmetrical amine-reactive electrophiles. For example, 1,3- and 1,4-phenylene diisothiocyanates were conjugated to amine congeners that were potent and selective A_1 AR ligands. The resulting reactive conjugates, i.e., the A_1 AR agonist *p*-DITC-ADAC **22** (Figure 2) and the A_1 AR antagonist *p*-DITC-XAC **28** (Table 1), effectively and irreversibly bound to the A_1 AR at very low concentrations (10 nM to 1 μM) [77].

We coupled radiolabeled bifunctional reagents designed for photoaffinity labeling to the same amine congeners. One example of such a cross-linking group is the PAPA moiety, as in **30** and **40**, which was readily iodinated and then converted to the photoactivatable *p*-azide [78]. Thus, XAC (available in its [^{3}H] form) served as the first high-affinity radiolabeled adenosine antagonist suitable for chemical or photochemical affinity crosslinking to the A_1 AR protein. This photoaffinity probe labeled the same receptor protein to which A_1 selective

agonists bind, as indicated by molecular weight determined by gel electrophoresis. A comparison of proteolytic digests of agonist- and antagonist-occupied receptor proteins identified fragments formed at cleavage sites, which differ in accessibility. In addition to the electrophilic isothiocyanate group, the sulfonyl fluoride group was also effective for ligand crosslinking to the ARs because of its reactivity with nucleophiles on proteins with a sufficient aqueous stability [79,80].

We also used chemically reactive bifunctional crosslinkers to prepare affinity labels from amine functionalized congeners for A_{2A} AR agonists. We found that *p*-DITC-APEC (2-[[2-[4-[2-[2-[(4-isothiocyanatophenyl)aminothiocarbonylamino]ethyl]aminocarbonyl]-ethyl]phenyl]ethylamino]-5'-N-ethyl-carboxamidoadenosine) **41** effectively and irreversibly bound to the A_{2A} AR at very low concentrations (~10 nM) [81]. Curiously, when this derivative was infused into the coronary artery of guinea pigs, there was a prolonged A_{2A} AR activation that could not be reversed by washing, leading to sustained vasodilitation.

We introduced trifunctional probes by simultaneously crosslinking a GPCR functionalized congener with a reporter group and a chemically reactive group, through a central symmetrically trisubstituted aryl unit (Figure 7) [82,83]. To further extend the use of isothiocyanates to covalently crosslink high-affinity ligands to the ARs, we prepared a series of analogues of *m*-DITC-XAC containing a third substituent in the phenyl isothiocyanate ring. Thus, the central aryl linker could consist of 1,3,5-triisothiocyanatobenzene (**67**, R = 5-NCS), although we also prepared some variants of conjugates containing 1,3,5-trichlorotriazine as a central aryl linker. This third substituent (R of **67**), when appropriately derivatized, modifed the physiochemical or spectroscopic properties of the conjugate. Thus, an amine congener reacted with a symmetrical trifunctional crosslinking intermediate bearing two isothiocyanate groups, which already contained a third substituent. We were thus able to synthesize derivatives of XAC containing hydrophilic, fluorescent, or reactive substituents, linked via an amide, thiourea, or methylene group in the 5 position of the central aryl ring, and found them to be irreversible inhibitors of the A_1 AR.

We examined the effects of modifying the 5-substituent on water solubility and on affinity. A conjugate of XAC and 1,3,5-triisothiocyanato-benzene, i.e., a trifunctional ligand intermediate (**68**, R = NCS), was 894-fold selective in binding to the rat A_1 AR. Reporter groups, such as fluorescent dyes and a spin label, were included as substituents in the third position of irreversibly binding analogues, which were designed for spectroscopic assays, histochemical characterization, and biochemical characterization of the receptor protein. We also designed and synthesized trifunctional ligands for the A_1 AR that contain a cleavable disulfide linkage in the chain, for chemically induced regeneration of the receptor-binding site for the purine by reduction of the disulfide [82].

Radioactive probes

Because functionalized congeners are used as a modular approach to ligand design, it is feasible to couple these derivatized pharmacophores to prosthetic groups for radiolabeling. For example, we used the active ester intermediate for the Bolton-Hunter reagent, *N*-succinimidyl-3-(4-hydroxy-phenyl)propionate, to incorporate a group for radioiodination both in simple functionalized congeners and in our trifunctional A_1 AR antagonists (Figure 7B) [83]. The resulting conjugate covalently labeled the receptor protein, which was readily detectable on a Western blot.

We also introduced other novel prosthetic groups for radiolabeling. We selected a prosthetic group for radiofluorination by the ease of replacement of benzyl bromides with nucleophilic ^{18}F-fluoride and applied it to derivatization of insulin for imaging of insulin receptors [84]. Other groups are now used for incorporating ^{18}F into peptides because of the

in vivo lability of benzyl fluorides [85]. Chelating groups capable of complexing radioactive metal ions were coupled to amine-functionalized congeners, e.g., **33**, with the retention of moderate affinity at the A_1 AR (Table 1) [4].

Immobilized AR ligands for affinity chromatography to isolate the receptor protein

Amine functionalized congeners of AR agonists and antagonists have been used as immobilized high-affinity ligands for both A_1 and A_{2A} receptors, for the purpose of affinity chromatography leading to the isolation of the receptors and purification to homogeneity [86–88]. For example, XAC was immobilized on a Sephadex column for isolation of the bovine A_1 receptor and for the isolation of the recombinant human A_{2A} receptor expressed in *E. coli*. The use of the agonist N^6-(4-aminobenzyl)adenosine for affinity chromatography allowed the isolation of the A_1 AR along with its associated G protein [89].

Functionalized AR ligands as spectroscopic probes

Fluorescent probes of high affinity for GPCRs are being explored as an alternative to the use of radioligands in binding assays and histochemical studies [90]. Fluorescent conjugates of both small-molecule ligands [91,92] and peptide ligands [93] of GPCRs have been used. A fluorescent-labeled naloxone has been used in confocal scanning laser microscopy to demonstrate specific binding to the μ-opioid receptor [92]. Moreover, more detailed information about the binding site and its microenvironment can be obtained from fluorescent ligands than from radioisotopes. For example, flow cytometry analysis and confocal laser-scanning microscopy of living cells showed that a fluorescent BODIPY (6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid) derivative of prazosin labels not only α1-adrenergic receptors on the cell surface, but also those localized inside the cell [94].

A conjugate of fluorescein isothiocyanate (FITC) with the amine functionalized congener APEC, which was a A_{2A} AR-selective agonist, has proven useful as an alternative to radioligands in receptor assays [95]. FITC-APEC **42** was of suitably high affinity for use in the quantitative characterization of ligand binding to the bovine A_{2A} AR. Fluorescence measurements provided a B_{max} value of 2.3 ± 0.3 pmol/mg protein and K_D value of 57 ± 2 nM. This observed specific binding was saturable and rapid in its rates of association and dissociation, and the parameters were comparable to those obtained in competition studies that used a tritiated agonist radioligand at the same receptor. The potencies of chemically diverse A_{2A} AR ligands (and control compounds of low AR affinity), estimated by inhibition of FITC-APEC binding, were also in good agreement with their potencies determined by radioligand-binding techniques. Schulte and coworkers characterized another fluorescent conjugate **43** of APEC, in which the fluorescent moiety consisted of Alexafluor488; they were able to follow internalization into cells (Table 1) [96]. Various other fluorescent derivatives have been prepared for the ARs through derivatization at sites on known ligands that are insensitive in receptor binding and are used for fluorescence correlation spectroscopy and other detection techniques [4,7,51,97–99]. Hill and colleagues have prepared and characterized such fluorescent probes of the ARs, including a fluorescent derivative of XAC **35** [7].

We and others have reported spin label probes and highly fluorinated analogues derived from ADAC and XAC and other purine AR ligands obtained by a similar attachment method [4,100]. For example, XAC was conjugated to the TEMPO moiety to yield **31**, which contains a nitroxyl radical, with the retention of high A_1 AR affinity [4]. Compound **32** contained a perfluorinated acyl prosthetic group. The intended use of these analogues

was receptor characterization by electron spin resonance (ESR) spectroscopy and fluorine-NMR, respectively [101,102].

Functionalized congeners of P2Y nucleotide receptor ligands

Although ARs constitute a mature field of medicinal chemistry, the P2Y nucleotide receptors are in the early stages of ligand development [29]. The currently defined eight subtypes of P2Y receptors fall into two structural and functional clusters of sequences (in general, low homology among subtypes), ligand preference, second messengers, and receptor sequence analysis [103]. The distribution of P2Y receptors is broad, and the therapeutic interests include antithrombotic therapy, modulation of the immune system, cardiac ischemia, neurodegeneration, diabetes, and treatment of cystic fibrosis and other pulmonary diseases [29,104–107].

$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptor subtypes belong to a structurally distinct cluster of P2Y receptor sequences and couple through $G\alpha_q$ to activate phospholipase C. The remaining three subtypes, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, couple via $G\alpha_i$ to inhibit adenylate cyclase. $P2Y_{12}$ receptors stimulate an important proaggregatory signal in platelets, $P2Y_{13}$ receptors occur in immunocytes and neuronal cells, and the $P2Y_{14}$ receptor that is found in dendritic cells is activated by UDP-glucose **8**. $P2Y_1$, $P2Y_2$, and $P2Y_{11}$ - $P2Y_{13}$ receptors are activated by adenine nucleotides, and $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{14}$ receptors are activated by uracil nucleotides. Pharmacological antagonism of the $P2Y_{12}$ receptor is widely used for antiplatelet therapy. We showed that a $P2Y_1$ antagonist can serve as an antithrombotic agent [104]. Other potential therapeutic approaches, involving other P2Y subtypes, have been aided by the availability of recently introduced pharmacological probes.

A variety of factors have tended to confound the pharmacological characterization of both native and recombinant P2Y receptors. These include the dependence of P2Y receptor classification on the use of unstable nucleotides as agonists and a lack of potent, selective, and bioavailable antagonists. In addition to a lack of selectivity for the different P2Y receptors, commonly used P2Y receptor agonists (and their metabolite nucleosides) and antagonists often have the potential to interact with other biological targets. Thus, new P2Y receptor ligands of clearly defined selectivity, chemical and biological stability, and bioavailability are needed. P2Y receptor ligands that would pass the blood-brain barrier when administered peripherally are desperately needed by neuroscientists to study the role of these receptors in the central nervous system.

We have explored functionalized congeners of $P2Y_1$ receptor agonists [108]. A functionalized chain was attached at the adenine C2 position of ATP derivatives. This design feature was based on the SAR of $P2Y_1$ receptor agonists, which indicates high potency of 2-thioethers compared with 2-amino or 2-oxo derivatives [109,110]. A tolerance for long extensions of this chain was found, by contrast to chain extension at the N^6 position of ATP, which eliminates $P2Y_1$ receptor recognition. Thus, in the putative binding site of the $P2Y_1$ receptor, only small hydrophobic groups in the N^6-region are tolerated; the C2 region has much less restricted tolerance of steric bulk. For example, N^6-(2-phenylethyl)-ATP was inactive at the $P2Y_1$ receptor; 2-(2-phenylethyl)thio ethers of ATP, such as **51**, were highly potent [33,108]. A biotinylated nucleotide analogue potentially suitable for specific $P2Y_1$ receptor isolation was reported [111]. A functionalized chain was attached as a 2-thioether in this agonist series.

We employed $P2Y_1$ receptor nucleotide antagonists that display similar SAR to the agonist series in a functionalized congener approach. The high-affinity $P2Y_1$ receptor antagonist MRS2500, a 3′,5′-bisphosphate derivative that contains an (N)-methanocarba ring system as a 2′-deoxyribose substitute, was subjected to the functionalized congener approach.

MRS2500 was effective in reducing thrombus formation *in vivo*, and it is more resistant to degradation than the related 9-riboside derivatives [104]. Although the substitution in this family at other positions decreases antagonist potency at the $P2Y_1$ receptor, a 2-alkynyl substitution of the adenine ring maintains antagonist potency. We have extended our search for antagonists of the $P2Y_1$ receptor by modifying the adenine 2 position with various long-chain alkynyl subtituents that retain receptor affinity [112]. Congener **52** was one such carboxylic acid derivative that was a potent $P2Y_1$ receptor antagonist (Table 1).

We prepared functionalized congeners of $P2Y_{14}$ receptor agonists that retain biological activity [113]. The most insensitive site for derivatization on the native ligand **8** is through the glucose moiety. In fact, the 6-carboxylate of UDP-glucuronic acid was found to be suitable for chain derivatization, e.g., by amide coupling to an *N*-acetyl-ethylenediamino group to form **53**.

Functionalized congeners of ligands for other GPCRs and non-G protein coupled receptors

Functionalized congeners of ligands for other GPCRs and non-G protein coupled receptors have been reported. This review includes a few representative studies on chain attachment and tethering of GPCR and non-GPCR ligands.

Portoghese and colleagues reported novel bivalent ligands for opioid and other receptors. Separate pharmacophores representing a δ-antagonist and a κ-agonist were tethered and found to bridge δ 2 and κ 1 opioid receptors [114]. These heterodimeric conjugates contained the δ-antagonist naltrindole and the κ-agonist ICI-199,441 tethered by oligoglycine linkers of variable length. Novel tethered bivalent ligands for two different classes of GPCRs were found to induce the direct association of μ-opioid and CCK2 (cholecystokinin) receptors [115]. These bivalent ligands appear to induce the association of these two divergent GPCR subtypes.

In our studies of biogenic amine neurotransmitter receptors, we explored muscarinic acetylcholine receptor agonists (Figure 8A) and antagonists with a functionalized congener approach [116–118]. We first used a chain extension method for muscarinic antagonists with the known antagonist pirenzepine **71** [117] and then extended our investigation to the more potent and selective M1 receptor antagonist telenzepine **73** (Figure 8B) [118]. Sites for chain derivatization were compared, and the attachment of a spacer chain to a distal piperazinyl nitrogen, common to both antagonists, was the generally insensitive site. We varied the chain at this position in the pirenzepine series **72** was varied and compared the binding affinities. We observed a progressive increase in affinity in the longer members of the series (6 or more methylenes). We applied the findings to the telenzepine series, and a derivative containing a 10-aminodecyl group (TAC, telenzepine amine congener, analogous to **72e** but in the telenzepine series) was highly potent in receptor-binding affinity and provided a nucleophilic functionality for further derivatization. The K_i value of TAC was 2.39 nM at the recombinant m1 receptor and 15.8 nM for the corresponding pirenzepine derivative.

We synthesized conjugates of TAC to contain prosthetic groups for radioiodination, protein crosslinking, photoaffinity labeling, and fluorescent labeling and biotin for avidin complexation. A *p*-aminophenyacetyl derivative for radioiodination and photoaffinity labeling had a K_i value of 0.29 nM at rat forebrain M1 muscarinic receptors; by contrast, the K_i value for telenzepine was 4.7 nM). A biotin conjugate displayed a K_i value of 0.60 nM at cardiac M2-receptors and a 5-fold selectivity for M2 compared with the M1 receptor. The high affinity of these derivatives makes them suitable for the characterization of muscarinic

receptors in pharmacological and spectroscopic studies, for peptide mapping, and for histochemical studies.

The telenzepine amine congener TAC has been used as the functionalized pharmacophore in other fluorescent probes of muscarinic receptors. For example, this approach recently resulted in a high-throughput screening method that uses live cells, made possible by the high-affinity fluorescent conjugate **74** of TAC with the cyanine dye Cy3B [119]. The same fluorescent ligand was used recently by Birdsall et al. for single-molecule fluorescent imaging of muscarinic receptors [120].

Fluorescent pirenzepine analogues labeled with BODIPY were synthesized for studying fluorescence resonance energy transfer between the antagonist ligand and muscarinic M1 receptors that were fused to enhanced green fluorescent protein [121]. Attachment of the fluorophore in these pirenzepine derivatives occurred through a functionalized chain at the 4-piperazine position. It appears that these analogues bridge the putative binding sites of the agonist acetylcholine and an allosteric modulator brucine.

A variety of other GPCRs have been studied using chain functionalization of small molecular ligands. For example, naltrexamine and 5′-guanidinonaltrindole have been linked through a polyamide spacer to form KDN-21, which is designed for interaction with heterodimeric delta-kappa opioid receptors [122]. Numerous other examples of linkage of GPCR pharmacophores for the simultaneous activation of heteromeric receptors [123,124] or homomeric receptors [125,126] have been described. Symmetric bivalent ligands have been described for serotonin and for dopamine receptors [125], muscarinic acetylcholine receptors [126], and for melatoninergic receptors [127]. Dual-acting drugs for activation of multiple receptors, even if not targeting GPCR dimers, have become important in drug development, for example, the antipsychotic agent ziprasidone was designed with separate pharmacophores to antagonize both the D2 dopamine receptor and the 5-HT_2 serotonin receptors [128]. Combined ligands for the H1 histamine receptor and the 5-HT_2 serotonin receptors have been studied [129].

Although GPCRs are a major focus of studies of functionalized congeners, application to other classes of cell surface receptors and even nuclear hormone receptors is feasible [130]. One of the earliest applications of a chain attachment approach to the study of receptors was carried out by Kosower and colleagues [131]. Biologically active barbital and amphetamine derivatives were appended with an alkyl chain that terminated in a chemically reactive moiety, such as a thiolreactive maleimide, designed for covalent bonding to the biopolymer with which these drugs interacted. The main mechanism of action of barbiturates is now known to be through the $GABA_A$ receptor chloride channel complex.

Antagonists of the $P2{\times}_7$ receptor, an ATP-gated ion channel, have also been reported. We functionalized modified tyrosyl derivatives that block the action of adenine nucleotides at the $P2{\times}_7$ receptor with extended chains at various positions [132]. We observed only moderate antagonistic potency for these derivatives.

Multivalent nanocarriers for AR functionalized congeners

We are using the multivalency of dendrimer conjugates to test for interaction with dimeric and higher-order multimeric GPCR assemblies, as have been detected [133]. Kim et al. recently reported the first poly(amidoamine) (PAMAM) dendrimer to which a functionalized congeners of a GPCR ligand was covalently attached [134]. Specifically, 31 moieties of the A_{2A} AR agonist CGS21680 **37** were coupled to a G3 (3^{rd} generation) PAMAM polyamine. This conjugate acted as a potent A_{2A} AR agonist and displayed characteristic antithrombotic activity. With multivalency of ligand substitution—e.g., 31 out

of 32 possible substitutions on a G3 PAMAM dendrimer, of varying relative orientation—there could be a greater likelihood of bridging GPCR dimers than by tethering only two pharmacophores. We are expanding the application of nanotechnology to the study of GPCRs, for example, in dendrimer conjugation to a wide variety of ligands for ARs [51,135] and other receptors. These dendrimer conjugates will be studied in diverse biological models.

We also employed the chemically reactive nucleoside A_{2A} AR agonist DITC-APEC (**41**, Figure 4) to derivatize the surface of G3 PAMAM dendrimers [126]. The resulting conjugates carried multiple copies of the agonist attached through a thiourea linkage, and they differed in the number of attachments and in the presence of a fluorophore or additional surface modification. *In vitro* radioligand competition experiments showed effective binding of these PAMAM-DITC-APEC dendrimer conjugates at the human A_{2A} and A_3 ARs with submicromolar K_i values and in their weak activity at the human A_1 AR. Furthermore, these nucleoside-loaded dendrimers exhibited an A_{2A} AR-mediated inhibitory effect on ADP-induced aggregation of human platelets.

To target the human A_1 AR with dendrimer derivatives, we selected the amine functionalized congener ADAC **20** for covalent coupling [51]. We used two variations on the linking chain: one was created by acylation of the terminal amine of **20**, and the other maintained an affinity favoring a free amino group (secondary) within the chain (i.e., **25** as an amine functionalized congener). The AR-binding profile of the latter derivative (MRS5183) unexpectedly indicated a shift toward A_3 AR selectivity, both in binding affinity and in functional potency. The key to this selectivity of >100-fold in both binding at the human A_3 AR (K_i app = 2.4 nM) and functional assays (EC_{50} = 1.6 nM in human A_3 AR-mediated inhibition of adenylate cyclase) was maintaining a free amino group (secondary) in an amide-linked chain.

We explored the interaction of dendrimer conjugates of GPCR ligands with their receptors through molecular modeling [136]. The theoretical possibility was investigated for bivalent binding to a homodimer of a GPCR of a dendrimer, covalently appended with multiple copies of a small ligand [25]. We constructed a molecular model of a G3 PAMAM dendrimer condensed with multiple copies of the potent A_{2A} AR agonist CGS21680 (Figure 2C). The dendrimer was bound to an A_{2A} AR homodimer. Two units of the nucleoside CGS21680 occupied the A_{2A} receptor homodimer simultaneously. The binding mode of CGS21680 moieties linked to the PAMAM dendrimer and docked to the A_{2A} receptor was similar to the binding mode of a monomeric CGS21680 ligand.

Conclusions

The development of functionalized congeners as selective ligands for ARs, P2Y nucleotide receptors, and other receptors has provided valuable new research tools and candidates for therapeutics. In general, medicinal chemistry has made possible and driven the biological exploration of the study of GPCRs that respond to purines and pyrimidines. Molecular modeling based on a rhodopsin template has provided structural insights into both binding and activation steps of the receptors, which have been adapted to structure-based design of ligands.

The functionalized congener approach, as introduced for GPCR drug design, involves structural variation at the distal unit of a preexisting ligand to improve the drug's pharmacological effects. The key pharmacophore unit of a small molecular ligand for a GPCR, especially Family A, generally binds within its heptahelical TM domain. In the case of functionalized congeners, the distal moiety of an appended chemically functionalized

chain may reside and potentially interact at or near the extracellular domain of the GPCR. Indeed, providing a secondary interaction by this approach can significantly enhance the affinity and selectivity of certain ligands. Such modulation of the pharmacological profile can be achieved at the distal unit through a variation of the linker length, functional group manipulation, or a combination of both techniques.

A_1, A_{2A}, and A_3 AR functionalized congeners have thus far yielded macromolecular conjugates, irreversibly binding AR ligands for receptor inactivation and crosslinking, radioactive probes that use prosthetic groups, immobilized ligands for affinity chromatography, and dual-acting ligands as binary drugs. PAMAM dendrimers act as nanocarriers for covalently conjugated AR functionalized congeners. These studies employ rational methods of ligand design derived from molecular modeling and templates. Thus, the design of novel ligands, both small molecules and macromolecular conjugates, for studying the chemical and biological properties of GPCRs has provided researchers with a strategy that is more versatile than the classical medicinal chemical approaches.

The review provides the historical background to functionalized congeners, as well as a summary of recent advances. In spite of the lack of that no drugs have yet evolved out of this approach, now several decades old, this field is now reenergized due to recent developments, such as the emerging concept of functional GPCR dimers. One possible reason for the lack of pharmaceuticals designed by this approach is that the molecular weight and H bonding character are increased, which is usually considered a disadvantage in conventional drug development. However, with the advent of biological therapeutic agents and other large molecular weight molecules in clinical use, the functionalized congener approach seems to have a new appeal. In fact, the full potential of such approaches as binary drugs and macromolecular conjugates has not been fully realized.

Abbreviations

ADAC	adenosine amine congener (N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]-anilino]carbonyl]methyl]phenyl]adenosine)
APEC	2-[[2-[4-[2-(2-aminoethyl)aminocarbonyl]ethyl]-phenyl]ethylamino]-5'-*N*-ethyl-carboxamidoadenosine
AR	adenosine receptor
BODIPY	6-(((4,4-difluoro-5-(2-thienyl)-4-bora-3a,4a-diaza-s-indacene-3-yl)styryloxy)acetyl)aminohexanoic acid
FITC	fluorescein isothiocyanate
CCK	cholecystokinin
CGS21680	2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine
CoMFA	Comparative Molecular Field Analysis
DHP	1,4-dihydropyridine
DITC	phenylene diisothiocyanate
GPCR	G protein–coupled receptor
ICI-199,441	2-(3,4-dichlorophenyl)-*N*-methyl-*N*-[(1S)-1-phenyl-2-(1-pyrrolidinyl)ethyl]acetamide hydrochloride

MRS2500	2-iodo-*N* ^{6}-methyl-(N)-methanocarba-2′-deoxyadenosine-3′,5′-bisphosphate
NECA	5′-*N*-ethylcarboxamidoadenosine
NK	neurokinin
PAMAM	poly(amidoamine)
PAPA	*p*-aminophenylacetyl group
SANPAH	*N*-succinimidyl 6-(4'-azido-2'-nitrophenylamino)hexanoate
SP	substance P
TAC	telenzepine amine congener (4,9-dihydro-3-methyl–4-[(4-(10-aminodecyl)-1-piperazinyl)acetyl]-10H-thieno[3,4-b][1,5]benzodiazepin-10-one)
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl
XAC	xanthine amine congener (8-[4-[[[[(2-aminoethyl)amino]-carbonyl]methyl]-oxy]phenyl]-l,3-dipropylxanthine)

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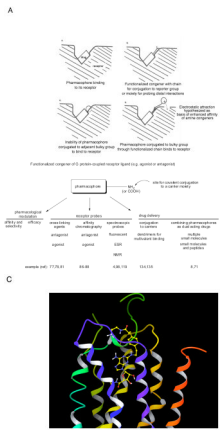


Figure 1.

A) Hypothetical interaction of adenosine functionalized congeners with the A_1 AR, as depicted in 1987. This figure is modified from ref. 4, to illustrate the historical development of this conceptual approach prior to the structural elucidation of GPCRs. B) Objectives and general considerations in functionalized congener approach to derivatizing ligands of GPCRs (applies to both small molecule agonists and antagonists). An amino group or carboxylic group is shown for conjugation, but those are only examples. The carrier moiety may be, for example, a prosthetic reporter group for radioactive or spectrospcopic detection, an electrophilic crosslinker for covalent receptor binding, or a macromolecular for targeting or immobilization. Many other approaches to conjugation chemistry are applicable. C) A three dimensional molecular model of APEC **39** bound to the human A_{2A} AR. The automatic docking of **39** to the recently published X-ray crystallographic structure (*22*) was carried out using the Schrodinger Glide XP program. The model shows that the functionalized chain of **39** protrudes into the extracellular regions of the receptor, as envisioned much earlier. The receptor helices are colored by residue position: N-terminus in red, TM 1 in orange, TM 2 in ochre, TM 3 in yellow, TM 4 in green, TM 5 in cyan, TM 6 in blue, TM 7 and C-terminus in purple.

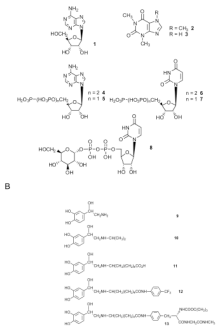


Figure 2.
Structures of several classes of GPCR ligands: A) purines and pyrimidines as the native ligands of the adenosine and P2Y nucleotide receptors, and B) the naturally occuring catecholamine norepinephrine **9** and synthetic chain-elongated analogues of isoproterenol **10**.

Figure 3.

Stepwise derivatization of chain-functionalized adenosine derivatives designed to maintain and optimize rat A_1 AR receptor binding affinity. The N^6 position was most suitable as a derivatization site for functionalized congeners that selectively bound to and activated the A_1 AR. *N*-Aryl substitution at that site was favored over *N*-alkyl substitution. The structural elements of the chain were added in stepwise fashion, with affinity optimized at each step.

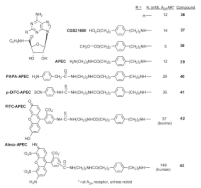


Figure 4.
Stepwise derivatization of chain-functionalized adenosine derivatives that were designed to maintain and optimize A_{2A} AR receptor binding affinity. The adenine C2 position was most suitable as a derivatization site for functionalized congeners that selectively bound to and activated the A_{2A} AR. 2-Alkylamino substitution was selected as a favorable linkage based on previous SAR and further structural probing.

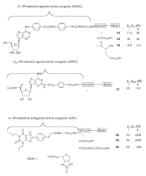


Figure 5.
Use of biotin-avidin methodology to detect and characterize the A_1 AR (using agonist ADAC and antagonist XAC) and the A_{2A} AR (using agonist APEC). In several cases, the length and chemical fiunctionality of the spacer chain were varied to find the optimal receptor affinity of the biotin conjugate, both alone and as its avidin complex. K_i values (nM) at one of the rat ARs in the absence (−) and presence (+) of excess avidin are given. N.D. – not determined.

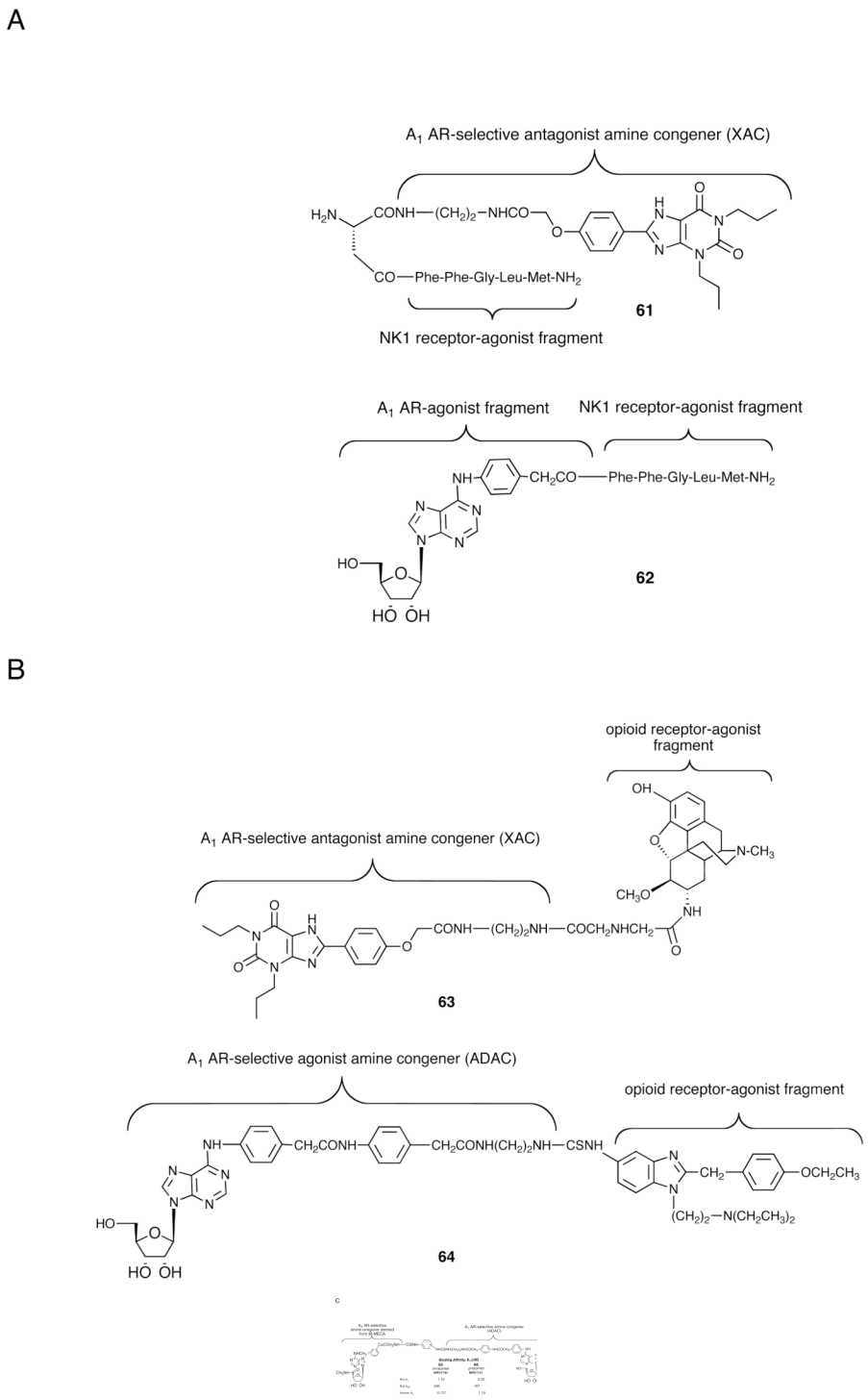


Figure 6.

Binary drugs for a given AR in covalent combination with ligands for another GPCR. A) Combination of A_1 AR-binding purines and substance P peptides as NK1 receptor ligands; B) Combination of A_1 AR-binding purines and non-peptide opioid receptor ligands; C) Combination of agonist ligands for A_1 and A_3 ARs, which have cardioprotective properties. High affinity binding to the A_{2A} AR remains excluded by this linking approach.

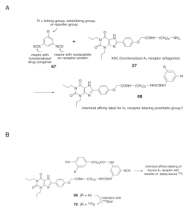
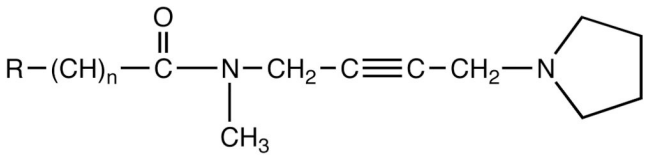
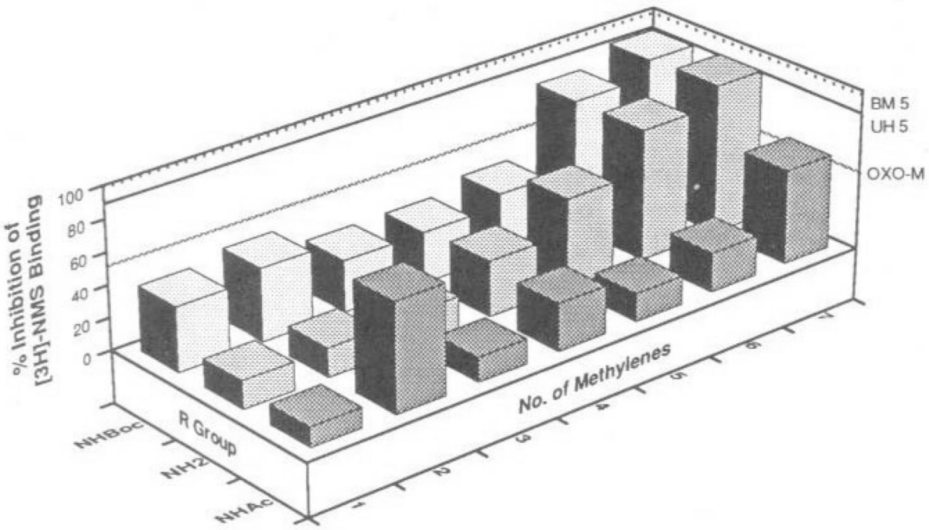


Figure 7.
Trifunctional reagents for covalent binding to ARs. A) General scheme for using a high affinity antagonist (XAC) to target a prosthetic group to the A_1 AR, at which it will bind covalently by virtues of the electrophilic isothiocyanate group, and B) Application of this scheme to a prosthetic reporter group for radioiodination.

A



R = NH_2, CH_3CONH, $(CH_3)_3COCONH$



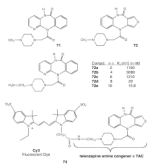
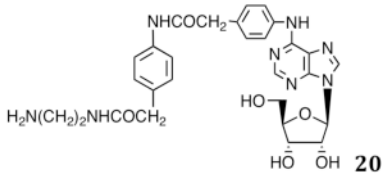
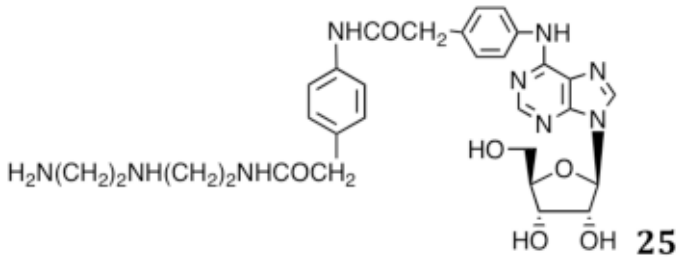
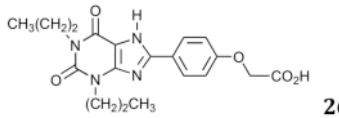
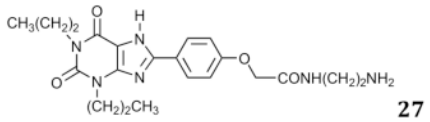


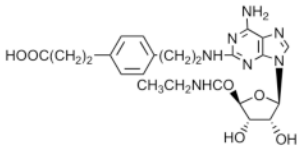
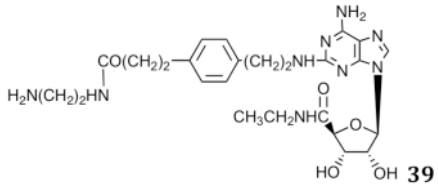
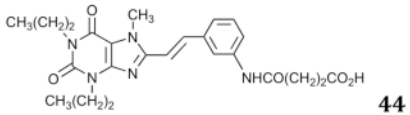
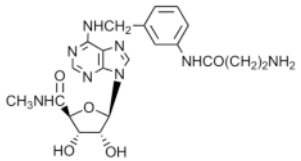
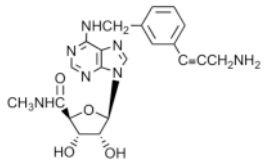
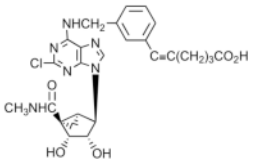
Figure 8.

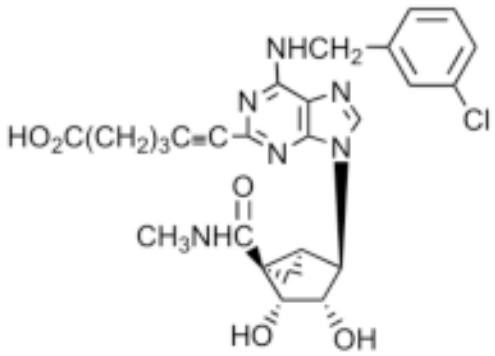
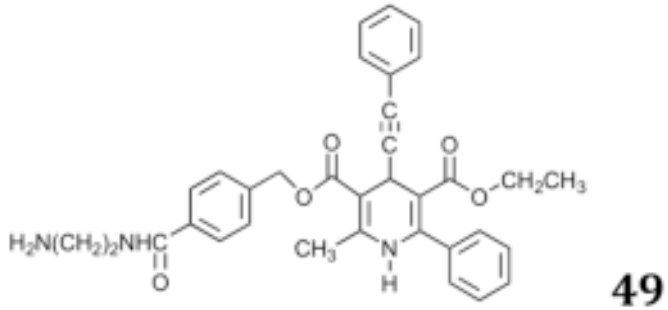
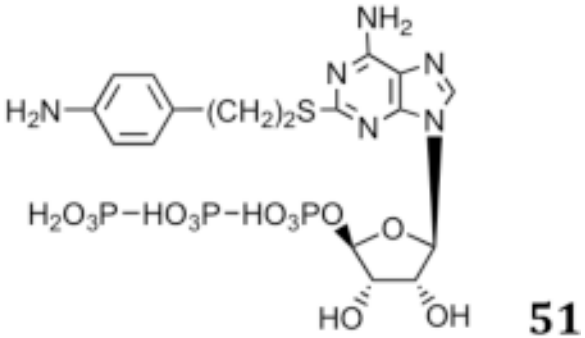
Functionalized congeners of ligands of the muscarinic acetylcholine receptors. A) Dependence of the inhibition of radioligand binding at the m3 receptor (SK-N-SH cell membranes) on the chain length and terminal chemical functionality in the agonist series of butynyl amides (116). The levels corresponding to inhibition by the pharmacological standards oxotremorine-M, *N*-methyl-*N*-[4-[1-(pyrrolidinyl)]-2-butynyl]acetamide (UH5), and *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)-acetamide (BM5) are shown (modified from reference [116]b). B) Muscarinic antagonists and their amine fucntionalized congeners (affinity of **72** shown at the recombinant rat m1 receptor expressed in A9L cells). Use of a functionalized congener of the muscarinic antagonist telenzepine to provide a high affinity fluorescent tracer (119).

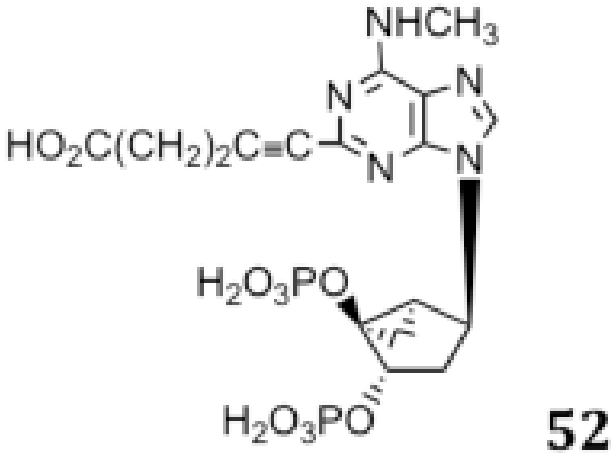
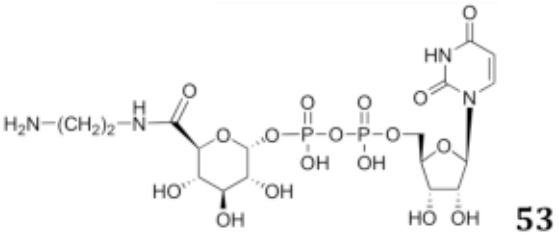
Table 1

Functionalized congeners for ARs and P2Y purine receptors and representative conjugates.

Structure	Conjugated to:	Name	Target Receptor and Function	Affinity K_i, nM (species)[a]	Ref.
 **20**	-	ADAC	A_1 AR agonist	0.85 (r)	2
	p-phenylenediisothiocyanate	*p*-DITC-ADAC **22**	A_1 AR agonist	0.47 (r)	76
	m-phenylenediisothiocyanate	*m*-DITC-ADAC **23**	A_1 AR agonist	0.87 (r)	76
	fluorescein isothiocyanate	F1TC-ADAC **24**	A_1 AR agonist	7.1 (r)	4
 **25**	-	MRS5169	A_1 AR agonist	43 (h); Found to be potent at hA_3 AR(9.5)	51
 **26**	-	XCC	A_1 AR antagonist	58 (r)	52
 **27**	-	XAC	A_1 AR antagonist	1.2 (r)	52
	p-phenylenediisothiocyanate	*p*-DITC-XAC **28**	A_1 AR antagonist	6.6 (r)	76,77
	m-phenylenediisothiocyanate	*m*-DITC-XAC **29**	A_1 AR antagonist	2.39 (r)	76,77
	p-amino-phenylacetic acid	PAPA-XAC **30**	A_1 AR antagonist	40 (r)	4
	TEMPO-isothiocyanate	TEMPO-XAC **31**	A_1 AR antagonist	4.9 (b)	4
	Perfluorobutyric acid	Heptafluoro -butyryl-XAC **32**	A_1 AR antagonist	0.83 (b)	4
	DTPA	DPTA-XAC 33	A_1 AR antagonist	3.25 (b)	4
	D-lysine	D-Lys-XAC **34**	A_1 AR antagonist	1.74 (r)	6

Structure	Conjugated to:	Name	Target Receptor and Function	Affinity K_i, nM (species)[a]	Ref.
	Bodipy-ε-amino-caproyl	XAC-BY630 **35**	A_1 AR antagonist	200 (h)	98
 **36**	-	CCS21680	A_{2A} AR agonist	14.1 (b)	55
 **39**	-	APEC	A_{2A} AR agonist	6.1 (b)	54
	p-amino-phenylacetic acid	PAPA-APEC **40**	A_{2A} AR agonist	43 (b), 23(r)	56
 **44**	-		A_{2A} AR antagonist	143 (r)	58
 **45**	-		A_3 AR agonist	23(r)	61
 **46**	-		A_3 AR agonist	7.32 (h) (*N*-acetyl deriv.)	46
 **47a**	-	MRS5168	A_3 AR agonist	17.1 (h)	62
	Amide with ethylene diamine	MRS5164 **47b**	A_3 AR agonist	5.21 (h)	62

Structure	Conjugated to:	Name	Target Receptor and Function	Affinity K_i, nM (species)[a]	Ref.
 **48a**	-	MRS5151	A_3 AR agonist	2.38 (h)	62
	Amide with ethylene diamine	MRS5166 **48b**	A_3 AR agonist	2.17 (h)	62
 **49**	-		A_3 AR antagonist	128(h)	63
	Biotin conjugate	**50**	A_3 AR antagonist	599 (h)	63
 **51**	-		$P2Y_1$ agonist	1.53 (EC_{50},t)	108

Structure	Conjugated to:	Name	Target Receptor and Function	Affinity K_i, nM (species)[a]	Ref.
 52	-	MRS2816	$P2Y_1$ antagonist	23(h)	112
 53	-		$P2Y_{14}$ agonist	496 (EC_{50},h) (*N*-acetyl deriv.)	113

[a] r (rat); h (human); b (bovine); t turkey. Expressed as K_i, unless noted. ND, not determined.