2012 Philip S. Portoghese Medicinal Chemistry Lectureship: Structure-Based Approaches to Ligands for G Protein-Coupled Adenosine and P2Y Receptors, From Small Molecules to Nanoconjugates [+]

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Abstract

Adenosine receptor (ARs) and P2Y receptors (P2YRs) that respond to extracellular nucleosides/tides are associated with new directions for therapeutics. The X-ray structures of the A_{2A} AR complexes with agonists and antagonists are examined in relationship to the G protein-coupled receptor (GPCR) superfamily and applied to drug discovery. Much of the data on AR ligand structure from early SAR studies, now is explainable from the A_{2A} AR X-ray crystallography. The ligand-receptor interactions in related GPCR complexes can be identified by means of modeling approaches, e.g. molecular docking. Thus, molecular recognition in binding and activation processes has been studied effectively using homology modeling and applied to ligand design. Virtual screening has yielded new nonnucleoside AR antagonists, and existing ligands have been improved with knowledge of the receptor interactions. New agonists are being explored for CNS and peripheral therapeutics based on in vivo activity, such as chronic neuropathic pain. Ligands for receptors more distantly related to the X-ray template, i.e. P2YRs, have been introduced and are mainly used as pharmacological tools for elucidating the physiological role of extracellular nucleotides. Other ligand tools for drug discovery include fluorescent probes, radioactive probes, multivalent probes, and functionalized nanoparticles.

Keywords

G protein-coupled receptor ; purines ; molecular modeling ; adenosine receptor ; P2Y receptor

I. Introduction: background on purine/pyrimidine receptors – new directions for therapeutics

Intense efforts by medicinal chemists in academia, government and industry have focused on the development of both agonist and antagonist ligands for the adenosine receptors (ARs) and for the P2Y receptors (P2YRs) that respond to extracellular nucleotides (Figure 1). All of these receptors belong to the Family A of G protein-coupled receptors (GPCRs). [1,2] The ARs are now at center stage in the exploration of general methodology for GPCR structure-based design. These purine/pyrimidine receptor families constitute 12 subtypes total,

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including the larger (P2Y) family that has two subgroups based on similarity of receptor structure and second messenger coupling. The P2YRs are activated by a range of native agonists, i.e. ATP, ADP, UTP, UDP, and UDP-sugars, while the four subtypes of ARs are more narrow in native agonist specificity, responding almost exclusively to extracellular adenosine. Of the hundreds of GPCRs under consideration for pharmaceutical development, these purine and pyrimidine receptors are among the most intriguing because of their widespread distribution and the dual use by nature of key important molecular mediators both intracellularly and for signaling purposes outside the cell. This molecular centrality tends to place the purine and pyrimidine receptors in important regulatory functions throughout the body. It has been noted that purinergic receptors are "the most abundant receptors in living organisms and appeared early in evolution". [3] ARs and P2YRs can be considered an important conserved vestige of the RNA world, which is also supported by the requirement, for activation of either class, of a $2'$-hydroxyl group present in the native ribonucleoside and ribonucleotide agonists.

Our basic research lab at NIH has introduced many of the important ligand tools for these GPCRs used by pharmacologists to determine the role of purine/pyrimidine signaling. Ligands of the ARs include clinical candidate drugs and typically have a well explored structure activity relationship (SAR). [4,5] However, P2YRs in some cases lack selective ligands altogether. [6] Also, many of the known P2YR ligands suffer from limited stability and bioavailability because of phosphate groups. In the P2YR field, only P2Y$_{12}$R antagonists as antithrombotic agents are well validated clinically. [7] Nevertheless, there are many new concepts, at various stages of development, of how to apply both ARs and P2YRs toward healing or diagnostic purposes. In general, to overcome the problem of widespread distribution of these two classes of GPCRs, novel drug delivery, [8] prodrug approaches, [9] allosteric modulation [10,11] and biased agonism [12] are needed to avoid ligand side effects. The pharmacokinetic and other reasons for failure of some clinical trials, even with receptor subtype-selective AR ligands, have been explained. [13]

A dendrogram arranged according to the degree of amino acid sequence homology of the twelve GPCRs that respond to extracellular nucleosides and nucleotides is shown in Figure 1A . Although the native ligands are chemically related, the ARs (similar to biogenic amine receptors) and P2YRs (similar to lipid receptors) are located on separate branches of the larger dendrogram of Family A GPCRs. [14] The four ARs (red traces) are coupled either to G$_i$ protein to inhibit the formation of cyclic AMP (A$_1$ and A$_3$ ARs) or to G$_s$ protein to stimulate the formation of cyclic AMP (A$_{2A}$ and A$_{2B}$ ARs). [1] There are three P2YRs (green traces; P2Y$_{12}$, P2Y$_{13}$ and P2Y$_{14}$) that are coupled preferentially to G$_i$ and five P2YRs (yellow traces; P2Y$_1$, P2Y$_2$, P2Y$_4$, P2Y$_6$ and P2Y$_{11}$) that are coupled preferentially to G$_q$ for activation of phospholipase C β (PLCβ). [2] G protein-independent signaling has also been studied within this family. [12,13b] The action of extracellular signaling nucleotides and nucleosides can be viewed in the context of a temporal sequence. Nucleotides released from intracellular sources by injury, stress, or from vesicles, transporters or hemichannels provide either an immediate or a delayed response to a local challenge to tissues or cells. [15] For example, a vesicular nucleotide transporter (VNUT) can regulate uptake of nucleotides into granules and their release. [11,16] Adenosine can also originate in intracellular sources. The released nucleotides can act on the same or on neighboring cells, either to open fast cation channels (P2XRs, adenosine $5'$-triphosphates) or for signaling on a slower time scale at metabotropic GPCRs (P2YRs, adenosine or uridine $5'$-triphosphates or $5'$-diphosphates). On a more prolonged time scale, the adenine nucleotides are converted by the sequential action of ectonucleotidases (for example, CD39 that hydrolyzes ATP/ADP and CD73 that hydrolyzes AMP) to the nucleoside adenosine for activation of the ARs. [11,16] A further metabolic step of conversion of adenosine to inosine by adenosine deaminase can create a secondary, weak agonist of the A$_3$ AR. [11,17] These transformations and sequential receptor

effects serve overall biological functions dictated by circumstances, such as cytoprotection, vascular adaptations and immune effects, which may be oppositely affected at different points in the time sequence. [18] The rapid effects of nucleotides on P2 receptors tend to boost the immune response, while the slower AR effects generally attenuate the immune response.

General features of adenosine as a GPCR ligand are shown in Figure 1B , in both the chemical and the biological context. The concept of separate portions of a molecule encoding clearly delineated message and address regions originated in the study of peptide GPCRs. [27]

We extend this structural division of labor within a given molecule from peptide sequences to a small molecule, i.e. adenosine. The planar adenine moiety is akin to the address region, because many adenine-like heterocycles are known to be AR antagonists [28] and also because the receptor subtype selectivity of adenosine congeners can be engineered largely by their substitution at the N^6 and C2 positions of adenine. In contrast, the three-dimensional ribose moiety with its built-in flexibility at sp ^{3}-hybridized atoms facilitates the conformational change of the receptor needed for activation. We know this role of the ribose from both the perspective of the ligand modification [29] and from X-ray crystallographic complexes of the A $_{2A}$ AR and its agonists. [20,21] In the biological realm, adenosine acting at ARs or nucleotide ligands acting at P2YRs can both be likened to adjusting the volume control on a radio, rather than flipping a power switch(with the multiplicity of signaling pathways that are differentially modulated, notwithstanding). There is a basal activation of many of these receptors that helps establish the set point to maintain homeostasis in a given tissue. [1,2] The stimulation or antagonism of the receptor can serve to counteract a signaling imbalance in a disease state to figuratively re-adjust the set point. The levels of expression and activity of these receptors can be greatly altered in diseased tissue, providing a justification for pharmacologic intervention. This is the basis of much of the intended therapeutic use of ligands of the ARs and of the P2YRs.

There are many currents therapeutic concepts at various stages of development for using selective adenosine agonists and antagonists. [1,5,30,31] For example, our lab has invented two agonists of the A $_3$ AR that have progressed to advanced clinical trials for autoimmune inflammatory disease and cancer. [32] A total of ten such trials at various phases (I – III) by Can-Fite Biopharma are currently listed in clinicaltrials. gov. These include trials for rheumatoid arthritis, psoriasis, dry eye syndrome, and hepatocellular carcinoma. The compounds have already been administered (orally) to >700 individuals, and there are indications of efficacy with no serious adverse effects.

This perspective will cover three modes of application of structural knowledge to the design of new GPCR ligands: 1. modification of known ligands or a search for new chemotypes to modulate a given receptor using newly available X-ray structures, such as A $_{2A}$ AR; 2. for other receptors in the same family, but not identical to a determined X-ray template, such as A $_1$ and A $_3$ ARs; 3. for receptors, such as P2YRs, more distantly related to X-ray templates. For the ARs, the luxury of having an A $_{2A}$ AR X-ray structure available empowers many studies of structure-based ligand design at this subtype. Both agonist-bound and antagonist-bound forms of the A $_{2A}$ AR are available, including one complex with inverse agonist 4-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5]triazin-5-yl-amino]ethylphenol **1** (ZM241,385, Figure 2A) having exceptionally high resolution showing details of water molecules and a sodium ion bound to an allosteric site on transmembrane helix (TM2). [19–23] For studying molecular recognition at the remaining ARs that have not been crystallized, homology modeling with the closely related template, i.e. A $_{2A}$ AR has proven successful. [24–26] The P2YRs still lack a close template for homology modeling, but studies are underway in that direction, i.e. to determine the structure of a P2YR.

II. Determination of structures of A $_{2A}$ AR complexes and relationship to the GPCR superfamily

The helical bundle of Family A GPCRs defines a cavity for the recognition of diverse ligands (Figure 2B). The precise chemical characteristics, size, shape, and accessibility to the extracellular milieu are tailored to each receptor family. [17] Nevertheless, the overall similarity of the architecture of these binding sites is evident in this graphical overlay. Within the rhodopsin-like Family A GPCRs, the transmembrane (TM) domain is more highly conserved than the ELs. Ligand binding occurs in the third of the TM region proximal to the exofacial side, with varying degrees of involvement of the ELs. [33] Despite variation in the orientation and depth of placement of orthosteric ligands of GPCRs, some residue positions are conserved in their role in coordinating the bound ligand (i.e. 6.55, using standard numbering convention [34]).

At present, the structure of the only member of the twelve purine/pyrimidine receptors to be solved is the A $_{2A}$ AR. [19 – 23] This receptor is a drug target for Parkinson's disease treatment with selective antagonists, such as nonxanthines **3a** and **3b** that are in advanced clinical trials. [35 , 121] The structure of the receptor was determined in multiple forms with different ligands. Both antagonist-bound and agonist-bound states of the receptor were solved initially by the Stevens group using the stabilizing fusion protein T4 lysozyme. [19 , 20] A $_{2A}$ AR complexes were crystallized by Heptares Therapeutics using receptors that are thermostabilized by systematic scanning mutagenesis (StaRs) include several other agonists and antagonists, including one with the antagonist 8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl)oxy]phenyl]-1,3-dipropylxanthine **2** (xanthine amine congener, XAC). [22] We introduced this high affinity ligand in 1985 as a functionalized congener for attachment to carriers and reporter groups without losing receptor recognition by coupling to the terminal amine. [36] Points of contact between the receptor and these ligands are shown in Figures 2B, C .

Characterization of molecular recognition at the ARs began long before their X-ray structural determination. Figure 2C outlines points along the historical progression of knowledge of the AR binding site(s), from the first molecular modeling, done by our lab in collaboration with Ad IJzerman (Leiden Univ.) in 1992. [37] Conserved residues and nucleoside ligand structures make it possible to apply knowledge gleaned about one AR subtype to the recognition at another subtype. Prior to the report of the crystallographic structure of the A $_{2A}$ AR, [19] the only method to represent the 3D structure of the ARs was through molecular modeling. [38] This approach relied on information from convergent sources: homology modeling of the protein and ligand docking, receptor mutagenesis (1995), [39] and both qualitative and quantitative SAR of ligands.

The progression of AR structural definition was greatly advanced by application of the neoceptor approach, which is a rationally designed receptor mutant that is orthogonally activated by a strategically modified agonist. [40 , 41] This approach is conceptually akin to RASSLs/DREADDs that have reengineered GPCR binding sites, except that the neoceptors begin with a rational design process for modification of both GPCR and tailored agonist ligands. [42] The first step in constructing neoceptors is to propose a binding hypothesis consistent with molecular modeling that predicts group proximity. The selective enhancement of affinity upon complementary favorable changes in the structures of a ligand and a receptor protein establishes a reliable anchor point for the hypothetical binding of the ligand. This was applied successfully first to the A $_3$ AR and later to the A $_{2A}$ AR (2005) by introducing an electrostatic pair to confirm proximity of the ribose 2′ and 3′-hydroxyl groups to a conserved His278 [7.43] in TM7. This H-bonding proximity later appeared in the agonist-bound A $_{2A}$ AR X-ray structures. [20 , 43]

We have reevaluated methods of ligand docking in light of the X-ray data, initially antagonist binding to the inactive state of the A $_{2A}$ AR and subsequently agonist binding. [45] The latter predicted structure approximated well the structure of an agonist-bound stable complex (2011). [20] A comparison of different docking approaches showed that even one extra constraint on the ligand position derived from physical measurements, such as a single H-bond implicated using site-directed mutagenesis, greatly improves the accuracy of in silico docking. [45] Our predicted model of docking of agonists in the A $_{2A}$ AR (2009), using the inactive state of the receptor as a template, almost exactly overlays the X-ray structure that was determined two years later. [20] The early modeling has evolved into a precise understanding of the separate processes of binding and activation; conformational changes of the ribose moiety are key to the activation process. [20] This is borne out in the general need in agonists (especially acting at the A $_3$ AR) for flexibility of the 5′ moiety of adenosine (CH $_2$ OH) or nucleosides resembling adenosine-5′- N -ethyluronamide **5** (NECA) with a CONH-small alkyl group, when present. [29]

Two distinct regions within the helical bundle of the ARs could be discerned in the early modeling, corresponding to the hydrophobic address and hydrophilic message regions around adenosine. The differences in the particular architecture of agonist- and antagonist-bound conformations have been analyzed in exquisite detail for the A $_{2A}$ AR and by analogy to other ARs. [20] The conformation of the A2AAR complex with 65, although lacking a G protein, displays features of an active-like conformation, e.g. spreading of the ionic lock. TMs 5, 6 and 7 engage in most of the conformation movement that leads to receptor activation, while the only pronounced movement within TMs 1–4 is a piston-like upward movement of TM3 to accommodate an agonist. The agonist-bound structure of A $_{2A}$ AR features a contracted ribose binding region, in which hydrophilic residues of the pocket are drawn toward H bonding groups of the ribose. The transformation of antagonist-bound to an agonist-bound structure of the A $_{2A}$ AR (a movie simulation in the Supporting Information of Xu et al. [20] is recommended for viewing) shows how residues in the hydrophilic regions of TM3 and TM7, primarily, approach the ribose moiety of the highly appended agonist 6-(2,2-diphenylethylamino)-9-((2R,3R,4S,5S)-5-(ethylcarbamoyl)-3,4-dihydroxytetrahydrofuran-2-yl)- N -(2-(3-(1-(pyridin-2-yl)piperidin-4-yl)ureido)ethyl)-9H-purine-2-carboxamide **6** (UK432097). This high affinity agonist from Pfizer was in clinical trials for chronic obstructive pulmonary disease with the expectation of a localized action in the lungs precisely because of its large size and extensive H bonding, which also contributed to A $_{2A}$ AR crystal stabilization. [13] The receptor-bound **6** is very similar to the structures of other bound AR agonists, obtained using StaRs. [21,42,47] There is a pattern of conserved H bonding of both the ribose and adenine moieties to key constraining amino acid residues, many of which are conserved in other AR subtypes. For example, the side chain amide of Asn254 $^{6.55}$ (using the nomenclature of Ballesteros and Weinstein [34]) acts as a stabilizing H bond donor to the adenine N7 and acceptor from N^6 H, consistent with the previously established SAR that did not tolerate 7-deaza or disubstituted N^6 analogues (Figure 2A). Agonist binding expels water from the ribose binding region and thus provides an entropic stabilization of the agonist-bound state consistent with earlier thermodynamic measurements. [46] This is consistent with the early observation that adenosine and its various monosubstituted analogues displayed exceptionally high affinity (nM range) in receptor binding, unlike some other receptors, such as muscarinic acetylcholine receptors, which feature more weak native agonist binding, i.e. μM. Agonist-specific changes include hydrophilic residues that approach the ribose and tighten around it like a belt, leading to characteristic movements of TM7 and TM6, similar to activation of opsin and the β $_2$ -adrenergic receptor. [48,49] His278 $^{7.43}$ moves inward to contact the 2′ and 3′ hydroxyl groups of ribose, and hydroxylic residues Thr88 $^{3.36}$ and Ser2.77 $^{7.42}$ are attracted to the 3′- and 5′-hydroxyl groups, respectively. Trp248 $^{6.48}$ undergoes a minor movement. EL3 is folded outward in the **6** -A $_{2A}$ AR complex as a consequence of a bulky N^6 substituent.

Thus, some of the movements are common with other GPCRs, and others movements are particular to the AR or even to a certain ligand, e.g. the outward movement of His264 in EL3. Most of the critical amino acid interactions in the hA $_{2A}$ AR crystal structures were predicted in mutagenesis/modeling studies. [20]

III. Use of GPCR structures for drug discovery

Several different approaches were used earlier to discover new AR antagonist ligands and to achieve receptor selectivity: a. Optimization of the affinity and selectivity of a known antagonist by empirical methods. An example is the elaboration of analogues of the prototypical naturally occurring and widely ingested xanthines, caffeine and theophylline, as AR antagonists beginning in the early 1980s. [36] b. Later, a search was conducted for new leads from the screening of structurally diverse chemical libraries for AR binding activity. The first examples were from small (by today's standards) libraries assembled from available flat heterocycles and many known bioactives. For example, the widely used antianginal and antihypertensive drug nifedipine bound to the A $_3$ AR at μM concentrations. From that effort came the identification of 1,4-dihydropyridines and flavonoids as AR antagonists that could be structurally optimized to eliminate the activity at their conventional target sites, e.g. Ca $^{2+}$ channels, and optimize their AR activity. [50] Consequently, the highly selective A $_3$ AR antagonist 1,4-dihydro-2-methyl-6-phenyl-4-(phenylethynyl)-3,5-pyridinedicarboxylic acid, 3-ethyl 5-(phenylmethyl) ester **4** (MRS1191), containing an elongated, rigid C4 substituent, was reported in 1996. [50] c. Combination of efficacy-lowering structural changes of nucleosides to convert a receptor subtype-selective agonist into an antagonist of similar selectivity. [29] This required a conceptual separation of the processes of binding and receptor activation, which we now know has a clear basis in the receptor structure. An example of the success of this approach is sterically constrained nucleoside analogues, such as (2 *R* ,3 *R* ,4 *S* ,5 *S*)-2-[*N* ^{6} -3-Iodobenzyl)adenos-9′-yl]-7-aza-1-oxa-6-oxospiro[4.4]-nonan-4,5-diol **17** (R ^{1} = I, MRS1292), or truncated nucleosides, such as (2 *R* ,3 *R* ,4 *S*)-2-(2-chloro-6-(3-iodobenzylamino)-9H-purin-9-yl)tetrahydrothiophene-3,4-diol (**19** , R ^{1} = I, R ^{2} = Cl, LJ1251) that act as selective A $_3$ AR antagonists. [52, 53] d. *In silico* screening of diverse libraries was carried out first based solely on ligand-defined pharmacophores and later on docking of the compounds to receptor structures or models. [51, 52]

The ARs can be considered an early example of the effective use of X-ray structures for both the selection of new chemotypes from chemical libraries and in the stepwise design of new ligands from known ligands. For example, biophysical mapping and fragment screening explored using StaRs, which can be stabilized in either agonist-preferring or antagonist-preferring conformations, are elegant and systematic approaches to this effort. [21, 47, 54]

The X-ray structure of the A $_{2A}$ AR has made possible a new paradigm in drug discovery for the ARs. Figure 3A is a flowchart of steps in the structure-based design approach that we have followed for the ARs and other GPCRs. [40] Structure-based virtual screening can be performed using the inactive A $_{2A}$ AR structure, which has proven useful for the discovery of new antagonists. Fragment-based approaches, in which only a portion of a known agonist is systematically replaced, have been reported for the discovery of novel agonists using an agonist-bound A $_{2A}$ AR structure. [20] This structure is representative of the activated state, especially in the vicinity of the bound ligand, because of the distancing of residues in the "ionic lock" for the inactive state, resemblance to other activated GPCR structures and the characteristic sodium-induced shift of agonist affinity observed pharmacologically, although it is not possible to directly determine the degree of activation of this structure because of the presence of the crystal-stabilizing T4 lysozyme in the 3 rd intracellular loop (IL3). Homology modeling is useful in extending both the antagonist-bound and agonist-bound

A $_{2A}$ AR structures to other AR subtypes or even other GPCR types. The reliability of these molecular models of GPCRs, in general, can be validated by comparing their predictions with the results of SAR and mutagenesis studies.

A. Library screening for nonnucleoside AR antagonists: ligand-based and target-based

Various recent publications have performed structure-based drug discovery of novel antagonists at the A $_{2A}$ AR. [51 , 55 – 59] Some of the novel ligands discovered by this process are shown in Figure 3B . In collaboration with Jens Carlsson (now at Stockholm Univ.) and Brian Shoichet (then at Univ. of California, San Francisco), we screened the ZINC library (http://zinc.docking.org/ , 1.6 million compounds at the time) and discovered novel chemotypes for the human A $_{2A}$ AR with a hit rate of 35%. [51]

Kolb et al. [26] found that the in silico screening of chemically diverse libraries at the A $_1$ AR had considerable overlap in reactivity with associated AR subtypes. Over two million lead-like compounds were docked in the A $_1$ AR homology models (based initially on the A $_{2A}$ AR inactive structure), and a screen of high-ranking molecules provided a hit rate of 21% of validated AR ligands, mostly of novel chemotype, but there was considerable cross reactivity at the A $_{2A}$ and A $_3$ ARs. The most potent hit **22** had a K $_i$ of 400 nM at the A $_1$ AR, but bound similarly at the A $_{2A}$ AR. Thus, X-ray-based in silico screening at GPCRs is likely to identify ligands for closely related families.

Structurally diverse screens have discovered new antagonists, but have been less successful at identifying novel agonists, except for a few reports such as fragment screening using target (StaRs) immobilized NMR screening and other biophysical methods. [60] A more narrowly defined 3D pharmacophore is needed for agonists than for antagonists, because of the more restrictive spatial and H bonding requirements of the flexible ribose moiety. In light of the multiple rearrangements of amino acid side chains in that region as shown in the X-ray structures, there is likely a family of conformations of the ligand in the ribose region that must be satisfied.

B. Design of new ligands by modification of known agonists and antagonists

1. Docking and modification of known ligands at the A $_{2A}$ AR — Most in silico screens using GPCR structures or homology models are for antagonists (including AR screens [51 , 55]), but a significant number of GPCR drugs are agonists (e.g. opioids, 5HT $_{1B}$ for migraines, etc.). Therefore, we need methods for rational design of agonists and their analogues that might have diverse pharmacological properties, including effector-biased agonism. [12 , 61]

Three regions of adenosine (C2, 5′, and N^6) have been modified structurally based on insights from each binding subpocket in the A $_{2A}$ AR X-ray structures. Each of these three regions, highly modified in past SAR studies, has its own considerations given the new structural insights, and different approaches have been applied: the C2 region has the most freedom of substitution, because it is open to the extracellular region; the 5′ region is a small pocket that is limited in size; and the N^6 region is intermediate in its ability to be extended. [4] The N^6 region is partly accessible to the extracellular space; there are also strict stereochemical considerations in proximity to the exocyclic amine. [62]

A recommended first step in establishing a binding model for predictive use for new derivatives is to see if the model explains the SAR of known ligands. Model docking of a test set of nucleoside agonists to an active form of the A $_{2A}$ AR explains structurally much of the previous, empirically-derived SAR. [24] In order to take into account the ELs, we used the X-ray structure of the **5** -A $_{2A}$ AR complex determined using StaRs that includes more of

these portions of the receptor. [21, 43] Three residues of the second extracellular loop (EL2) seem to be in proximity of important terminal functionality on several adenosine agonists that are extended at the C2. The terminal carboxylate group of 2-[p-(2-carboxyethyl)phenyl-ethylamino]-5′- N-ethylcarboxamidoadenosine (CGS21680, **11a**) is predicted to interact with K153 [EL2]; the terminal alkylamino group of the corresponding ethylenediamine derivative **11b** (APEC) is predicted to interact with E169, a residue in the C-terminal half of EL2 that folds into the main adenosine binding region. The same carboxylate group is shared with the exocyclic adenine in many non N^6-substituted analogues, and it was shown by earlier mutagenesis to be important in ligand recognition. [63] It was evident previously that addition of terminal amines led to enhanced potency of both AR agonists and antagonists, but the structural basis of such functionalized congeners was unknown. [36] The terminal isothiocyanate group of affinity label **11c**, made by elongation of an amine-functionalized A$_{2A}$ AR agonist **11b** with a bifunctional cross-linking moiety, p-phenylene diisothiocyanate (DITC), is predicted to react covalently with K150 [EL2] to form a thiourea anchor on the receptor. This terminal isothiocyanate group is required for potent irreversible inhibition of the A$_{2A}$ AR by **11c** and its consequent prolonged vasodilation of the coronary artery that is not diminished upon washout. [64]

Based on the docking modes, we prepared a variety of amino acid conjugates of **11a** by amide formation at the terminal carboxylate. [24] The interactions of the carboxylate group of a high affinity D-His conjugate **12** with both K150 [EL2] and K153 [EL2] and of the imidazole ring with E169 [EL2] were predicted. [24] Now various predicted interactions can be tested using site-directed mutagenesis.

Fragment-based searching of new AR agonists was performed by Katritch et al. [65] The focus was on the sterically restricted 5′ region, which consists of an N-ethylcarboxamide in **5**. Substitutions of the amine component of the amides were screened in silico at an active state of the A$_{2A}$ AR [20] using the ICM molecular modeling package, [66] with the restriction of MW <150, and the resulting hits included both known and novel fragments at the 5′ amide. A receiver operating characteristic (ROC) curve, reflecting the degree of predictive accuracy of the model for selecting genuine ligands from a training set, validated the use of an agonist-bound A$_{2A}$ AR structure to select true agonists. An alternative template in silico that was adapted from the inactive A$_{2A}$ AR was less successful for retrieving agonists. Novel 5′-carboxamide moieties were also identified, for example, a pyrazole amide **9a** was predicted to be favored in interaction with this region of the receptor. When the ligand was synthesized, it proved to be a moderately potent A$_{2A}$ AR agonist with a K$_i$ value of 230 nM. Gain of affinity at the A$_1$ AR was noted for some of the derivatives such as an oxetane **9b**, which was explained by putative H bonding to Asn [5.42]. The change in a single residue at 5.46 between A$_{2A}$ AR and A$_1$ AR from Cys to Trp enlarged that region sterically by pushing TM5 away from TM3 and TM4. This allows a larger 5′-carboxamido group of partial agonist **9b** to be accommodated in the A$_1$ AR, leading to a K$_i$ value of 12 nM.

2. Ligands for receptors closely related to the X-ray template: A$_1$ and A$_3$ ARs — The effects of truncation of hydrophilic functionality in the 'message' region of adenosine derivatives were analyzed pharmacologically. A correlation of changes at the N^6 position in two parallel series of nucleosides has revealed that the A$_3$ AR affinity is better preserved than A$_1$ AR affinity in 4′-truncated nucleoside analogues. [62] Diverse N^6-alkyl andarylalkyl groups that favor A$_1$ AR affinity in the ribose series were applied to the 5′-truncated series of (N)-methanocarba analogues. At the A$_3$ AR affinity was largely maintained, while at the A$_1$ AR there was a loss of affinity of generally two orders of magnitude. The structural basis for this difference between the subtypes was probed using homology modeling. Recognition of agonists at the A$_3$ AR is less dependent on H-bonding groups about the 5′ position of ribose than is ligand recognition at the A$_1$ AR. This

correlates with the H-bond donating ability of His250 $^{6.52}$ in the A $_{2A}$ AR that is conserved in the A $_1$ AR (His251 $^{6.52}$) but is absent in the A $_3$ AR (replaced by Ser247 $^{6.52}$ that is predicted not to be in direct contact with the ligand).

Activation of the A $_1$ AR in the truncated (N)-methanocarba series is highly variable, ranging from full efficacy to low efficacy partial agonism depending on subtle differences in N^6 substitution. [62] Thus, there is compensation at a remote region of the receptor for the loss of an important element of recognition and receptor activation in the 5′ region, as shown by docking of many nucleosides in an A $_1$ AR homology model, derived from the closely related X-ray crystallographic structure of A $_{2A}$ AR. We are now studying this N^6 interaction in more detail. Agonist (1 R ,2 R ,3 S ,4 R ,5 S)-4-(2-chloro-6-((dicyclopropylmethyl)amino)-9 H -purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol **10** (MRS5474) is a truncated N^6 -dicyclopropylmethyl (N)-methanocarba analogue that fully and selectively activates the A $_1$ AR because of the addition of the N^6 -dicyclopropylmethyl group. [62] Even minor deviations from the structure of **10** lose the pharmacologically favorable combination of A $_1$ AR affinity, selectivity and efficacy. According to our homology modeling, there are two separate subpockets in the A $_1$ AR for branched N^6 substituents, but only one of these subpockets exists in the A $_3$ AR. This is our structural hypothesis for the A $_1$ /A $_3$ selectivity of **8** . In light of this pharmacologically optimized structure having more drug-like physical properties, **10** occupies a sweet spot as an A $_1$ AR agonist among nucleoside structures.

<u>Nucleoside derivatives as optimized A $_3$ AR ligands :</u> Although the two prototypical agonists of the A $_3$ AR of nanomolar affinity have shown numerous selective actions in model systems and apparently in humans, as evidenced by the lack of cardiovascular side effects at low to moderate doses of N^6 -(3-iodobenzyl)-5′- N -methylcarboxamidoadenosine **13** (IB-MECA), [32] questions of their pharmacological selectivity remain. There are examples of these two agonists activating other AR subtypes; including A $_1$, A $_{2A}$ and even the low affinity A $_{2B}$ AR depending on concentration. [67] High concentrations (~30 μM) of 2-chloro- N^6 -(3-iodobenzyl)-5′- N -methylcarboxamidoadenosine **14** (Cl-IB-MECA) [32] have been noted to have AR-independent actions that affect survival, for example blockage of cell cycle progression at G1/S and G2/M transitions. [68] Consequently, we embarked on an effort to enhance the A $_3$ AR selectivity by further structural modification of this series of nucleosides.

To address the question of cross reactivity of these agonists at unrelated sites, we collected data for biological activities of these agents, as listed in the PubChem publicly available database. [69] The results provided an initial indication that the relatively few off target and unexpected interactions (i.e. not related to ARs) occur only at high μM concentrations (Figure 4A). Therefore, the action of **13** and **14** would appear to be relatively selective for the ARs with respect to diverse cellular pathways.

Progress in the systematic molecular refinement of A $_3$ AR-selective nucleoside ligands can be outlined chronologically. Representative structures are shown in Figure 3B , and this diagram is not exhaustive of the total SAR of the A $_3$ AR, which involves numerous research labs. [38 , 70] The logic behind changes in each region highlighted is dissected separately.

a. The need for small amide moieties for AR binding in general was established early in the process. The superiority of a 5′- N -methyluronamide in **15** for achieving selectivity for the A $_3$ AR was reported in 1994. [71]

b. In a comparison of arylalkyl groups, an unbranched N^6 -benzyl group was best suited for selectivity for the A $_3$ AR, although considerable affinity was seen with

other groups such as N^6-(2-phenylethyl). 3-Chloro or 3-iodo substitution (**15** , R ^{1}) was also associated with high A $_3$ AR affinity.

c. Substitution with sulfur in place of 4′-oxygen in **18** as a means of preserving or enhancing A $_3$ AR activity was discovered in collaboration with the lab of Prof. Lak Shin Jeong at EWHA University. [72] Initially, it seemed that sulfur substitution was more geared for maintaining A $_{2A}$ AR affinity, [73] but in an extensive SAR exploration at the A $_3$ AR it became clear that the 4′-thio modification was especially applicable to this subtype.

d. Pharmacological theory teaches that structural modifications of an agonist ligand resulting in partial agonism, if sufficiently substantial, can be combined to achieve antagonism. There is a continuum of degrees of efficacy that implies antagonism at its lowest point, i.e. the ligand binds competitively with the native agonist but cannot induce the necessary conformational change for receptor activation. We and others have combined individual changes that produce partial agonists in a known series of agonists, notably at A $_3$ AR or P2Y $_1$ R, to remove the residual activation of the receptor. [29 , 52 , 61 , 74 , 75] For example, the conversion of selective nucleoside agonists of the A $_3$ AR into selective antagonists may be effected by modification of the ribose moiety or by addition of suitable groups at the C2, N^6 or C8 positions of adenine.

One of the effects of truncation of adenosine derivatives at the 4′ position, e.g. **19** , is that A $_3$ AR agonists are converted into antagonists. [52] Numerous nucleosides exist at the boundary of A $_3$ AR agonism and antagonism. [29] Truncation of the 5′ group, i.e. removal of either CH $_2$ OH for native 7-riboside structures or a 5′- N-uronamide in **4** -like analogues, tended to preserve the affinity but not efficacy at the A $_3$ AR. Thus, it was possible to generate a wide range of selectively binding A $_3$ AR antagonists. An advantage of this modification in the design of antagonists in the nucleoside series in comparison to chemically diverse antagonists was the closer agreement in A $_3$ AR affinity at different species.

A comparison of the A $_{2A}$ AR X-ray structure with residues in the A $_1$/A $_3$ binding sites around the ribose moiety, which is important for the activation process, helps explain the effects of truncation. [20 , 24 , 25 , 62] The A $_3$ AR is less dependent on the 5′ region for recognition (likely related to the absence of His250 $^{6.52}$), but still dependent on remaining hydrophilic residues for activation. The effects of truncation on recognition at the various AR subtypes can be understood in terms of interactions with specific amino acid residues in the binding site and subsites (Figure 5). Hydrophilic residues are especially important in this process. The effects on binding and activation can be analyzed separately in conjunction with changes in the ligand structure.

e. In order to improve upon the selectivity and potency of the A $_3$ AR agonists, we examined the conformational factors of ribose in receptor recognition. Ring constrained substitutes for ribose were incorporated showing that the A $_3$ AR greatly favors the (N) conformation as in **16** . The (N)-methanocarba, pioneered by Victor Marquez for antiviral nucleosides, [76] was incorporated into adenosine and its A $_3$ AR-selective derivatives and found to be conducive to enhanced selectivity. The opposite isomer, which maintains a South (S) conformation of the ribose-like ring was >100-fold less potent at the A $_3$ AR. Thus, (N) lock of the ring more closely approximates an active conformation of ribose when bound to the receptor. Other forms of conformationally locked substitutions for the tetrahydrofuryl ring were incorporated in nucleosides for binding to the A $_3$ AR, [77] and the (N)-methanocarba is the most successful in enhancing pharmacological properties.

A$_3$ AR antagonist **17** has a conformationally-locked spirolactam that maintains selectivity at the A$_3$ AR but precludes receptor activation. [29]

f. The extended, rigid C2-arylalkynyl group in **20** was found to be compatible with both the (N)-methanocarba ring system and the preferred substituted N^6-benzyl group. This C2 modification was previously introduced in native ribose-containing nucleosides by Cristalli and coworkers, [78] but its enhancing effect seems not to be additive in combination with large N^6-benzyl and other groups unless the (N)-methanocarba ring system is present. This combination of relatively rigid substitutions of adenosine created a dilemma in the receptor modeling and docking of this series. A homology model was constructed based on the close template of the agonist complex of the A$_{2A}$ AR. [20] In order to maintain the conserved stabilizing H-bonds within the hydrophilic ribose binding regions, it was necessary to shift TM2 away from the ligand to avoid a steric clash with the C2 substituent. [25] This shift was justified by using as template for the TM2 alone structures of several other GPCRs in active forms, i.e. the β$_2$ adrenergic receptor and opsin. [48, 49] In those structures, the position of TM2 is displaced outward from its position in the A$_{2A}$ AR. Thus, a hybrid homology model that takes advantage of two templates for different regions of the receptor was suitable for docking of the new series of phenylethynyl derivatives **20**. [25]

g. The affinity of this series was sufficiently robust to accommodate a range of N^6 substitutions. Small groups, such as N^6-methyl, are associated with high affinity at the human A$_3$ AR, with substantial reductions noted at the rat and mouse A$_3$ AR, at least in the 9-riboside series with species dependent affinities differing by as much as 100-fold. [25] In the case of rigid multiply-substituted full agonist **5**, the ratio of affinities at human to rat was ~10-fold. N^6-Benzyl substitution in this series provided a closer correspondence of affinities at the human and mouse A$_3$ ARs. One such agonist was (1S,2R,3S,4R,5S)-4-(6-((3-chlorobenzyl)amino)-2-((3,4-difluorophenyl)ethynyl)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide **20** (R^3 = 3-chlorobenzyl, R^4 = 3,4-difluoro, MRS5698), which is bioavailable and has in vivo activity.

h. The 5′-N-methyluronamide could be deleted without losing hA$_3$ AR selectivity in the C2-arylalkynyl series as in **21**, similar to previous truncation in other series of adenosine derivatives, but it limited the range of substitutions possible to maintain high affinity at the A$_3$ AR. The 5′-N-methyluronamide serves as an important stabilizing anchor of the hydrophilic ribose-binding region of the ARs. However, its loss can be tolerated if suitable compensatory features of the truncated nucleoside ligands maintain A$_3$ AR affinity. For example, A$_3$ AR selectivity of **21** is associated with small N^6 groups (i), such as methyl, ethyl, and methyloxy, but not larger groups, such as N^6-benzyl or 2-phenylethyl in **21**. As found earlier, [51] a consequence of the loss of the flexible 5′-N-methyluronamide was reduction in the efficacy in activation of the A$_3$ AR. In some cases, the efficacy was sufficiently suppressed that the 4′-truncated derivatives were essentially full antagonists with respect to inhibition of adenylate cyclase (e.g. **21**, R^3 = (CH$_2$)$_2$Ph, R^4 = H, K$_i$ 20 nM). The low efficacy could be modulated with changes in the C2-arylalkynyl group, such that some of the derivatives (e.g. **21**, R^3 = CH$_3$O, R^4 = H, 30% efficacy, K$_i$ 13 nM) were selective partial agonists of the human A$_3$ AR. GPCR partial agonists might have superior pharmacological properties to full agonists for in vivo application. [44, 79] Thus, there is a pronounced interrelationship of substitution at various sites on the adenosine structure with respect to their effects on AR affinity, selectivity and efficacy. Underlying this analysis is the

observation that interactions of specific regions of potent AR ligands could be effectively modeled through ligand docking to the appropriate receptor.

3. Ligands for receptors more distantly related to current X-ray structural templates: P2YRs — P2YRs have a hopeful future for drug discovery, although the medicinal chemistry is more challenging than for the ARs. The correspondence of the native mononucleotide agonists to the eight subtypes of P2YRs is shown in Figure 1A . Naturally occurring dinucleotides, such as diadenosine tetraphosphate (Ap $_4$ A), also bind to various P2YRs. Synthesis and purification of large numbers of nucleotide derivatives is more labor intensive, and pharmacological evaluation is subject to uncertainty due to possible instability and poor availability of these ligands. Library screening of chemical libraries to identify new antagonist leads has so far only succeeded for a few of the P2YR subtypes (such as **43** , Figure 6). [80] In recent years, the modeling of P2YRs is also more difficult than for some other GPCRs, because of the lack of a close template. Concerning a template selection for P2YRs, this family is most closely related to CXCR4 among the X-ray structures currently existing. [81] Also worthy of consideration for their structural similarity to the P2YRs are the opioid receptors and a protease activated receptor (PAR1). [82 , 83] CXCR4 has already served as a suitable template for several of the P2YRs, [84] based 25.6% sequence identity in TMs and a Pro kink in same position in TM2 (2.58). As a result, the binding cavity in the CXCR4-based P2Y $_{12}$ R model is relatively extended and solvent-exposed. The disulfide between the N-terminus and EL3 found in the CXCR4 structure is possible due to the presence of analogous Cys residues but the does not seem to be necessary for P2Y $_{12}$ R activation. [120]

We have studied the structure and function of the entire family of P2YRs through sequence analysis, molecular modeling, site-directed mutagenesis of one subtype, and extensive SAR probing of agonist and antagonist ligands. Novel selective agonists and antagonists of the less explored P2YRs include nucleotides and uncharged or nonhydrolyzable compounds. Only P2Y $_1$ and P2Y $_{12}$ Rs have extensively explored SAR of competitive antagonists, while selective nucleotide agonists have been reported for P2Y $_1$, P2Y $_2$, P2Y $_4$, P2Y $_6$, and P2Y $_{14}$ Rs. Nucleotide agonists have been effectively converted into selective antagonists by functional group manipulation at P2Y $_1$ (5′-diphosphates to 3′,5′-bisphosphates) and P2Y $_{12}$ Rs (5′-diphosphates to 5′-triphosphates).

The conformationally locked methanocarba analogues of the nucleotides have delineated clear conformational preferences for the subtypes linked to PLC, the P2Y $_1$ R-like subfamily. (1′R,2′S,4′S,5′S)-4-(2-iodo-6-methylamino-purin-9-yl)-1-[(phosphato)-methyl]-2-(phosphato)-bicyclo[3.1.0]hexane (MRS2500, **36**), a selective P2Y $_1$ R antagonist of the (N)-methanocarba class, [85] was the first subnanomolar antagonist of this receptor and serves as a widely used probe for in vitro and in vivo studies, [86 , 87] for example, to test the hypothesis that such antagonists are effective antithrombotics. Although various adenosine bisphosphate derivatives were shown to antagonize the P2Y $_1$ R, the conformational lock of **36** greatly enhances its affinity, selectivity and antithrombotic properties. Stability in vivo is also enhanced, because of reduced hydrolysis of the 5′-phosphate by 5-nucleotidase due to the (N) conformation. A similarly locked 5′-diphosphate derivative, [[(1 R ,2 R ,3 S ,4 R ,5 S)-4-[6-amino-2-(methylthio)-9 H -purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester **35** (MRS2365) is a potent full and selective agonist of the P2Y $_1$ R.

The P2Y $_2$ R and P2Y $_4$ R, which both respond to 5′-triphosphates, are difficult to distinguish pharmacologically, but recently selective agonists were introduced with the aid of molecular modeling. [88 , 89] The 2′-amino group on UTP analogues such as selective P2Y $_2$ R agonist **38** was predicted, using a rhodopsin-based model, to interact with two

residues of the receptor by both H and amine-π bonding. Phosphonate **37** appears to be an allosteric partial agonist that is selective for the $P2Y_2R$, which normally requires a $5'$-diphosphate for activation. [117] The elongated N^4-arylalkoxy group of selective $P2Y_4R$ agonist N^4-phenylpropoxycytidine-$5'$-triphosphate **39** (MRS4062) was predicted to occupy an extracellular loop region that is more spacious in this subtype than in the $P2Y_2R$, using CXCR4-based homology models. [89]

The requirement by the $P2Y_6R$, which is normally activated by UDP, of a South (S) conformation of ribose, unusual for the P2Y family, was demonstrated through receptor modeling and docking, followed by the confirmatory synthesis of (S)-methanocarba-UDP **40**. [118] The preference for the (S) conformation is so pronounced that the corresponding (N)-methanocarba-UDP is completely inactive. Certain dinucleoside triphosphates such as P^1-(uridine $5'$-)-P^4-(N^4-methoxycytidine $5'$-)triphosphate **41** (MRS2957) are $P2Y_6R$-selective agonists.

In the $P2Y_{12}R$-like receptor subfamily, linked to inhibition of cAMP accumulation, we have not identified the preferred conformation of the ribose moiety. The SAR of UDP and UDP-glucose derivatives as agonists at the $P2Y_{14}R$ has been explored, to identify methylene phosphonate derivative **42** as a subnanomolar agonist. [115] An initial conclusion that UDP antagonizes the $P2Y_{14}R$ was later modified to characterize UDP generally as a potent agonist. The agonist glucose moiety has been functionalized for coupling to carriers by amine coupling to the carboxylate of the glucuronic acid moiety, which was predicted using molecular modeling to protrude to the extracellular region.

IV. Novel ligand tools for drug discovery – fluorescent probes, radioactive probes, multivalent probes, and functionalized nanoparticles

We have also concentrated on the introduction of specialized ligand probes for GPCR characterization that access multiple regions of a single receptor or that can possibly bridge multiple receptors. The SAR studies discussed so far are geared toward recognition by monomeric receptors, but it is recognized that stable dimers of GPCRs can have altered pharmacology. [90] The two protomeric receptor proteins in a pair communicate allosterically, typically through negative cooperativity, [119] to affect ligand binding and activation. The heterodimerization of the $A_{2A}AR$ and the dopamine D_2R occurs in the striatum. The $A_{2A}AR/D_2R$ is a well-validated heterodimer that is thought to be important in regulation of striatal pathways in the brain governing movement. [92] Ligands that have a preference for this or another ($A_1AR/A_{2A}AR$) heterodimeric pair are sought as drugs for the CNS disorders, such as Parkinson's disease. [91]

GPCR dimers have been studied by molecular modeling [93] and pharmacologically. [91] We have designed multivalent AR ligands that are intended to be able to adopt a geometry suitable for bridging protomers of dimeric (and possibly higher order aggregates) of GPCRs. The theoretical docking of a multivalent dendrimeric conjugate of an $A_{2A}AR$ agonist to the homodimer receptor complex was demonstrated. [93] A fluorescent adduct of agonist **11b** with Alexa Fluor 532 served as a probe to follow the allosteric modulation within $A_{2A}AR/D_2R$ heterodimers using real-time FRET. [92] Recently, we introduced fluorescent probes for characterizing the A_3AR and the $A_{2A}AR$, antagonist **44** and agonist **46** (demonstrated using flow cytometry) and antagonist **45** (demonstrated using fluorescence polarization), respectively (Figure 7A). [94, 95, 122] The fluorophores in each case were predicted by molecular modeling to form specific associations with the extracellular regions of the receptors that would enhance the affinity. Thus, the GPCR affinity/selectivity of a given conjugate is only partially dependent on the pharmacophore and is greatly influenced by the

presence of distal groups on the ligand. The agonist fluorescent probes were shown to internalize with the receptor by agonist-induced processes.

Macromolecular adenosine and P2Y receptor ligands have been shown to interact with and activate or antagonize their receptors. We introduced G PCR Li gand De ndrimer (GLiDe) conjugates to interact with receptor aggregates and to act as 'smart drugs'. [96] The attachment of GPCR functionalized congeners to tree-like polyamidoamine (PAMAM) dendrimer carriers has greatly increased selectivity and affinity in novel multivalent and multifunctional probes. PAMAM dendrimers have versatile chemical functionality and properties that are compatible with biological systems. Dendritic unimolecular micelles containing tethered A$_{2A}$ AR agonists and PEG (polyethylene glycol) chains were fully active in receptor activation and consequent inhibition of platelet aggregation, and the effects of pendant PEG groups on receptor recognition were relatively minor. The altered kinetics of the residual thrombotic effect is an example of qualitative differences between the monomeric and multivalent ligands. Blocking free amino groups reduced internalization. A series of conjugates of xanthine antagonist **2** tethered from PAMAM dendrimers of systematically varied stoichiometry indicated a more than additive effect of increasing ligand density on the AR affinity, which is suggestive of cooperativity of binding due to multiple binding sites on the membrane surface. [97] Nucleoside conjugates of PAMAM G4 (generation 4) dendrimers were synthesized by click chemistry ([2+3]cycloaddition of azide and alkyne) were potent A$_3$ AR agonists and induced potent cardioprotection. [98] Fluorescent and other reporter groups incorporated into these nucleotide conjugates provide new assay and imaging possibilities. Fluorescent imaging of the A$_3$ AR expressed in cultured cells was demonstrated. The receptor is not evenly distributed over the cell surface, suggesting clustering or aggregation, as is characteristic of GPCRs.

AR ligands have been covalently tethered from solid nanoparticles and shown to interact with and in some cases activate ARs. The design of adequate tethers (length, functionality, hydrophobicity, etc.) to allow small GPCR ligands on solid nanoparticles to retain receptor affinity is challenging. For example, quantum dots are solid particles of defined size and stable fluorescent properties, and they may be chemically functionalized for ligand attachment. In order to achieve aqueous solubility of highly fluorescent quantum dot conjugates of A$_{2A}$ AR agonists (**47** , Figure 7B), it was necessary to add a highly polar, multivalent spacer consisting of a PAMAM dendron. [99] The presence of excess carboxylate groups maintained the nanoparticle conjugates in solution, which enabled the measurement of affinity at the A$_{2A}$ AR. Gold nanoparticles (AuNPs) are being explored as drug carriers in the treatment and diagnosis of cancer and other diseases. Their physical properties can help target drugs, for example by active targeting or by accumulation in solid tumors by passive processes. An AuNP conjugate of XAC (**48** , Figure 7C) as a nonselective antagonist demonstrated binding of the functionalized solid nanoparticle to the A$_{2A}$ AR, but only when a solubilizing phosphate chain was also present on the AuNP. [100] These conjugates are now suitable for further study of ARs in vitro and in vivo.

V. New directions for CNS and peripheral actions of improved AR and P2Y ligands

In the past, most of the clinical trials of AR agonists and antagonists for drug therapy have failed, partly due to nonselectivity, lack of efficacy, poor availability or the appearance of toxic side effects. [11] Although there are representative selective agents for each of the four AR subtypes, it is desirable to have AR ligands that are more drug-like in their physicochemical properties. We recently identified a nucleoside derivative that acts as an A$_1$ AR agonist with a distinct CNS-protective effect. Compound **8** inhibited seizures in the

mouse corneal kindled seizure model with an IC $_{50}$ of 3 mg/kg at both 36 Hz and 48 Hz, which is indicative of possible application to treatment-resistant seizures. [62]

A $_3$ AR agonists are in trials as a targeted therapy for autoimmune diseases and cancer. The development of agonists as potential therapeutic agents acting through ARs is appealing despite the risk of desensitization of the effect and subsequent receptor internalization induced by GPCR agonists. [32] A $_3$ AR is over expressed in cancer and inflammatory cells, whereas low or no expression is found in normal cells. The mechanism of action of a prototypical A $_3$ agonist **13** in models of autoimmune inflammatory disease has been ascribed apoptosis of proinflammatory cells (for example synoviocytes in rheumatoid arthritis), reduction in TNFα and its associated signaling, and preservation of normal white blood cells. The high receptor expression is reflected in the peripheral blood mononuclear cells of the patients. In a clinical study of patients with rheumatoid arthritis treated with the A $_3$ AR agonist **13** , a direct correlation was found between receptor expression at baseline and the patients' response to the drug. Treatment with A $_3$ AR agonist **14** inhibits the development of hepatocellular carcinoma in an animal experimental model via modulation of the Wnt and NF-κB signaling pathways, resulting in apoptosis of the tumor cells, and initial patient data is encouraging. [101]

Recently elucidated therapeutic directions include use of A $_3$ AR agonists to treat chronic neuropathic pain. [102] Three different A $_3$ AR agonists, including one of the more selective (N)-methanocarba derivatives (**16** , R ^{1} = I, R ^{2} = Cl), were active in vivo. A $_3$ AR activation prevented the formation of the pain and also reduced the effects once it developed. The protective effects were demonstrated in two mouse models: chronic constriction injury and cancer chemotherapeutic agents having three different mechanisms (oxaliplatin, paclitaxel and bortezomib). This could provide a life-saving advantage, for instance allowing continued treatment for cancer in affected patients. The A $_3$ AR agonists not only reduced pain transiently when administered after it developed, but prevented its development.

Civan and coworkers have used our selective A $_3$ AR agonists and antagonists and receptor knockout mice to define an effect of this receptor on the formation of aqueous humor. [103] They elucidated the mechanism by which blocking an A $_3$ AR coupled to chloride inflow in the nonpigmented epithelial cell layer can lower intraocular pressure. Thus, topically applied A $_3$ AR antagonists that penetrate the cornea are promising for glaucoma treatment.

Development of P2YR agonists and antagonists for therapeutic applications is generally not as advanced as for the ARs, but nevertheless hold great potential for disease treatment. However, the use of potent P2Y $_{12}$ R antagonists as 'blockbuster' antithrombotic agents is well validated with three approved pharmaceutical agents. ADP activates both receptors on the platelet surface to induce components of aggregation, i.e. platelet shape change in response to P2Y $_1$ R and platelet adhesion, activation, and thrombus expansion and stability following P2Y $_{12}$ R activation. P2Y $_1$ R antagonists have shown efficacy for thrombosis in various in vivo models and possibly could impede atherosclerosis. [86] New, uncharged drug-like P2Y $_1$ R antagonists have been reported. [124]

Among other newer ideas for P2YR-based therapeutics are: agonists of P2Y $_1$ R and P2Y $_6$ R and antagonists of P2Y $_{13}$ R and P2Y $_{14}$ R for diabetes, [104–108] P2Y $_2$ R agonists for cardiac ischemia and neurodegenerative disease, [109, 110] P2Y $_4$ R agonists for cardiovascular disease and constipation. [85, 111] In the context of diabetes, activation of the P2Y $_6$ R increases insulin release in mouse beta islets and beta cell lines. P2Y $_6$ R agonists protect mouse pancreatic MIN6 β $^-$ cells, skeletal muscle, and receptor-expressing astrocytes against TNF-induced and ischemia-induced apoptosis. Activation of the P2Y $_6$ R lowers

intraocular pressure; thus, such agonists are of interest for treating glaucoma. [112] P2Y $_6$ R is also a target for neurodegeneration; the P2Y $_6$ R occurs in microglial cells, where it serves as a stimulus to induce phagocytosis. [113] The P2Y $_{14}$ R receptor was found to facilitate the release of histamine from mast cells and its genetic knockout increased insulin sensitivity in target tissue. Thus, selective P2Y $_{14}$ R antagonists could be a target for asthma or diabetes. [108], [114]

VI. Conclusions

Much of the data on AR ligand structure from early SAR studies, now is explainable from the A $_{2A}$ AR X-ray crystallography. The ligand-receptor interactions in related GPCR complexes can be identified by means of modeling approaches, e.g. molecular docking. These insights can be used to guide the refinement of known ligands or the identification of entirely new hits. Efficacy of the native ligands as well as affinity can be systematically modulated to provide new selective antagonists, with an understanding of the receptor interactions involved in binding and activation. In this manner, new A $_3$ AR and P2Y $_1$ R antagonists have been designed. New promising directions for selective AR ligands include A $_3$ AR agonists for chronic neuropathic pain and improved A $_{2A}$ AR antagonists for CNS-protective action. Selective ligands of P2YRs are of interest for treating immune, endocrine, neurodegenerative, and other conditions. The challenge of discovering new drug-like molecules to bind with high affinity to P2YRs has only been overcome for two of the subtypes, P2Y $_1$ and P2Y $_{12}$ Rs, but the expectation of increased structural knowledge promises major advances in the SAR of P2YRs.

Acknowledgments

Abbreviations used

AR	adenosine receptor
cyclic AMP	adenosine $3',5'$-cyclic monophosphate
AuNP	gold nanoparticles
CNS	central nervous system
EL	extracellular loop
FRET	fluorescence resonance energy transfer
GPCR	G protein-coupled receptor
NECA	$5'$-N-ethylcarboxamidoadenosine
PAMAM	polyamidoamine
P2YR	P2Y receptor
ROC	receiver operating characteristic
SAR	structure-affinity relationship
StaRs	thermostabilized mutated receptors
TM	transmembrane helix
XAC	xanthine amine congener

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Biography

Kenneth A. Jacobson, Ph.D. as Chief of the Laboratory of Bioorganic Chemistry and the Molecular Recognition Section at the National Institute of Diabetes and Digestive and Kidney Diseases, NIH explores structure and pharmacology of G protein-coupled receptors. He received a B.A. from Reed College and Ph.D. in Chemistry at the University of California, San Diego. He was a Bantrell Fellow at Weizmann Institute of Science, Dept. of Organic Chemistry before joining the NIH. Recent awards include the 2003 Hillebrand Prize, appointment to the Senior Biomedical Research Service of NIH, the 2009 Sato Award and 2009 Pharmacia-ASPET Award in Experimental Therapeutics. Dr. Jacobson is an American Chemical Soc. Fellow, served as ACS Medicinal Chemistry Division Chair and is in the Division's Medicinal Chemistry Hall of Fame.
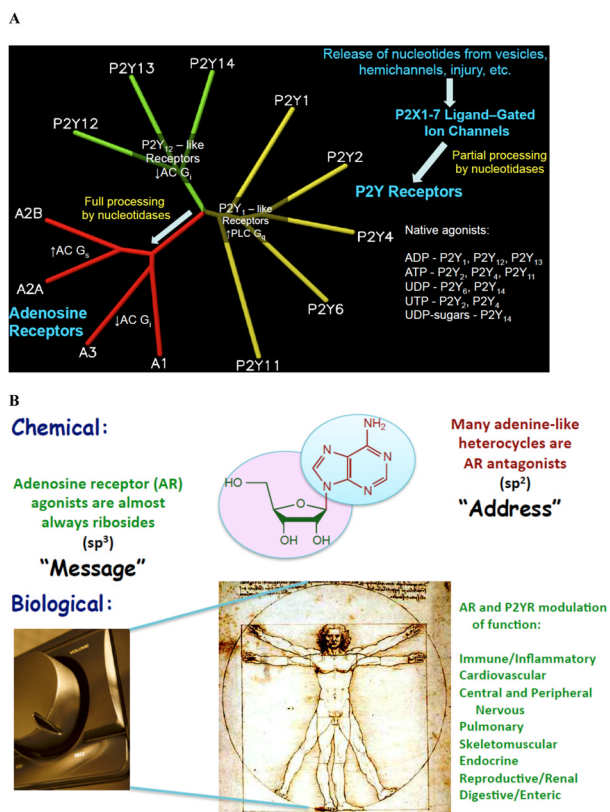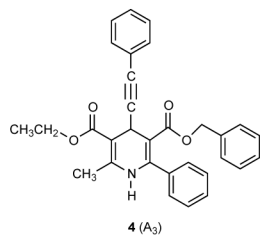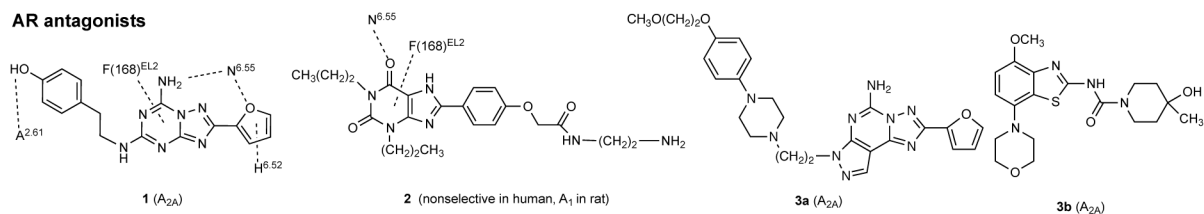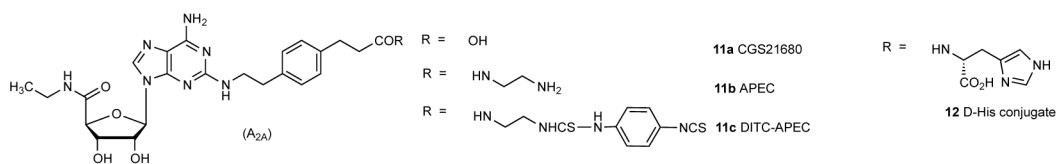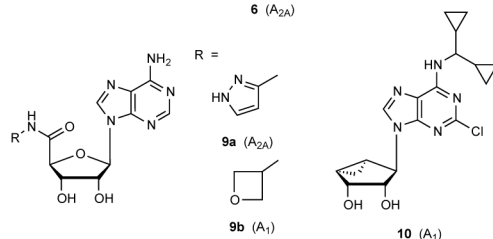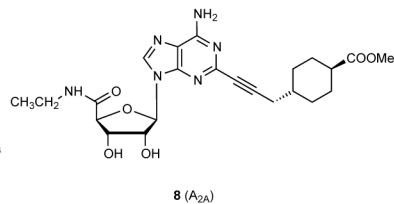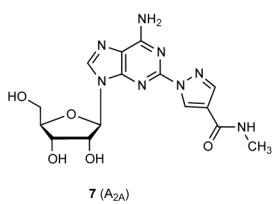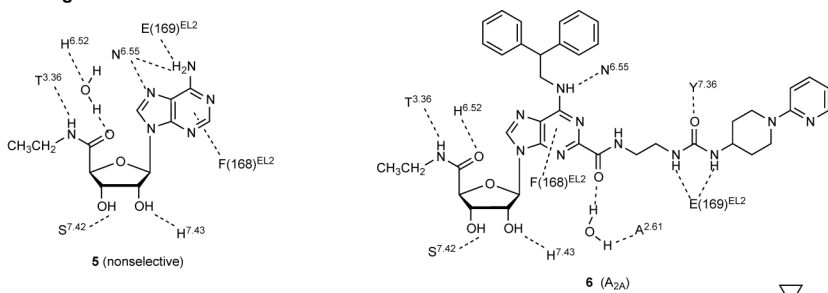
Figure 1 .
A. The time-dependent progressive activation (white arrows) of receptors for nucleosides and nucleotides on the cell surface. The 4 subtypes of ARs and 8 subtypes of P2YRs are arranged in a dendrogram according to their sequence homology. Although the ligands have commonality, the ARs and 8 subtypes of P2YRs are on distant branches of the larger dendrogram of Family A GPCRs. B. General characteristics of adenosine as a GPCR ligand and its receptors as therapeutic targets in both chemical (A) and biological (B) contexts.
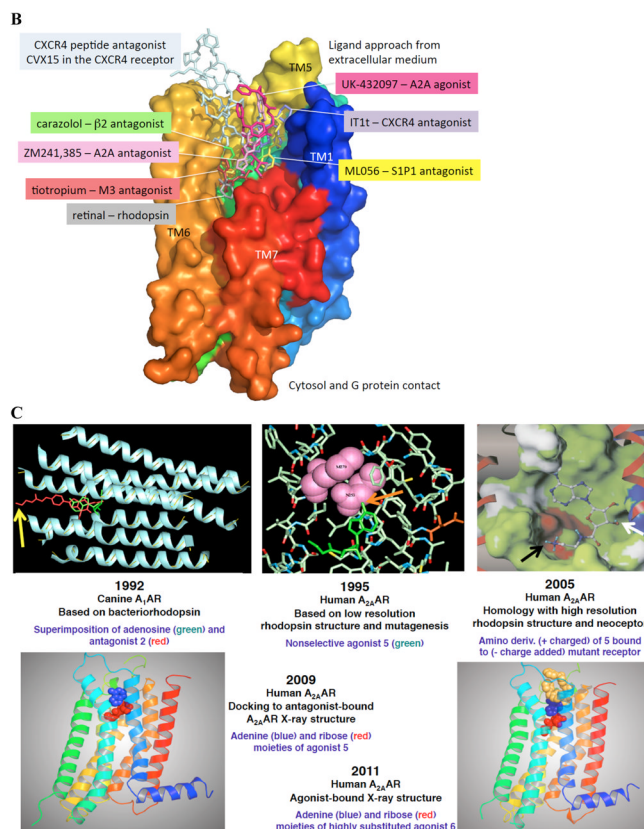
A

AR antagonists

AR agonists

1 (A$_{2A}$)

2 (nonselective in human, A$_1$ in rat)

3a (A$_{2A}$)

3b (A$_{2A}$)

4 (A$_3$)

5 (nonselective)

6 (A$_{2A}$)

7 (A$_{2A}$)

8 (A$_{2A}$)

R =

9a (A$_{2A}$)

9b (A$_1$)

10 (A$_1$)

(A$_{2A}$)

R = OH   **11a** CGS21680

R =   **11b** APEC

R =   **11c** DITC-APEC

R =

12 D-His conjugate

Figure 2 .

A. Structures of selected AR ligands discussed in the text, including those that have been co-crystallized with the A $_{2A}$ AR (**1** , **2** , **5** , and **6**). Dashed lines indicated the major H-bonding and π-bonding contacts between the receptor and ligand present in X-ray structures. Other ligands shown include pharmacological probes (**4** and **9–12**) and: advanced clinical candidates **3a** and **3b** for Parkinson's disease; diagnostic agents for myocardial perfusion imaging **7** (approved and in trials for sickle cell anemia and ischemic conditions) and **8** (clinical candidate). B. The helical bundle of Family A GPCRs defines a cavity for the recognition of diverse ligands. The phospholipid bilayer is not shown. Figure courtesy of Stefano Costanzi (American Univ., Washington, DC). C. Historical progression of knowledge of the AR binding site(s). [19 , 20 , 37 , 39 , 41 , 45] Arrows in upper panels indicate the following interactions: Yellow, position of terminal amino group intended for covalent linking [37] ; orange, H-bonding interaction predicted between the exocyclic amine of adenine and a conserved Asn6.55 [39] ; white, proximity of 3′ and 2′-hydroxyl groups with conserved His7.43 predicted using a neoceptor approach [41] . Lower panels: left, predicted docking of agonist 5 using the antagonist-bound X-ray structure [19 , 45] ; right, actual position of agonist 6 in X-ray structure. [20 , 24]

A

B
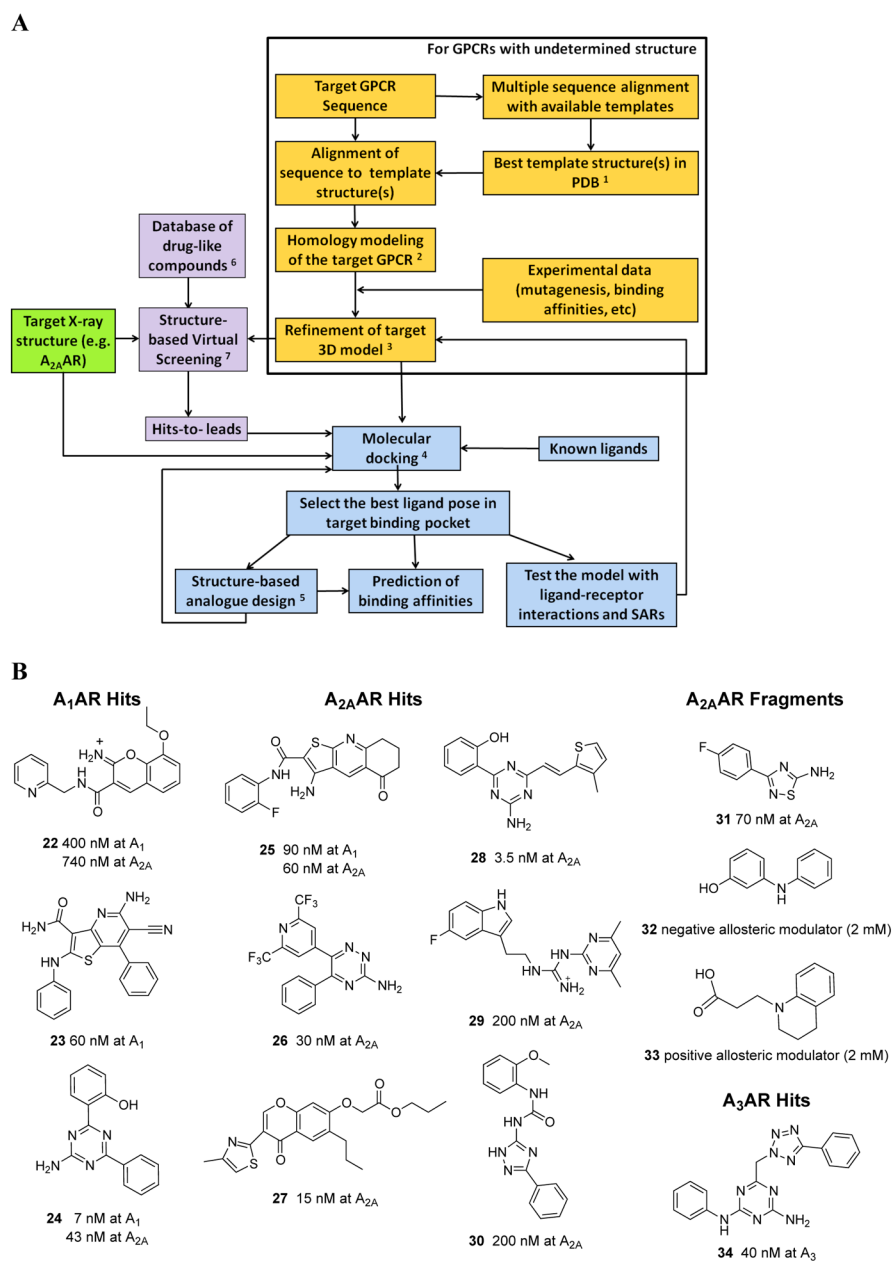
Figure 3 .

A. A flowchart depicting current modeling and docking approaches for closely related GPCR structures (Reprinted from ref. 40 , Copyright (2013), with permission from Elsevier). The key to footnotes:

1. Selection of template is based on sequence identities, conserved key residues, binding site similarities, consideration of disulfide bridges, and shared structural features.

2. DSModeler, Prime, ICM, and MOE are commercial software packages widely used for homology modeling with the structure-based alignment and the template structure as inputs and root main square deviation (RMSD) of Cα, side chain, and heavy atoms as criteria for homology modeling success.

3. Ligand-supported homology modeling, energy minimization or/and molecular dynamics serve to relax unfavorable contacts.

4. Automated docking in rigid binding site, induced-fit docking, or docking by Monte Carlo simulations may be used. Among the available docking software packages are: AutoDock, DOCK, Glide, GOLD, ICM, and OEDocking. SAR information can help to identify the binding pocket in the receptor.

5. Modification of the lead compounds is based on the property of the binding site to attain binding and specificity for the target protein. Approaches include: fragment addition, fragment replacement, connection of fragments with new scaffolds, and other techniques.

6. Database preparation with several physical and chemical filters, e.g. number of rotatable bonds in the compounds, polar surface area, and application of variations of Lipinski's rule-of-five based on lipophilicity, hydrophobicity, and molecular weight.

7. Structure-based virtual screening (SBVS) with high-throughput docking of compound database to the 3D structure of the target. Glide, Gold, ICM, AutoDock are common docking tools for SBVS. The post-docking analysis for the selection of the hits to be tested includes shape complementarity, cluster analysis, consensus scoring, geometric analysis, and visualization of binding modes."

B. Representative structurally diverse antagonists and fragments that bind to ARs as reported: **22** [26], **23** [55], **24** [58], **25** [55], **26** [21a], **27** and **28** [59], **29** [2?], **30** [123], **31 – 33** [60], and **34** [26].
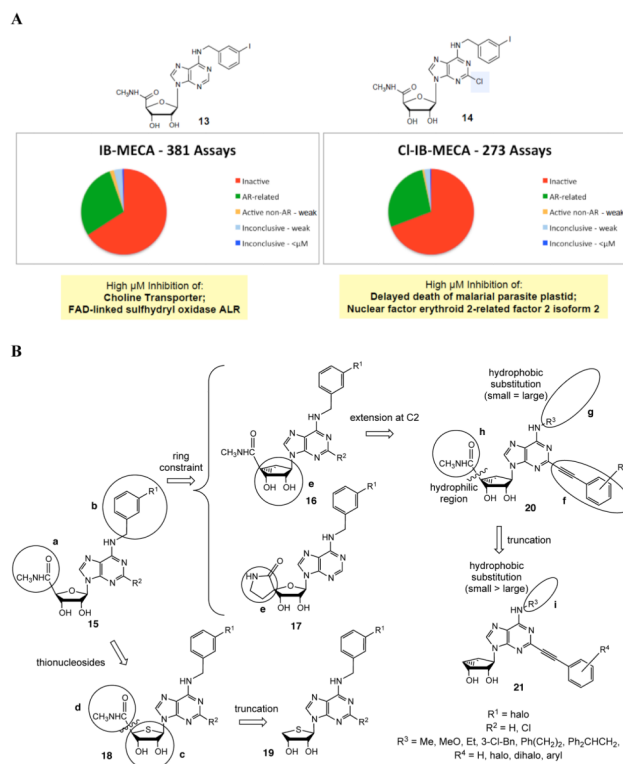
Figure 4 .
A. The structures of two prototypical A $_3$ AR agonists (**13** , IB-MECA, CF101) and cancer (**14** , Cl-IB-MECA, CF102), which are in clinical trials, and a survey of biological activities listed in the PubChem database. These compounds are in clinical trials for a variety of autoimmune inflammatory diseases (**13**) and cancer (**14**). Representative examples of off-target effects at high concentration are given. B. Chronological progression of the key steps in the design of nucleosides as ligands of the A $_3$ AR. The letters labeling specific chemical functionality are described in the text.
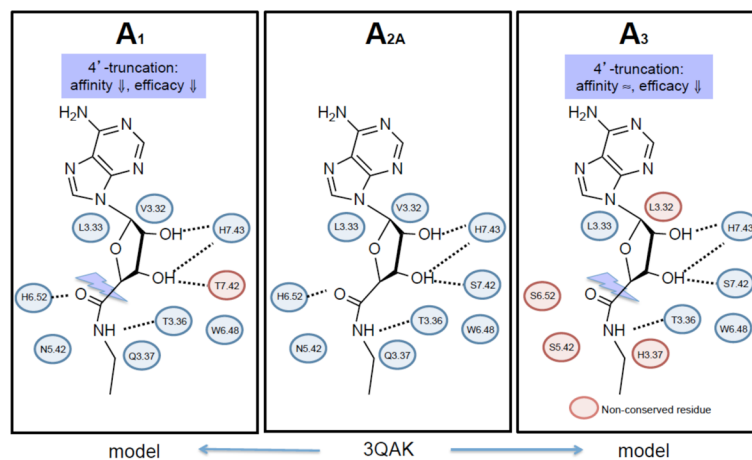
Figure 5 .

A comparison of the amino acids residues lining the subpockets of the ARs that are important for recognition of the ribose moiety. 3QAK is an X-ray structure of the **6** -A $_{2A}$ AR complex, [20] which in this figure is adapted to **5** . The interactions of 3QAK were generalized by homology to A $_1$ AR and A $_3$ AR models (blue residues are conserved), and the general pharmacological effects of truncation are indicated. Many of these residues were detected as important in ligand recognition using site directed mutagenesis prior to the elucidation of the X-ray crystallographic structure.
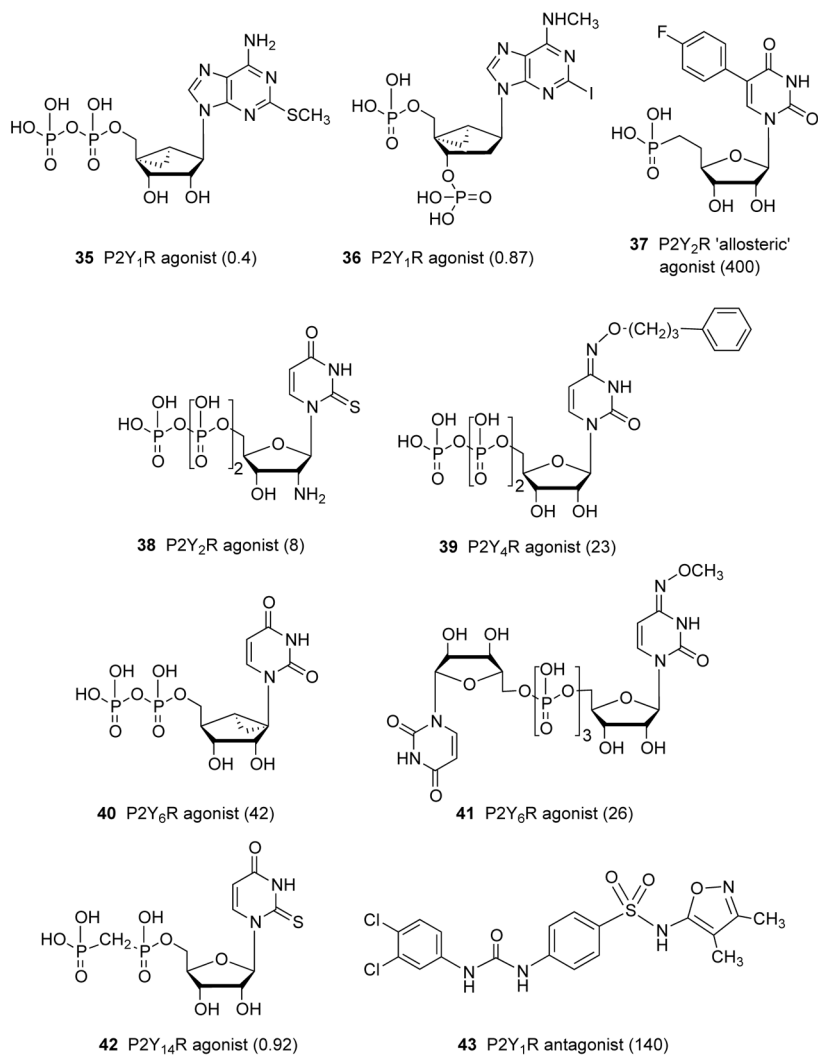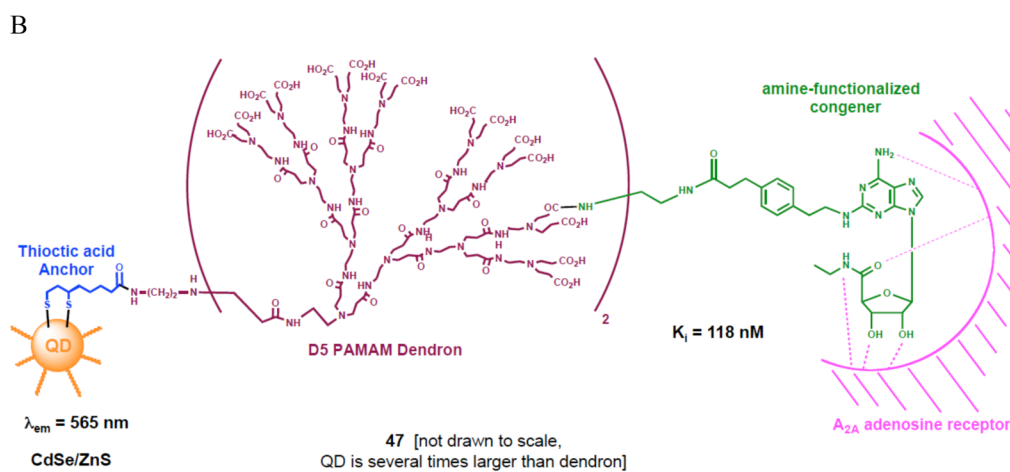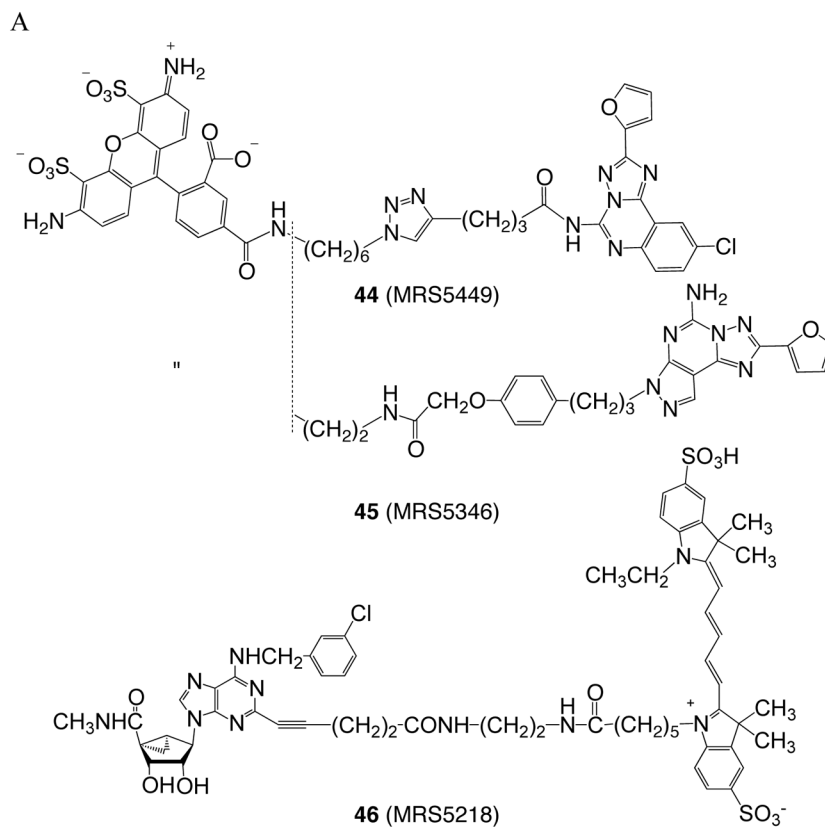
Figure 6 .
Representative P2YR ligands from the NIH laboratory. [6], [80], [88], [89], [115], [118] EC $_{50}$ or
IC $_{50}$ values (nM) at the appropriate receptor are indicated. Partial agonist **37** displays 43%
maximal efficacy. [117]

A

44 (MRS5449)

45 (MRS5346)

46 (MRS5218)

B

amine-functionalized
congener

Thioctic acid
Anchor

QD

λ_{em} = 565 nm

CdSe/ZnS

D5 PAMAM Dendron

K_i = 118 nM

A_{2A} adenosine receptor

47 [not drawn to scale,
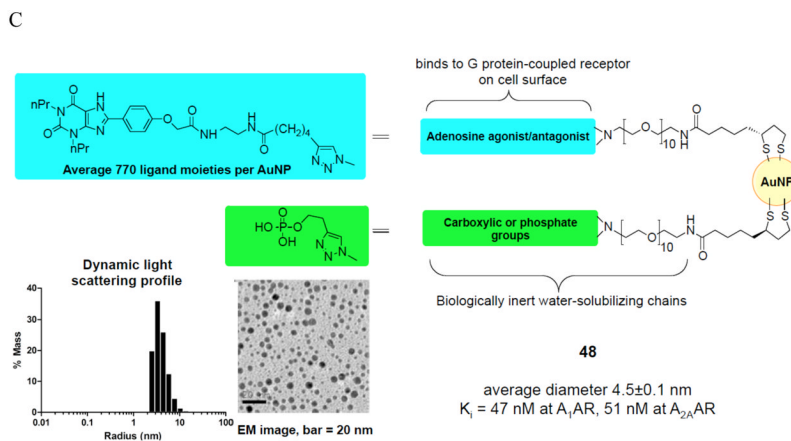QD is several times larger than dendron]

C

Figure 7 .
Specialized GPCR ligands linked to reporter groups or that are covalently bound to carriers in a manner that retains activity. (A) Recently reported fluorescent probes of the ARs, Alexa Fluor 488 conjugates **44** and **45** (antagonists of A 3 and A 2A ARs, respectively) and cyanine dye (Cy5) conjugate **46** (agonist of A 3 AR). [94 , 95] Tethering strategically functionalized AR ligands from quantum dots (B) and gold nanoparticles (C). [99 , 100]