Molecular Mechanisms of Adenosine A_1 Receptor Allosteric Enhancers

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ABSTRACT

Allosteric enhancers of the adenosine A_1 receptor amplify signaling of orthosteric agonist ligands. Allosteric enhancers are appealing drug candidates because their activity requires that the orthosteric site be occupied, thereby conferring specificity to stressed or injured tissues that produce adenosine.

In chapter 2, we explore the mechanism of allosteric enhancer activity. We examine AE activity on several A_1 receptor constructs, including (1) species variants, (2) species chimeras, (3) alanine scanning mutants and (4) site-specific mutants. These findings are combined with homology modeling of the A_1 receptor and *in silico* screening of an allosteric enhancer library. The binding modes of docked allosteric enhancers correlate with the known structure-activity relationship, suggesting that these allosteric enhancers bind to a pocket formed by the second extracellular loop, flanked by residues S150 and M162. We propose a model in which this vestibule controls the entry and efflux of agonists from the orthosteric site, and agonist binding elicits a conformational change that enables allosteric enhancer binding. This model provides a mechanism for the observations that allosteric enhancers slow the dissociation of orthosteric agonists but not antagonists.

In chapter 3, we describe several observations that characterize the mechanisms by which AEs function: (1) Reducing agents such as dithiothreitol (DTT), reduced glutathione (GSSG) and tris(2-carboxyethyl)phosphine (TCEP) can completely block and slowly ($t_{½}$ = 10 min) reverse AE activity without chemically modifying AEs;
(2) Mutations occluding an A_1R disulfide bond pocket (C80-C169) reduce AE activity;

(3) Hydrogen peroxide elicits a resistance to GTPγS-indiced decoupling, similar to AEs; (4) compound screening of disulfide oxidizing agents revealed that aryl disulfides have AE activity; and (5) mutations rendering the disulfide more accessible introduce engineered AE sensitivity to the AE-insensitive $A_{2A}R$. Evaluation of protein structures reveals this disulfide region may be dynamic upon ligand binding. AE binding may prevent this change in conformational states. Chapter 2 identifies an AE binding pocket in ECL2. Chapter 3 suggests that AE activity is derived from a second, independent site: a pocket near the C80-C169 disulfide bond connecting ECL1 and ECL2.

DEDICATION AND ACKNOWLEDGEMENTS

I would like to dedicate this dissertation to my family, whose support has made this dissertation possible.

My wife, Natalie Kennedy (née Negrey), Ph.D. (GSAS ’09, ’12) has been a profound influence over the last five years. She has never paused in her support of me, whether analyzing or critiquing (and ultimately strengthening) arguments, being a reliable companion and inspiration, helping paint the house, or ensuring we move to a place where yard work is not an option.

My immediate family has also been very supportive. My parents, Penny Kennedy and Ronald Kennedy have always encouraged my success and given me the tools to be successful. Combined with my sister, Sarah Norton, Ph.D. (Virginia Commonwealth University, 2012), my family has been a consistent set of role models, ultimately reminding me to work hard, do my best and not take myself too seriously. A unique component of the inspiration I derive from my family comes in the form of my nieces Kendall and Lindsay Norton, whose infant curiosity reminds me why I was originally enamored with science.

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Chapter 1:

Introduction

Abstract

G protein-coupled receptors (GPCRs) are attractive drug targets due to their recognition of diverse ligands and their ability to induce changes to intracellular signaling. Allosteric ligands bind outside of the orthosteric, or endogenous ligand, binding site and have the potential to instill receptor subtype specificity and other clinical benefits. Allosteric enhancers (AEs) amplify adenosine A_1 receptor (A_1R) orthosteric agonist ligand binding and signaling, a process dependent upon occupation of the orthosteric site. This property likely permits AEs to specifically target tissues containing or actively releasing adenosine ((2*R*,3*R*,4*S*,5*R*)-2-(6-amino-9*H*-purin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol) in clinical settings. Chemically, AEs are 2-aminothiophenes or the later discovered 2-aminothiazoles. Since the discovery of 2-aminothiophene AEs 23 years ago, several discoveries have enlightened the unique mechanism by which AEs confer increases in efficacy, but not potency, to orthosteric agonists.

Introduction

Chemical compounds acting as allosteric modulators were first proposed by Jacques Monod, who in 1963 theorized that natural selection would have likely evolved mechanisms of allosteric modulation (1). Today we know several ions, proteins and small molecules act as allosteric modulators in sensing and feedback signaling (2). Allosteric agents elicit these effects through a diverse array of sites and mechanisms and can be generally characterized by their function, as most commonly act to inhibit or potentiate

target activity. Accordingly, they are classified as “negative” or “positive,” respectively. Allosteric agents have a preferable clinical profile, displaying saturable effects that can reduce overdose, and endogenous ligand dependence that can induce site and event specificity.

Several allosteric modulators are currently clinically available. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and maraviroc (3) are currently used to combat HIV. Another allosteric modulator, cinacalcet is a calcium-mimetic(4). The benzodiazepines and barbituates are central nervous system depressants used clinically as sedatives, hypnotics, anxiolytics, and anticonvulsants.

Allosteric Modulation of GPCRs

The study of allosteric modulators for clinical indications has become increasingly popular in the last decade as researchers and clinicians identify new mechanisms of activity and therapeutic advantages.

Muscarinic Receptors

The five muscarinic receptor subtypes (M_1-M_5) can influence several biological processes and are thought to be potential therapeutic targets. For example, activation of the M_1 subtype is thought to be a potentially beneficial for conditions including schizophrenia and Alzheimer’s disease (5). Until very recently, pharmacologists and chemists relied on subtle differences in the conformation of the muscarinic receptor orthosteric binding pocket to introduce selectivity. However, due to the high sequence

identity and nearly uniform topology of muscarinic receptor orthosteric pockets, compounds targeting this site exhibited adverse effects attributable to off-target activation of the M_2 and M_4 subtypes (6).

Allosteric modulators targeting muscarinic receptors deliver subtype specificity (6,7). Specifically, TBPB [1-(1′-2-methylbenzyl)-1,4′-bipiperidin-4-yl)-1***H***-benzo[***d***]imidazol-2(3***H***)-one] preferentially targets the M_1 receptor through an allosteric site (5), and the M_1 receptor has at least two allosteric sites (8,9). More broadly, GPCR subtypes must retain a certain degree of topological conservation to bind the same orthosteric ligand. Thus, nearly all GPCRs are candidates that can benefit from allosteric modulation.

Site of Activity

Two muscarinic receptor allosteric sites have been proposed. Mutagenesis experiments have identified residues in the second extracellular loop (ECL2) and the amino terminus of TM7 that affect allosteric ligand activity in the muscarinic M_2 acetylcholine receptor. These residues include E172, D173, E175 and Y177 in ECL2 and N419, W422 and T423 in TM7 (10). The role of ECL2 residue Y177 plays in allosteric modulation has been particularly well studied: chimeric M_2/M_5 muscarinic receptors and point mutations identified that Y177A reduced potency of negative allosteric modulators (11-13).

Adenosine Receptors (ARs)

Of the four AR subtypes, A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R, allosteric modulators have been identified for all except the $A_{2B}R$. $A_{2B}R$ has not been well evaluated by virtue of not having a suitable agonist radioligand to develop screening assays.

Sodium modulates the activity of several GPCRs, including A_1R, $A_{2A}R$ and A_3R (2). The sodium allosteric site was recently confirmed in a high resolution $A_{2A}R$ crystal structure (PDB ID: 4eiy), residing under the orthosteric pocket in the receptor core (14). Sodium ions and amiloride analogues comprise all known $A_{2A}R$ allosteric modulators (15), and the sodium site has also been found to be the binding site of $A_{2A}R$ (16,17) and A_3R-targeting (15,18) amilorides. Amiloride derivatives are the most ubiquitous and best characterized GPCR allosteric agents, with targets including: A_1R, $A_{2A}R$, A_3R, adrenoceptors α_1, α_{2A}, α_{2B} and dopamine D_2 receptor (2,19,20). However the non-selective nature of amiloride derivatives precludes their clinical applicability.

Like many allosteric agents, those targeting the ARs require endogenous ligand to function. Such endogenous ligand-dependent agents potentially contain two additional clinically beneficial properties: site- and event-specificity. When endogenous ligands are not uniformly present, allosteric modulators dependent on endogenous ligand can act in a site-specific manner, effectively reducing the risk of off-target effects. Only locations with endogenous ligand present will be sensitive to the allosteric modulator. In physiological or pathophysiological events that increase the concentration of endogenous ligands, allosteric modulators will target receptors only during the period of increased endogenous ligand concentration. For example, adenosine is released locally as a result of

injury and is rapidly degraded. Allosteric modulators targeting ARs will selectively target receptors in this area, but only for the duration of the event (21).

Molecular Design and Discovery of AR Allosteric Modulators

Chemical Classes and Structure Activity Relationships of A_1R AEs

In 1990, 2-aminothiophene compounds were discovered to increase specific binding of ^{3}H-cyclopentyladenosine to A_1R (**Figure 1**)(22). An initial structure activity relationship (SAR) study published alongside the characterization of AE activity demonstrated that 2-amino-3-benzoylthiophenes are best optimized for activity (23). The SAR study posits that a hydrogen bond forms between the 2-amino and 3-benzoyl group, and switching that bond to interact with the receptor is a possible mechanism of AE activity.This study also demonstrates that the 1-position sulfur, along with the 2-amino and carbonyl of the 3-benzoyl group constitute an essential hydrophilic region (**Figure 1d**). Building upon these findings and theories, Bruns, et al. discovered that hydrophobic groups built off the thiophene 3-position increase activity, culminating in the identification of PD 81,723 as prototypical AE (23). PD 81,723, a 2-aminothiophene with trifluromethyl meta-substituted on the benzoyl group, and methyl groups at the 4- and 5-position (**Figure 1a**), is now the canonical AE and the standard of comparison.

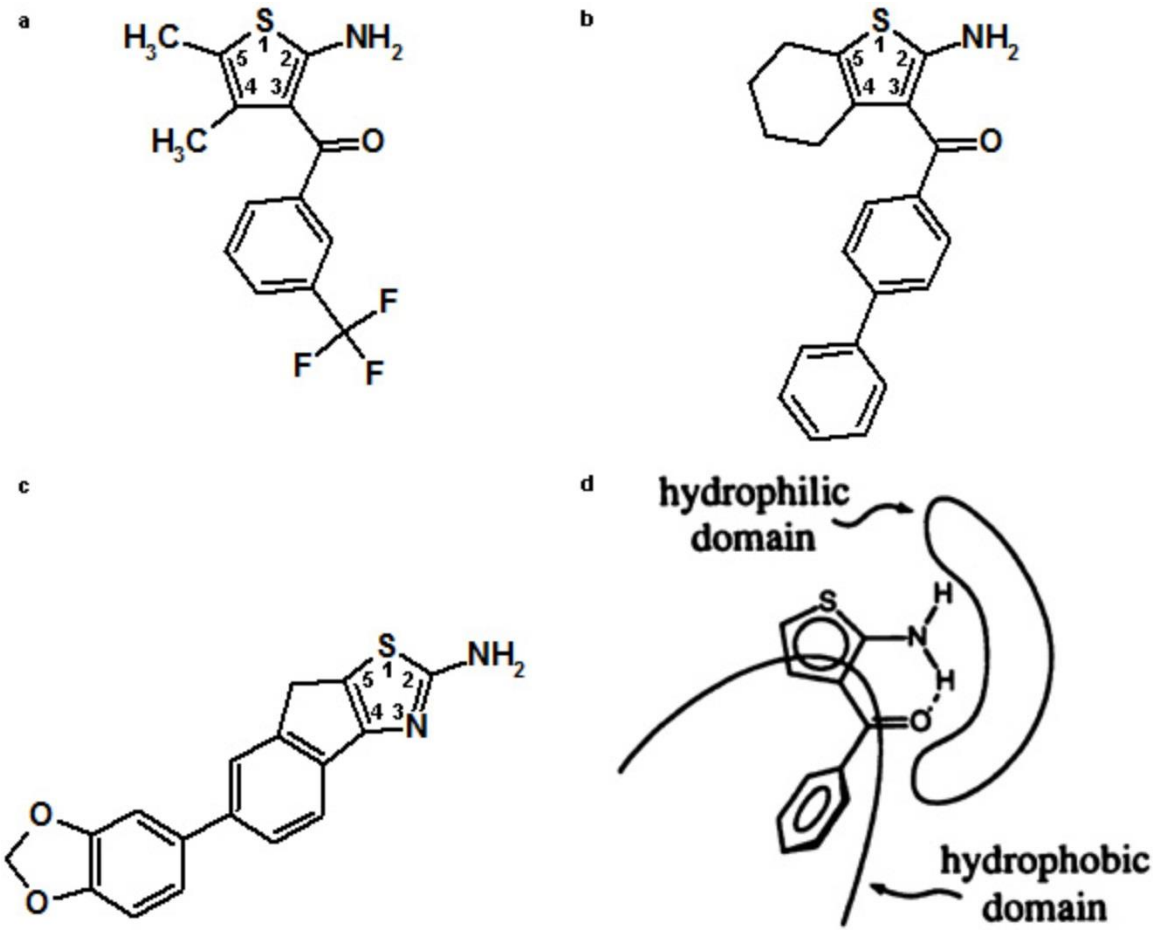


Figure 1: Chemical structures of AEs (a) PD 81,723, (b) ATL525 and (c) 1-277. (d) Predicted 2-aminothiophene hydrophobic and hydrophilic domains. Republished from (23). Numbers indicate positions in 2-aminothiophene and 2-aminothiazole rings.

Subsequent studies have sought to determine the biological and chemical determinants of AE activity. Subsequent investigations of AE molecular development evaluated the 3-, 4- and 5-positions of the thiophene ring, as any alteration to the 1- or 2-positions greatly reduced activity. Substituting halides directly on the 3-benzoyl aromatic ring increased activity, and introducing a ring connecting the thiophene 4- and 5-positions increased activity (24) (**Figure 1b**). Combining these traits resulted in molecules with increased potency (24,25). These findings were soon extended to provide new insights into the mechanism of AE activity when a directly proportional relationship was discovered between 4-5 ring size and AE activity was discovered (26).

After identifying the optimal substitutions on the 2-aminothiophene ring, 2-aminothiazoles were identified as a novel class of AEs. 2-aminothiazoles lack the 3-benzoyl group entirely, replacing the 3-position carbon with nitrogen (**Figure 1c**). Functional groups must be added at the 4-5 positions of 2-aminothiazoles, as the 1-position is an essential sulfur atom, the 2-position is substituted with an essential amino group and the 3-position is a nitrogen atom (27,28). 2-aminothiazoles were discovered to possess higher affinity, and called into question the 2-amino-3-benzoylthiophene AE pharmacophore. However, as the 2-amino-3-benzoylthiophene electron-rich carbonyl group was replaced with an electron-rich nitrogen atom in 2-aminothiazoles, the chemical properties of the compounds remain spatially consistent. SAR and electron density modeling revealed that a 5-member, sulfur-containing ring, with a 2-amino group and high electron density near the 3-position is the AE pharmacophore (28). However, molecules lacking large 3-, 4- and 5- substitutions were shown to have very poor activity,

despite retaining the hydrophilic pharmacophore, indicating the hydrophobic domain is also essential (23-25).

Allosteric Modulation of A_3R

Allosteric modulators targeting A_3R have been identified to increase binding of agonist radioligand. While several chemical classes of positive allosteric modulators (PAMs) targeting A_3R have been identified, two prototypical chemical classes have been identified: 3-(2-pyridinyl)isoquinolines and1H-imidazo-[4,5-c]quinolin-4-amines. Other A_3R chemical classes include 2,4-disubstituted quinolones, endocannabinoid 2-arachidonylglycerol and Brilliant Black BN. Several structure-activity relationship studies have informed the molecular design and therapeutic development of A_3R PAMs (29). While specific residues in several receptor domains have been implicated in A_3R PAM activity, no studies have demonstrated the molecular mechanism or binding site of A_3R PAMs (29).

Physiology and Pharmacology of A_1R AEs

Physiological Investigations of the Mechanisms of AE Activity

When first discovered, AEs were noted for their ability to increase the absolute binding of orthosteric agonists, resulting in an increase in intracellular signaling efficacy (22,30). Since then, the mechanism of this effect has been informed. AEs stabilize the active, agonist- and G protein-bound conformation of the receptor, experimentally demonstrated by increased ^{35}S-GTPγS (guanosine 5-[γ-thio]triphosphate; an R-G complex decoupling

agent) binding to the receptor for a given concentration of agonist and an increase the amount of agonist radioligand (^{125}I-ABA; ^{125}I-N^6-4-aminobenzyladenosine) bound at a given GTPγS concentration (30). If the increase in agonist binding was due to general receptor stabilization, both agonist K_d and B_{max} would be positively modulated. However, Figler, et al. demonstrated that AEs do not alter agonist K_d and that agonist k_{on} is slowed by AE. As $K_d = k_{off}/k_{on}$, they were able to conclude that AEs increase the k_{off} of agonists (30). With the kinetic and physiological mechanisms of AE activity delineated, several experiments sought to enumerate specific residues and receptor domains necessary for AE activity.

The first two mutations identified to render A_1R insensitive to AEs, T277A in TM7 and G14T in TM also substantially affected orthosteric ligand binding. T277A also has a profound impact on agonist binding and G14T stabilizes the receptor active state (31-33). Due to the effects on agonist binding, these residues are not thought to be components of the allosteric binding site.

Subsequently, more comprehensive mutagenesis strategies were employed to identify A_1R domains essential for AE activity. Swapping the domains responsible for interacting with G proteins (the third intracellular loop (ICL3) and the C-tail) between AE-sensitive A_1R and relatively AE-insensitive $A_{2A}R$ creates two informative chimeras. The first is A_1R with an $A_{2A}R$ ICL3 and C-terminus. These receptors are AE sensitive but couple to $A_{2A}R$ partner $G_{\alpha s}$. The second chimera is AE-insensitive, $A_{2A}R$ with A_1R ICL3 and C-terminus. This receptor coupled to A_1R G-protein partner $G_{\alpha i}$ (34). While these

chimeric replacement experiments did not alter AE sensitivity or identify a binding domain, they preclude ICL3 and the C-tail as binding site components.

More recently, in addition to mutagenesis, researchers have explored "bitopic" ligands – orthosteric and allosteric pharmacophores tethered together – to delineate allosteric binding sites. In A_1R, such a study identified the ideal radius between the orthosteric and allosteric sites as the length of a 9-carbon chain (35). Structural modeling was used to interpret these experiments and to deductively identify ECL2 as a potential site of AE binding in A_1R. These models demonstrated that ECL2, as the largest ECL, is most capable of accommodating AE within a 9-carbon radius of the orthosteric binding site (35). Ultimately, neither this scheme, nor mutagenesis, was able to delineate specific residues, a specific AE binding site or mechanism of activity.

As a result of the experimental difficulties encountered in identifying the AE binding site, many investigations have employed mathematical modeling to identify protein domains necessary for activity (19,36-38). Mathematical models have helped interpret several possible ligand-receptor binding behaviors, with authors concluding the A_1R allosteric site is likely along the path followed by a ligand to reach the orthosteric site (39). Likewise, transition state modeling determined that allosteric modulators follow a concerted, or MWC allosteric mechanism (36,37). While unable to identify specific residues or protein domains, knowledge of the mathematical possibilities substantially informs the search for potential molecular mechanisms of AE activity in ways experiments frequently cannot.

Molecular Mechanisms of GPCR Activation and AE Activity

Understanding the molecular mechanisms of orthosteric agonist binding and receptor activation can assist the clinical development of AEs by delineating potential mechanisms of AE agonist dependence and receptor-G protein coupled conformational stability. A recent molecular dynamics simulation study predicts that ligands pass through a predocking vestibule between ECL2 and ECL3 to enter the orthosteric binding pocket of the β_2 adrenergic receptor (40). Although there are no known adrenergic receptor allosteric modulators, these authors proposed the predocking vestibule as a potential location for GPCR allosteric modulation.

Recently, we proposed a model in which a possible A_1R homolog of this vestibule controls the entry and efflux of agonists from the orthosteric site. Our model of this site suggests that it is flanked by human A_1R residues S150 and M162 (**Figure 2**). Included in the modeled bonding pose is a hydrogen bond between S150 and the 2-amino group on the thiophene ring, consistent with the previous SAR findings that the 2-amino group increases AE activity. Our structural modeling has been greatly informed by the recent publication of several GPCR X-ray crystal structures, nuclear magnetic resonance (NMR) and other biophysical studies identifying specific mechanisms of GPCR activation (41-44).

For example, we evaluated agonist- and antagonist-bound $A_{2A}R$ structures and observed that agonist binding elicits a conformational change that may enable AE binding to the ECL2 pocket. Importantly, this conformational change may result in the

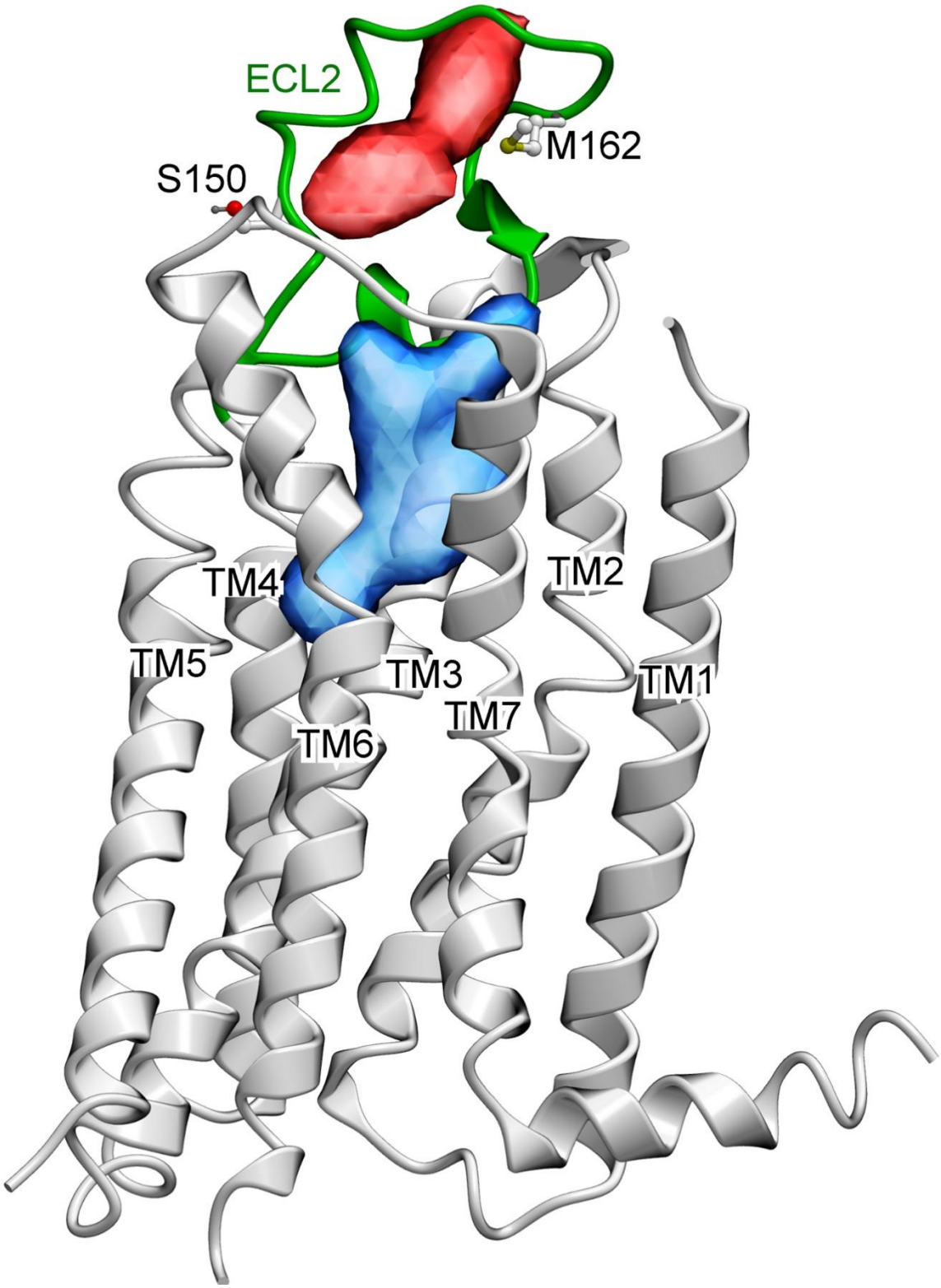

ECL2
M162
S150
TM4
TM2
TM5
TM3
TM1
TM7
TM6

Figure 2: hA_1R homology model based on the high-resolution crystal structure of $hA_{2A}R$ (PDB ID: 3qak): backbone (grey), ECL2 (green). Residues S150 and M162, identified as being involved in AE signaling by site-directed mutagenesis in ECL2 (green) are shown as sticks. Ligand binding pockets were identified using ICM PocketFinder, including the orthosteric site (blue surface) and a pocket in ECL2 large enough to accommodate hA_1R AEs (red surface).

appearance of the binding site only upon agonist binding. These findings allowed us to generate a model that provides a mechanism for the observations that AEs slow the dissociation of orthosteric agonists but not antagonists.

Our proposed AE binding mode also explains other known AE properties, including the molecular structure. The 4- and 5- positions of the thiophene ring extend over the orthosteric binding site, which we theorize acts to trap the orthosteric ligand in place, delineating a mechanism that would follow the observed effects of increasing B_{max}, but not altering the K_d of orthosteric agonist (30). The SAR-determined large hydrophobic domain off the 3- position of the thiophene ring appears to be stabilized in the hydrophilic extracellular space by several hydrophobic ECL2 residues which form a pocket around the AE, while the site is within the 9-carbon chain radius proposed by the bivalent ligand experiments (35).

Previous studies have implicated ECL2 as an AE binding site. In addition to the bitopic ligands (35), another study concluded that mutation of ECL2 residues W156 and E164 in A_1R modified activity of PD 81,723 (45). The case for an AE binding pocket in ECL2 is further bolstered by data suggesting ECL2 is an important mediator of ligand binding in the glucagon-like peptide-1 receptor (GLP-1R). These authors demonstrated that ECL2 regulates orthosteric agents, but not GLP-1R allosteric modulators (46).

Cumulating the results of these experiments suggest that the computationally predicted predocking vestibule (40) may be conserved between several GPCRs. However, the vestibule may have slightly different positions and functions between different GPCRs, acting as a predocking site for orthosteric ligand between ECL2 and

ECL3 in adrenergic receptors, but defined more by ECL2 in GLP-1R. Likely, A_1R and the M_2 muscarinic receptor are not the only GPCRs subject to ECL2-mediated allosteric modulation.

Clinical Indications of A_1R AEs

Several physiological studies have been conducted *in vivo* and *ex vivo* to evaluate the clinical relevance of AEs. The first animal experiments conducted with AEs demonstrated that AEs increase the S-H interval, slowing the heart rate of hearts treated *in situ* and isolated from guinea pigs (47). Successive experiments on rat atria demonstrated that PD 81,723, coadministered with agonist CPA (N^6-cyclopentyladenosine), resulted in a more potent chronotropic and inotropic effect than CPA alone (48). These effects were confirmed on isolated guinea pig hearts, demonstrating that AEs enhance the A_1R-mediated effects of adenosine on S-H interval, but do not impact the $A_{2A}R$-mediated effect on coronary dilation (49,50).

Several studies have evaluated the effects of AEs on ischemic preconditioning (IPC). IPC is a phenomenon where repeated periods of transient ischemia protect tissues from subsequent, prolonged ischemia, and was attributed to be an A_1R-mediated event at about the same time AEs were first introduced as potential new drug candidates (51). The first of these study evaluating the effects of AEs on IPC determined that PD 81,723 did not enhance cerebral IPC in the gerbil (52). However, also using PD 81,723, a separate group determined that AEs reduced the IPC threshold in dogs (53). Despite this early

work evaluating the potential benefits of AEs in IPC and other cardiac conditions, clinical development of AEs has focused on other indications.

Recently, a phase II clinical study (www.clinicaltrials.gov ID: NCT00809679) evaluating the analgesic efficacy and safety of AE T-62 for subjects with postherpetic neuralgia was conducted, marking the first clinical trial of an allosteric modulator targeting ARs. The study was terminated as some subjects experienced asymptomatic, transient elevation of liver transaminases. Notably, transaminase elevation is frequently specific to chemical compounds, not drug classes. For example, nonsteroidal anti-inflammatory drug (NSAID) sulindac accounts for most NSAID transaminase elevation (54). The T-62 trial did not report cardiovascular complications, retaining the potential for future AE therapeutics.

Clinically administered AEs may offer several advantages, including capitalizing on the rapid physiological degradation of adenosine. As a result of degradation, adenosine does not travel far from the location of release, such as an injury, further enabling AE site- and event specificity. A final potential benefit of AEs is the prospect that they will distribute favorably compared to A_1R agonists. A_1R agonists are generally derived from adenosine, but AEs are chemically distinct, enabling them to access body locations adenosine-derived compounds do not (55). These findings and theories, if proven, combine to suggest AEs will be important future clinical candidates.

Concluding Remarks

AEs retain the potential to be beneficial therapeutics as they are thought to possess several clinically beneficial traits including subtype specificity, decreased risk of overdose and adverse events, as well as specificity for sites and pathophysiological events. However, several concerns remain to be alleviated. Chiefly, effects on cardiac pacing may preclude development for non-cardiovascular indications, or may prevent use in patients with cardiac or inflammatory conditions. Some of these fears have subsided with the conclusion of the first phase II AE clinical trial, which did not report cardiovascular complications. A second factor that has slowed clinical development of AEs has been the lack of a known binding site, such that new chemical compounds could be developed to reduce the current micromolar potency of the best AEs. The recent discover of the AE binding site holds great implications for the molecular mechanism of action: sterically trapping agonist in the orthosteric binding pocket. This site may only be revealed by the agonist-bound receptor conformation. Combined, these findings hold the potential to improve the AE pharmacological profile by enabling the design of highly potent chemical compounds with reduced risk of contraindications. With the advent of such compounds, the therapeutic implications of AEs are significant. For the first time, a drug could be administered and be distributed throughout the entire body, but only elicit effects at a specific location or point of injury.

Abbreviations

Adenosine	(2*R*,3*R*,4*S*,5*R*)-2-(6-amino-9*H*-purin-9-yl)-5-(hydroxymethyl) oxolane-3,4-diol
A_1R	adenosine A_1 receptor
ARs	adenosine receptors
^{125}I-ABA	^{125}I-N^6-4-aminobenzyladenosine
AE	Allosteric enhancer
CPA	N^6-cyclopentyladenosine
ECL	Extracellular loop
GLP-1R	glucagon-like peptide-1 receptor
GPCR	G protein-coupled receptor
GTPγS	guanosine 5-[γ-thio]triphosphate
ICL3	third intracellular loop
IPC	ischemic preconditioning
NMR	nuclear magnetic resonance
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NSAID	nonsteroidal anti-inflammatory drug
PAM	positive allosteric modulator
R-G complex	receptor-G protein complex
SAR	structure-activity relationship
TM	transmembrane domain

Chapter 2:

The Second Extracellular Loop of the Adenosine A_1 Receptor Mediates Activity of Allosteric Enhancers

Introduction

G protein-coupled receptors (GPCRs) are expressed throughout the body and regulate a broad range of physiological actions through transmembrane signaling and coupling to heterotrimeric G proteins (56,57). As a result, GPCRs are the most targeted protein class in modern therapeutics (58). However, only a small fraction of known GPCRs have been targeted, leaving much room for new drug development through reverse pharmacology.

Allosteric modulators of GPCRs bind outside the conventional orthosteric ligand-binding site and elicit either a negative (negative allosteric modulators, NAMs) or positive (positive allosteric modulators, PAMs) effect on transmembrane signaling and receptor coupling. Adenosine receptors (ARs) are a family of GPCRs for the nucleoside adenosine, which consists of four members: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. PAMs of the adenosine A_1 receptor (A_1R) are also known as allosteric enhancers (AEs). A number of AEs have been identified, primarily targeted to the A_1R subtype (22). Herein, we identify the A_1R AE binding site and suggest a mechanism by which these compounds act.

AEs decrease the dissociation kinetics of pre-bound orthosteric agonists, and have no effect on the binding kinetics of orthosteric antagonists (22,30). A prerequisite for AE activity is occupancy of the orthosteric site by an agonist. This property makes AEs appealing as drug candidates because they act selectively in tissues actively releasing adenosine, such as a site of injury. For example, AEs of A_1Rs protect the heart (53), brain (59,60), and kidney (61) from ischemia reperfusion injury, inhibit lipolysis (62,63) and

decrease neuropathic pain (64,65). An additional advantage of AEs is that their selectivity for tissues that generate adenosine may obviate the limitation of A_1R orthosteric agonists, which produce heart block as a dose-limiting side effect.

Identification of the molecular determinants of AE activity has the potential to advance mechanistic studies and clinical development. Mathematical modeling is one approach that has been used to gain mechanistic insight into allosteric enhancers (19,36-38). In general, the models involve simplified systems including the receptor, orthosteric ligand and the allosteric enhancer. Such modeling predicted that the A_1R allosteric site resides along the path followed by a ligand to reach the orthosteric site (39). While unable to identify specific residues or protein domains, the mathematical models provide guidance for the design of experiments. Nevertheless, despite 23 years of research since the initial discovery of A_1R AEs (22), a detailed understanding of their mechanism of action remains largely unknown.

Drug development of AEs has also been impeded in part by difficulties in studying their physiological actions *in vivo*. In previous studies, AE activity was reported to vary *in vitro* and *in vivo* among species such as human, mouse, guinea pig (47,50), dog (53), and rat (22). However, many of these investigations used assays that do not distinguish AE activity from competitive antagonist activity, which is also possessed by AE compounds to a variable extent. Consequently, the measured activities were a composite of allosteric and competitive antagonist effects. To obviate this issue, kinetic methods are considered the most sensitive and direct measurement of allosteric modulation of GPCRs (66).

An additional impediment to the drug development of AEs is that their binding sites have not been precisely determined. GPCRs possess seven transmembrane domains, three intracellular and three extracellular loops, an extracellular N-terminus and an intracellular C-terminus. Residues in each of these domains affect allosteric modulation (21,67). In the muscarinic receptors, allosteric sites have been identified in the second extracellular loop (ECL2) (11) and near TM6 and ECL3 (68). However, allosteric sites are not necessarily conserved between GPCR subfamilies so that allosteric targeting of each receptor is an individual pursuit (66,69).

For adenosine receptors, a study using orthosteric agonists tethered to AEs (so-called "bitopic ligands") suggested that the ECL2 of A_1R may be an AE binding region (35). In addition, a recent study showed that mutation of ECL2 residues W156 and E164 in A_1R modified AE activity (45). Our studies sought to define the AE binding site of A_1R in more detail.

Results and Discussion

Overall strategy

To comprehensively explore the AE binding site in A_1R, we examined the activity of AEs on (1) species variants, (2) species chimeras, (3) alanine scanning mutants and (4) targeted site-specific mutants. To yield more accurate measurements of AE activity, we used a kinetic assay that is not influenced by competitive antagonism (26,30). In addition, our test compound, ATL525, is a highly efficacious AE with limited antagonist activity.

To quantify AE activity, we used a system that "scores" AE activity, with results ranging from 0-100, where 0 represents no effect of the AE on orthosteric agonist dissociation kinetics, and 100 represents equilibrium binding, or no orthosteric agonist dissociation. Reported pharmacological parameters were calculated from curves fit to the raw scores. GTPγS-insensitive binding, such as that scored in this assay, is a unique reporter for AE activity that is minimally affected by ^{125}I-ABA binding affinity. AE affinity, cooperativity with agonist and changes in receptor-G protein coupling are all inter-related in every assay system.

Agonist dissociation experiments using ATL525 allowed us to evaluate the effects of AEs directly on the receptor, not the whole cell, which results in a more precise evaluation of AE binding. Due to the effects of competitive antagonism, the measurement of dissociation kinetics gives the most pure assessment of AE activity. We also determined the EC_{50} of AEs as a measure of AE binding affinity. The EC_{50} is reported as an index of AE affinity, which has never been experimentally reported. All reported mutations were evaluated for changes in GTP sensitivity, and none was observed.

The structural interpretation of our mutagenesis and activity experiments was guided by an A_1R homology model based on the X-ray crystal structure of the agonist-bound $hA_{2A}R$ (PDB ID: 3qak) (70). Further computational analysis used the ICM PocketFinder algorithm (71), and the identified allosteric binding pocket was refined using the Automated Ligand-guided Backbone Ensemble Receptor Optimization (ALiBERO) protocol (72-74). Taken together, these analyses defined an AE binding

pocket in ECL2, and we propose that AEs function by occupying this vestibule and blocking agonist dissociation from the high affinity (R*) state of the receptor.

AE activity does not correlate with overall sequence identity between species

We first compared A_1R sequence variability between a number of species with the relative activities of the potent AE, ATL525, which lacks antagonist activity (**Figure 1**, inset) (30). Experiments on the dissociation kinetics of orthosteric agonist revealed a range of AE activities among these species (**Figure 1**). Agonist dissociation from the rhesus monkey A_1R is the slowest, followed by human and chicken, with dog, rat and mouse being the least affected by ATL525. The A_1R sequence variability, relative to monkey, are: human: 0.3% (1 residue of 327), mouse and rat: 4.9% (16/327); dog: 6.1% (20/327); and chicken: 19.4% (63/325). The order of AE activities does not correlate with overall sequence variability. This finding suggests that AE activity is governed by specific amino acids in discrete binding or signaling domains.

Chimeric mutagenesis indicates that residues 150 and 162 mediate AE activity

Since AE activity varies among species, we created species chimeras to identify residues responsible for AE activity differences. Analysis of the sequence alignments between species revealed that the ECL2 (residues 147-175) and the C-terminus (residues 291+) regions of the protein contain the highest sequence variability (**Figure 2A**). On the basis of previous studies of receptor species chimeras, the C-terminus and third

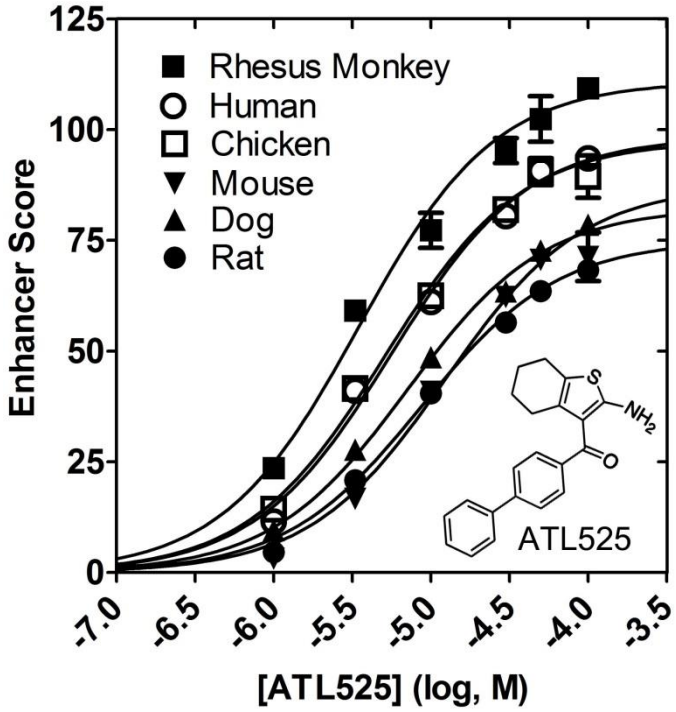


Figure 1. Variation in interspecies AE activity. Enhancer activity score (0-100) among species is plotted against concentration of ATL525; (■) rhesus monkey, (○) human, (□) chicken, (▼) mouse, (▲) dog, (●) rat. Each point is the mean ± SEM. *Inset:* chemical structure of ATL525.

intracellular loop are not involved in AE activity (34). However, as noted above, there is evidence that AEs bind to ECL2 (35), and hence, we focused our attention on this region.

Excluding the chicken A_1R, only ECL2 residues 147-162 are variable among species, and we created a set of chimeras between the human and dog receptors spanning this region (**Supplemental Table 1**). Our experiments identified two human to dog mutations that reduced the activity of AEs on hA_1Rs to that of dog A_1R: S150G and M162G (**Figure 3B**). When compared to hA_1R, activity on the dA_1R is decreased by 17.7 ± 1.3 enhancer score points. AE score on the hA_1R-dECL2 chimera decreases by 29.6 ± 0.34 ($p < 0.0001$), S150G decreases by 27.9 ± 0.88 ($p < 0.001$), and M162G decreases by 16.9 ± 2.2 ($p < 0.01$) enhancer score points.

The species differences in AE activity are not solely due to these two residues, since introducing the reciprocal mutations in dog A_1R did not restore activity. To further investigate this result, we built a homology model of hA_1R based on the crystal structure of agonist-bound $hA_{2A}R$ (PDB ID: 3qak) (70). In the hA_1R homology model, residues 150 and 162 reside at opposite sides of ECL2, potentially defining the boundary of a binding site (**Figure 3A**).

Alanine Scanning of ECL2 confirms that residues 150 and 162 mediate AE activity

To further define the role of ECL2 in AE activity, we conducted an alanine scan, in which blocks of four consecutive residues from all three hA_1R ECLs were mutated to alanine (**Figure 2B** and **Supplemental Table 2**). In particular, these experiments were designed to identify conserved residues between dog and human A_1R that alter AE

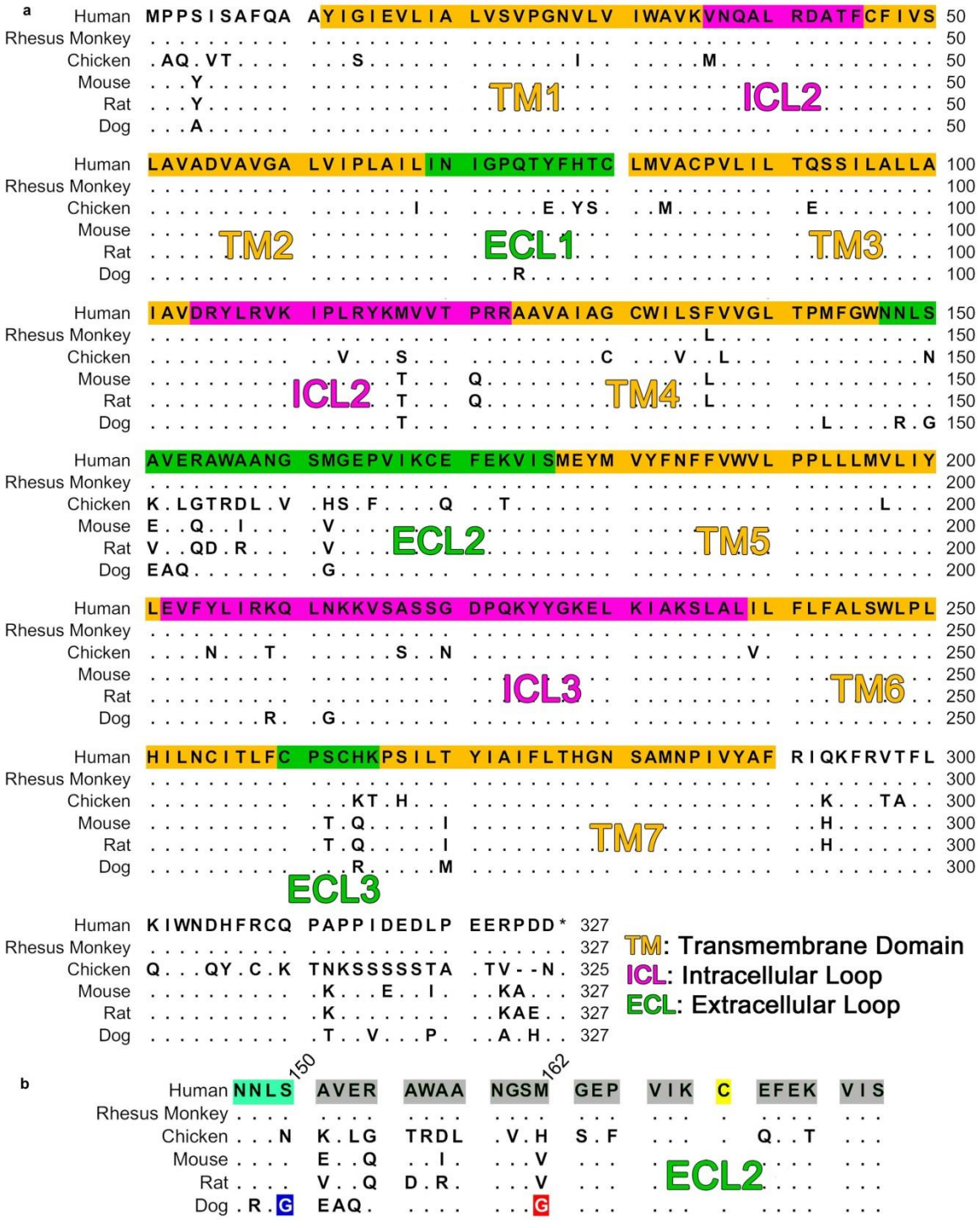

a
Human MPPSISAFQA AYIGIEVLIA LVSVPGNVLV IWAVKVNQAL RDATFCFIVS 50
Rhesus Monkey 50
Chicken .AQ.VT.... ...S...... ....I.. .....M.... 50
Mouse ...Y...... 50
Rat ...Y...... 50
Dog ...A...... 50
TM1
ICL2
Human LAVADVAVGA LVIPLAILIN IGPQTYFHTC LMVACPVLIL TQSSILALLA 100
Rhesus Monkey 100
Chicken ....I.. .....E.YS. ..M....... .E........ 100
Mouse .....TM2... ....ECL1... ....TM3... 100
Rat .....TM2... ....ECL1... ....TM3... 100
Dog ....R...... 100
Human IAVDRYLRVK IPLRYKMVVT PRRAAVAIAG CWILSFVVGL TPMFGWNNLS 150
Rhesus Monkey ....L.... 150
Chicken .....V...S... ....C...V..L... ....N 150
Mouse .....ICL2..T... Q......... .....TM4..L... 150
Rat .....ICL2..T... Q......... .....TM4..L... 150
Dog ....T.... ....L....R.G 150
Human AVERAWAANG SMGEPVIKCE FEKVISMEYM VYFNFFVWVL PPLLLMVLIY 200
Rhesus Monkey 200
Chicken K.LGTRDL.V .HS.F....Q ..T..... ....L... 200
Mouse E..Q..I... .V.....ECL2.. ....TM5.... 200
Rat V..QD.R... .V.....ECL2.. ....TM5.... 200
Dog EAQ..... .G...... 200
Human LEVFYLIRKQ LNKKVSASSG DPQKYYGKEL KIAKSLALIL FLFALSWLPL 250
Rhesus Monkey 250
Chicken ....N...T. .....S..N ....V.... 250
Mouse ....ICL3..... ....TM6... 250
Rat ....ICL3..... ....TM6... 250
Dog .....R... .G...... 250
Human HILNCITLFC PSCHKPSILT YIAIFLTHGN SAMNPIVYAF RIQKFRVTFL 300
Rhesus Monkey 300
Chicken .....KT.H... ....K...TA.. 300
Mouse .....T.Q....I .....TM7..... ....H..... 300
Rat .....T.Q....I .....TM7..... ....H..... 300
Dog .....R....M 300
ECL3
Human KIWNDHFRCQ PAPPIDEDLP EERPDD* 327
Rhesus Monkey 327
Chicken Q...QY.C.K TNKSSSSSTA .TV--N. 325
Mouse .....K...E..I. ..KA... 327
Rat .....K..... ..KAE.. 327
Dog .....T..V...P. ..A.H.. 327
TM: Transmembrane Domain
ICL: Intracellular Loop
ECL: Extracellular Loop
b
150
162
Human NNLS AVER AWAA NGSM GEP VIK C EFEK VIS
Rhesus Monkey ....
Chicken ...N K.LG TRDL .V.H S.F ... . Q..T ...
Mouse .... E..Q ..I. ...V ... ...
Rat .... V..Q D.R. ...V ... ... ECL2 ....
Dog .R.G EAQ. ....G ... ...

Figure 2. **a**, hA_1R amino acid sequence alignment of the species analyzed for AE activity. Dots (·) indicate conserved residues. Dashes (-) indicate gaps. ECL: Extracellular loops, green; ICL: Intracellular loops, magenta; TM: Transmembrane domains, yellow. Residue numbers are indicated at the end of each row for each species. **b**, Summary of ECL2 mutations. Blocks of three and four amino acids denote groups of residues mutated in alanine scans. Positions 150 (blue) and 162 (red) were identified by swapping residues between species.

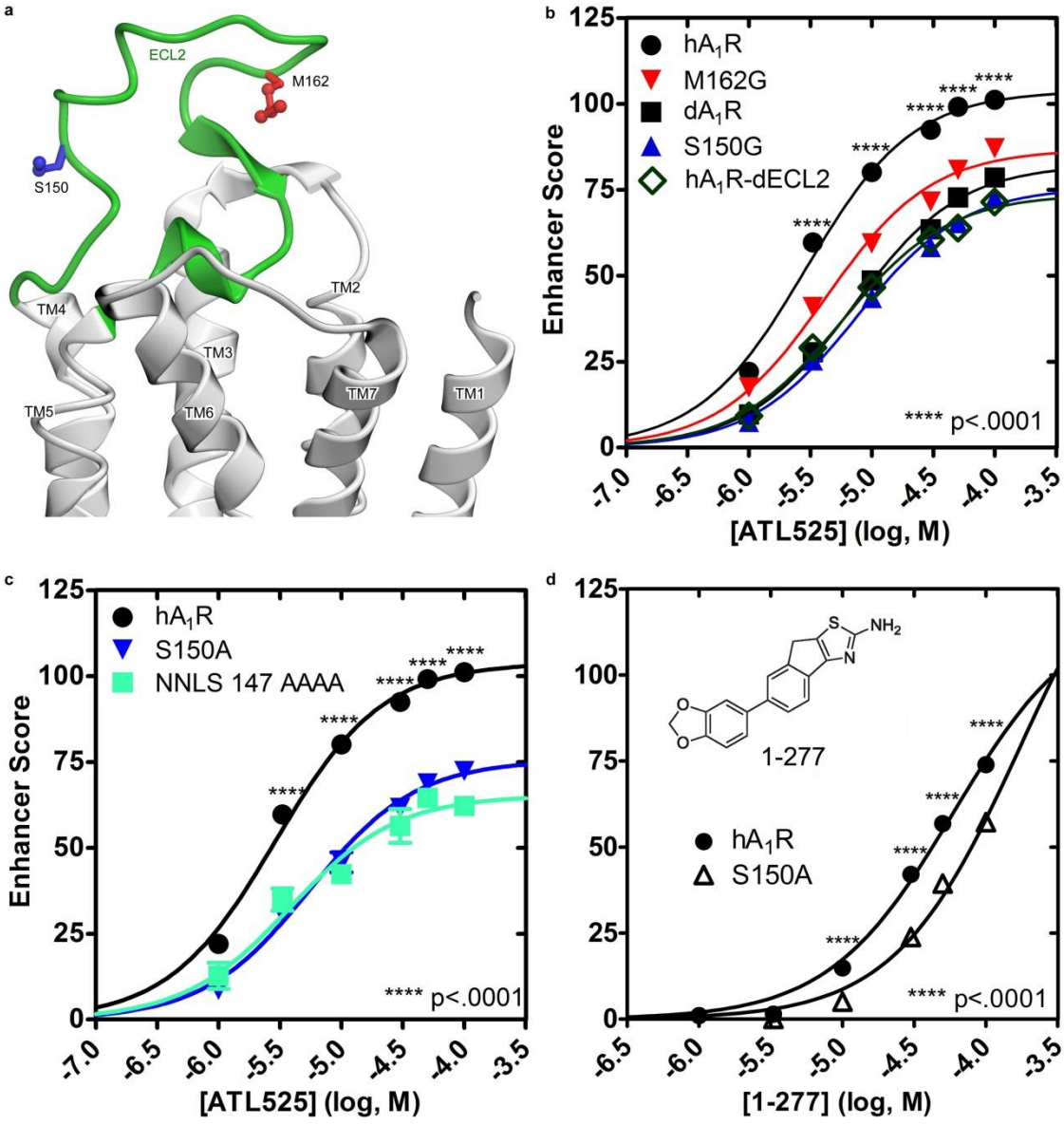

a
ECL2
M162
S150
TM2
TM4
TM3
TM7
TM1
TM5
TM6
b
hA1R
M162G
dA1R
S150G
hA1R-dECL2
Enhancer Score
**** p<.0001
[ATL525] (log, M)
c
hA1R
S150A
NNLS 147 AAAA
Enhancer Score
**** p<.0001
[ATL525] (log, M)
d
1-277
hA1R
S150A
**** p<.0001
[1-277] (log, M)

Figure 3. Mutation of residues S150 or M162 decreases AE activity. **a**, hA_1R homology model based on $hA_{2A}R$ structure (PDB ID: 3qak): backbone (grey), ECL2 (green), S150 (blue), and M162 (red). **b**, ATL525 AE scores (0-100). (●) hA_1R, (▼, red) hA_1R M162G, (■) dog A_1R, (▲, blue) hA_1R S150G, (◊) hA_1R-dECL2 (hA_1R background with dA_1R ECL2 residues). **** $p<0.0001$. **c**, Activity of hA_1R S150A and hA_1R NNLS 147 AAAA compared to hA_1R. (●) hA_1R, (▼, blue) hA_1R S150A, (■, cyan) hA_1R NNLS 147 AAAA. **** $p<0.0001$. Data plotted ± SEM. **d**. AE dose response curves for the 2-aminothiazole, 1-277. (●) hA_1R, (△), hA_1R S150A. **** $p<0.0001$. Each point is the mean ± SEM. *Inset:* structure of 1-277.

activity. Two of the mutants caused large decreases in AE activity: NNLS 147 AAAA and NGSM 159 AAAA. Our initial experiments used transient transfections, and we also generated stable cell lines of these two mutants, as well as mutants in which residues 147-150 (NNLS) were individually mutated to alanine. AE activity measurements revealed that only the S150A mutation significantly decreased AE activity compared to human, with AE sensitivity similar to NNLS 147 AAAA (**Figure 3C**). The involvement of ECL2 residue M162 was also confirmed by alanine scanning. The NGSM 159 AAAA mutation reduced AE activity to a similar extent as the M162G species chimera mutant. The efficacy of AEs is significantly reduced by these mutations compared to native hA_1R. Maximum AE activity decreased for NNLS 147 AAAA by 37.3 ± 6.3 ($p < 0.0001$) and for S150A by 28.4 ± 2.6 ($p < 0.001$). Compared to hA_1R the S150A mutation shifts the EC_{50} from 2.9 μM ± 0.19 to 5.5 μM ± 0.36 ($p < 0.05$), suggesting that this mutation affects the ability of the AE to occlude the orthosteric binding pocket more than to affect AE binding affinity. A recent alanine scanning study (45) employed an indirect yeast growth reporter assay that is susceptible to antagonist activity of AEs, effects of agonist-AE cooperativity and signal amplification (discussed in (75)). Nevertheless, these experiments showed that the mutations W156A and E164A in ECL2 decreased the effects of the AE PD 81,723. Agonist dissociation measurements with receptor chimeras and alanine mutants demonstrated that mutation of hA_1R residue S150 to either G or A significantly decreases the activity of ATL525 (**Figure 3B**). The decrease in AE activity was not additive with M162G, suggesting that S150 and M162 both participate in AE binding.

Identification of ECL2 residues S150 and M162 as mediators of AE activity can potentially explain the A_1R subtype specificity of 2-aminothiophene AEs. The A_1R and the AE-insensitive $A_{2A}R$ differ in 19 out of 34 residues in ECL2. In addition, the A_1R has only one disulfide bond in ECL2, whereas the $A_{2A}R$ contains three. As a result, the $A_{2A}R$ ECL2 is likely to have reduced conformational flexibility compared to ECL2 in the A_1R, and this constraint may impede AE binding compared to the A_1R.

ECL2 mutagenesis affects the activities of two chemical classes of AEs

The first described A_1R AEs were 2-aminothiophenes, exemplified by PD 81,723. Thereafter, more efficacious compounds were developed such as ATL525 (24-26). We recently demonstrated that a second class of AEs, 2-aminothiazole compounds, also possess AE activity (28). We evaluated the AE activity of the 2-aminothiazole compound 1-277 (**Figure 3D**, inset) on native hA_1R and the S150A and M162G mutants. Although the M162G mutation had no effect on the AE score of 1-277, the S150A mutation decreased the 1-277 AE score, similar to ATL525 (**Figure 3**). Since AEs from both chemical classes display reduced activity on receptors bearing the mutation S150A, this residue may interact with the common feature between these structurally different chemical classes: a 2-amino substituted, sulfur-containing, five-membered ring. The general inference is that these two classes of AEs likely share a common A_1R binding site.

Molecular modeling, in silico screening and docking simulations identify an AE binding pocket in ECL2

On the basis of the involvement of S150 and M162 in AE activity, we sought to further investigate the structural details of AE binding using molecular modeling, *in silico* screening and docking simulations. A potential pocket that included residues S150 and M162 in ECL2 was identified in our hA_1R homology model using the ICM PocketFinder algorithm (**Figure 4A,D** red surface) (71). The proposed ECL2 binding site is a solvent exposed cleft that is accessible to AEs. Notably, similar pockets formed by ECL2 were present in homology models of A_1Rs from several other species (**Supplemental Figure 4**).

Comparison of the agonist-bound crystal structure of $A_{2A}R$ with the inactive, antagonist-bound structure reveals a distinctive coupled movement between the antiparallel β-sheets in ECL1 and ECL2 and TM3. In the antagonist bound structure of $A_{2A}R$ (PDB ID: 4eiy (14)), TM3 contains a kink (**Figure 5**, blue); however, in the agonist bound structure (PDB ID: 2ydv (76)) TM3 is straightened in an outwards, piston-like movement of ~2.5 Å (**Figure 5**, orange), breaking contacts with TM5 and TM6. At the same time, agonist binding results in a repositioning of the β-sheets adjacent to TM3 in ECL1 and ECL2 (**Figure 5**, black arrow). It should be noted that the coupled movement of the antiparallel β-sheets and TM3 was observed in both the thermostabilized (76) and the fused T4 lysozyme (70) agonist-bound $A_{2A}R$ structures. This mechanism may also be

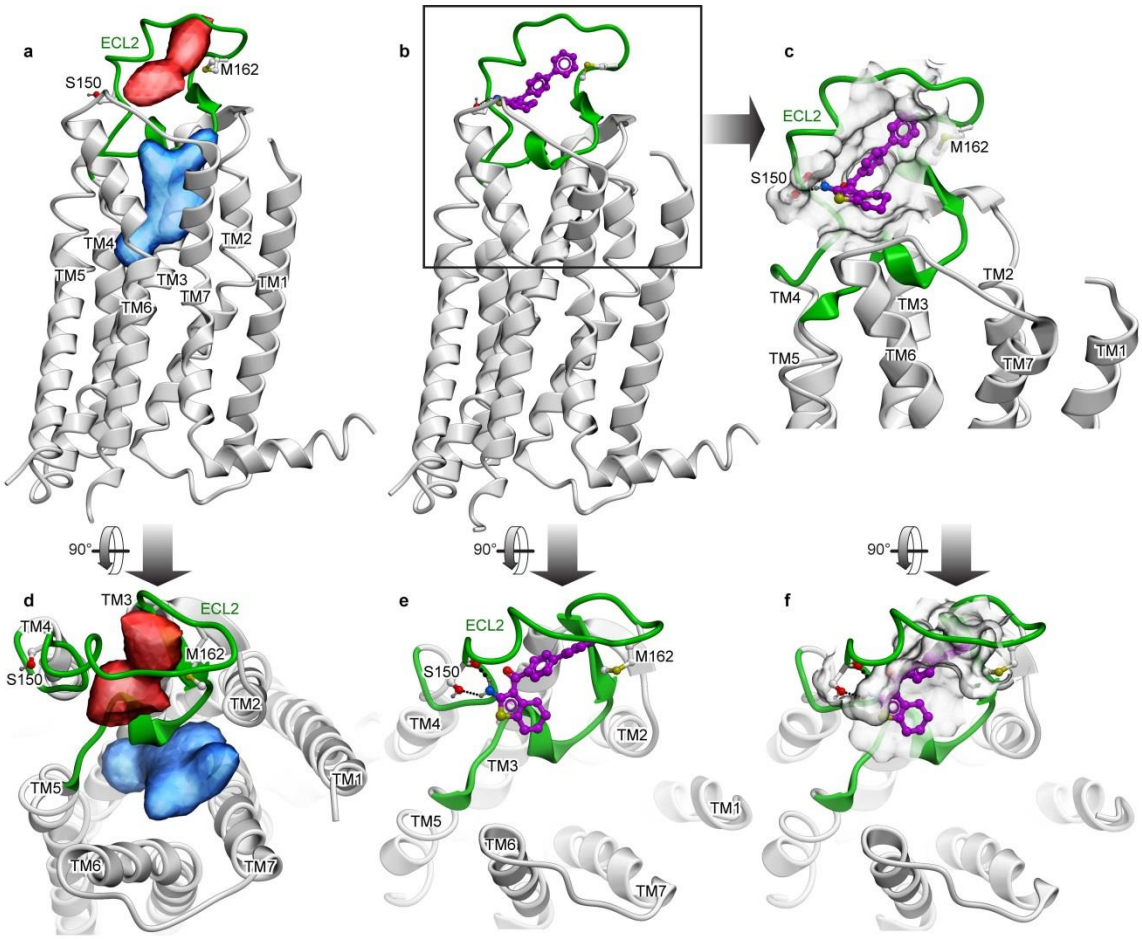

a
ECL2
S150
M162
TM4
TM2
TM5
TM3
TM1
TM7
TM6
b
c
ECL2
M162
S150
TM2
TM4
TM3
TM5
TM6
TM7
TM1
90°
90°
90°
d
TM3
ECL2
TM4
M162
S150
TM2
TM1
TM5
TM6
TM7
e
ECL2
S150
M162
TM4
TM2
TM3
TM1
TM5
TM6
TM7
f

Figure 4. ATL525 docked to the hA_1R homology model. **a**, hA_1R homology model (grey) based on the high-resolution crystal structure of $hA_{2A}R$ (PDB ID: 3qak). Residues S150 and M162, identified as being involved in AE signaling by site-directed mutagenesis in ECL2 (green) are shown as sticks. Ligand binding pockets were identified using ICM PocketFinder, including the orthosteric site (blue surface) and a pocket in ECL2 large enough to accommodate hA_1R AEs (red surface). **b**, Allosteric enhancer, ATL525 (ball and stick), docked into the hA_1R ECL2 binding pocket of the ALiBERO-optimized hA_1R homology model (ribbon). **c**, Enlarged view of ATL525 docked into the hA_1R homology model. **d**, **e** and **f**, are extracellular views of **a**, **b** and **c**, respectively, perpendicular to the plane of the membrane. AE atoms colored according to atom type; carbon: purple, sulfur: yellow, nitrogen: blue, oxygen: red. Dotted lines depict hydrogen bonds between ATL525 and S150.

involved in the activation mechanism of A_1R. Thus, AE binding to the proposed allosteric site in ECL2 (**Figure 5b**, red surface) may affect the conformational equilibrium of TM3 in hA_1R and bias the receptor towards the active state. While further insights into this coupled mechanism for hA_1R will require crystal structures of the active and inactive states of hA_1R, we speculate that a conformational switch in the receptor upon orthosteric agonist binding accounts for the differential effects on association and dissociation kinetics.

To improve our model of the AE binding site, the hA_1R homology model was refined using the ALiBERO protocol (73). ALiBERO uses elastic-network normal mode analysis to generate multiple binding site conformations and virtual ligand screening to identify models that best discriminate between "active" and "inactive" compounds. For this analysis, a library of known A_1R AEs ("actives") (21), as well as compounds that had little or no AE activity ("inactives") (23) was used (**Supplemental Table 3**). The ability to distinguish active from inactive compounds in virtual ligand screening is correlated with increased accuracy in predicting atomic contacts within ligand binding sites (72,73,77). Consistent with the crude character of the initial hA_1R homology model, the putative ECL2 pocket did not recognize the known AEs, where the Normalized SQuare-root Area Under Curve (NSQ_AUC) of only 1.8 is close to a random NSQ_AUC value of 0. After model optimization using ALiBERO, the best receptor conformation ensemble recognized the known AEs with an NSQ_AUC of 89.8 (approaching the ideal of 100), indicating that the refined models could better predict atomic contacts between A_1R and AEs.

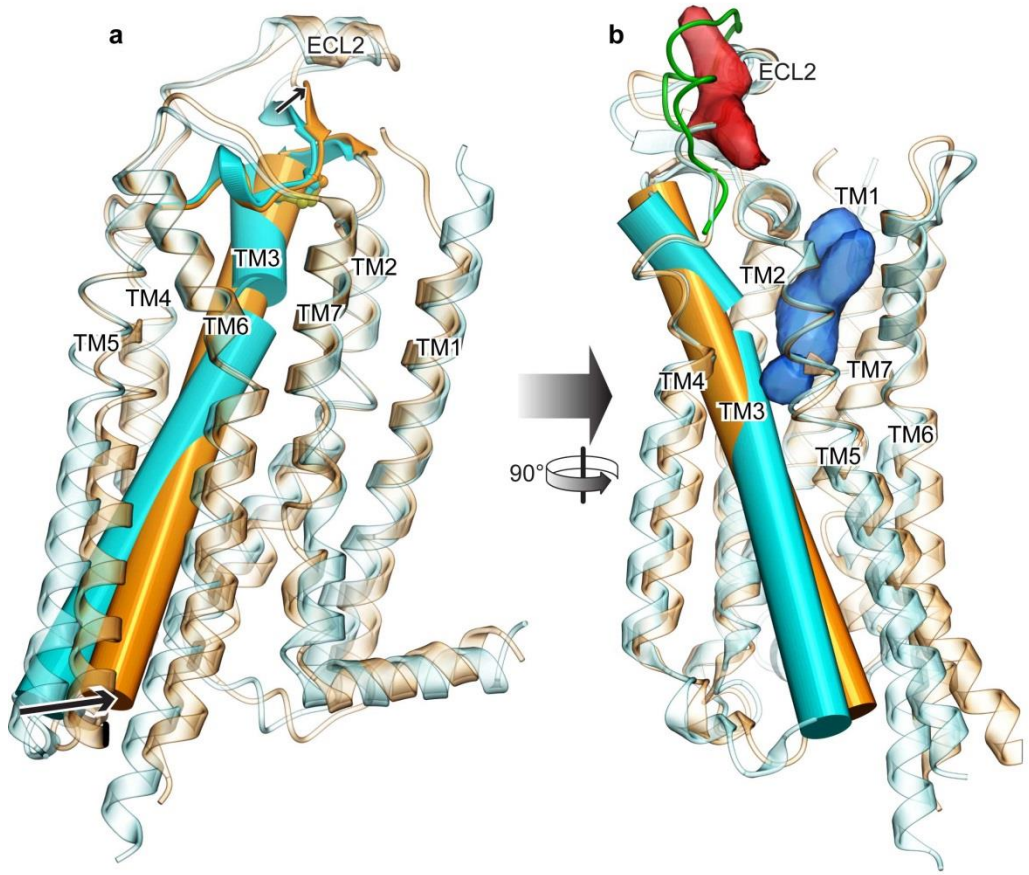


Figure 5. Superimposed, representative $A_{2A}R$ X-ray crystal structures (ribbons), with bound agonist (PDB ID: 2ydv (76), TM3 orange cylinders) and antagonist (PDB ID: 4eiy (14); TM3 blue cylinders). Black arrows indicate movement of TM3 and ECL2 from the antagonist-bound state (blue) to the agonist-bound state (orange). **a, b** Two views showing the $A_{2A}R$ orthosteric site (blue surface), the model of ECL2 for hA_1R (green ribbon) and the proposed allosteric site (red surface) superimposed onto the $A_{2A}R$ crystal structures. Conserved disulfide bond shown as sticks.

Docking PD 81,723 and ATL525 into the optimized receptor conformations illustrated how AEs could bind to the proposed allosteric site formed by ECL2 (**Figure 4B-F** and **Supplemental Figures 5 and6**). Superposition of the docked poses of ATL525 and PD 81,723 revealed a similar binding mode for the two AEs, including the presence of a hydrogen bond between the 2-amino group and S150, a residue independently implicated in AE binding in mutagenesis experiments (**Figure 3B** and **3C**). The 4- and 5-positions of the thiophene are solvent exposed and the 3-benzoyl group is directed toward the back of the site formed by ECL2. Docking calculations with PD 81,723 demonstrated a similar binding mode.

The increased length of ATL525 versus PD 81,723 (~12 Å and ~9 Å, respectively), may account for its greater AE activity. Specifically, ATL525 can extend further over the orthosteric binding site and form additional van der Waals contacts with the proposed allosteric pocket (**Supplemental Figure 6**). In addition, increasing the size of the fused ring at the 4- and 5-positions increases AE activity (23). More recent studies showed that large substituents at the 4- and 5-positions also enhance AE activity (78,79). These observations provide two possible explanations to account for differences in the AE activity of ATL525 and PD 81,723: the ability of ATL525 to form additional A_1R-AE interactions and an increased ability to trap agonists in the orthosteric binding pocket, thereby preventing exit from the receptor. Orthosteric agonist trapping is likely bestowed by substitutions on the 4- and 5- positions of the thiophene ring.

Mechanistic and functional implications

The binding modes of AEs are consistent with the established structure-activity relationship (SAR) (23), in which the 2- and 3-positions of the thiophene are restricted to an amino group and a carbonyl-containing substituent, respectively, and various alkyl and aryl substituents are tolerated at the 4- and 5-positions (**Supplemental Figure 7**). A key feature of 2-aminothiophenes is an intramolecular hydrogen bond between the 2-amino and the 3-keto groups, creating a ring coplanar with the thiophene ring (23). The hydrogen bonding interaction to S150 may explain why acylation of the 2-amino group results in loss of AE activity (23). The docked poses of the AEs in the potential AE binding site were consistent with the established SAR. The 2-amino group formed a hydrogen bond to S150, the 3-benzoyl group was directed toward the back of the pocket and the 4- and 5-positions of the thiophene were solvent exposed (**Figure 4E**). This docked conformation of ATL525 may explain the diversity of alkyl and aryl substituents that are tolerated in these positions (23,80).

We note that the hA_1R ECL2 site is similar to a computationally predicted ligand entry vestibule comprised of ECL2 and ECL3 in the β_2-adrenergic receptor (β_2AR). Alprenolol, a non-selective β-adrenergic receptor antagonist, was predicted to pass through several metastable states in this vestibule, as it enters the β_2AR orthosteric site (40). The proposed ECL2 AE pocket in A_1R may serve a similar function. The orthosteric agonist-bound receptor conformation may have a less accessible vestibule than the antagonist bound conformation, since agonists dissociate from A_1Rs much more slowly than antagonists with comparable equilibrium binding affinity (81). Upon binding, AEs

may sterically interfere with the exit of agonists and thereby slow ligand dissociation. This notion is supported by the observation that bulkier allosteric ligands, created by inserting larger cyclic linkers between the 4- and 5- positions on the thiophene ring, exhibit higher activity (26). In addition, this explanation is consistent with previous observations that AEs increase the B_{max} of orthosteric agonist ligands (30). Comparison of the agonist and antagonist bound crystal structures of $A_{2A}R$ (**Figure 5**), demonstrates that agonist binding results in a conformational switch of TM3 and the antiparallel β-sheets in ECLs 1 and 2, and we suggest that this coupled movement facilitates AE binding to ECL2, locking the agonist in the orthosteric binding pocket until the AE dissociates.

The functional consequence of AE binding to active, receptor-G protein complexes is an apparent increase in the efficacy and duration of agonists. Site-directed mutagenesis and molecular modeling studies suggest that the AEs bind to a pocket in ECL2 that is flanked by S150 and M162 in the hA_1R. We propose that AEs function by occupying the identified ECL2 vestibule, thereby impeding agonist dissociation. The identification of the ECL2 vestibule provides an unprecedented opportunity to use pharmacological and structural data to guide the development of new AEs for hA_1R (82-84).

Materials and Methods

Radioligand Binding

Radioligand binding was performed as previously described (26,30). We used an AE activity assay that measures ligand dissociation and therefore is not complicated by AE antagonist activity, as the receptor is pre-bound to orthosteric ligand. Receptors (10 µg in 50 µL) and the A_1R-specific agonist ^{125}I-ABA (0.5 nM in 50 µL; ^{125}I-*N*^{6}-(3-iodo-4-aminobenzyl)adenosine) are brought to equilibrium binding by a 120 min incubation at ambient temperature. At this concentration ^{125}I-ABA specifically binds to A_1R (**Supplemental Figure 1**). In the kinetic assay that we used (30), we observed that the effects of allosteric enhancer were directly related to the time of incubation. For each assay, the AE was added for a consistent period of time (10 min). Ten min was selected because it was sufficient for the AE to bind to A_1R, but sufficiently short that any effects on the equilibrium binding of the pre-bound, orthosteric agonist radioligand were minimized.

Finally, 50 µL containing 50 µM guanosine 5-[γ-thio]triphosphate (GTPγS) and 100 µM xanthine amine congener (XAC) are added for 15 min, which is sufficient to evaluate the AE-induced stability to GTPγS-induced dissociation. XAC is a non-specific AR antagonist that is added to ensure that ^{125}I-ABA does not re-associate with the receptor. The residual binding is adjusted to a 100 point scale, giving a unitless value for the enhancer activity. An enhancer score of "0" is fully decoupled (GTPγS and XAC with no added AE), and a score of "100" is equilibrium binding (no added AE, GTPγS, or

XAC). The AE score was measured at the end of the 10 min incubation period, in which case the score ranged from 0 to 100.

Statistical Analysis

AE activity measurements were conducted in triplicate on cell lysates from the species variants or receptor mutants. Each lysate was derived from at least two parallel-derived stable cell lines or at least three independent transient transfections. Results were compared by 2-way ANOVA at each concentration point and fitted for EC_{50} and maximal AE activity (regression line asymptote) in Prism 5.0 (Graphpad). Direct comparisons (log EC_{50} or maximum AE activity) were made using the Student's *t*-test, in which three to five experiments were averaged. Curves were also compared by the extra sum-of-squares F test in Prism 5.0. Error was presented as ± SEM.

A_1R Mutagenesis

Human and dog A_1R cDNAs were subcloned into the pDoubleTrouble vector (hexahistidine and FLAG peptide-tagged CLDN10B vector) (85) for stable expression in mammalian cells. Mutagenesis was performed using QuickChange Lightning® and/or QuickChange Multi Lightning® (Agilent Technologies). Primers were synthesized per Agilent guidelines. All mutations were confirmed by sequencing (Genewiz). The A_1R affinity for ^{125}I-ABA was not affected significantly by the reported mutations.

4x Alanine Scan of ECLs

The mutations introduced into the A_1R-pcDNA3.1+ background were NIGP 70 AAAA; QTY 74 AAA; FTH 77 AAA; NNLS 147 AAAA; AVER 151 LAAA; AVER 151 QAAA; AWAA 155 LALL; AWAA 155 GANH; NGSM 159 AAAA; GEP 163 AAA; VIK 166 AAA; PS 261-2 AA, HK 264-5 AA; C260A and C263A (**Supplemental Table 1**). Receptor mutants were transiently transfected into HEK293 cells using Lipofectamine® 2000 (Invitrogen) per the manufacturer's instructions. To allow sufficient time for protein expression, cells were lysed and prepared for binding 72 hr post-transfection.

Generation of Stable Cell Lines

Plasmids were purified with NucleoBond® Xtra Midi kit (Macherey-Nagel), and receptor mutants were transfected stably into HEK293 cells using Lipofectamine® 2000. Cells were selected for plasmid expression with G418 (1 mg/mL; Inalco), screened for A_1R expression by agonist (^{125}I-ABA) radioligand binding +/- adenosine-5′-*N*-ethylcarboxamide (NECA) as a measure of non-specific binding. HEK293 cells were cultured with 10% CO_2 at 37°C in DMEM (Invitrogen) containing 10% fetal bovine serum (Gemini Bio-products) and 1% Antibiotic/Antimycotic (Invitrogen). Cell lysates were prepared by repeated (10-12) passes through a 28.5 gauge needle (BD Scientific) at 4°C in a hypotonic solution (10 mM HEPES, pH 7.4) containing 2 U/mL adenosine deaminase (Roche) (30,85). Radioligand binding was conducted as previously reported with identical reagents and materials (30). Several A_1R mutants were created in cell lines

to delineate specific residues responsible for differences between human and dog A_1R (**Supplemental Tables 2)**. Single alanine mutants created to identify specific residues involved in binding were: N147A, N148A, L149A, S150A (**Supplemental Table 3**). K_D and B_{max} data were determined for each mutation or cell line (**Supplemental Table 4**).

Allosteric Enhancers

Synthesis and characterization of AE 6-(3,4-methylenedioxyphenyl)-8*H*-indeno[1,2-*d*]thiazol-2-ylamine hydroiodide (1-277) has been reported previously (compound 3ab) (28). AEs 2-amino-4,5,6,7-tetrahydro-benzo[*b*]thiophen-3-yl)biphenyl-4-yl-methanone (ATL525) (26) and 1-277 were evaluated at concentrations < 100 μM. Concentrations > 100 μM require DMSO levels known to disrupt the radioligand binding assay. It is difficult to determine if AEs have direct agonist effects because cells and membranes are frequently contaminated with low levels of adenosine. It is clear AEs produce much stronger effects in the presence of orthosteric agonists than in their absence. ATL525 displays minimal antagonist effects, as previously demonstrated (30).

Molecular Modeling

Ligand preparation, sequence alignment, homology modeling, docking and analyses were carried out in ICM version 3.7-3a (Molsoft L.L.C., La Jolla, CA) (86,87). A multiple sequence alignment was generated between hA_1R, $A_{2A}R$, $A_{2B}R$, A_3R and A_1R for the species of interest (dog, mouse, rat, chicken and rhesus monkey; (**Supplemental Figure 2**). Building of the initial homology model of hA_1R was based on the high-

resolution, agonist-bound, crystal structure of the adenosine $A_{2A}R$ receptor (PDB ID: 3qak) (70), after removal of the T4-lysozyme insertion. The backbone conformations of the well-aligned regions were inherited from the template, while the insertions and deletions were modeled by exhaustively searching a library of PDB fragments for loops of similar length and termini orientation. The loop searches were performed for the following regions in hA_1R: A155 to G163 (P149 to H155 in $hA_{2A}R$), which were disordered in the template and L211 to Q223 (L208 to R222 in $hA_{2A}R$), which was replaced by the T4-lysozyme in the template. The loop fragments were sampled and minimized in the context of the model to find an optimal conformation for each loop. The model was then subjected to extensive side-chain sampling and refinement.

Potential ligand binding sites in the initial hA_1R homology model were predicted using the ICM PocketFinder algorithm (71). Residues that were identified as surrounding the potential AE binding site in ECL2 were used to define the binding site for docking (F77, N148, E153, A157, M162, G163, V166, I167, K173). The model was subjected to refinement and evaluation using the Automated Ligand-guided Backbone Ensemble Receptor Optimization (ALiBERO) algorithm (73). This algorithm searches the conformational space of the proposed binding site in the initial hA_1R homology model by Elastic Network Normal Mode Analysis (EN-NMA) of the neighboring backbone and side chain atoms. ALiBERO evaluates multiple generated conformations for their compatibility with the activity of known AEs. For this evaluation, we used a set of 58 compounds (**Supplemental Table 5**) that were previously characterized for A_1R allosteric modulator activity: 33 “active” compounds (21) and 25 “inactive” compounds

(23). (Inactive compounds were defined as chemicals with A_1R enhancement of less than 10%.) The library of known active and inactive compounds was screened against 100 ALiBERO-generated receptor conformations using the ICM ligand docking and scoring module (**Supplemental Figure 3**), and a receiver-operating characteristic curve was built for each receptor conformation. The NSQ_AUC (72,88) was also calculated. The ability of the receptor conformations to discriminate active compounds from inactive compounds in virtual ligand screening (higher NSQ_AUC) correlates with increased reliability of the model. Using ALiBERO, the five receptor conformations that contributed to the ensemble with the highest NSQ_AUC were re-subjected to the EN-NMA sampling procedure, and this was repeated four times to further optimize the hA_1R homology models. The receptor conformations from the final ensemble with the highest NSQ_AUC were visually inspected and the docked binding modes of the AEs were compared with the SAR of known AEs. Based on visual inspection of the receptor-ligand complexes, the model of hA_1R in complex with a 2-aminothiophene AE that satisfied the known SAR was retained for further docking studies.

To evaluate the species differences for A_1R, ECL2 in the hA_1R homology model was mutated to ECL2 for each species of interest (dog, mouse, rat, chicken and rhesus monkey). The complexes underwent minimization where the 2-aminothiophene was tethered to its initial position (tzWeight=0.1), “soft” van der Waals terms were used (vwMethod=2), and side-chains within 8 Å were minimized for up to 10,000 iterations,

mimicking the induced fit effect of ligand binding. The AEs PD 81,723 and ATL525 were computationally docked into the proposed allosteric site in ECL2 of all species, and their interactions with the receptor were assessed.

Acknowledgements

This work was supported by: NIH grants R01 HL048908 (MY), R01 HL056111 (JL), R01 GM071872, U01 GM094612, and U54 GM094618 (RA). The authors thank Dr. Manuel Rueda for assistance with ALiBERO and Dr. Irina Kufareva for useful discussions and for critically reading the manuscript. We thank Drs. Kevin Lynch and Ray Stevens for helpful discussions.

Abbreviations

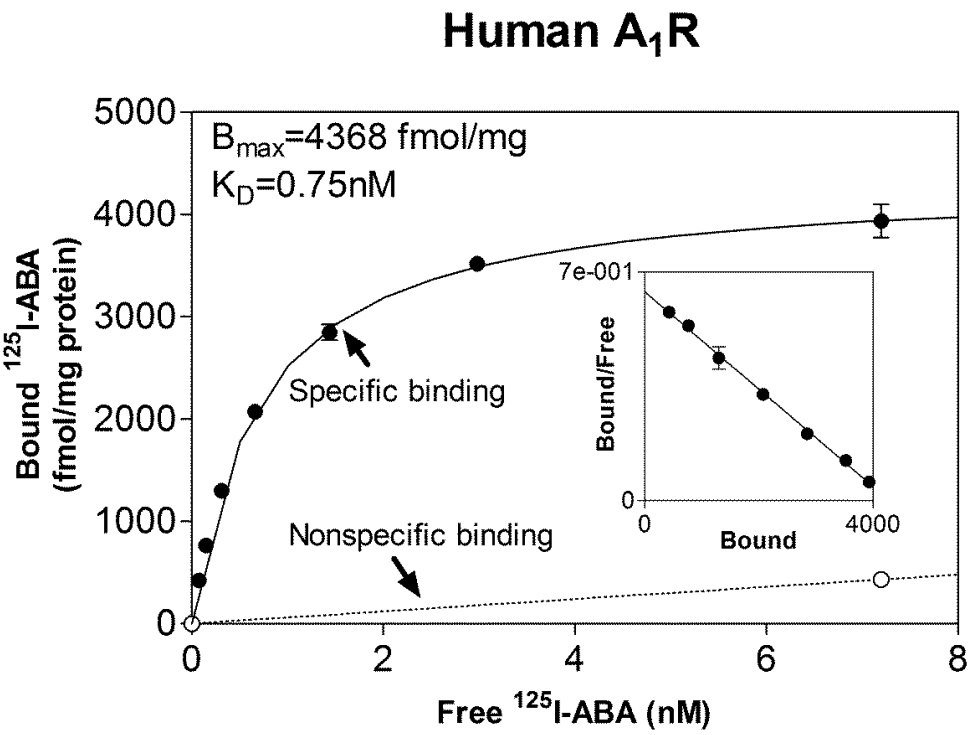
A_1R	Adenosine A_1 receptor
AE	Allosteric enhancer
AR	Adenosine receptor
ATL525	2-amino-4,5,6,7-tetrahydro-benzo[*b*]thiophen-3-yl)biphenyl-4-yl-methanone
ALiBERO	Automated ligand-guided backbone ensemble receptor optimization
β_2AR	β_2-adrenergic receptor
CPA	^{3}H-N^6-cyclopentyladenosine
dA_1R	dog A_1R
ECL2	2nd extracellular loop
GPCR	G protein-coupled receptor
GTPγS	Guanosine 5-[γ-thio]triphosphate
hA_1R	human Adenosine A_1 Receptor
^{125}I-ABA	[^{125}I]N^6-(3-iodo-4-aminobenzyl)adenosine

NECA	adenosine-5′-*N*-ethylcarboxamide
NSQ_AUC	Normalized square-root area under curve
PD 81,723	(2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluromethyl)-phenyl]-methanone
4-41	Bis-(2,2'-*N*,*N*-piperdinecarboxyamidephenyl)-disulfide
1-277	6-(3,4-Methylenedioxyphenyl)-8*H*-indeno[1,2-*d*]thiazol- 2-ylamine hydroiodide
SAR	Structure-activity relationship
VLS	Virtual ligand screening
XAC	Xanthine amine congener

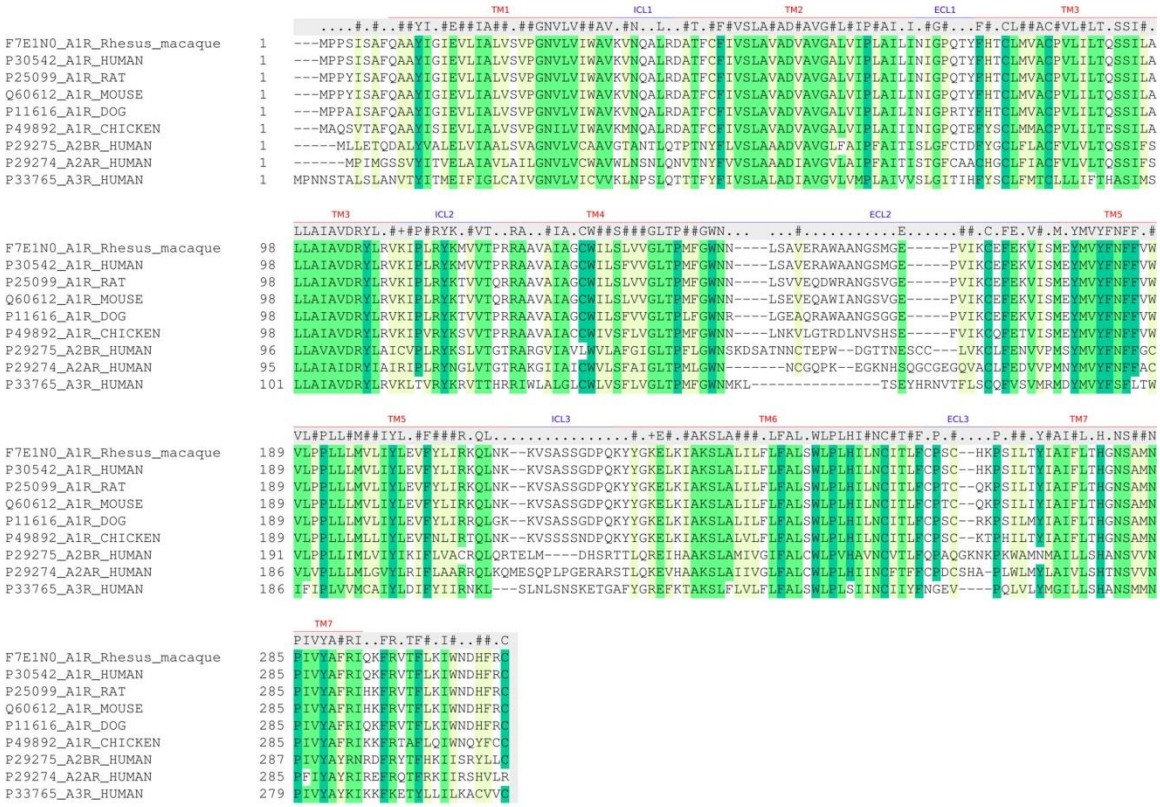
Author Contributions

Susan Leonhardt, Michael Purdy and I designed, introduced and verified the hA_1R mutations. Heidi Figler, Robert Figler, Melissa Marshall and I deigned and conducted pharmacological studies. Fiona McRobb, guided by Ruben Abagyan, conducted molecular modeling and docking experiments. Mahendra Chordia synthesized 1-277. Joel Linden, Robert Figler and I developed and implemented the AE kinetic assay. Fiona McRobb wrote the portions of the manuscript corresponding to her experimental results, primarily assisted by Mark Yeager and Joel Linden. Mark Yeager was responsible for overall project strategy and management.

Supplementary Figures

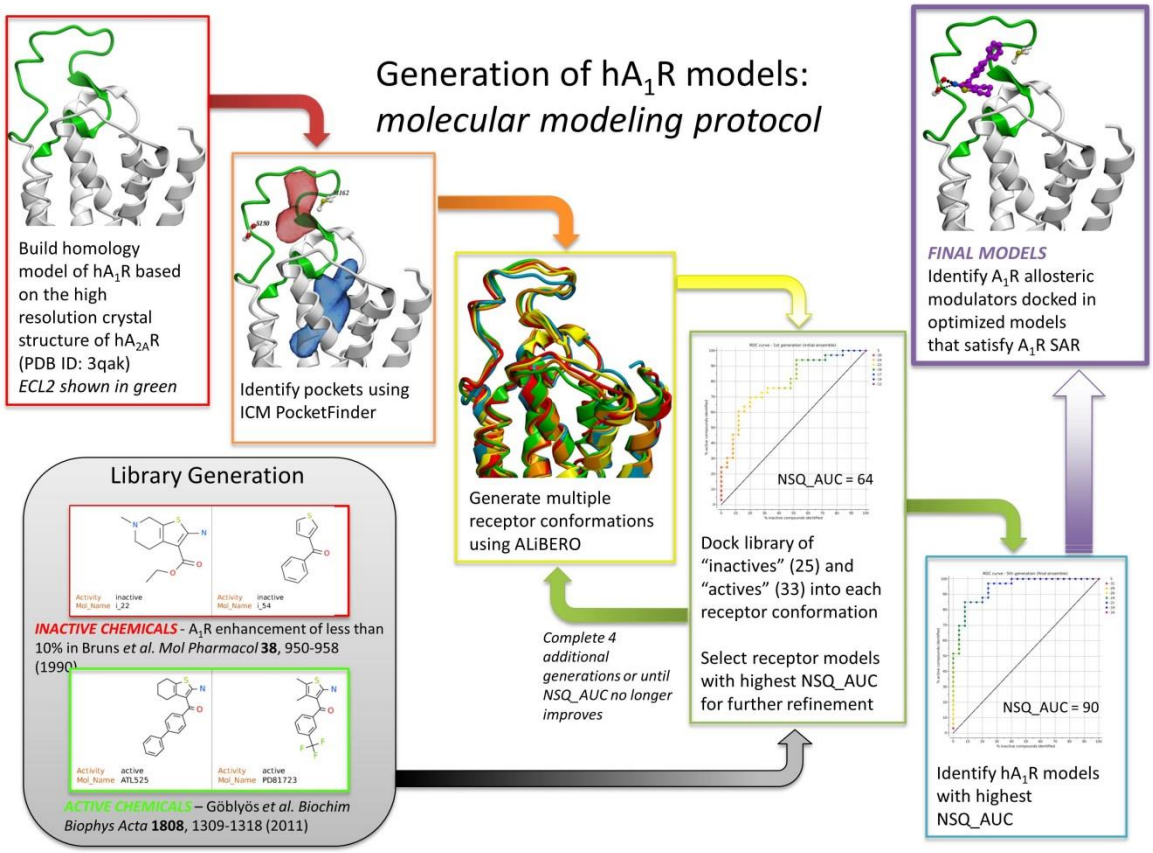


Supplemental Figure 1. Concentration curve of specific ^{125}I-ABA binding to lysates of HEK293 cells artificially expressing A_1R. (●) Specific (total – non specific) binding (○) Non-specific binding (^{125}I-ABA + 50μM NECA). **Inset**: Scatchard transformation of concentration curve data. Linearity indicates a single binding site. The *x*-intercept is approximately the B_{max}, and the slope is approximately $-1/K_D$. If there were multiple binding sites, these data would be curvilinear, allowing us to conclude that ^{125}I-ABA detects only binding to recombinant receptors in these cells. Furthermore, untransfected HEK cells show no detectable expression of A_1R and no specific binding of ^{125}I-ABA.



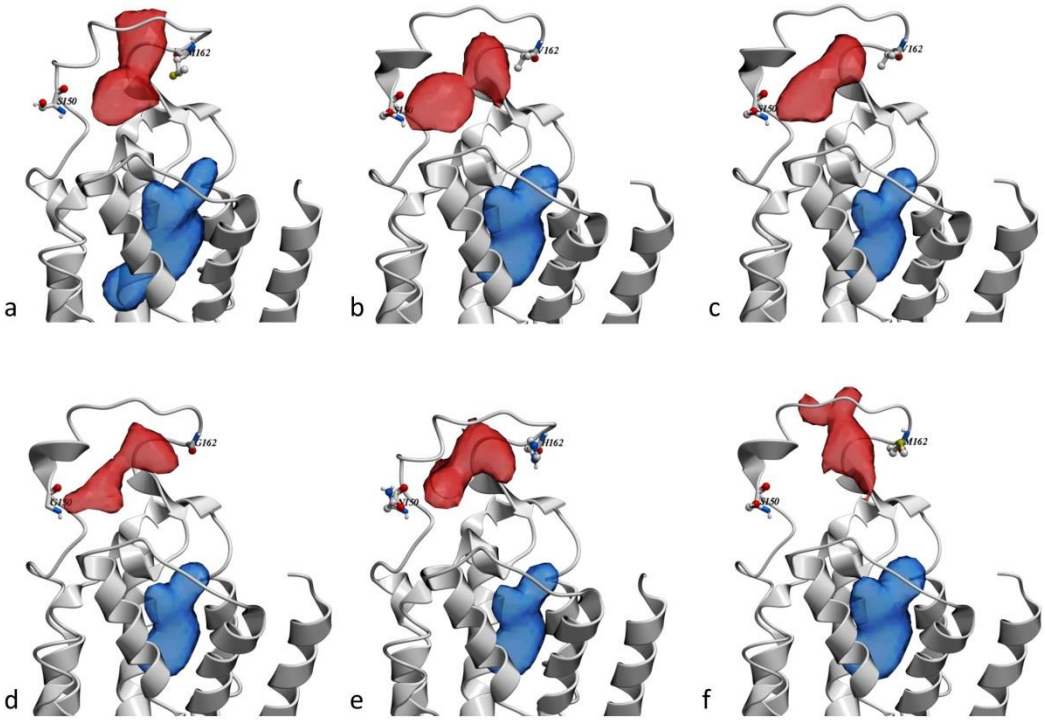
Supplemental Figure 2. Multiple sequence alignments of the hA_1R, other human adenosine receptors (A_{2A}R, A_{2B}R and A_3R) and A_1R of different species (Rhesus monkey, rat, mouse, dog and chicken).

Figure prepared by Fiona McRobb.



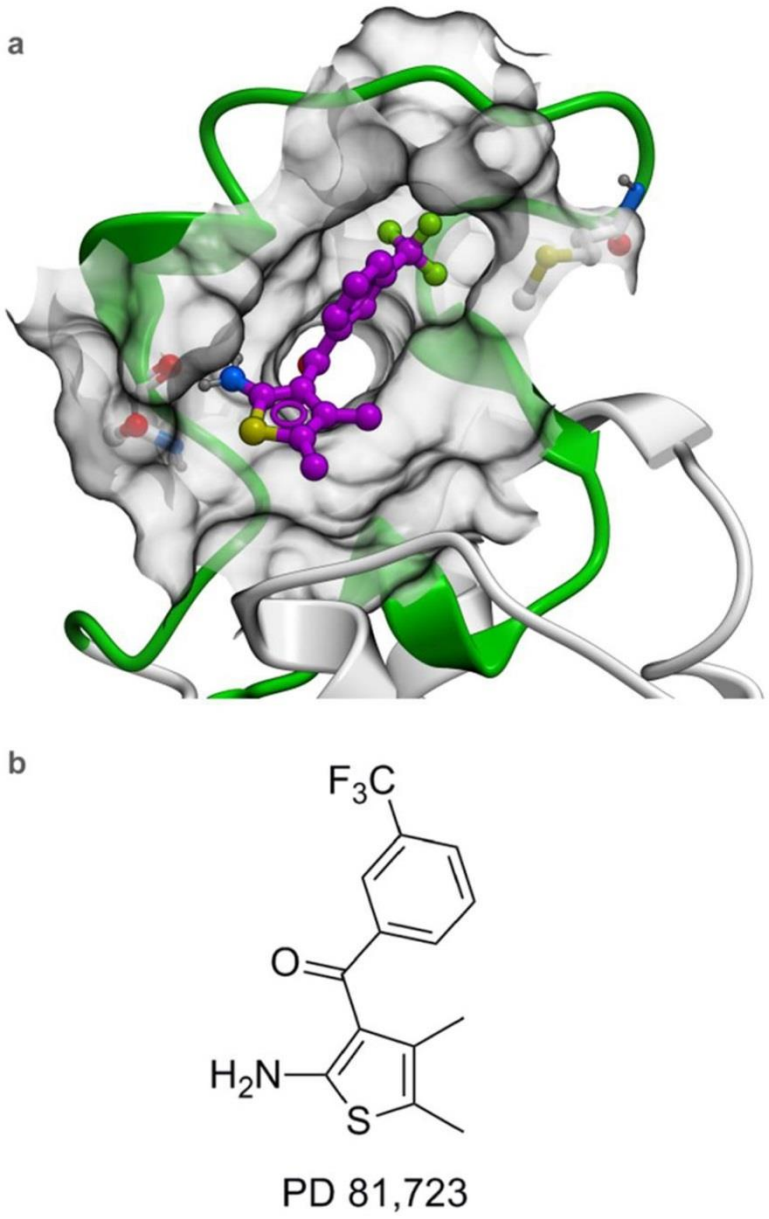
Supplemental Figure 3. Schematic of the molecular modeling protocol employed for the generation and refinement of the homology models of hA_1R.

Figure prepared by Fiona McRobb.



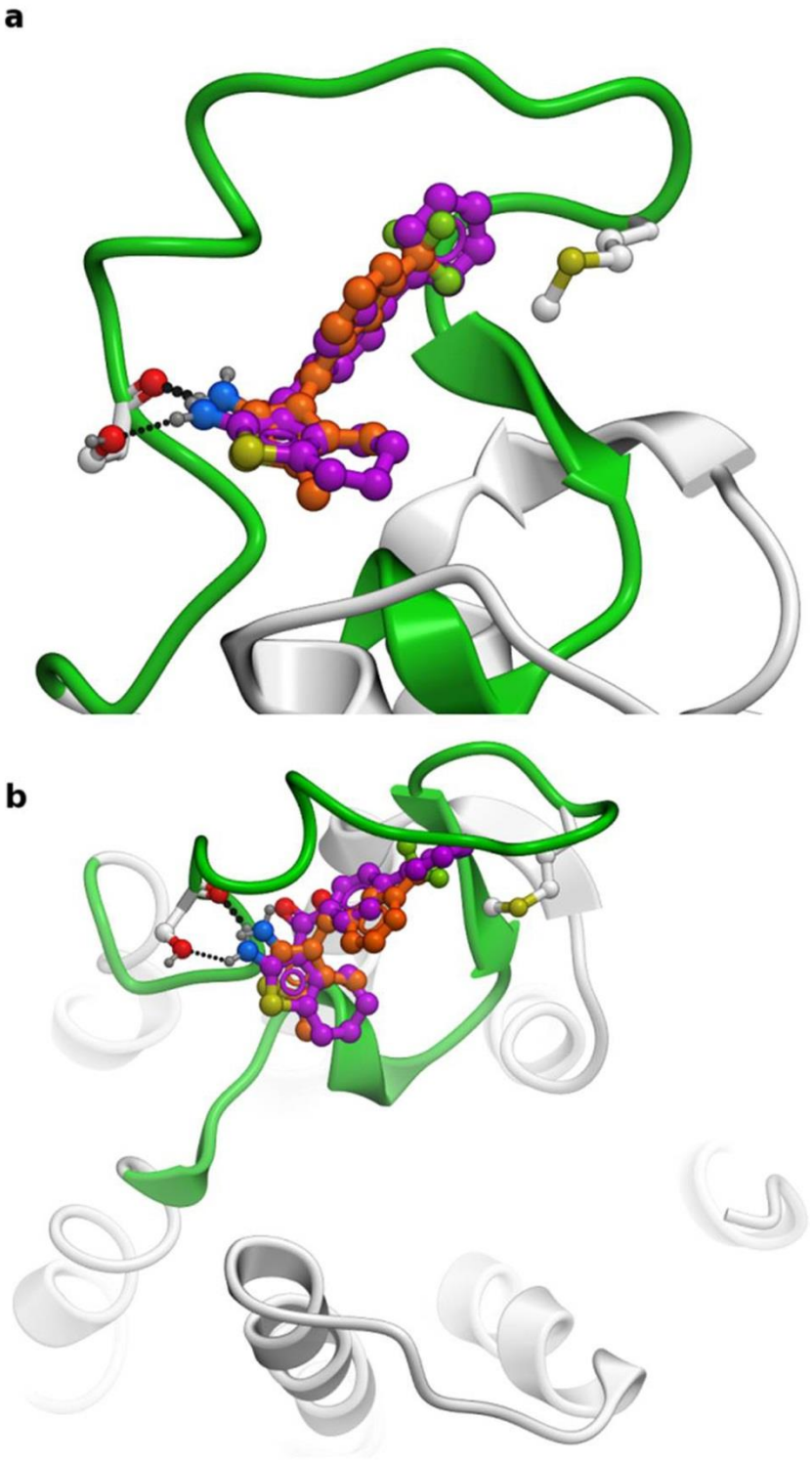
Supplemental Figure 4. The AE and orthosteric binding pockets were predicted using ICM PocketFinder (71). The orthosteric site (blue surface) and a pocket in ECL2 were large enough to accommodate hA_1R AEs (red surface) in homology models of different species: **a**, human; **b**, mouse; **c**, rat; **d**, dog; **e**, chicken; **f**, rhesus monkey. Residues corresponding to the positions of S150 and M162 are shown as grey sticks.

Figure prepared by Fiona McRobb.



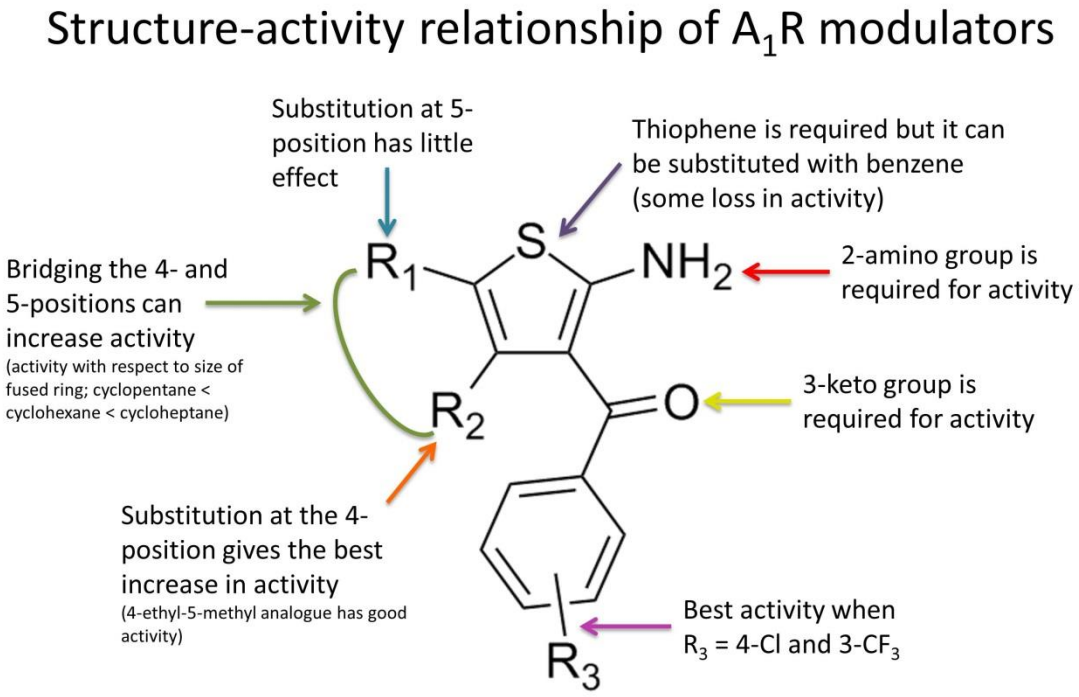
Supplemental Figure 5. **a**, PD 81,723 (colored by atom type; carbon: purple, sulfur: yellow, nitrogen: blue, oxygen: red) docked in the ECL2 pocket (green ribbon, grey surface). **b**, Chemical structure of PD 81,723.

Figure prepared by Fiona McRobb.


a
b

Supplemental Figure 6. **a,** Overlay of PD 81,723 (colored by atom type; carbon: orange) and ATL525 (colored by atom type; carbon: purple) docked to the hA_1R model (ribbon, ECL2 highlighted in green, S150 and M162 shown as grey sticks). Hydrogen bonds between AEs and hA_1R are shown in black. **b,** Top view of **a**.

Figure prepared by Fiona McRobb.



Bruns, R. F. *et al. Molecular Pharmacology* **1990**, *38*, 950–958.

Supplemental Figure 7 Structure-activity relationship of A_1R AEs(23) describing effects of chemical substitutions around the AE pharmacophore: 2-amino-3-benzoylthiophene. Figure prepared by Fiona McRobb and Dylan Kennedy.

Mutant and Primer name	Primer sequence
dog A_1R (human 148-153)	5'-ccccgctgttcggctggaacaatctgagcgcggtggagcgggcctgg-3'
_antisense	5'-ccaggcccgctccaccgcgctcagattgttccagccgaacagcgggg-3'
Dog A_1R G162M	5'-cggccaacggcagcatgggcgagcccgtgat-3'
_antisense	5'-atcacgggctcgcccatgctgccgttggccg-3'
human A_1R (dog 148-153)	5'-gacccctatgtttggctggaacaggctgggtgaggcgcagcgggcctggg-3'
_antisense	5'-cccaggcccgctgcgcctcacccagcctgttccagccaaacataggggtc-3'
human A_1R M162G	5'-agccaacggcagcgggggggagcccgtg-3'
_antisense	5'-cacgggctccccccccgctgccgttggct-3'
Dog A_1R G150S	5'-ctggaacaggctgagcgaggcgcagcggg-3'
_antisense	5'-cccgctgcgcctcgctcagcctgttccag-3'
human A_1R S150G	5'-gctggaacaatctgggtgcggtggagcgg-3'
_antisense	5'-ccgctccaccgcacccagattgttccagc-3'
Also generated using primers above and evaluated:	
Dog A_1R G150S, G162M	
Human A_1R S150G, M162G	
Human A_1R (dog ECL2): N148R, S150G, A151E, V152A, E153Q, M162G	
Dog A_1R (human ECL2): R148N, G150S, E151A, A152V, Q153E, G162M	

Supplemental Table 1. Primers used to generate 4x alanine scanning mutations. Left column: mutation introduced. Right column: primer used (forward over reverse).

Mutant and Primer Name	Primer Sequence
NIGP 70 AAAA	5'-tccccctcgccatcctcatcgccgctgcggcacagacctacttccacacc-3'
Reverse	5'-ggtgtggaagtaggtctgtgccgcagcggcgatgaggatggcgaggggga-3'
QTY 74 AAA	5'-tcctcatcaacattgggccagcggccgccttccacacctgcctcatgg-3'
Reverse	5'-ccatgaggcaggtgtggaaggcggccgctggcccaatgttgatgagga-3'
FTH 77 AAA	5'-cattgggccacagacctacgccgccgcctgcctcatggttgcctg-3'
Reverse	5'-caggcaaccatgaggcaggcggcggcgtaggtctgtggcccaatg-3'
NNLS 147 AAAA	5'-ctgacccctatgtttggctgggccgctgcggctgcggtggagcgggcctgggc-3'
Reverse	5'-gcccaggcccgctccaccgcagccgcagcggcccagccaaacataggggtcag-3'
AVER 151 LAAA	5'-cccctatgtttggctggaacaatctgagtctagcggcggcggcctgggcagccaa-3'
Reverse	5'-ttggctgcccaggccgccgccgctagactcagattgttccagccaaacatagggg-3'
AVER 151 QAAA	5'-tatgtttggctggaacaatctgagtcaggcggcggcggcctgggcagcc-3'
Reverse	5'-ggctgcccaggccgccgccgcctgactcagattgttccagccaaacata-3'
AWAA 155 LALL	5'-ggaacaatctgagtgcggtggagcggctagcgttactaaacggcagcatgggggagcccgtgat-3'
Reverse	5'-atcacgggctcccccatgctgccgtttagtaacgctagccgctccaccgcactcagattgttcc-3'
AWAA 155 GANH	5'-tctgagtgcggtggagcggggcgcgaatcacaacggcagcatgggggagc-3'
Reverse	5'-gctcccccatgctgccgttgtgattcgcgccccgctccaccgcactcaga-3'
NGSM 159 AAAA	5'-gcgggcctgggcagccgccgccgccgcgggggagcccgtgatc-3'
Reverse	5'-gatcacgggctcccccgcggcggcggcggctgcccaggcccgc-3'
GEP 163 AAA	5'-caacggcagcatggcggcggccgtgatcaagtgc-3'
Reverse	5'-gcacttgatcacggccgccgccatgctgccgttg-3'
VIK 166 AAA	5'-gcagcatgggggagcccgcggccgcgtgcgagttcgagaaggt-3'
Reverse	5'-accttctcgaactcgcacgcggccgcgggctcccccatgctgc-3'
EFEK 170 AAAA	5'-gggagcccgtgatcaagtgcgcggccgcggcggtcatcagcatggagtacat-3'
Reverse	5'-atgtactccatgctgatgaccgccgcggccgcgcacttgatcacgggctccc-3'
VIS 174 AAA	5'-gatcaagtgcgagttcgagaaggccgccgccatggagtacatggtctacttca-3'
Reverse	5'-tgaagtagaccatgtactccatggcggcggccttctcgaactcgcacttgatc-3'
PS,HK 261-2,264-5 AA,AA	5'-gcatcaccctcttctgcgcggcctgcgccgcgcccagcatccttacct-3'
Reverse	5'-aggtaaggatgctgggcgcggcgcaggccgcgcagaagagggtgatgc-3'
CC260,263AA	5'-catcaccctcttcgccccgtccgcccacaagcccagc-3'
Reverse	5'-gctgggcttgtgggcggacggggcgaagagggtgatg-3'

Supplemental Table 2. Primers used to generate species chimera mutations and other mutations introduced using these primers.

Mutant	Primer name	Primer
N147A	N147A	5'-cccctatgtttggctgggccaatctgagtgcggtgg-3'
	N147A_antisense	5'-ccaccgcactcagattggcccagccaaacatagggg-3'
N148A	N148A	5'-ctatgtttggctggaacgctctgagtgcggtggagc-3'
	N148A_antisense	5'-gctccaccgcactcagagcgttccagccaaacatag-3'
L149A	L149A	5'-ccctatgtttggctggaacaatgcgagtgcggtggagc-3'
	L149A_antisense	5'-gctccaccgcactcgcattgttccagccaaacataggg-3'
S150A	S150A	5'-gtttggctggaacaatctggctgcggtggagcgg-3'
	S150A_antisense	5'-ccgctccaccgcagccagattgttccagccaaac-3'

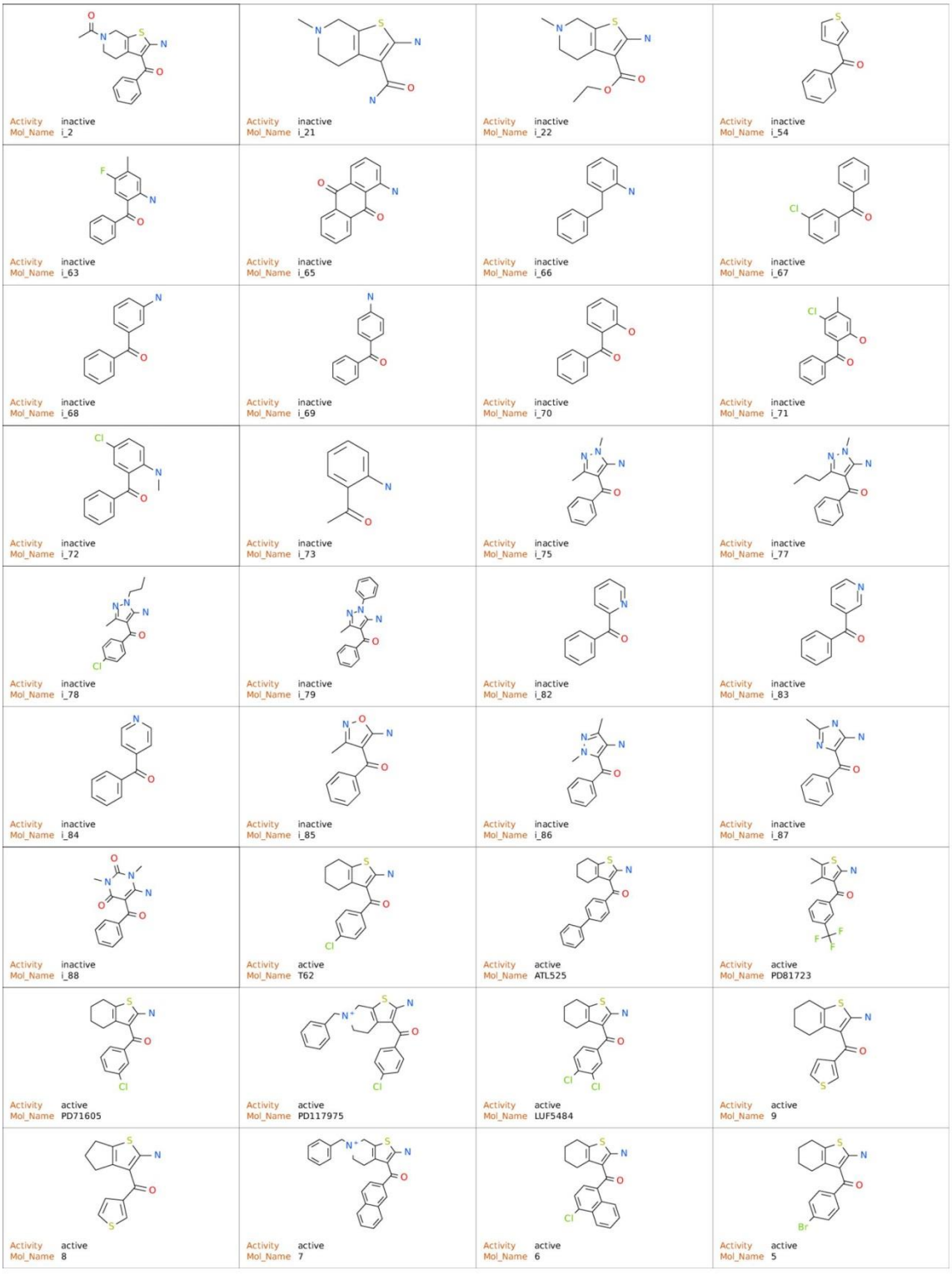
Supplemental Table 3. Mutations generated and primers used for single alanine scan.

	*K*D (nM)		Bmax (fmol/mg)		
	Mean	SEM	Line 1	Line 2	N
human A1 R	3.401	0.4554	981.4	818.6	2
human (dog ECL2)	1.073	0.529	328.5	1728	2
human M162	1.434	0.8549	1837	972	2
human S150G	1.475	0.5361	4105	234.3	2
human NNLS 147 AAAA	1.961	0.807	1481	1677	2
human S150A	1.625	0.8404	665.1	3251	2
dog	3.339	2.065	497.1	134.5	2
rhesus money	0.932	0.104	1644	339.2	2
mouse	0.6943	0.0693	3914	3686	2
rat	0.7455	0.3635	1889	3158	2

* chicken A1 receptor was previously described: Aguilar, et al., 1995.

Aguilar J, Fulong T, Durand I, Green R. (1995) Isolation and characterization of an avian A1 adenosine receptor gene and related cDNA clone. *Biochem J* **307**: 729-734.

Supplemental Table 4. Pharmacological properties (K_D and B_{max}) of mutants. K_D presented as a mean of two independently derived cell lines. Independent B_{max} values presented for each cell line.


Activity inactive
Mol_Name i_2
Activity inactive
Mol_Name i_21
Activity inactive
Mol_Name i_22
Activity inactive
Mol_Name i_54
Activity inactive
Mol_Name i_63
Activity inactive
Mol_Name i_65
Activity inactive
Mol_Name i_66
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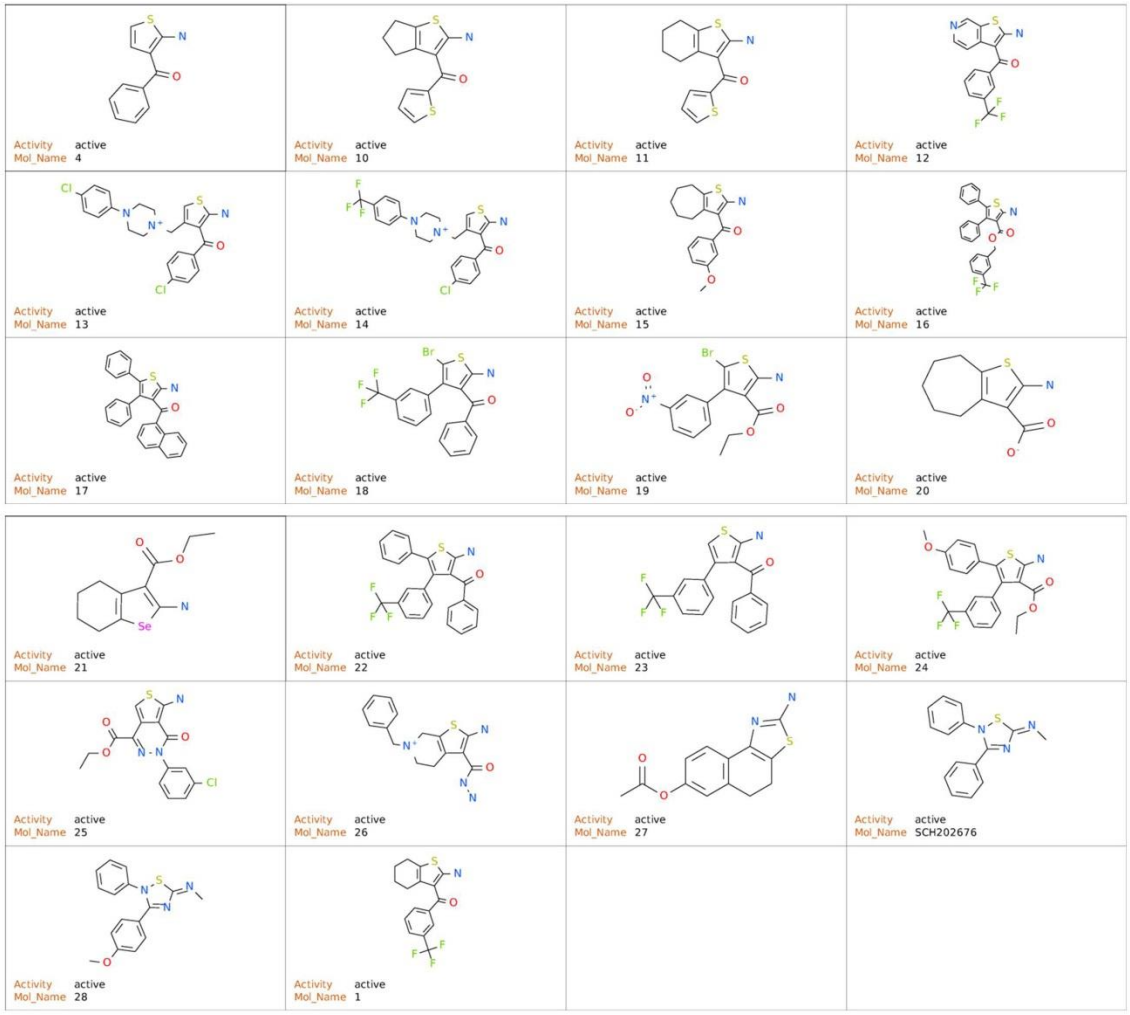

Supplemental Table 5. The database of 58 compounds that were previously characterized for A_1R AE activity (25 inactive(23) and 33 active compounds(21)). These compounds were used for virtual screening during ALiBERO to optimize the receptor conformations.

Figure prepared by Fiona McRobb.

Chapter 3:

Oxidizing Agents Function as Allosteric Enhancers of the Adenosine A_1 Receptor

Introduction

Allosteric modulation is a promising therapeutic strategy that has gained pharmacological validity. Allosteric modulators bind at sites distinct from the "orthosteric," agonist-binding site and can be characterized as positive allosteric modulators (PAMs) or negative allosteric modulators (NAMs), increasing or decreasing target signaling, respectively. Allosteric modulators targeting several GPCRs have been identified and hold several therapeutic advantages compared to conventional orthosteric ligands, including site and event specificity (2).

Adenosine receptors (AR) are a GPCR subfamily responsible for adenosine signaling. The subfamily is comprised of four subtypes: A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R. Activation of $G_{\alpha i}$-coupled A_1R, expressed in cardiac myocytes and pharmacologically targeted for its effects in the atrioventricular node, mediates reduced cyclic AMP accumulation and elevated potassium channel conductance to elicit negative chronotropic and dromotropic effects in the heart (89-91). Additionally, A_1R functions via several mechanisms to produce an array of other physiological effects, including protection of cardiac tissues after injury or stress (92) and facilitation of angiogenesis (93).

Compounds that act as PAMs of A_1R are termed AEs (22). Previously, we discovered that AEs stabilize A_1R-G protein complexes and developed an assay method to score enhancer activity on a scale from 0 to 100 based on their ability to prevent the rapid dissociation of agonist radioligand in response to guanosine 5-[γ-thio]triphosphate (GTPγS) (30). *In vivo* experiments with AEs demonstrate that some 2-aminothiophenes appear to be functional enhancers of A_1R-mediated physiological effects such as negative

dromotropic cardiac actions (48-50) and reducing allodynia in response to peripheral nerve injury (94,95).

Recently, we found that AEs bind to a pocket within ECL2 of A_1R (**Chapter 2**). The ECL2 binding site is topologically distinct to that evaluated in this study (**Figure 1**). We identified this second site while investigating the effects of AEs on the dissociation kinetics of agonist radioligand ^{125}I-aminobenzyladenosine (^{125}I-ABA) from A_1R, observing that AE activity is abolished by the addition of several reducing agents. Based on these observations we conducted the experiments presented in this study. First, we exhibit that the A_1R disulfide bond (C80-C169), conserved among 78.9% of GPCRs, is surface exposed in our structural model (96). Second, we characterize the sensitivity of AE activity to mutations that occlude this "disulfide bond site." Third, as a result, we suspected oxidation may play a role in AE activity and used this observation to synthesize a new chemical class of AEs termed aryl disulfides and identified that oxidizing compound hydrogen peroxide (H_2O_2) mimics AEs. Fourth, we determined that exposing the disulfide bond site on $A_{2A}R$ increases AE sensitivity of that receptor. Finally, we employed these experimental observations to interpret the previously identified dynamic nature of TM3, within the disulfide bond site, upon ligand binding (**Chapter 2**).

The conserved disulfide bond (A_1R residues C80 and C169) connects the first and second extracellular loop domains (ECL1 and ECL2), constraining the movement of ECL2 by folding it over itself along the edge of the top, extracellular portion of the receptor, resulting in the AR-conserved residue F171 being oriented in the orthosteric

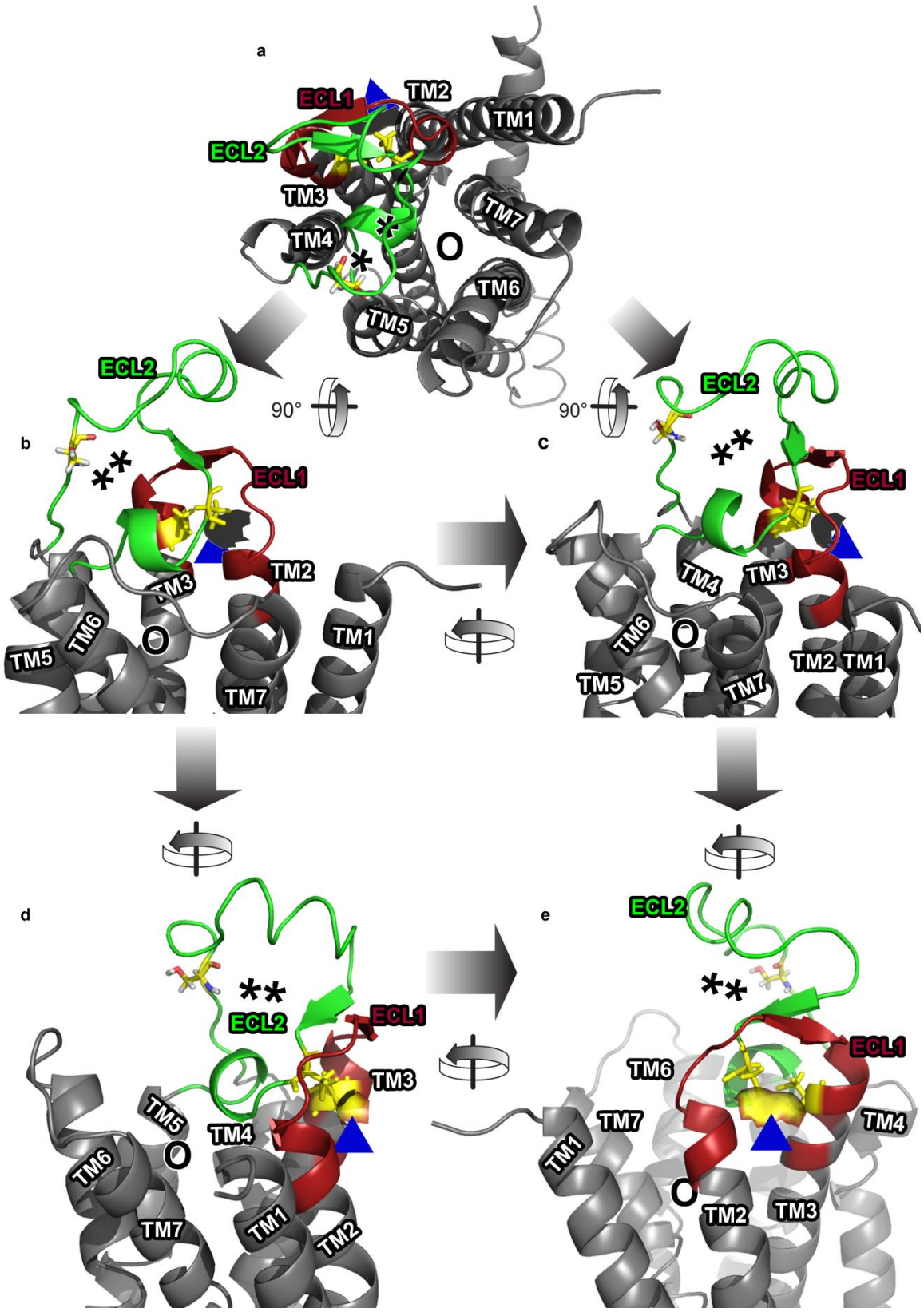
a
ECL1
TM2
TM1
ECL2
TM3
TM7
TM4
TM6
TM5
90°
b
ECL2
ECL1
TM2
TM3
TM6
TM5
TM1
TM7
c
ECL2
ECL1
TM4
TM3
TM6
TM2
TM1
TM5
TM7
d
ECL2
ECL1
TM3
TM5
TM4
TM6
TM7
TM1
TM2
e
ECL2
ECL1
TM6
TM7
TM4
TM1
TM2
TM3

Figure 1: Relative topological locations of AE binding sites on an A_1R homology model of $A_{2A}R$ structure (PDB ID: 3qak)(70), shown from the (**a**) top and (**b-e**) several side angles. ECL1 (red) and ECL2 (green). Receptor backbone (grey). Asterisks indicate general location of ECL2 binding site. Blue triangle indicates general location of ECL1 binding site. "O" indicates orthosteric binding pocket. Residue S150 depicted as sticks, colored by atom (carbon: yellow, oxygen: red, nitrogen: blue, hydrogen: grey). Disulfide bond pair C80 and C169 (yellow) depicted as sticks. The surface of just these two residues is rendered, most apparent in (**e**) and colored yellow.

binding pocket, where it participates in π-bonding with adenosine and other orthosteric ligands (ECL2 topology can be observed in **Figure 1**) (97). Breaking this disulfide bond renders the receptor unable to bind ligands (97,98).

ECL2 is known to influence orthosteric ligand binding, and the AE binding site we recently identified residues near the disulfide bond, within the fold of ECL2 and above the orthosteric binding pocket. In **Chapter 2**, we propose that AEs binding to this site block the exit from the A_1R binding pocket of orthosteric agonists. In this study, we suggest that AE activity is derived through a second, ECL1 site near the C80-C169 disulfide (**Figure 1**).

To interact at two sites, AEs must conserve two chemically distinct pharmacophores. Evidence for two pharmacophores within the AE molecule can be evaluated from the activity of previously synthesized AEs. The first identified AEs belong to the 2-aminothiophene chemical class (22,23). These compounds increase agonist binding by decreasing the rate of orthosteric agonist, but not antagonist, dissociation from the A_1R (30). Subsequently, several new series of 2-aminothiophene AEs with improved potency and efficacy have been developed, although unmodified 2-aminothiophene is not an efficacious AE (24-26,99-103). Subsequently, a second chemical class of A_1R AEs was identified: 2-aminothiazoles (27,28). Herein, we introduce a third class of AEs: aryl disulfides, aromatic disulfides containing bis-ortho-urea functions. Aryl disulfides have greater potency than compounds comprising other AE chemical classes, but lack the conserved chemical characteristics. Compounds from

these classes are differentially sensitive to binding pocket mutations and assist our efforts to delineate the distinct pharmacophores.

Results

The activity of AEs is eliminated by reducing agents

The actions of highly effective A_1R AEs ATL525 ((2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)biphenyl-4-yl-methanone; **Figure 2a**), a 2-aminothiophene, and 1-285 (6-(4-(dimethylamino)phenyl)-8H -indeno[1,2- d]thiazol-2-amine-hydroiodide; **Figure 2b**), a 2-aminothiazole (28), are completely blocked when added alongside reducing agents DTT, reduced human glutathione (rhGT) or TCEP (**Figure 3a and 3b**). For these experiments, AE activity is defined as preventing rapid, GTPγS-induced dissociation of agonist radioligand ^{125}I-ABA from lysates of HEK293 cells stably transfected with recombinant human A_1R (hA_1R). The effect of 5 mM DTT on AE activity is not surmountable, even when AEs are added at 50x their ED_{50} concentrations (**Figure 3c**).

Sensitivity to DTT was evaluated in a kinetic study, revealing that receptors preincubated with DTT are not affected by subsequent exposure to AE. In the control condition, the $t_{½}$ of 1-285 activity is ~20 min (**Figure 3d**). Combined, these findings suggest that AE binding to A_1R is not rate-limiting for AE activity. Kinetic experiments evaluating the reversal of AE activity by adding DTT and GTPγS to A_1R pre-incubated

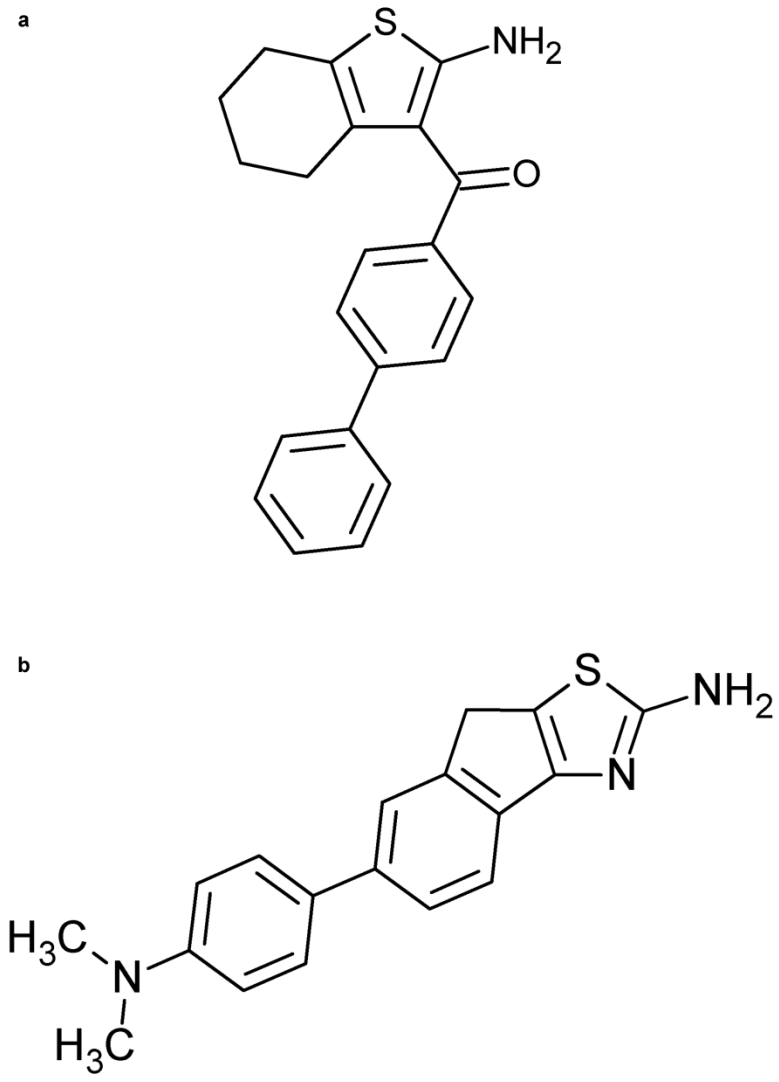

Figure 2: Chemical structures of (**a**) ATL525 and (**b**) 1-285.

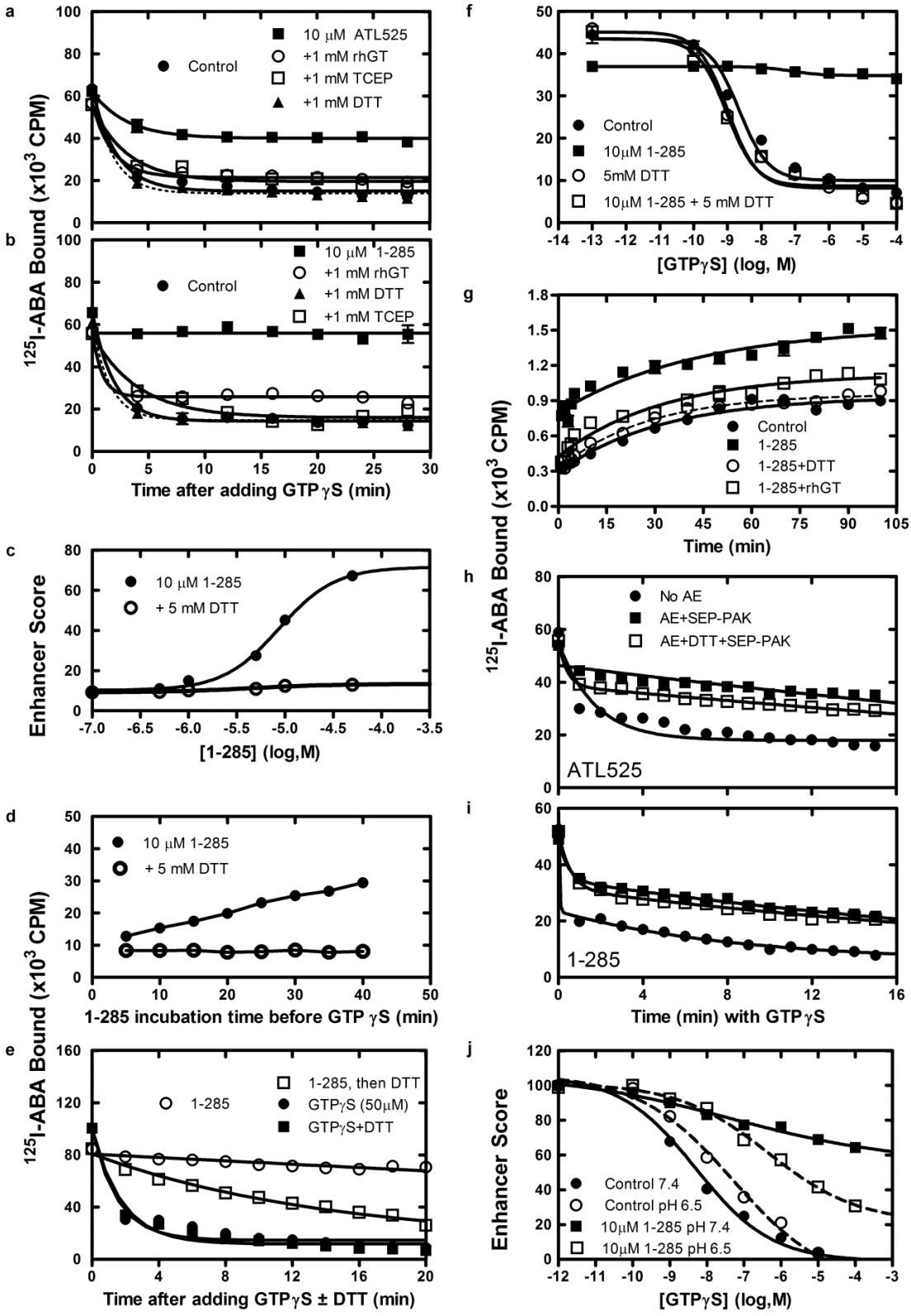
a
b
c
d
e
f
g
h
i
j
Control
10 μM ATL525
+1 mM rhGT
+1 mM TCEP
+1 mM DTT
10 μM 1-285
125I-ABA Bound (x10^3 CPM)
Time after adding GTPγS (min)
Enhancer Score
10 μM 1-285
+ 5 mM DTT
[1-285] (log,M)
1-285 incubation time before GTP γS (min)
1-285
1-285, then DTT
GTPγS (50μM)
GTPγS+DTT
Time after adding GTPγS ± DTT (min)
10μM 1-285
5mM DTT
10μM 1-285 + 5 mM DTT
[GTPγS] (log, M)
1-285
1-285+DTT
1-285+rhGT
Time (min)
No AE
AE+SEP-PAK
AE+DTT+SEP-PAK
ATL525
Time (min) with GTPγS
Control 7.4
Control pH 6.5
10μM 1-285 pH 7.4
10μM 1-285 pH 6.5

Figure 3: Thiol reducing agents eliminate AE Activity. (**a**) Effect of ATL525 ± thiols DTT, rhGT and TCEP on time course of GTPγS-induced agonist dissociation. (**b**) Similar to **a**, using 2-aminothiazole 1-285. (**c**) Effect of DTT on AE activity over several concentrations of 1-285. (**d**) Effect of 1-285 incubation time on AE activity, and the relative sensitivity to DTT. (**e**) Receptors at equilibrium binding treated as indicated. 15 min 1-285 incubation time, where indicated. 50 μM GTPγS added to all conditions at 0 min. (**f**) Concentration response curve for GTPγS ± 1-285, ± DTT treatment. (**g**) Total ^{125}I-ABA binding to CHO-K1 cells stably expressing human A_1R pretreated for 30 min ± 1-285, then treated with reducing thiols DTT, rhGT and TCEP at 0 min. (**h**) ATL525 and (**i**) 1-285 mixed with DTT, purified on a SEP-PAK column and used in these experiments to determine if AEs are chemically modified by reducing agents. (**j**) Effect of pH on AE activity GTPγS sensitivity at pH 6.5 or 7.4 ± 1-285. Higher pH favors oxidizing conditions.

Data compiled by Heidi Figler.

with AE and ^{125}I-ABA demonstrate that AE effects are slowly depressed (**Figure 3e**), so we confirmed that DTT does not affect GTPγS sensitivity (**Figure 3f**). As AEs also increase equilibrium ^{125}I-ABA binding in the absence of GTPγS, we determined that DTT and rhGT negate the AE-induced increase of ^{125}I-ABA binding to intact HEK293-hA_1R cells (**Figure 3g**). Similar results were observed in parallel experiments with 2-aminothiophene AEs.

AEs are not modified by reducing agents

DTT and other reducing agents may prevent AE activity by modifying A_1R residues, preventing oxidative reactions, or chemically modifying AE compounds. To distinguish between these possibilities, we incubated AE compounds ± DTT. We then removed DTT by passing the mixture over a C18 Sep-Pak column and washing with water. Residual AE (~58%) was eluted in methanol, dried and reconstituted to the original concentration. When AE activity was assayed, ATL525 (**Figure 3h**) and 1-285 (**Figure 3i**) retained their full activity, allowing us to conclude that the effect of reducing agents is not due to chemical modification of the AE.

Elevating pH increases AE activity

As a result of our experiments with reducing agents, we sought to identify a role of oxidation in receptor activation. If receptor stability does correlate with oxidative reactions, it should be slowed by lowering pH because protonated thiols are resistant to disulfide bond formation. In order to test this hypothesis, we adjusted the radioligand

binding assay buffer to pH 6.5 and 7.4. AE activity is attenuated at more acidic pH, indicating a potential role of oxidation in receptor activation and AE activity (**Figure 3j**).

The GPCR-conserved disulfide bond is surface-exposed on an A_1R homology model

As part of our previous work identifying an AE binding site in ECL2 (**Chapter 2**), we generated an A_1R homology model from an agonist-bound human $A_{2A}R$ structure (PDB ID: 3qak) (70). We inspected this model to identify locations or residues that may be involved in oxidation-mediated receptor stability. The A_1R homology model displays surface exposure of the conserved ECL1-ECL2 (C80-C169) disulfide bond (**Figure 4a and 4b**) (98). In our model, the conserved disulfide resides within a pocket with sides defined by structural domains (residues) TM2 (L68), ECL1 (P73, T75, T79) and TM3 (V83). This pocket is not present in the template structure (human $A_{2A}R$; PDB ID 3qak) (70). We identified two $A_{2A}R$ residues responsible for occluding this pocket on the $A_{2A}R$ crystal structure: F70 and I80. In the A_1R, these residues are P73 and V83, respectively (**Figure 4c**). In addition to being a smaller residue, P73 enlarges the pocket by swinging the ECL1 protein backbone away from the disulfide bond.

Mutations Occluding the A_1R Disulfide Bond Pocket Reduce Activity of ATL525

Residues encircling the disulfide bond pocket were mutated to tryptophan (L68, P73, T75, T79, V83) or the $A_{2A}R$ homolog phenylalanine (P73F) or isoleucine (V83I). Each mutation is predicted to have at least one rotomer configuration predicted to

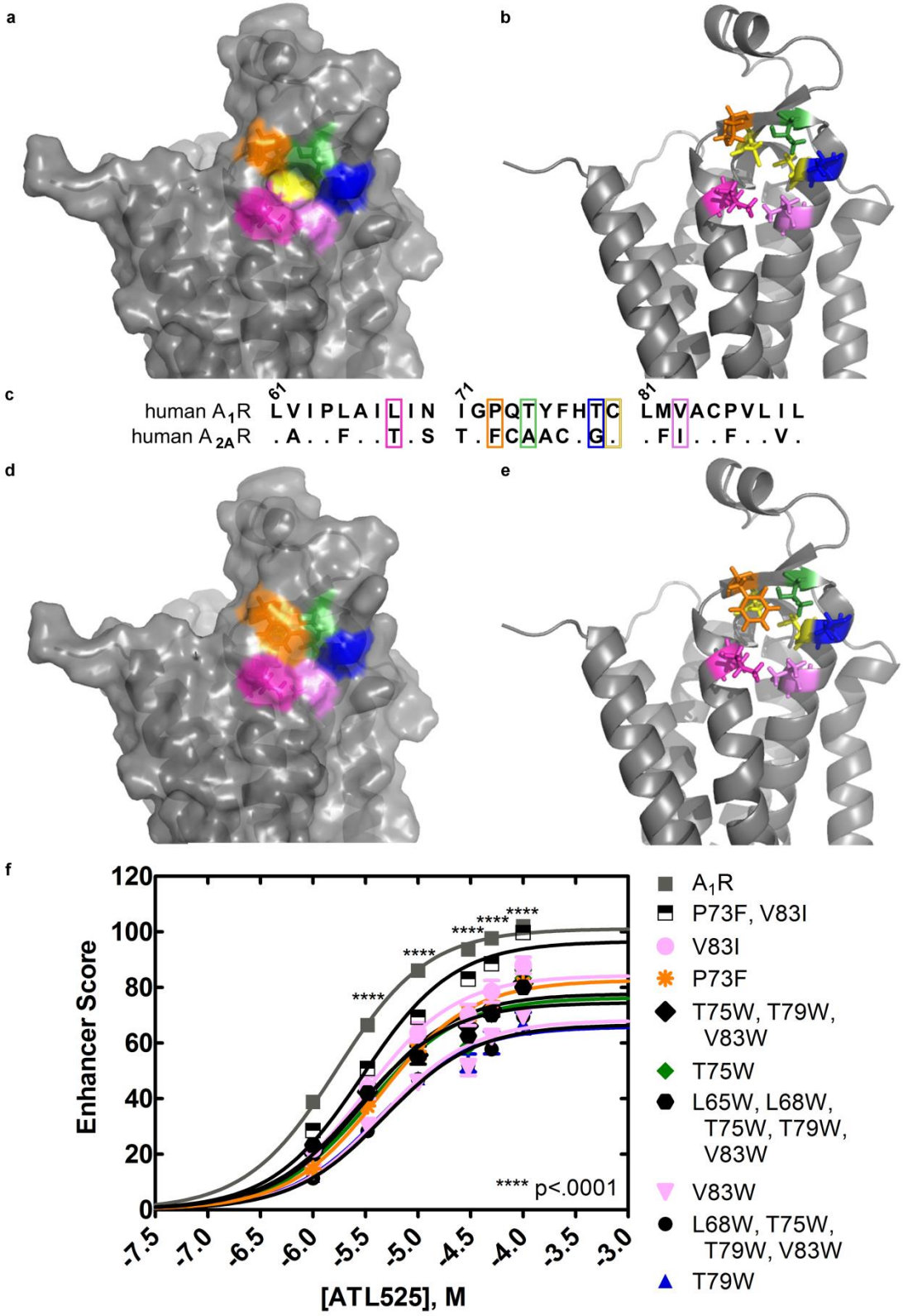
a
b
c
61
71
81
human A1R LVIPLAILIN IGPQTYFHTC LMVACPVLIL
human A2AR .A..F..T.S T.FCAAC.G. .FI..F..V.
d
e
f
Enhancer Score
120
100
80
60
40
20
0
-7.5
-7.0
-6.5
-6.0
-5.5
-5.0
-4.5
-4.0
-3.5
-3.0
[ATL525], M
**** p<.0001
A1R
P73F, V83I
V83I
P73F
T75W, T79W, V83W
T75W
L65W, L68W, T75W, T79W, V83W
V83W
L68W, T75W, T79W, V83W
T79W

Figure 4: The GPCR-conserved disulfide bond is surface-exposed in the human A_1R homology model. Depiction of disulfide bond surface-exposing pocket (**a**) Surface and (**b**) Cartoon and stick depiction of $A_{2A}AR$-based (PDB ID: 3qak)(70) A_1R homology model delineating the disulfide bond pocket with pocket-lining residues color coded: L68 (Pink), P73 (Orange), T75 (Green), T79 (Blue), C80 and C169 (Yellow), V83 (Light Pink). (**c**) Sequence alignment comparing A_1R with AE-insensitive $A_{2A}AR$. Human A_1R residue numbers as indicated. (**d**) Surface rendering and (**e**) Cartoon and stick depiction of P73F mutation introduced into the A_1R homology model. (**f**) Effects of mutations predicted to occlude the disulfide-exposing pocket on activity of ATL525 in the enhancer scoring assay. **** $p<.0001$

partially or fully occludes the disulfide bond (for example: **Figure 4d and 4e**). We observe that each of these mutations significantly reduced the AE activity of ATL525 (**Figure 4f**). At one position, V83, we separately introduced two mutations: V83I and V83W. The bulkier mutation, V83W, displayed decreased AE activity compared to V83I.

Aryl Disulfides Function as A_1R Allosteric Enhancers

Based on evidence suggesting oxidative reactions stabilize the agonist-bound A_1R conformation, we screened disulfide-containing compounds for AE activity. We found that aryl disulfides containing bis-ortho-urea functions have AE activity, slowing ^{125}I-ABA dissociation from HEK293-hA_1R cell lysates. The most potent member of this class was 4-41 (Bis-(2,2'-*N*,*N*-piperdinecarboxyamidephenyl)-disulfide) (**Figure 5a**). AEs prolong of the action of CPA to lower cAMP following addition of a receptor-saturating concentration of the antagonist cyclopentyltheophylline (CPT, 10 μM), demonstrating that 4-41, 1-285 and ATL525 all function in live, intact HEK293-hA_1R cells (**Figure 5b**).

Reactive Oxygen Species Stabilize the Active Conformation of A_1R

To further evaluate the role oxidative reaction play in receptor activation, we incubated A_1R with H_2O_2, a reactive oxygen species generated at sites of ischemia reperfusion injury (104). In the presence of H_2O_2, we observe a significantly increased AE score, indicating a more stable A_1R-G protein complex (**Figure 6a and 6b**). This

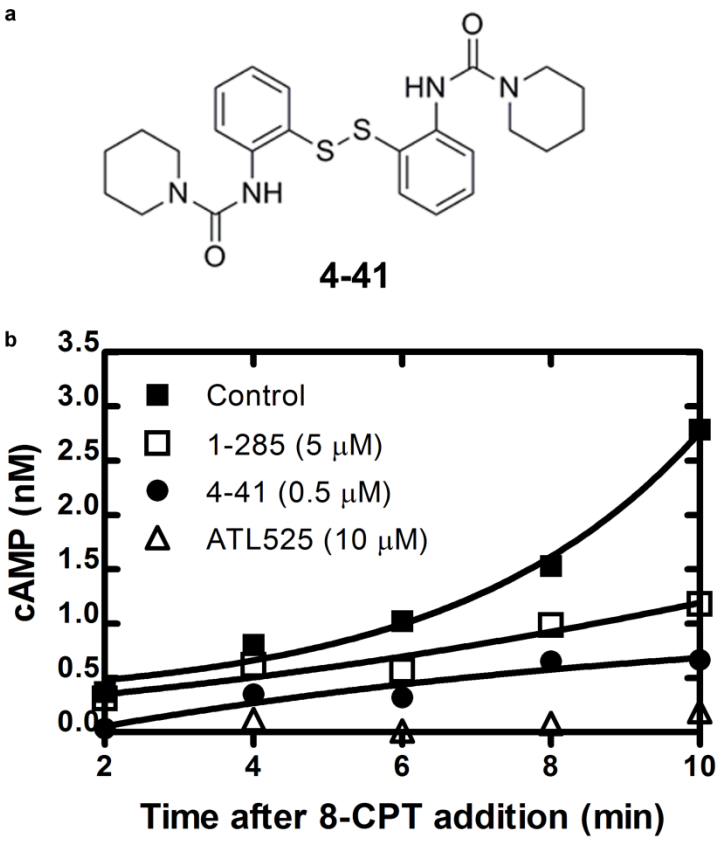

Figure 5: Aryl disulfide compounds act as allosteric enhancers. (**a**) Chemical structure of 4-41. (**b**) cAMP levels of whole cells following cAMP assay. Briefly, whole cells were treated with phosphodiesterase inhibitor rolipram +/- agonist, followed by incubation +/- AE and finally treated with antagonist, facilitating an increase in intracellular cAMP levels. Therefore, Lower levels of cAMP are indicative of higher AE activity (see Methods for full experimental protocol).

Data in (**b**) compiled by Heidi Figler.

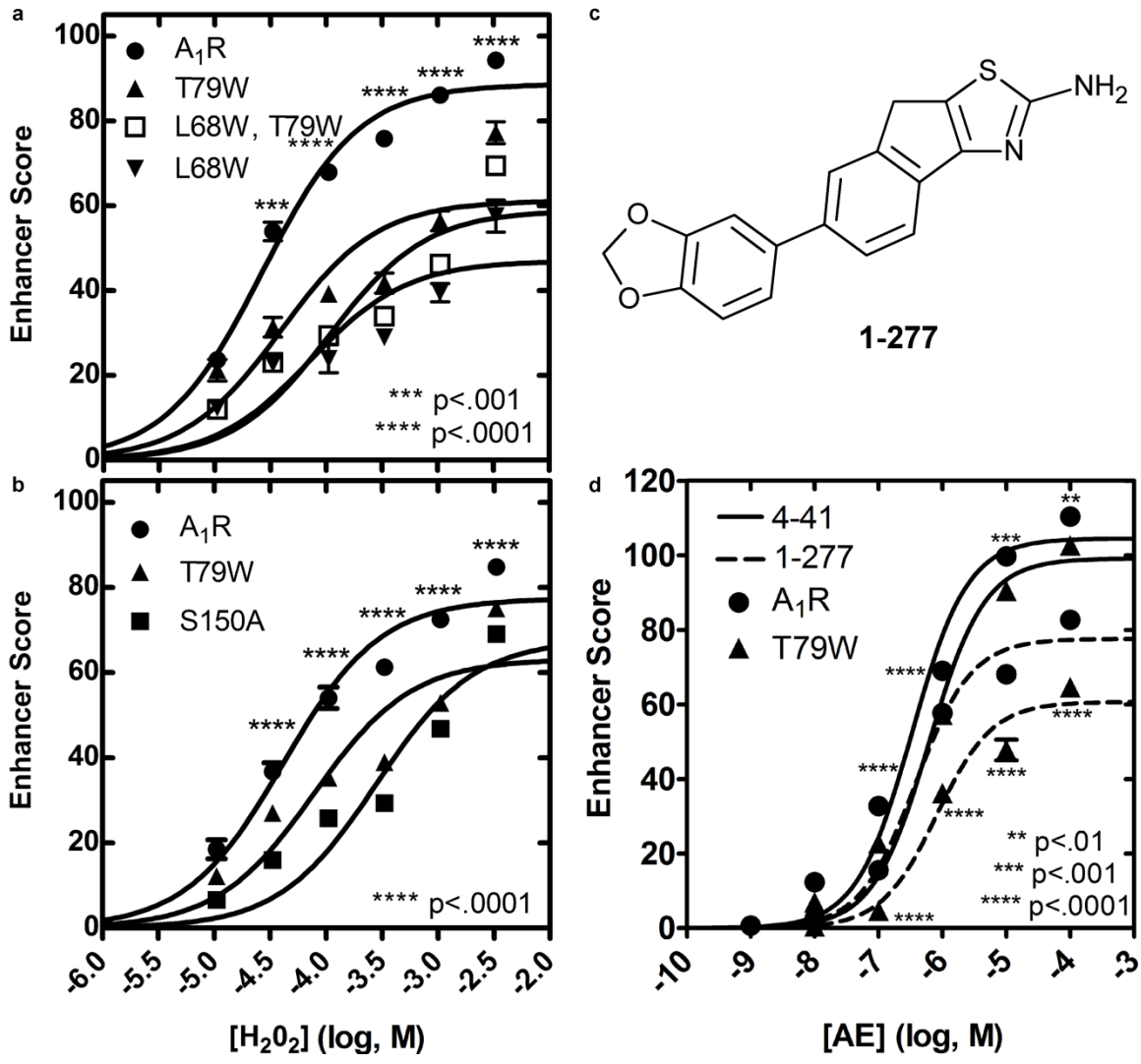
a
Enhancer Score
A1R
T79W
L68W, T79W
L68W
*** p<.001
**** p<.0001
b
Enhancer Score
A1R
T79W
S150A
**** p<.0001
[H2O2] (log, M)
c
S
NH2
N
O
O
1-277
d
Enhancer Score
4-41
1-277
A1R
T79W
** p<.01
*** p<.001
**** p<.0001
[AE] (log, M)

Figure 6: H_2O_2 stabilizes the active conformation of A_1R. Residual binding following H_2O_2 treatment and GTPγS-induced decoupling on (**a**) (●) A_1R, (▲) A_1R T79W, (□) A_1R L68W T79W (▼) A_1R L68W. (**b**) (●) A_1R, (▲) A_1R T79W, (■) A_1R S150A. **** p<.0001, *** p<.001 A_1R compared to mutations. (**c**) 2-aminothiazole 1-277 chemical structure. Disulfide pocket mutations reduce the activity of other classes of AEs. (**d**) Activity of aryl disulfide 4-41 (solid line) and 2-aminothiazole 1-277 (dashed line) in AE activity assay on (●) A_1R and (▲) T79W mutant

activity was decreased by pocket-occluding mutations L68W and/or T79W, indicating that these residues either prevent H_2O_2 from accessing a catalytic site, or that the disulfide bond pocket is mechanically important for AE activity. We continued to explore the effects of H_2O_2 by analyzing the effects of a mutation recently identified to mediate AE activity in ECL2: S150A. We observed that H_2O_2 activity is decreased in the presence of this mutation, indicating residue S150 is generally important to AE function (**Figure 6b**). S150 may be a residue key to integrating conformational changes resulting from G protein or agonist binding. 2-aminothiophene AEs interact directly with S150 (**Chapter 2**) and may prevent movement directly, but oxidative AEs may act at a remote site, their activity less able to be conveyed in the absence of the integrative S150 residue.

Mutations Occluding the Disulfide Bond Pocket Reduce the Activity of AEs

To better characterize the molecular components necessary for AE activity at the disulfide bond, we evaluated 2-aminothiazole 1-277 (6-(3,4-dimethoxyphenyl)-8H-indeno[1,2-d]thiazol-2-amine hydroiodide) (**Figure 6c**) and aryl disulfide 4-41 with T79W, the mutation that caused the largest reduction of 2-aminothiophene activity (**Figure 4f**). We observed that T79W resulted in reduced potency and efficacy of 1-277 and modestly reduced potency of 4-41, indicating that mutations occluding this site decrease activity of all three AE chemical classes (**Figure 6d**).

Two A_1R Residues Confer AE Activity to $A_{2A}R$

The $A_{2A}R$ crystal structure 3qak identifies two residues that occlude the disulfide bond: F70 and I80 (**Figure 7a and 7b**). We introduced the homologous A_1R residues in these positions with $A_{2A}R$ mutations F70P and I80V (**Figure 7c**). Combined, these two mutations are predicted expose the disulfide bond to the receptor surface when introduced into the 3qak background (**Figure 7d and 7e**). Based on the hypothesis that the disulfide bond pocket is important for AE activity, we evaluated whether these two mutations result in an increase in the activity of ATL525 on $A_{2A}R$-enriched S*f*9 cell lysates. In this is assay, we observed that both F70P and I80V mutations are required to increase AE activity in $A_{2A}R$, potentially because the disulfide bond is not rendered sufficiently surface-accessible by individual mutations (**Figure 7f and 7g**). We continued to evaluate the importance of the disulfide binding site-exposing F70P and I80V mutations by evaluating if these mutations were able to confer sensitivity to other chemical classes of AEs. We observed F70P and I80V combined to increase 1-277 activity 73%, but had no effect on aryl disulfide 4-41 (**Figure 7g**).

$A_{2A}R$ Disulfide-Exposing Mutations Increase H_2O_2 Activity

We reasoned that if H_2O_2 acts as an A_1R AE (**Figure 6**), it may also function on $A_{2A}R$. H_2O_2 activity was identified in $A_{2A}R$ and increased by the F70P and I80V mutation set (**Figure S1**). H_2O_2 activity on human $A_{2A}R$ can be evaluated in our enhancer scoring assay and has a score of 45.5 +/- 2.0. Combining F70P and I80V results in an increased score of 69.8 +/-2.3 ($p<.0001$), representing a 53% increase in activity. With

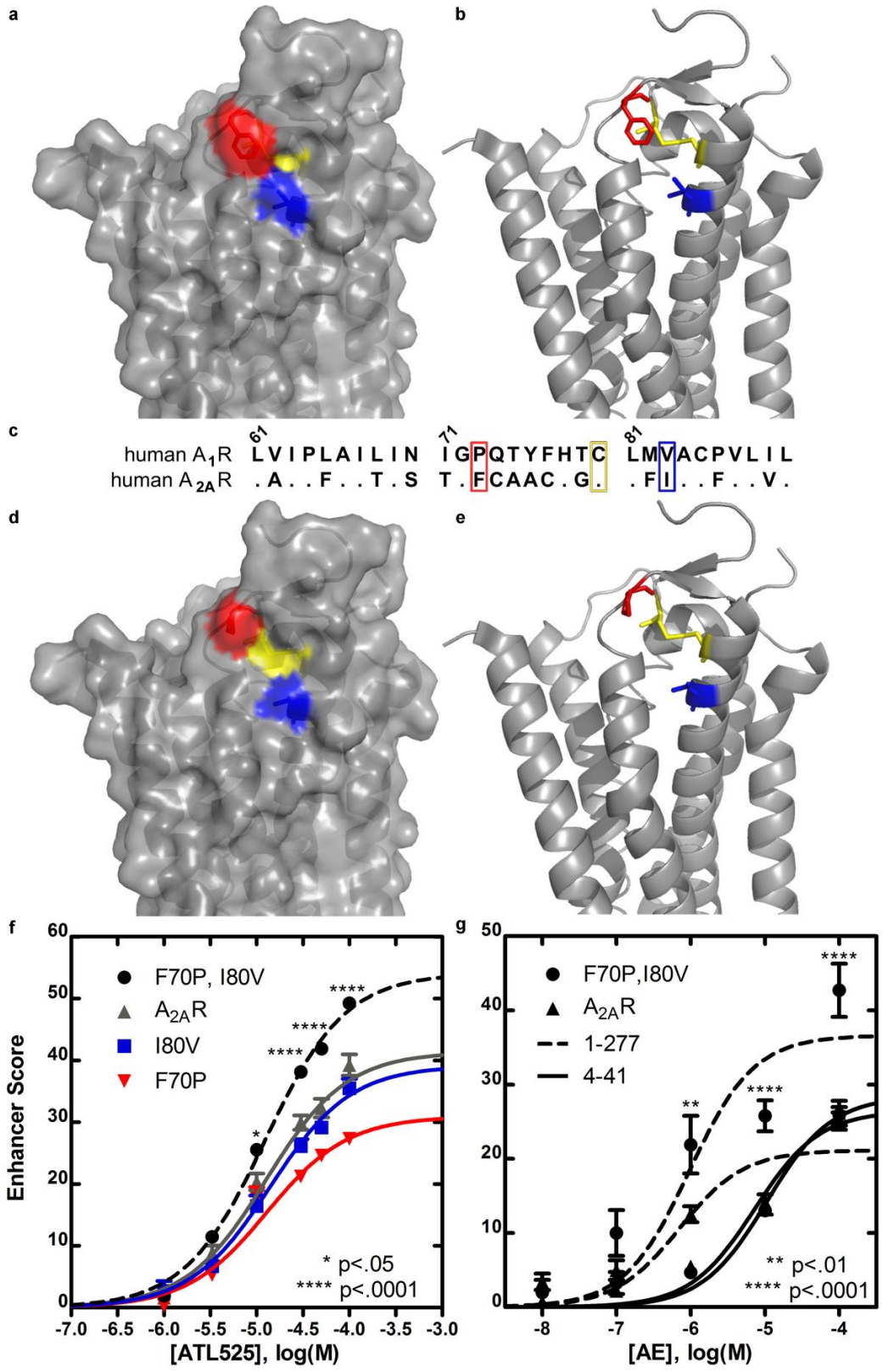
a
b
c
61
71
81
human A1R LVIPLAILIN IGPQTYFHTC LMVACPVLIL
human A2AR .A..F..T.S T.FCAAC.G. .FI..F..V.
d
e
f
F70P, I80V
A2AR
I80V
F70P
Enhancer Score
[ATL525], log(M)
* p<.05
**** p<.0001
g
F70P,I80V
A2AR
1-277
4-41
[AE], log(M)
** p<.01
**** p<.0001

Figure 7: Introduction of two A_1R residues in $A_{2A}R$ increase 2-aminothiophene and 2-aminothiazole AE activity. (**a**) Surface depiction of $A_{2A}R$ (PDB ID: 3qak)(70). Residue F70 (red), I80 (blue) and C77-C166 (yellow). (**b**) Cartoon depiction of A., with F70, I80, C77 and C166 depicted in sticks. (**c**) Sequence alignment of A_1R and $A_{2A}R$; A_1R numbering. (**d**) Simulated depiction of $A_{2A}R$ mutations F70P (red) and I80V (blue). Structure is otherwise identical to (**a**). (**e**) Cartoon depiction of (**d**). C77, C166 and mutations P70, V80 depicted in sticks. (**f**) Effect of mutations I80V and F70P on activity of ATL525 and (**g**) 1-277 and 4-41. Concentrations presented as (log, M) in **f** and **g**.

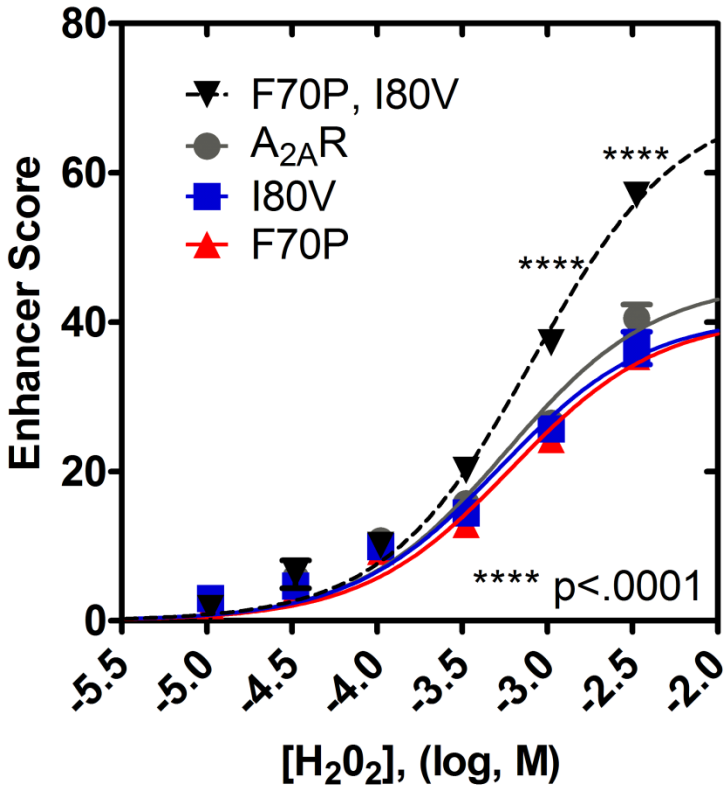

Figure S1: H_2O_2 activity on $A_{2A}R$ is also increased by F70P and I80V: effect of mutations I80V and F70P on activity of H_2O_2.

only the F70P mutation to surface expose the disulfide bond, the H_2O_2 score is 40.79 +/- 1.4 (ns). With only the I80V mutation the H_2O_2 score is 40.9 +/- 2.1 (ns). EC_{50} values were not altered by the mutations.

Several additional molecular contact points are likely not restored by these two mutations. Comparing the relative size of AEs to the surface exposure of the disulfide bond suggests that AEs interact with additional residues around the bond if they bind here. AEs are also likely not as efficacious on mutated A_{2A}Rs due to poor conservation of other binding site(s), including the ECL2 binding site. To our knowledge, this is the first reported instance of a GPCR allosteric site being engineered into another receptor.

Discussion

Understanding the mechanisms by which PAMs stabilize GPCRs will aid in the design of new, targeted therapeutic agents, the use of allosteric compounds for structural determinations and the translation of AEs to therapeutic uses. Herein, we show that AEs function by a reducing agent-sensitive mechanism that can be mimicked by H_2O_2 (**Figure 3a-i, 6a and 6b**). The GPCR-conserved disulfide bond that is essential for orthosteric ligand binding may be important for AE activity, as mutations to occlude the A_1R disulfide decrease AE activity, while reducing agents eliminate AE activity and mutations that expose the bond in A_{2A}R increase activity of normally A_1R-specific AEs (**Figures 3a-i, 4, 6d and 7**) (96,97). Previously, we identified an AE binding site in ECL2 of A_1R from which AEs block dissociation of orthosteric ligands. The disulfide bond binding site described herein is between TM2, ECL1 and TM3, on the outside of the A_1R structure

and distinct from the ECL2 site (**Figure 1,** blue triangle). Based on our current discoveries, we were able to synthesize a new class of AEs: aryl disulfides (**Figures 5 and S2**).

Three chemical classes of AEs have now been identified: 2-aminothiophenes, 2-aminothiazoles and aryl disulfides. Prior to our discovery of aryl disulfides, AEs were known to all share a five membered ring with a sulfur residue (position 1) and 2-amino group (28). While the structure of aryl disulfides is dissimilar to 2-aminothiophenes and 2-aminothiazoles, the structures of 2-aminothiophenes and 2-aminothiazoles also differ greatly, building substituents from opposite sides of the five member, sulfur-containing ring (**Figure 2**) (28).

Several experiments have probed the AE pharmacophore. Compounds lacking a sulfur (replaced with nitrogen or carbon) atom have greatly reduced activity compared to similar sulfur-containing compounds (23). Replacing the sulfur with selenium, however, increases AE activity (105). Based on our previous work, we speculate that residual, low level activity of AE compounds lacking sulfur (such as compounds 55-74 in (23)) can be attributed to binding to ECL2, functioning to block ligand exit by blocking the orthosteric agonist exit vestibule (**Chapter 2**). The discrepancy between sulfur, selenium, nitrogen and carbon atoms may be a result of atomic size or oxidation potential (105). Selenium is a more efficient oxidant than sulfur. However, AE activity also directly correlates with the atomic radius of these atoms.

The size of these atoms may be the key to AEs utilizing the disulfide bond pocket to stabilize the receptor. Upon inspection of $A_{2A}R$, we observed an interesting point of difference between the agonist and antagonist bound crystal structures of $A_{2A}R$ is the conformation of a section of TM3 directly adjacent to, and subjected to, our mutations (**Figures 5f, 6E and 6F**). In the antagonist bound structure of $A_{2A}R$ (PDB ID: 4eiy (14)), TM3 contains a kink (**Figure 8**, blue); however, in the agonist bound structure (PDB ID: 2ydv (76)) TM3 is straight (**Figure 8**, orange). The root mean square deviation (RMSD) between the C_α atoms in the two TM3 helices is ~2.5 Å. TM3 is a critical link between the orthosteric binding site and heterotrimeric G protein. Identifying this conformational change introduces the potential that the disulfide bond pocket sterically facilitates TM3 movement. TM3 connects to ICL2, which makes extensive contacts with $G_{\alpha s}$ in the crystal structure of the agonist-bound β_2 adrenergic receptor-$G_{\alpha s}$ complex (41). These structural changes may underlie the agonist-bound conformational selectivity of AEs (22,30). These results support a global hypothesis of AE activity where 2-aminothiophene AEs interact directly with ECL2 residue S150 to promote conformational stability and block agonist efflux from the orthosteric pocket, while H_2O_2 and aryl disulfides act at remote locations, their signals conveyed through conformational hubs, such as S150. Residues such as S150 may be analogous to proposed "hot spot" residues important for orthosteric binding in GPCRs (106). "Hot spot" residues were identified by statistical analysis correlating GPCR residue chemistry and position with ligand chemistry and activity. The positions identified were predominantly between TM2, TM3 and ECL2 (near the disulfide bond). In our proposed mechanism, mutation of

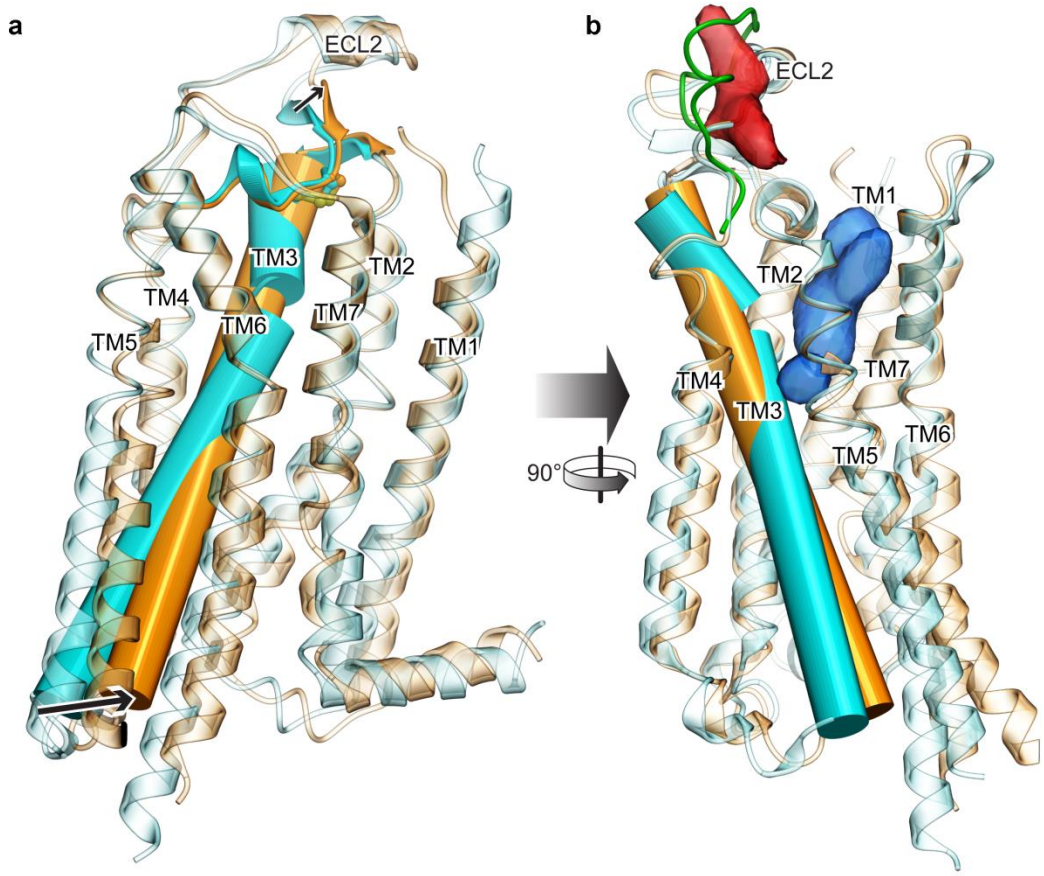

Figure 8. Superimposed ribbon models of representative $A_{2A}R$ X-ray crystal structures (grey ribbons) with bound agonist (PDB ID: 2ydv(76), TM3 orange cylinders) and antagonist (PDB ID: 4eiy(14); TM3 blue cylinders). Black arrows indicate movement of TM3 from the antagonist-bound state (blue) to the agonist-bound state (orange). (**a**) side view in which the orange lock shows the location of potential allosteric binding site, (**b**) top view showing $A_{2A}R$ residues 147-162, excluding flexible region of ECL2 for clarity. Conserved disulfide bond shown as sticks.

Figure prepared by Fiona McRobb.

conformational hub, or allosteric“hot spot” residues such as S150, results in a decrease in the activity of allosteric modulators. Oxidative AEs likely interact with multiple sites given the promiscuity of reactive oxygen species and small magnitude of activity reduction by disulfide bond pocket mutations on aryl disulfide compounds (**Figure 6d**).

Combining our conformational switching (**Figure 8**) and our other experimental results, we have developed three possible mechanisms of how AEs utilize the disulfide bond site:

1) AEs bind at this site and prevent the TM3 switch to the kinked conformation. In this scenario, replacing sulfur with selenium serves to better sterically inhibit the conformation change, while smaller atoms would be less suitable to do so. In this scenario, oxidation reactions are presumed to mimic AEs through a separate mechanism at a separate site.
2) AEs form an oxidative adduct, such as a thiol-disulfide exchange bond, with the surface-exposed disulfide bond residue C169. The bound AE constrains TM3 in the helical conformation. Oxidative species may also follow this mechanism, or may mimic AEs via a separate mechanism at a distinct site.
3) AEs function by catalyzing the formation of the C80-C169 disulfide bond which serves to stabilize the helical conformation of TM3.

By any mechanism, locking TM3 in a straight conformation may lock the receptor in the agonist bound state, blocking communication between the intracellular and extracellular sections of the receptor, stabilizing the conformational state of the receptor and resulting in a larger population of activated receptors without altering the orthosteric ligand K_D.

Our experiments probing the introduction of the disulfide bond site to the $A_{2A}R$ can also inform our mechanistic interpretation. While only one mutation around the A_1R disulfide bond site is necessary and sufficient to reduce AE activity (**Figure 4f**), both F70P and I80V mutations are necessary to introduce activity to $A_{2A}R$ (**Figure 7f** and **S1**). Coupled with our TM3 kink findings, these results suggest that access to the disulfide bond is essential to AE activity, and preventing TM3 kinking may be a secondary effect of AE binding, if also an essential component of AE activity.

While our results demonstrate the importance of the disulfide bond site, they do not demonstrate the full effect of this site. Incomplete pocket occlusion from rotomer switching and ECL1 movement prevents the mutations from blocking the disulfide bond at all times. The fraction of time these mutations spend blocking the pocket likely contributes to the variable inhibition of AE activity between mutations.

H_2O_2 and other reactive oxygen species are well known participants in the injury and stress response pathways. Herein, we identify that H_2O_2 modulates activity of a GPCR, a new mechanism by which reactive oxygen species alter cellular signaling and an addition to known injury response pathways influenced by oxidation, including cell migration, hyperplasia, inflammation, blood vessel relaxation, and apoptosis (107). The physiological consequences of oxidation-mediated A_1R active state stabilization may be especially relevant in cardiac tissues and at other injury sites. For example, ROS generated during cardiac ischemia and reperfusion injury may actually facilitate A_1R activation, resulting in physiological protection as A_1R activation promotes negative chromotropic and dromotropic effects on the heart, decreasing cardiac oxygen demand.

There are many therapeutic and pathophysiological implications of our results. First, they provide a novel mechanism by which oxidative signaling may function to modulate GPCRs. Second, pharmacological activation of the ARs is potentially beneficial for several conditions. While early pharmacological targeting of the ARs focused on cardiac functions, new indications have been discovered, improving tissue protection. Third, we have conclusively demonstrated that disulfide bond exposure increases AE activity. Combined, these factors demonstrate the potential for A_1R-targeted compounds, such as AEs, lacking cardiovascular contraindications.

Materials and Methods

Radioligand Binding

Radioligand binding was performed on receptor-enriched cell lysates as previously reported (26,30) (**Chapter 2**). We used an AE activity assay that measures ligand dissociation and therefore is not complicated by AE antagonist activity as the receptor is pre-bound to agonist. Receptors (10 µg in 50 µl) with 1.0 U/mL adenosine deaminase (ADA) and A_1R-specific agonist ^{125}I-ABA (0.5 nM in 50 µl; [^{125}I]*N*^{6}-(3-iodo-4-aminobenzyl)adenosine) are brought to equilibrium binding by 120 min incubation in 10 mM HEPES buffer (pH 7.4, except as indicated in **Figure 3j**). AE is added for 10 min, (30 min in A_{2A}R experiments) a period of time observed to yield the greatest distinction between mutations. Finally, 50 µl containing 50 µM non-hydrolysable GTP analog and physiological mimic guanosine 5-[γ-thio]triphosphate (GTPγS) and 100 µM xanthine amine congener (XAC), a non-specific AR antagonist, are added for 15 min (Such as in **Figure 3a**), 15 min are sufficient to evaluate the AE-induced resistance to GTPγS-induced dissociation. Alternatively, XAC and GTPγS are added over a time course to evaluate AE activity (such as **Figures 3b**, **3c and 3d**) XAC is added to ensure ^{125}I-ABA does not re-associate with receptor. The residual binding can be scored on a 100 point, unitless scale. "0" is residual binding in decoupling conditions: GTPγS and XAC without AE. "100" is equilibrium binding, only receptor and ^{125}I-ABA; no AE, GTPγS, or XAC. Scores greater than 100 are indicative of an (AE-induced) increase in equilibrium binding that occurs during the incubation of the AE and ^{125}I-ABA, before the addition of XAC and GTPγS. The scoring procedure was used in **Figures 3c, 3j, 4f, 6a, 6b, 6d, 7f and 7g**).

Assays were terminated by washing cell lysates three times and filtering through a 96 well plate, drying, and counting ^{125}I by gamma counter. Three experiments were conducted in triplicate and evaluated cell lysate from cells expressing human A_1R +/- mutations from ≥ two parallel-derived, stable cell lines. Reducing agents were added either with AE or with GTPγS, as indicated. Results were fit by "One site – specific binding" and compared by the extra-sum-of-squares F test for EC_{50} (potency) and maximal AE activity (efficacy) in Prism 5.0 (Graphpad).

All radioligand binding assays were performed in 96 well format with Multiscreen® HTS FC type C, 1.2 micron glass filters plates, purchased from Millipore (Billerica, MA). Washings and filtration of radioligand-bound 96-well plates were performed under vacuum on Brandel filtration device (Brandel Inc. Gaithersburg, MD). The cell lysates from each well on the 96 well plates are punched in the tube with Millipore multiscreen punching instrument (Billerica, MA).

Allosteric Enhancers

Synthesis and characterization of 1-277 [Compound 3ab](28); 1-285 [compound 3m],(28) and ATL525 are reported earlier (26,34), synthesis of 4-41 is described herein (**Figure S2**). All AEs were evaluated at concentrations less than 100 μM, as concentrations greater than 100 μM require solvent levels known to disrupt the radioligand binding assay. Fresh 10 mM stock solutions of AEs were prepared in dimethylsulfoxide (DMSO) daily, or stored frozen in small aliquots to avoid repeated freeze thawing.

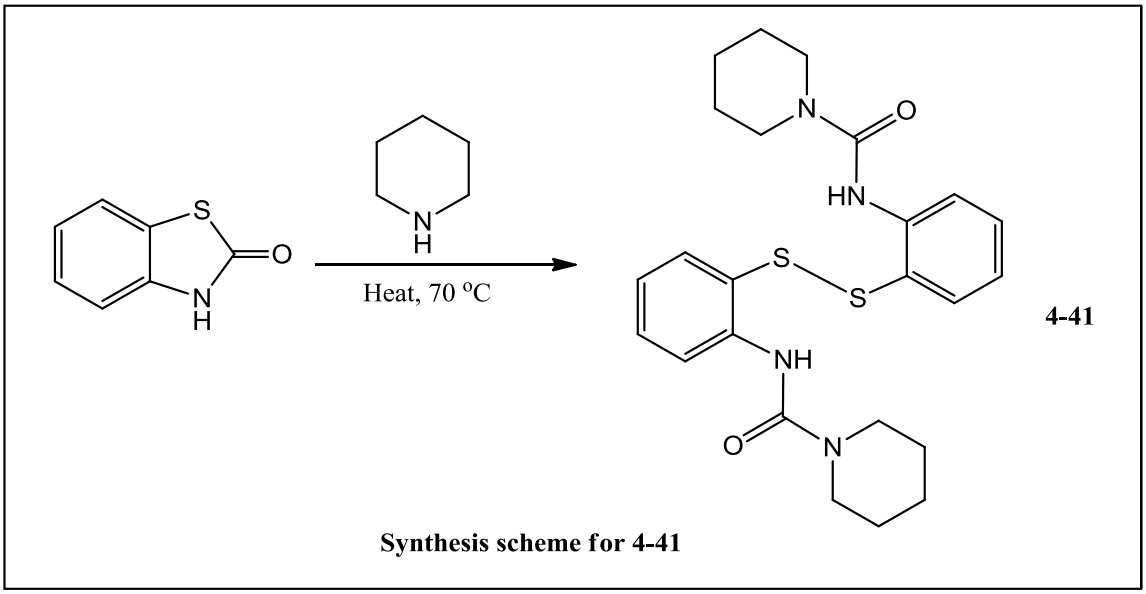

Figure S2: Synthesis of 4-41. Thiazolone (302 mg, 2 mmol) was mixed with excess of piperidine (1.7 g, 20 mmol) and heated at 70 ^{o}C in a vial for 16 hr. After cooling, the reaction mixture was diluted with ethyl acetate (10.0 mL) and washed with HCl (1 N, 5 mL). The ethyl acetate layer was separated, washed with water, dried over Na_2SO_4 and concentrated under reduced pressure to yield a viscous yellow solution. The compound was purified by column chromatography over silica gel using 10% ethyl acetate: hexane eluent to yield pure 4-41, as characterized by ^{1}H and ^{13}C NMR and mass analysis. Figure prepared by Mahendra Chordia.

Independent incubations of AE with DTT followed by SEP-PAK purification

10 mM stocks of 1-285 and ATL525 were diluted to 20 mM in 2.0 ml of dH_2O. A scan was completed to determine the UV_{max} for both compounds; UV_{max} for 1-285 was 309 nm, ATL525 was 280 nm. Absorbance was read for each sample; 1-285 A=0.246, D1 A=0.517) Each sample was split in half and 5 µl of 1.0 M DTT was added to 1.0 ml of each sample (final concentration was 5.0 mM). Samples of 10 µM AE ± DTT were incubated for 30 min at room temperature and then applied to 1.0 ml C18 SEP-PAK cartridges (Waters) that had been prewashed with 5.0 ml of dH_2O. DTT (and ~40% AE) was eluted by addition of 5.0 ml of dH_2O. AE was eluted in 1.0 ml 100% MeOH, evaporated to dryness and resuspend in 1.0 ml of 10 mM HEPES buffer (pH 7.4) and added to AE assays. Recovery of 1-285: A= 0.144, ~58%, ATL525: A=0.325, ~63%. Binding assays were performed as detailed above.

cAMP Assay

*S*tably transfected human A_1R-expressing CHO-K1 cells were collected by removing the culture medium, incubating with EDTA in PBS buffer for 10 min and washing twice with PBS saline. Cells were resuspended in PBS buffer with 10mM HEPES pH 7.2 and 2 U/ml of ADA. Cells were incubated with phosphodiesterase inhibitor forskolin to increase cAMP levels (30 min). Agonist cyclopentyladenosine (CPA) was added for 30 min, reducing cAMP levels. These were followed by antagonist 8-cyclopentyltheophylline (8-CPT) and AE. Therefore, lower levels of cAMP are indicative of higher AE activity.

A_1R Mutagenesis

Human A_1R was subcloned into the pDoubleTrouble vector (hexahistidine and FLAG peptide-tagged CLDN10B vector) (85) for expression in mammalian cells. Mutagenesis was performed using QuickChange Lightning® and/or QuickChange Multi Lightning® (Agilent Technologies). All mutations were confirmed by sequencing (Genewiz). P73F and V83I were generated to mimic AE-insensitive A_{2A}R. The evaluated single mutations are: P73F, T75W, T79W, V83I, V83W. The evaluated combination mutants are: L65W, L68W, T75W, T79W, V83W; L68W, T75W, T79W, V83W; T75W, T79W, V83W; P73F, V83I. All mutations were introduced in a human background. We were unable to generate a functional, ligand binding receptor with a lysine-glutamic acid salt bridge in place of the disulfide bond. Therefore, as direct mutation of C80 or C169 creates a receptor unable to bind adenosine or other orthosteric ligands, steric occlusion (i.e. tryptophan) mutagenesis is the best method to evaluate the role of the GPCR-conserved disulfide bond, (97,98).

Generation of Stable Cell Lines

All human A_1R constructs were stably expressed in HEK293 cells. Cell lines were selected for receptor expression with G418 (1.0 mg/ml; Inalco), as previously reported (**Chapter 2**).

Graphics

Surface rendering and mutagenesis modeling was completed in The PyMOL Molecular Graphics System 1.5.0.4 (Schrödinger, LLC).

Acknowledgements

ABA was a gift from Dr. Susan Daluge (GlaxoSmithKline, Research Triangle Park; NC), $Na^{125}I$ was purchased from Amersham Biosciences (Piscataway, NJ). ^{125}I-ABA was synthesized by iodination of ABA as reported (108). ADA was from Roche Diagnostics (Indianapolis, IN).

Abbreviations

1-277	6-(3,4-dimethoxyphenyl)-8H-indeno[1,2-d]thiazol-2-amine hydroiodide
1-285	(6-(4-(dimethylamino)phenyl)-8H-indeno[1,2-d]thiazol-2-amine-hydroiodide)
4-41	Bis-(2,2'-*N*,*N*-piperdinecarboxyamidephenyl)-disulfide
ADA	adenosine deaminase
A_1R	adenosine A_1 receptor
$A_{2A}R$	adenosine A_{2A} receptor,
ATL525	(2-amino-4,5,6,7-tetrahydro-benzo[b]thiophen-3-yl)biphenyl-4-yl-methanone
CPA	cyclopentyladenosine
8-CPT	8-cyclopentyltheophylline
^{125}I-ABA	^{125}I-aminobenzyladenosine
DDM	dodecyl-β-maltoside
DMSO	dimethylsulfoxide

DMF	dimethylformamide
DTT	dithiothreitol
GTPγS	guanosine 5-[γ-thio]triphosphate
H_2O_2	hydrogen peroxide
rfGT	reduced human glutathione
RMSD	root mean square deviation
TCEP	tris(2-carboxyethyl)phosphine
XAC	xanthine amine congener (XAC)

Author Contributions

I conducted the majority of experiments, all figures except Figure 8 and S1 and prepared the text. Heidi Figler conducted the experiments in Figures 3 and 5 and generated preliminary figures. She, under the direction of Joel Linden was the first to discover that H_2O_2 acts as an AE on ARs. Mahendra Chordia synthesized 4-41. William McIntire and Susan Leonhardt edited the manuscript. Melissa Marshall and Susi Srinivasan provided extensive technical assistance developing cell lines and conducting the radioligand binding assay. Fiona McRobb generated Figure 8. Ruben Abagyan oversaw Figure 8 comparisons, and Michael Purdy quantitatively assessed Figure 8. Mark Yeager assisted the conceptual development of the project. Joel Linden edited the manuscript and oversaw the conceptual development of the project.

Chapter 4:

Conclusions

CONCLUSIONS

Over several decades, numerous allosteric modulators that act on several receptors and channels have been identified. Many of the mechanisms by which these modulators elicit their effects remain unknown. Herein, the understanding of AEs, a subset of GPCR allosteric modulators, is expanded by: 1) identifying and describing how AE ATL525 traps ligands in the orthosteric pocket, 2) demonstrating that oxidation mimics AE activity in A_1R and $A_{2A}R$, 3) demonstrating that reducing agents eliminate activity of 2-aminothiophene and 2-aminothiazole AEs, and 4) discovering a mechanism to introduce AE activity in the relatively AE-insensitive $A_{2A}R$.

AEs were first described in 1990 by two publications that served as our best understanding of the mechanisms of AE activity for over a decade (22,23). These reports determined two components of ligand chemistry necessary for AE activity: hydrophilic and hydrophobic domains (See **Introduction Figure 6d** and associated text). Successive studies revealed that only the hydrophilic domain was conserved in active AE molecules (28), although separate, non-conserved hydrophobic domains were still necessary for activity. Chemically, the hydrophilic component is composed of a five-member ring with a sulfur atom in the 1-position, an amino group bound to the 2-carbon, and high electron density in the 3-position (nitrogen atom or carbon bound to an (oxygen-containing, electron-rich) carbonyl). The lipophilic component of the molecule was initially observed to bestow greater activity with large 3- substitutions, such as a benzoyl group (23). Later, carbon rings bridging the 4- and 5-position were discovered to increase AE activity proportionally with their size (24). These chemical groups were theorized to increase the

number of molecular contacts between these AEs and A_1R, however the potency of AEs has remained remarkably consistent, counter to the general expectation of compounds with greater molecular contacts.

Summary of Results

We observed that two non-overlapping sites on A_1R have a profound influence on AE activity, each with a different influence on the actions of AEs. From the first site, in ECL2, AE binding can trap agonists in the binding pocket. This result explains the previous observation that AEs increase B_{max} but do not alter K_D. Meanwhile, mutations to a site near ECL1, the disulfide bond site, also substantially reduce AE activity. Many of the residues mutated are directly adjacent to TM3, which we discovered to possess separate conformations between agonist- and antagonist-bound receptors. The observations at the disulfide bond site suggest that if AEs bind at both sites, they would possess two pharmacophores and explain why the sulfur residue is conserved, but not modeled to participate in molecular interactions with the A_1R ECL2.

We modeled that the hydrophilic component 2-amino group is immediately adjacent to residue S150 of ECL2 in human A_1R. Mutation S150A substantially reduces AE activity. We identified residue S150 by three experimental and interpretive procedures: 1) species scanning mutagenesis, evaluating non-identical residues attributing to variable activity between species, 2) alanine scanning mutagenesis of the ECLs, and 3) computational modeling and ALiBERO structural refinement. ALiBERO optimizes the A_1R structure, associating modeled AE binding with experimentally

determined activity. These methods demonstrate that, when bound to ECL2, AEs rest within the ECL2 fold, a binding pocket conserved between A_1R from several species and is similar and potentially homologous to other GPCRs, such as those predicted in the β_2-adrenergic receptor (40) and GLP-1R (46). In these GPCRs, this pocket is an important "pre-docking" site for orthosteric ligands; ligands bind in this site prior to entering the orthosteric pocket. In A_1R, this pre-docking site is exploited by AEs. Upon binding to this site, we propose that AEs trap bound orthosteric ligands, preventing their exit (**Chapter 2, Figure 4**). In fact, our computationally docked ligand poses reveal that bulky chemical groups and large rings bridging the 4 and 5 positions of the thiophene ring, extend toward the orthosteric pocket, further blocking routes of ligand exit. These results correlate with previous experimental findings (26). Based upon our findings, new AEs can be designed to specifically target this site, maximizing activity.

One particularly interesting component of AE activity is the degree to which AE compounds binding to extracellular domains are able to confer changes to intracellular receptor function, principally stabilizing the binding of G proteins. One component of the mechanism by which ECLs can confer intracellular changes may lie in the structural interpretation of the AE preference for agonist- over antagonist-bound A_1R; AEs do not effect antagonist function or binding, only that of agonists (22,30). Evaluation of antagonist-bound $A_{2A}R$ structures (such as PDB ID: 4eiy (14)) demonstrate a rotation and kink in TM3 that is absent in agonist-bound structures (such as PDB ID: 2ydv (76)) (**Chapter 2, Figure 5** and **Chapter 3, Figure 8**). This movement results in an altered orientation of ECL1 and movement of ECL2 via the GPCR-conserved disulfide bond.

This movement in ECL2 may prevent AE binding. Likewise, due to this structural change in TM3, the immediately adjacent ECL1 disulfide bond site may only be surface-exposed in the agonist-bound conformation. Upon AE binding, TM3 may not be able to resume the "kinked" conformation, essentially locking the receptor in the agonist-bound conformation. Curiously, the TM3 rotation is not present upon agonist binding to other GPCRs, such as the β_2-adrenergic receptor, which has no known allosteric modulators. By extension, the TM3 rotation and kink may be a mechanism by which AEs are specific for A_1R.

While identifying the binding site in ECL2 is a great advance in understanding the molecular mechanisms of AEs, it does not fully explain conserved AE chemistry and SAR: namely, there are no chemical interactions predicted between A_1R and the thiophene sulfur residue. Previous results demonstrated that AEs containing selenium, a larger atom with greater oxidizing potential than sulfur, increase AE activity (105), while smaller carbon or nitrogen decrease activity (23). Combined, these results pose three potential mechanisms of how AEs utilize the disulfide bond site:

1) AEs bind at this site and prevent the TM3 switch to the kinked conformation. In this scenario, replacing sulfur with selenium serves to better sterically inhibit the conformation change, while smaller atoms would be less suitable to do so. In this scenario, oxidation reactions are presumed to mimic AEs through a separate mechanism at a separate site.
2) AEs form an oxidative adduct, such as a thiol-disulfide exchange bond, with the surface-exposed disulfide bond residue C169. The bound AE constrains

TM3 in the helical conformation. Oxidative species may also follow this mechanism, or may mimic AEs via a separate mechanism at a distinct site.

3) AEs function by catalyzing the formation of the C80-C169 disulfide bond which serves to stabilize the helical conformation of TM3.

By any mechanism, locking TM3 in a straight conformation may lock the receptor in the agonist bound state, blocking communication between the intracellular and extracellular sections of the receptor, stabilizing the conformational state of the receptor and resulting in a larger population of activated receptors without altering the orthosteric ligand K_D.

We continued our investigations to identify that H_2O_2 acts as an AE (**Chapter 3, Figure 6**), and that AE activity is entirely sensitive to reducing agents (**Chapter 3, Figure 3**). From this observation, we were able to engineer the development of a new chemical class of AEs: aryl disulfides (**Chapter 3, Figures 5 and 6**). Curiously, aryl disulfide 4-41 was insensitive to ECL2 mutagenesis, but was modestly sensitive – along with the more pronounced sensitivity of 2-aminothiophene and 2-aminothiazole AEs – to mutations designed to occlude surface exposure of the disulfide bond formed between A_1R residues C80 and C169 and conserved among 78.9% of GPCRs (96).

The disulfide bond is exposed on the surface of an A_1R homology model based on agonist-bound $A_{2A}R$ structure (PDB ID: 3qak (70)). Disulfide bond surface exposure on A_1R is attributable to A_1R residues V83 and P73. P73 swings ECL1 away from covering the disulfide bond, exposing it on the outside of the molecule (**Chapter 3, Figure 4**). These residues are I80 and F70 in $A_{2A}R$. Together, they serve to sterically occlude the bond in that receptor. Introduction of A_1R mutation T79W, also designed and predicted

to occlude the disulfide bond, results in a decrease of potency, as observed by a higher EC_{50}, of all chemical classes of AEs: 2-aminothiophenes, 2-aminothiazoles, aryl disulfides and H_2O_2, although the effect on aryl disulfides is modest by comparison.

The final remaining component of the AE chemical structure that is not fully explained is the large, hydrophobic nature of substitutions to the 3-position of 2-aminothiophenes. These moieties are absent in 2-aminothiazoles, which contain a nitrogen atom at the 3-position (**Introduction, Figure 6c** and **Chapter 3, Figure 2b**). However, when aligning the sulfur and five-member ring of the highest activity 2-aminothiophenes and 2-aminothiazoles, large hydrophobic groups are attached to the 3-positions of the thiophene ring and the 4-position of the thiazole ring, demonstrating a requirement for hydrophobicity in the space near these positions (28). Within the ECL2 pocket, hydrophobic groups likely serve as contact points with A_1R residues. The ECL2 site is an enclosed hydrophobic pocket, enabling binding of hydrophobic molecules in the aqueous extracellular environment. Hydrophobic character of the AE may also increase activity at the disulfide bond site by allowing AEs to partition into the phospholipid bilayer: the polar sulfur, 2-amino group and electron rich 3-position can partition into the hydrophilic domain of the bilayer, while the rest of the molecule, being hydrophobic, can partition with the acyl chains of the bilayer (**Introduction, Figure 6d**).

Another finding with wide reaching implications is that two point mutations sensitized the relatively AE-insensitive $A_{2A}R$ to AEs, conclusively demonstrating that AEs and H_2O_2 act through the ECL1 site. This is the first instance of introducing the activity of an allosteric modulator to a GPCR (**Chapter 3, Figure 7**). Previously, similar

experiments have used chimeric proteins to evaluate allosteric sites. For example, in the muscarinic receptor GPCR subfamily, the 100x selectivity of alkane-bisammonium and caracurine V-type allosteric ligands for the M_2 receptor compared to the M_5 receptor is nearly entirely removed by two point mutations: Y177G and T423H (11). Similarly, the sodium allosteric binding site of the kainate receptor GluK2 was engineered to become a high affinity divalent cation binding site by a single point mutation: M739D (109). In addition to identifying oxidative regulation of A_1R (**Chapter 3**), our methods are the first to engineer an increase in GPCR sensitivity to allosteric modulation by small molecules or oxidative species (**Chapter 3, Figure 7**). These results define a site that may play a role in diseases potentially attributed to oxidative stress, including Alzheimer's disease, Parkinson's disease and diabetes mellitus.

Introducing the oxidation-sensitive mutation set may prove very important for studying agonist-bound GPCRs. Wider scale engineering of catalytic binding sites may have implications for drug screening, crystallization and other biochemical methods.

Potential Implications

Our results support a global hypothesis of AE activity in which 2-aminothiophene AEs interact directly with ECL2 residue S150 to promote conformational stability and block agonist efflux from the orthosteric pocket, while H_2O_2 and aryl disulfides act at remote locations, their signals conveyed through conformational hubs, such as S150. Upon mutation of residues such as S150, activity of H_2O_2 decreases (**Chapter 3, Figure 6d**). If residue S150 is a conformational hub, 2-aminothiophene AEs may stabilize it by

directly interacting with it. In such a scenario, mutation S150A creates a less efficacious hub, resulting in a decrease in the activity of H_2O_2, which is not large enough to occlude ligand efflux from the orthosteric binding site.

A comprehensive evaluation of mutagenesis data from the two allosteric sites suggests that if AEs interact with both sites, the ECL1-disulfide bond is a lower affinity interaction than the ECL2 site. This is supported by results demonstrating the correlation between AE incubation time and activity, and the slow reduction of activity upon the addition of DTT (**Chapter 3, Figure 3d and 3e**). This assessment is also sustained qualitatively: while the AE is predicted to be surrounded by ECL2 residues at the ECL2 site, the disulfide bond site offers minimal depth or dimension for interaction between AE and A_1R. Comparing our mutagenesis results, an EC_{50} increase is observed by mutagenesis at the disulfide bond site, but not the ECL2 site. If activity is derived from both sites, mutagenesis disrupting only the lower affinity will change the EC_{50} and activity, while only changing the higher affinity site will be observed only as a decrease in activity. Our results also suggest that binding to the disulfide bond site is not the rate limiting step of AE activity at this site. In these experiments, baseline activity is observed that may be attributed to AE activity from other sites (**Chapter 3, Figure 3e and Chapter 2, Figure 3c and 3d**).

Concluding Statements

The two sites identified and characterized may not be the only A_1R sites important for AE activity. Several groups have identified agonist-bound A_1R-independent effects of AEs (110-113). While we have identified and characterized two sites and mechanisms of AE activity, additional sites and mechanisms indicate that AEs are more promiscuous agents than previously thought. Cumulatively, all binding modes and mechanisms of AEs are likely not yet known.

Recent work on the β_2-adrenergic receptor helps conceptualize the number of potential sites and mechanisms from which allosteric modulators can alter receptor activity. Agonist binding to the β_2-adrenergic receptor was demonstrated to stabilize the extracellular half of the TM domains. Binding of G protein to the receptor stabilized the intracellular half (43). The ICLs are stabilized by G protein binding (41), leaving the extracellular loops as the only protein domain not stabilized when GPCRs are associated with G proteins. However, our results suggest that AEs may stabilize the A_1R-G protein complex by reducing the conformational flexibility of ECLs and thus reducing receptor movement. Essentially, any receptor domain can potentially alter activity.

While AEs were identified over two decades ago, knowledge of the specific molecular mechanisms facilitating AE activity enables the clinical development of AEs by allowing custom, targeted development of compounds optimized for these sites and mechanisms of action. Further experimentation may identify other GPCRs sensitive to these mechanisms, enabling the development of allosteric modulators preserving these molecular mechanisms, but specific for other receptors. The findings presented herein

advance our understanding of GPCRs and enlighten our understanding of the mechanisms of intramolecular activation. Combined, these results demonstrate how far pharmacology and the receptor concept has come since the time of Claude Bernard and G.G Stokes, but also reveal that there are still several receptor-based therapeutics waiting to be discovered. Ultimately, these results will facilitate therapeutic targeting of GPCRs by allosteric modulators, potentially resulting in the design of more GPCR-targeting therapeutics, advancing therapeutic strategies, ultimately improving human welfare and improving clinical outcomes.

Appendix:

Historic Rationale and Additional Background

Foundations of Receptor Theory in Biological Research[1]

Pharmacology, Greek for "the study of drugs," relies on one fundamental concept: drugs target receptors to elicit their effects. In the history of scientific research, the receptor concept is relatively young – John Newport Langley first described "receptive substances" in 1905 (116) after several decades of experiments suggesting drugs actions are elicited by receptors.

One of the first such experiments was reported in 1856 when Claude Bernard experimentally demonstrated the concept of receptors. He found that the poison curare is only effective when used on an arrow, not when given by mouth. Today, we know that this distinction occurs because curare cannot be absorbed by the digestive system. Bernard's experiments demonstrate that drugs and poisons must have access to specific body locations to properly function. His descriptions of "American toxins" were recently translated from his native French (117).

Soon after Bernard, in 1865, G.G. Stokes observed that oxygen introduced or removed from blood causes spectral changes to blood, indicating the formation of a complex between oxygen and a "colouring matter." Today we understand the "colouring matter" to be the protein hemoglobin (118).

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[1] Two sources assisted the conceptual development of this section:

114. Limbird, L. E. (2005) *Cell Surface Receptors: A Short Course on Theory and Methods*, 3rd ed., Springer Science+Business Media, Inc., New York,

115. Rang, H. P. (2006) The receptor concept: pharmacology's big idea. *Br J Pharmacol* **147 Suppl 1**, S9-16.

Paul Ehrlich is generally acknowledged to have established the biological concept of receptors. He discovered that lead and dyes are absorbed differentially between organs, suggesting the presence of an entity responsible for this result. (These studies were conducted between 1878 and 1905 and are reviewed with historical context (119).) In 1878, Ehrlich proposed "a definite chemical character of the cell" that was necessary for the reaction of dye with the cell. John Newport Langley was the first to use the word "receptor," in 1905, having previously referred to a physiological matter which forms "compounds" with pilocarpine and atropine in 1878. However, Ehrlich was not immediately convinced of the receptor concept. Only in 1907 – two years after Langley first proposed the presence of "receptive substances" – did Ehrlich acknowledge that drugs must target invading organisms with greater affinity than the host. Others were not convinced for reasons of diction. Another early Pharmacologist, H.H. Dale considered the word "receptor" as "speculative and a cloak for ignorance" (115). However, Dale contributed to the development of the receptor theory in his work on the physiological actions of ergot, having demonstrated the adrenalin reversal phenomenon and the muscarinic and nicotinic actions of acetylcholine. His findings on these topics were reported in 1906 (120).

The first hypothesis correlating receptor occupancy and response was made in 1926 by A.J. Clark, who proposed that receptor occupancy is directly proportional to response (121). In the 1960s, E.J. Ariëns and R.F. Furchgott refuted this hypothesis, observing that binding and response were not always directly proportional (122,123). In 1937, Gaddum derived equations to quantify the effects of two drugs competing to bind

at the same receptor – essentially describing the effects of antagonists (124). H.O. Schild later further developed the quantification of ligand binding when he introduced the Schild Plot in 1947, establishing the concept of ligand affinity for receptor (125).

Several of these theories and findings have brought about the drugs and therapeutic agents we know today by enabling development of the field of pharmacology. The first Department of Pharmacology was established by the University of Michigan in 1891, years after the first evidence of biological receptors, yet a decade before the receptor concept was given the name we know today. That department was chaired by John Jacob Abel, who co-founded the *Journal of Biological Chemistry* in 1905 and founded the *Journal of Pharmacology and Experimental Therapeutics* in 1909. The timing of the department's founding also coincided with a period of advocacy for food and drug safety legislation, capped by the 1906 adoption of the Pure Food and Drugs Act, signed by President Theodore Roosevelt as a response to publication of Upton Sinclair's *The Jungle*. This act established food and drug regulatory functions which were a precursor of the Food and Drug Administration. The act was also known as the Wiley act, attributed for a quarter century of advocacy and development by Harvey Wiley, Chief Chemist at the US Department of Agriculture from 1883-1912 (126).

Therapeutic Implications of the GPCR Superfamily

G protein coupled receptors (GPCRs) are expressed throughout the body and have a diverse array of effects on human physiology. As receptors that span the plasma membrane, GPCRs are mediators of extracellular signaling molecules, responsible for relaying signals to the intracellular compartment. These features have combined to make GPCRs the single largest class of drug targets. GPCRs comprise 26.8% of the 324 proteins targeted by therapeutic agents (58).

Conventionally, GPCRs have been pharmacologically targeted by chemical compounds acting as agonists, partial agonists, inverse agonists or antagonists. These compounds bind in the orthosteric pocket – the same location as endogenous, native ligand – to elicit effects on receptor function. These compounds act independently of, and frequently in competition with endogenous ligand (127).

The effects ligands can exert on receptors are governed by pharmacodynamics and pharmacokinetics. Pharmacodynamics are evaluated in several ways, including ligand activity at the desired target, undesirable drug effects, the duration of action, and receptor binding properties (K_d and B_{max}). Pharmacokinetics, are the "ADME" properties: absorption, distribution, metabolism, excretion. Due to these properties, different compounds targeting the same receptor can have vastly different effects on physiology (127).

The variation of therapeutic outcomes of just one receptor can be observed in the case of addictive medicines. In the example of diacetylmorphine, commonly known as heroin, a user administers the drug allowing quick access to the bloodstream: injection,

"snorting," or smoking. The drug rapidly enters the brain, where it is converted to morphine, and targets the μ-opioid receptor, a GPCR (128). Stimulation of the μ-opioid receptor initially presents the "high" associated with drug use, but can lead to dependence as the μ-opioid receptor is involved in the neurological reward pathway (129). In cases of dependence, patients are treated with methadone to prevent withdrawal symptoms (130-134). Methadone also acts as an agonist on μ-opioid receptors, but has a much greater half-life ($t_{½}$) than diacetylmorphine (15-60 hours, compared to ~5 minutes). Treatment of patients addicted to heroin with methadone can decrease the neurological (addiction) and pain (from withdrawal) impulses for heroin by providing a gradually decreasing stimulation to the μ-opioid receptor (135,136).

However, addiction is not the only complication of drug use. Overdose can occur when heroine is co-administered with alcohol. The physiological mechanism for overdose rests in μ-opioid receptors increasing signaling of the neurotransmitter γ-aminobutyric acid (GABA) (137). When co-administered with alcohol or other depressants, GABA stimulation can lead to fatal levels of suppressed breathing. In such cases, administration of more μ-opioid receptor agonist, such as methadone, is detrimental. A favorable therapeutic outcome is reached by reducing μ-opioid receptor signaling. Overdose must be treated by targeting the same receptor, with μ-opioid receptor antagonists such as naloxone or naltrexone (138). These antagonists are administered to rapidly remove μ-opioid stimulation and restore normal breathing patterns (139).

The μ-opioid receptor is one of the approximately 791[2] GPCRs that function in the human body. Of these 791, which account for roughly 2% of the protein-coding genome, a significant proportion (391) is involved in olfactory and pheromone sensing (141). Of the remaining, several have unknown ligands (termed orphan receptors) or are not currently targeted by therapeutics (140).

There are approximately 324 proteins in the body that are targeted by therapeutics (58). A plurality, 87 (26.8%), of these drug targets are GPCRs, although the majority of GPCRs have not yet been targeted by therapeutics. Consequently, targeting GPCRs by reverse pharmacology remains a reasonable path to drug discovery. Development of GPCR-targeting therapeutics retains residual challenges. Some GPCRs have not been targeted because their physiological properties, including endogenous ligand, function or expression location, have not been enumerated. Other GPCRs are difficult to target for pharmacological or biochemical reasons, such as chemical compounds that do not specifically target the protein of interest. Generally, these ligands either bind another receptor because the receptor binding pockets are similar between receptors, or because the chemical compound also targets other receptors. However, new technologies to screen chemical libraries, virtually screen computational libraries and optimize ligand

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[2] Other sources list the number of GPCRs as "over 800":
127. Laurence L. Brunton, e., and John S. Lazo, K. L. P., associate editors. (2012) *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 12 ed., McGraw-Hill Companies, New York
Or that the number of GPCRs has been identified, without citing a number:
140. Foord, S. M., Bonner, T. I., Neubig, R. R., Rosser, E. M., Pin, J. P., Davenport, A. P., Spedding, M., and Harmar, A. J. (2005) International Union of Pharmacology. XLVI. G protein-coupled receptor list. *Pharmacol Rev* **57**, 279-288).

chemistry for specific biological compartments have raised hope that many of the approximately 313 GPCRs that are not currently therapeutically targeted soon will be, allowing new mechanisms of therapeutic intervention.

The Adenosine Nucleoside

Physiologically, the adenosine nucleoside participates in several essential biochemical processes. In energy transfer, adenosine is the chemical foundation of adenosine triphosphate (ATP), the primary energy carrier in biology. Adenosine is also a chemical component of cyclic adenosine monophosphate (cAMP), an essential component of intracellular signaling. Adenosine also acts directly as a neurotransmitter to promote sleep, and in numerous other physiological processes via one of four adenosine receptors (ARs).

Pharmacologically, adenosine is administered to dilate the coronary arteries and restore oxygen supply to the heart during cardiac ischemia. By a separate mechanism, adenosine also slows electrical conduction through the AV node, converting paroxysmal supraventricular tachycardia (PSVT) and restoring normal heart rhythms (142).

Adenosine ((2*R*, 3R, 4S, 5R)-2-(6-amino-9*H*-purin-9-yl)-5-(hydroxymethyl)oxolane-3,4-diol; **Figure 1b**) is an endogenous purine nucleoside, conventionally defined as an adenine molecule (**Figure 1a**) connected by a β-N_9-glycosidic bond to ribofuranose. Attachment of phosphate to the 5’ carbon of adenosine results in generation of AMP, attachment of two phosphate groups yields ADP and three,

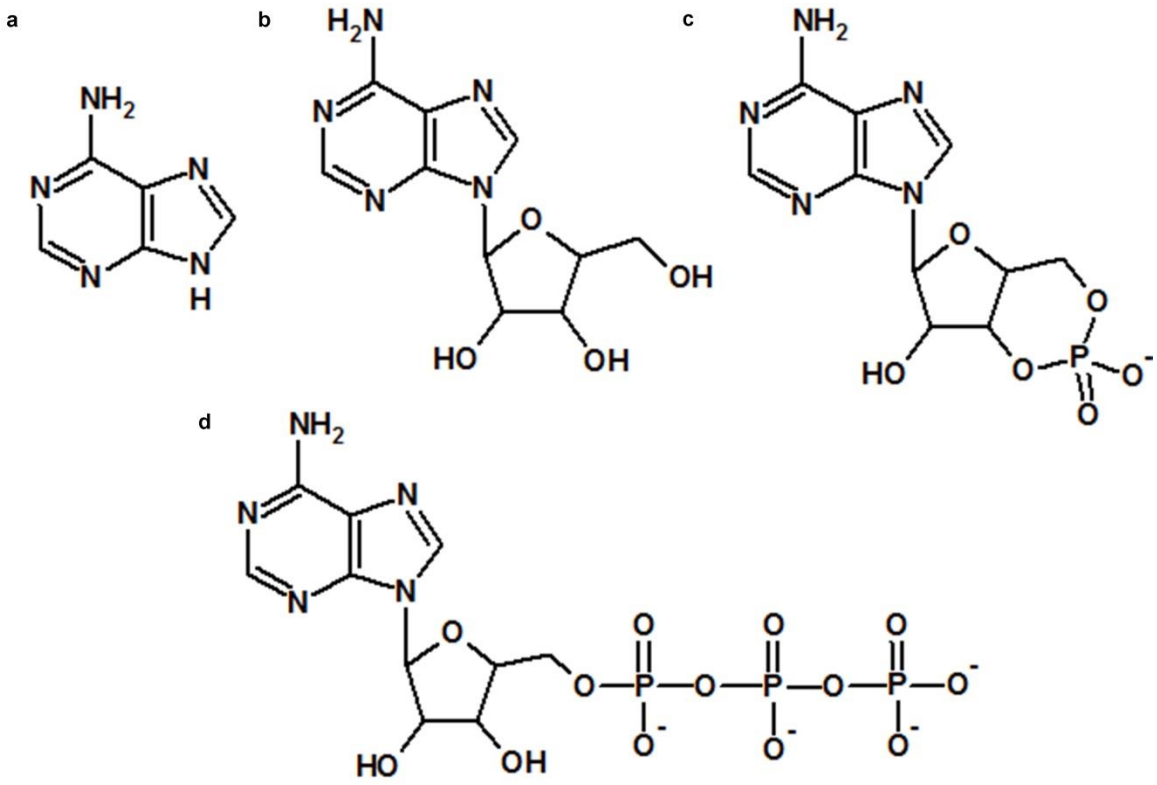

Figure 1: Chemical structures of (a) adenine, (b) adenosine, (c) cAMP and (d) ATP.

ATP (**Figure 1d**). Dual linkage of the AMP phosphate to the 3' and 5' positions yields cAMP, a widely utilized intracellular signaling molecule (**Figure 1c**) (143).

Socially, adenosine signaling is a component of the mechanism of the most widely used psychoactive substance in the world, caffeine (144). Caffeine and theophylline, the similarly acting compound found in tea and cocoa beans (145), are non-specific antagonists of ARs, a GPCR subfamily with numerous functions throughout the body. Caffeine and theophylline belong to the AR-antagonizing chemical class of methylxanthines. The effects of caffeine and theophylline on wakefulness are derived from blocking the neurotransmitter actions of adenosine.

Historically, the scientific discovery of adenosine dates to 1927 when A.N. Drury and A. Szent-Györgyi of the University of Cambridge identified a disturbance in cardiac rhythm when they injected extracts of bullock cardiac tissues into a guinea pig (146). By successive rounds of purification, the active agent of this activity was determined to be an adenine-based compound.

The involvement of adenosine in the dilation of coronary arteries was first proposed by Robert Berne*[3] in 1963 (147). Over time, his hypothesis has become known as "The Adenosine Hypothesis," and launched generations of adenosine research. Specifically, Berne's hypothesis was based on his experimental finding that an isolated heart subjected to hypoxic conditions resulted in a decrease in coronary vascular

[3] Asterisks (*) indicate this individual was a faculty member at the University of Virginia.

resistance and the release of inosine and hypoxanthine – consecutive products of adenosine degradation (adenosine is converted to inosine by adenosine deaminase and inosine to hypoxanthine by nucleoside phosphorylases). Further experiments revealed that adenosine could also dilate the coronary arteries, leading Dr. Berne to hypothesize that hypoxia results in adenosine release from myocardial cells, and this adenosine results in the dilation of coronary arteries.

Dr. Berne's lab produced two of the preeminent adenosine researchers in former postdoctoral fellows Drs. Joel Linden* and Luis Belardinelli*. Both initiated research that inspired successful adenosine-based drug discovery programs and biotech companies. Dr. Linden formed Adenosine Therapeutics, LLC (ATL), while Dr. Belardinelli joined CV Therapeutics (CVT). Combined, these companies represent all the current clinically approved and Phase III AR-targeting drugs, excepting adenosine itself, which was scientifically guided to the clinic based upon the research of Dr. Berne. ATL and CVT also account for all but one currently proceeding AR-targeting Phase III trials. CVT was purchased by Gilead, while ATL was purchased by Clinical Data, which in turn was purchased by Forest Laboratories.

Over time, the indications for ARs have stealthily increased from coronary dilation to also include treatment for PSVT and recently a wide range of other indications (55). Several of these indications are discussed in great detail in later sections.

The Adenosine Receptor Subfamily of GPCRs

Discovery of cardiovascular effects of adenosine led to much scientific investigation and experimentation of these phenomena. Over time, this experimentation demonstrated that methylxanthines act as competitive antagonists of adenosine in the heart (148) and brain (149,150), which promoted the hypothesis that adenosine binds to receptors. After further characterization, including the chemical synthesis of numerous adenosine analogs, A_1 and A_2 receptors were described, with A_1 decreasing intracellular cAMP concentrations and A_2 increasing these levels.

Today, it is known that adenosine derives its activity from interacting with four ARs. These GPCRs were identified by pharmacological characterization and confirmed as genomic information became available. They are named adenosine A_1 receptor (A_1R), $A_{2A}R$, $A_{2B}R$ and A_3R. Coronary dilation has been attributed to agonist stimulation of the $A_{2A}Rs$ in the coronary arteries, while conversion of PSVT rhythm by adenosine is attributed to A_1Rs expressed in the AV node.

AR function is analogous to other GPCRs as a result of their similar structure. GPCRs have seven transmembrane domains (TMs) connected by three intracellular (ICL) and three extracellular loops (ECLs). The N-terminus is extracellular, and the C-terminus is intracellular (**Figure 2**). Upon binding of agonist and G proteins, GPCRs undergo several changes, including a quarter turn rotation and large movement on the intracellular side of TM6 away from the midline of the molecule and TM3 and TM5. Generally, the agonist stabilizes the extracellular half of the TMs, while G_α stabilizes the intracellular half. These structural modifications were elegantly displayed in the crystal structure of

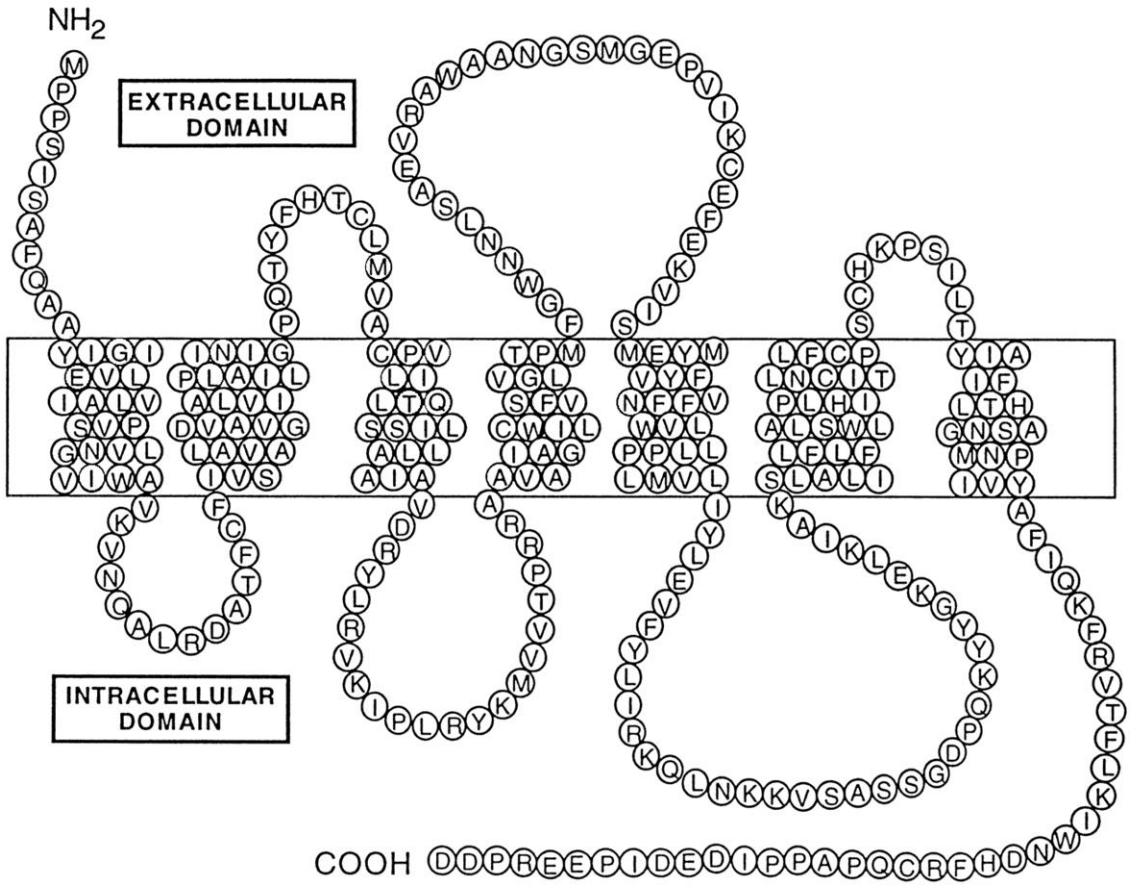

Figure 2: Conserved GPCR topology and structure can be seen in the example of the adenosine A_1 receptor. Transmembrane domains enclosed in rectangular box.

Figure adapted from (32). This research was originally published in *Journal of Biological Chemistry*. S.A. Rivkees, H. Barbhaiya, A.P. IJzerman. Identification of the adenine binding site of the human A_1 adenosine receptor. *J Biol Chem*. 1999 Feb 5; 274(6):3617-21. © the American Society for Biochemistry and Molecular Biology.

the β_2-adrenergic receptor in complex with G proteins (41) and subsequent experiments evaluating intramolecular dynamics following agonist binding (43).

Receptor activation results in intracellular changes that produce physiological modifications. In Dr. Berne's lab, Drs. Linden and Belardinelli made several important observations describing the physiological effect of ARs, including describing that adenosine counters the activity of adrenergic receptors in myocardia (151) and whole hearts (152). Later, they demonstrated that A_1R is downregulated and desensitized upon treatment with selective A_1R agonist R-PIA((-)hydroxyphenylisopropyladenosine), a finding made possible by Dr. Linden's development of several radioligands specific for ARs, including ^{125}I-ABA (^{125}I-*N*^{6}-4-aminobenzyladenosine) and ^{125}I-R-PIA. Many of these compounds display increased selectivity for A_1R, greatly reducing non-specific binding. Their development facilitated the study of A_1R in the heart, where it is expressed at much lower levels than in the brain (108,153).

Most GPCRs also contain a conserved extracellular disulfide bond between the short ECL1 (between TM2 and TM3) and the longer ECL2 (between TM4 and TM5). This disulfide bond is conserved in 78.9% of all GPCRs (96), and all the ARs. In the ARs, this bond is essential for immobilizing a phenylalanine residue that forms a side of the orthosteric binding pocket and participates in π-stacking interactions with the adenine ring structures (97). These recent structural observations have confirmed A_1R cysteine-scanning mutagenesis demonstrating that the only the cysteine residues essential for ligand binding are the two participating in this disulfide bond (98).

GPCR Mechanism of Action and Classification

The mechanism of action of GPCRs (127) is well characterized in textbooks, the literature and in classrooms. Briefly, GPCRs act via a cyclical mechanism, undergoing conformational changes upon the binding agonist that better allow them to accommodate G protein binding on their intracellular side. G proteins α, β and γ oligomerize with the receptor to form the heterotrimeric G protein complex, also known as the receptor-G protein (R-G) complex. G_{α} is a slow GTPase, which binds the inactive conformation of the receptor. Agonist binding to the receptor enables G_{α} activation: GDP dissociates from G_{α} and is replaced with GTP. This process activates G protein signaling and dissociation of G_{α} from the receptor. Over time, G_{α} hydrolyzes GTP to GDP to terminate signaling, and is again sequestered to receptor (**Figure 3**). There are multiple subtypes of each G_{α}, G_{β} and G_{γ}, enabling diverse intracellular signaling effects via several second messengers. For the studies described herein, the distinction between $G_{\alpha i}$ and $G_{\alpha s}$ are most critical. $G_{\alpha s}$ activation results in an increase of intracellular cAMP by coupling to and stimulating adenylyl cyclase. $G_{\alpha i}$ operates through the contrary: decreasing the output of cAMP by adenylyl cyclase. Of the ARs, A_1R and A_3R are $G_{\alpha i}$-coupled, while $A_{2A}R$ is $G_{\alpha s/olf}$-coupled and $A_{2B}R$ is $G_{\alpha s/q}$-coupled.

GPCRs are conventionally divided into three classes: A, B and C. These subgroups were determined by phylogenic similarities (154). Class A GPCRs are "Rhodopsin-like," and constitute ~85% of known GPCRs (662 members). Class A can be subdivided into the olfactory/pheromone receptors (391 members) and the endogenous

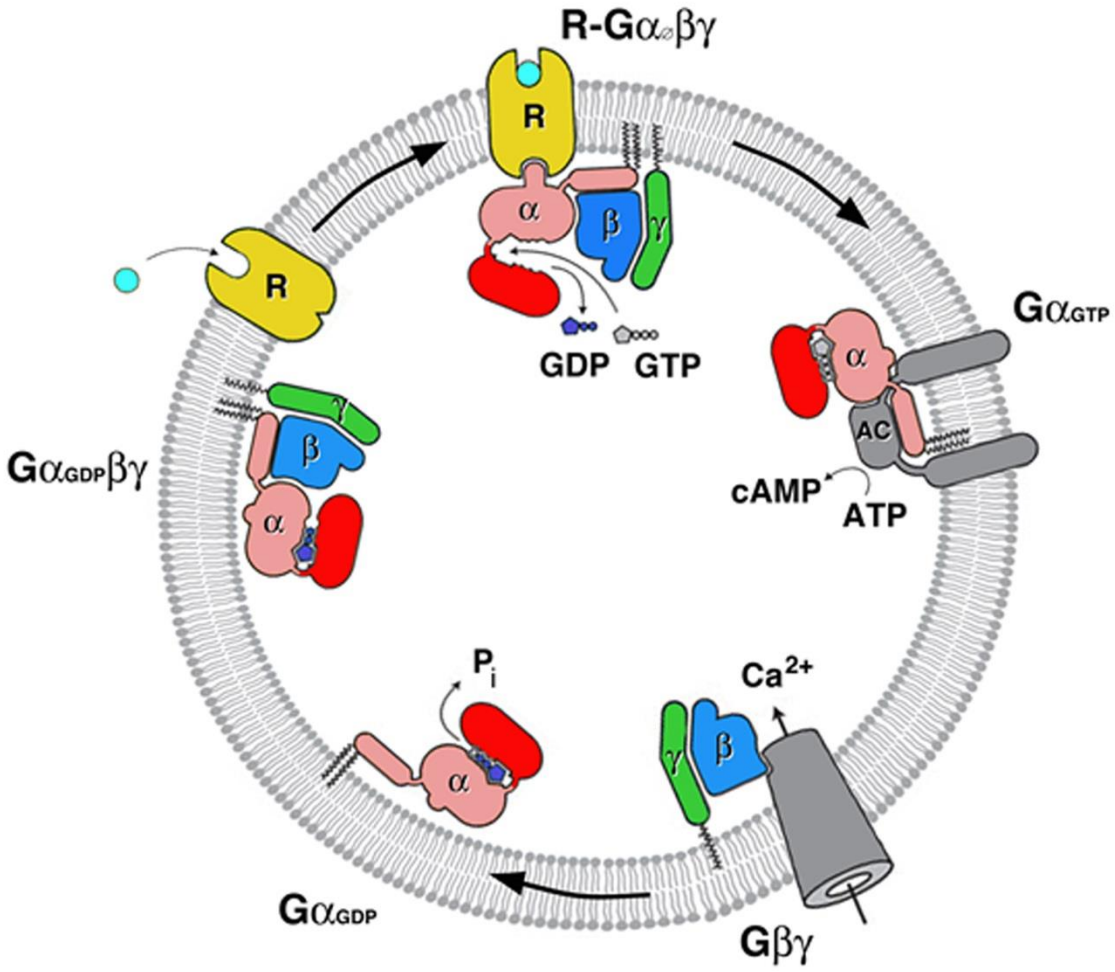

Figure 3: The GPCR activation cycle. R: Receptor, α, β, γ: G proteins, AC: Adenylyl cyclase, P_i: inorganic phosphate produced from GTP hydrolysis to GDP by G_α. Subscript after G_α denotes guanine nucleotide binding state: GTP-bound, GDP-bound or unoccupied. Light blue: GPCR ligand (e.g., adenosine).Calcium channel and AC activation depicted as representations of G protein signaling. Adapted from (41) by Kelly Dryden, Susan Leonhardt, William McIntire and Michael Purdy.

ligand and orphan receptors (271 members). Class B GPCRs are "secretin-like," and contain 15 members. Class C GPCRs contain 22 members and are termed "glutamate receptor-like."

A History of GPCR Research with Emphasis on Contributions from the University of Virginia[4]

The importance of both the mechanisms and indications of GPCR modulation started to become clear in the 1980s when G proteins were discovered as the conduit of GPCR signal transduction (155). This work built upon Dr. Earl Sutherland's discovery that hormones, specifically epinephrine, act via second messengers. Dr. Sutherland received the 1971 Nobel Prize in Physiology or Medicine "For his discoveries concerning the mechanism of the action of hormones," only to be followed in 1994 by Dr. Alfred Gilman* and Martin Rodbell "For their discovery of G-proteins and the role of these proteins in signal transduction in cells." Essentially, Dr. Gilman characterized G proteins (156-164), and Dr. Rodbell demonstrated the involvement of GTP in cell signaling (165-169). Combined, these awards acknowledged and foreshadowed the trend toward GPCR-targeting therapies and the increasing ability of scientists to focus on and evaluate drugs targeting GPCRs.

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[4] Asterisks (*) indicate this individual was a faculty member at the University of Virginia.

Dr. Sutherland's lab was very popular in the late 1960s. Both Drs. Gilman and Ferid Murad*, another future Nobel Laureate, aspired to work with Dr. Sutherland, who was departing Case Western Reserve University for Vanderbilt University. In the end, both Drs. Gilman and Murad were co-mentored by Dr. Sutherland and his younger collaborator, Dr. Theodore Rall*. Dr. Rall, who likely shares as many publications (eighteen) with more Nobel Laureates (three) before their prizes were awarded than any other scientist, collaborated with Drs. Sutherland and Murad to identify adenylyl cyclase (170-173). Dr. Rall also contributed to the discovery of cAMP (172) and identified factors affecting cAMP accumulation (174,175). Notably, Dr. Rall proposed the existence of and made discoveries essential for the identification of ARs (149,176). For his part, Dr. Gilman veered outside of G protein work during his postdoctoral studies with 1968 Nobel Laureate in Physiology or Medicine Dr. Marshall Nirenberg, who discovered the genetic code, before returning to Charlottesville, VA in 1971 as an Assistant Professor of Pharmacology at the University of Virginia. Dr. Murad received the 1998 Nobel Prize in Physiology or Medicine with Drs. Robert Furchgott and Louis Ignarro "For their discoveries concerning nitric oxide as a signaling molecule in the cardiovascular system," demonstrating the continued importance understanding the mechanisms of coronary artery relaxation and contraction have on greater human health.

With the intermolecular mechanisms of GPCR activation well characterized and the predominance of GPCR-targeting therapeutics in current medicine, much recent research has focused on new ways to capitalize on the large number of GPCRs that are not pharmacologically targeted. This research has included evaluating the structural basis

of GPCR pharmacology and activation. As a climax to their careers evaluating GPCRs, the 2012 Nobel Prize in Chemistry was awarded to Drs. Robert Lefkowitz and Brian Kobilka "For studies of G protein-coupled receptors." Dr. Kobilka is perhaps best known for solving the second crystal structure of a GPCR (β_2-adrenergic receptor) (177), and solving the structure of the β_2-adrenergic receptor in complex with G proteins, revealing several notable structural differences resulting from agonist and G protein binding (41). Dr. Lefkowitz was the first to clone the genes for eight adrenergic receptors, eventually enabling his lab to identify that GPCRs have several structural similarities, including how they bind ligand between the TM domains – a finding also enabled by his development of radioligands specific for the adrenergic receptors (178-180). These findings are cornerstones of the experiments described herein examining pharmacologically-induced modifications of orthosteric ligand binding.

The ascension of GPCRs as the largest individual class of therapeutic targets has correlated with three Nobel Prizes (1971, 1994 and 2012). These prizes have certified GPCR research as one of the predominant fields of scientific research. Future experiments will likely better delineate GPCR activation mechanisms and physiological activity, leading to more high level achievements and recognition. Likewise, the number of untargeted GPCRs may foreshadow the development of several new GPCR-targeting therapies.

Indications of Pharmacological Modification of GPCRs and ARs

GPCRs are therapeutically targeted to treat several extremely diverse conditions. Even among the AR subfamily, indications for agonists alone vary from reducing neurological pain (A_1R, A_3R) to tolerance of hypoxia (A_1R), wakefulness ($A_{2A}R$), vasodilation ($A_{2A}R$), vascular integrity ($A_{2B}R$), vasoconstriction (A_1R), reduced heart rate (A_1R), airway contraction (A_3R), inhibition of neurotransmitter release (A_1R), inhibition of insulin/glucagon release (A_1R), and decreased renal blood flow and tuboglomerular feedback (A_1R) (55). However, even these are not an exhaustive list, although they involve numerous organs, organ systems and physiological and pathophysiological states and conditions.

Currently, CVT $A_{2A}R$ agonist Regadenoson is the only approved adenosine-derived compound in the clinic, approved as a myocardial perfusion imaging agent. There are several adenosine-derived compounds in clinical trials, including Forest Laboratories' $A_{2A}R$ agonists ATL146e (phase III), ATL1222 (preclinical), and ATL313 (preclinical) for indications of myocardial perfusion imaging, acute inflammatory conditions and ophthalmic disease, respectively. CVT also has A_1R agonist Tecadenoson in Phase III clinical trials for the indication of PSVT. ATL844, an $A_{2B}R$ antagonist, is being pursued by Forest Laboratories for indications including asthma and/or diabetes. Preladenant was the only compound in Phase III clinical trials not developed by ATL or CVT (it was being developed by Merck & Co.), for the indication of Parkinson's disease. Despite positive Phase II trial results (181), Preladenant was discontinued in May, 2013 after no

drug effect was observed compared to placebo (www.clinicaltrials.gov ID: NCT01227265). Preladenant is an $A_{2A}R$ antagonist (182).

While these potential and realized AR indications are diverse, they also highlight some of the complications of clinical use of full agonists and antagonists. For example, if a clinician desired to decrease insulin release by targeting A_1R with an agonist, he or she would assuredly run into contraindications of reduced heart rate and vasoconstriction. However, unlike full agonists or antagonists, allosteric modulators possess several properties which may reduce such complications.

Mechanisms of Allosteric Modulation

As previously described, modern mechanisms of therapeutically modifying drug target activity generally involve chemical compounds targeting the endogenous ligand binding site (also called the orthosteric site). Molecules targeting this site can modify the target protein by either activating, as agonists or partial agonists, or blocking agonist activity, as antagonists or inverse agonists. Upon administration, drugs distribute to certain tissues or areas based on their biochemical properties. Upon reaching sufficient concentrations in these compartments, agonists and antagonists act on the receptors they target. Clinically, in addition to achieving desired effects, this property can result in off-target effects. For example, if a drug is targeting a receptor in the kidney, it will likely modify signaling of that receptor in the heart. This results in certain receptors involved in essential physiological functions, such as cardiovascular or neurological homeostasis, being restricted from pharmacological treatment regardless of the potential beneficial

effects such treatment may have for certain pathophysiological conditions or disease states.

An alternative mechanism of altering drug target activity is via allosteric regulation. The term "allostery" is derived from the Greek for "other object." True to their name, allosteric compounds act at sites outside of the orthosteric binding site. Allosteric agents elicit their effects through a diverse array of allosteric sites, and therefore utilize several mechanisms to elicit changes in target activity. However, allosteric compounds can be generally characterized by their function, as most commonly act to inhibit or potentiate target activity, and are accordingly classified as "negative" or "positive," respectively. Upon binding, positive allosteric modulators (PAMs) change the conformation of the target protein to promote signaling, resulting in an increase of orthosteric ligand affinity or efficacy. Conversely, negative allosteric modulators (NAMs) inhibit receptor activation and will decrease target affinity or efficacy.

Assays and Evaluation Parameters to Characterize Allosteric Modulators: Characterizing the functional mechanisms of allosteric modulators is essential for their clinical development. With several binding modes and functions, developing assays to evaluate the function of allosteric modulators is a challenging component of their study, yet essential to identify new allosteric modulators and evaluate their pharmacological and physiological properties. Historically, allosteric modulators have been identified and characterized by their ability to alter the pharmacological properties of orthosteric site radioligands. Such studies are conducted on receptors at a steady-state, equilibrium

bound to orthosteric ligand, an assay scheme that allows experimenters to discriminate changes to orthosteric ligand binding properties.

Generally, PAMs potentiate agonist binding and sensitivity by decreasing K_d (66). NAMs generally reduce both affinity (K_d) and efficacy (B_{max}) (83). However, not all allosteric modulators follow these patterns. For example, AEs increase absolute binding of orthosteric agonists, resulting in an increase in intracellular signaling efficacy, but AEs do not alter their K_d (22,30). Allosteric modulators can also promote receptor oligomerization (183).

Aside from static evaluation of agonist pharmacological binding properties, allosteric modulators can also be evaluated with kinetic assays to characterize the effects of allosteric modulation on receptor binding properties such as the orthosteric ligand on and off rates, k_{on} and k_{off}. Such kinetic assays have profound utility, as they increase the experimental sensitivity: conventional experiments evaluating K_d and B_{max} are limited by the magnitude of change of a regression line, which can be difficult to statistically differentiate (66). However, kinetic dissociation experiments can be quantified between two points: equilibrium bound receptor (100%) and fully dissociated receptor (0%). Allosteric modulators that slow dissociation fall between 0% and 100%, while modulators that accelerate dissociation reach "0" sooner, allowing innate dissociation to be scaled between 0% and 100% (114). A similar assay is used herein, whereby receptors are brought to equilibrium binding, pre-loaded with AE and dissociated with guanosine 5-[γ-thio]triphosphate (GTPγS) (**Figure 4**). As AEs stabilize A_1R, the "score" falls between GTPγS-dissociated, residual binding ("0"), and equilibrium binding ("100")

(30,99). Extending this method over several concentrations of AE, we were able to evaluate the subtle effects of mutagenesis on AE activity. These comparisons would not have been possible to statistically evaluate by comparing pharmacological binding properties of orthosteric ligand, such as EC_{50} shifts or changes in efficacy.

Radioligand binding assays are the most direct method to evaluate the effects of allosteric modulators on orthosteric ligand binding, as they measure the most elementary interaction of allosteric modulator activity, that of the orthosteric ligand. To better predict the viability of drug candidates, several functional assays have been developed to assess allosteric modulator activity. Several of these assays measure second messengers, such as cAMP, or orthosteric ligand signaling-dependent growth of yeast. However, when identifying a binding site, such methods lack the resolution necessary to identify small, mutation-induced activity changes. Additionally, as such endpoints do not directly measure orthosteric ligand binding, they may be complicated by other signaling pathways and/or the indirect (and often biologically amplified) nature of the measurement. Due to these reasons, functional assays are important tools to evaluate the efficacy of a drug, but are not as suitable for determining an allosteric binding site.

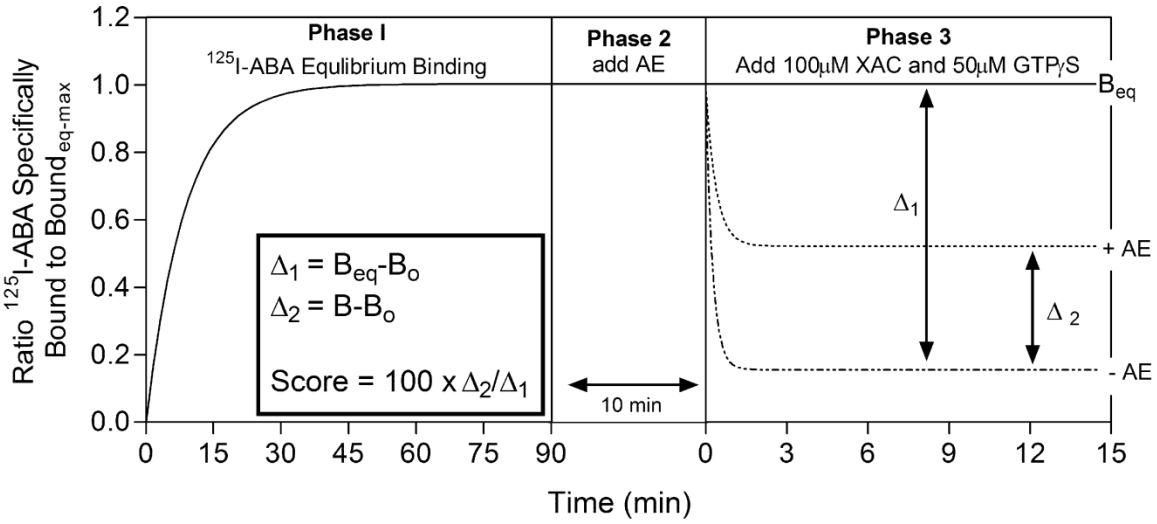

Figure 4: Experimental protocol for the AE activity assay measuring the conformational stability conferred by AEs. Adapted from (26,30).

The Molecular Basis of GPCR Allosteric Modulation – Protein Domains Implicated in Activity

In spite of the many difficulties of evaluating allosteric modulators, the potential pharmacological benefits have, thus far, outweighed the complications. Several research groups have evaluated GPCRs to identify the binding sites and mechanisms of action of allosteric modulators (reviewed: (21,55,66,83,183,184)).

A number of techniques have been used to identify allosteric binding sites. Two of the most common are targeted and scanning mutagenesis, designed to identify and disrupt molecular interactions between the receptor and allosteric ligand. Among GPCRs, mutagenesis experiments demonstrated that ECL2 residue Y177 is an important residue for allosteric modulation in muscarinic M_2 acetylcholine receptors. Chimeric M_2/M_5 muscarinic receptors and point mutations identified that Y177A reduced potency of NAMs (11-13).

In ARs, A_1R mutagenesis identified that T277A in TM7 and G14T in TM1 render A_1R insensitive to AEs, however T277A also makes a profound impact on agonist binding and G14T stabilizes the active state, indicating that these residues are likely not components of the AE binding site (31-33). Another report describes a more comprehensive mutagenesis strategy employed to identify A_1R domains essential for AE activity. Swapping the domains responsible for interacting with G proteins (the third intracellular loop (ICL3) and the C-tail) between AE-sensitive A_1R and relatively AE-insensitive $A_{2A}R$ creates two informative chimeras. The first is A_1R with an $A_{2A}R$ ICL3 and C-tail. These receptors are AE sensitive but couple to $A_{2A}R$ partner $G_{\alpha s}$. The second

chimera is AE-insensitive, chimeric $A_{2A}R$ with an A_1R ICL3 and C-tail. Coupling to G proteins is also reversed, as this receptor coupled to A_1R G-protein partner $G_{\alpha i}$ (34). While these chimeric replacement experiments did not alter AE sensitivity or identify a binding domain, they preclude ICL3 and the C-tail as binding site components.

A more conclusive mutagenesis strategy modifies the selectivity of the allosteric binding site. Thus far, this strategy has been utilized on two GPCRs: the kainate receptor GluK2 (109) and the muscarinic receptor M_5 (11). The GluK2 receptor was sensitized to allosteric modulation by divalent cations Ca^{2+} and Mg^{2+} by the single point mutation M739D. This mutation removes sensitivity to positive allosteric modulator and monovalent cation sodium (185). A similar strategy was used in the muscarinic receptors. Alkane-bisammonium and caracurine V type allosteric ligands are ~100x selective for the M_2 receptor over the M_5 receptor. Mutation of M_2 to M_5 residues, Y177G and/or T423H, nearly entirely removed the M_2 selectivity. Herein, I introduce the AE binding site into the $A_{2A}R$, the first documented introduction of a GPCR allosteric site (**Chapter 3**).

More recently, in addition to mutagenesis, researchers have explored "bivalent" ligands – orthosteric and allosteric pharmacophores tethered together – to delineate allosteric binding sites. In A_1R, such a study identified the ideal radius between the orthosteric and allosteric sites as the length of a 9-carbon chain (35).

Identifying binding sites, mechanisms of action and the chemical modulators themselves have presented several experimental difficulties. As a result, many research groups have turned to modeling, both mathematical and structural, to identify protein domains necessary for activity (19,36-38). Mathematical models have helped interpret

several possible ligand-receptor binding behaviors, with authors concluding the A_1R allosteric site is likely along the path followed by a ligand to reach the orthosteric site (39). Likewise, transition state modeling determined that allosteric modulators follow a concerted, or MWC allosteric mechanism (36,37). Structural modeling was used to interpret the bivalent ligand experiments and to deductively identify ECL2 as a potential site of AE binding in A_1R. These models demonstrated that ECL2, as the largest ECL, is most capable of accommodating AE within a 9-carbon radius of the orthosteric binding site (35). Structural modeling has been greatly informed by the recent publication of several GPCR X-ray crystal structures, nuclear magnetic resonance (NMR) and other biophysical studies identifying specific mechanisms of GPCR activation (41-44).

Clinical Applications of Allosteric Modulators

Several allosteric modulators are currently clinically available, with several additional compounds being brought to market for new indications. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) and miraviroc are currently used to combat HIV. Another allosteric modulator, cinacalcet is a calcium-mimetic. The benzodiazepines and barbituates are central nervous system depressants used clinically as sedatives, hypnotics, anxiolytics, and anticonvulsants.

The predominant clinically marketed class of NAMs is NNRTIs. NNRTIs inhibit function of the human immunodeficiency virus (HIV) reverse transcriptase (RT), an RNA-dependent DNA polymerase essential for the replication of retroviruses, such as HIV. Functionally, RT binds RNA and synthesizes a complementary DNA strand via

incorporation of deoxynucleoside triphosphate (dNTP) molecules. Conventional RT inhibitors are nucleoside analogs, and block RT function by preventing dNTP from binding in the active site. The general RT structure can be best understood if one imagines RT "grabbing" the nucleic acids with a (human) hand. NNRTIs function at a site ~10Å from the active site and prevent the RT "thumb" domain from clamping down the nucleic acids in the active site (comprised of the "thumb," "palm" and "finger" domains) (**Figure 5**). NNRTIs are thought to act as a wedge and prevent the grabbing action. This was elegantly shown in an NNRTI-bound crystal structure – the first of an allosteric modulator in complex with its target (186).

Another allosteric modulator used clinically to combat HIV infection is maraviroc. Maraviroc prevents HIV entry into T-cells and macrophages by binding to CCR5, a chemokine-sensing, class A GPCR essential for HIV entry into human cells. As an allosteric modulator, maraviroc does not directly block the CCR5 residues necessary for entry, but rather binds to a distinct site from where it induces structural changes disrupting the geometry of several points of contact between CCR5 and HIV protein gp120 (3,187-190).

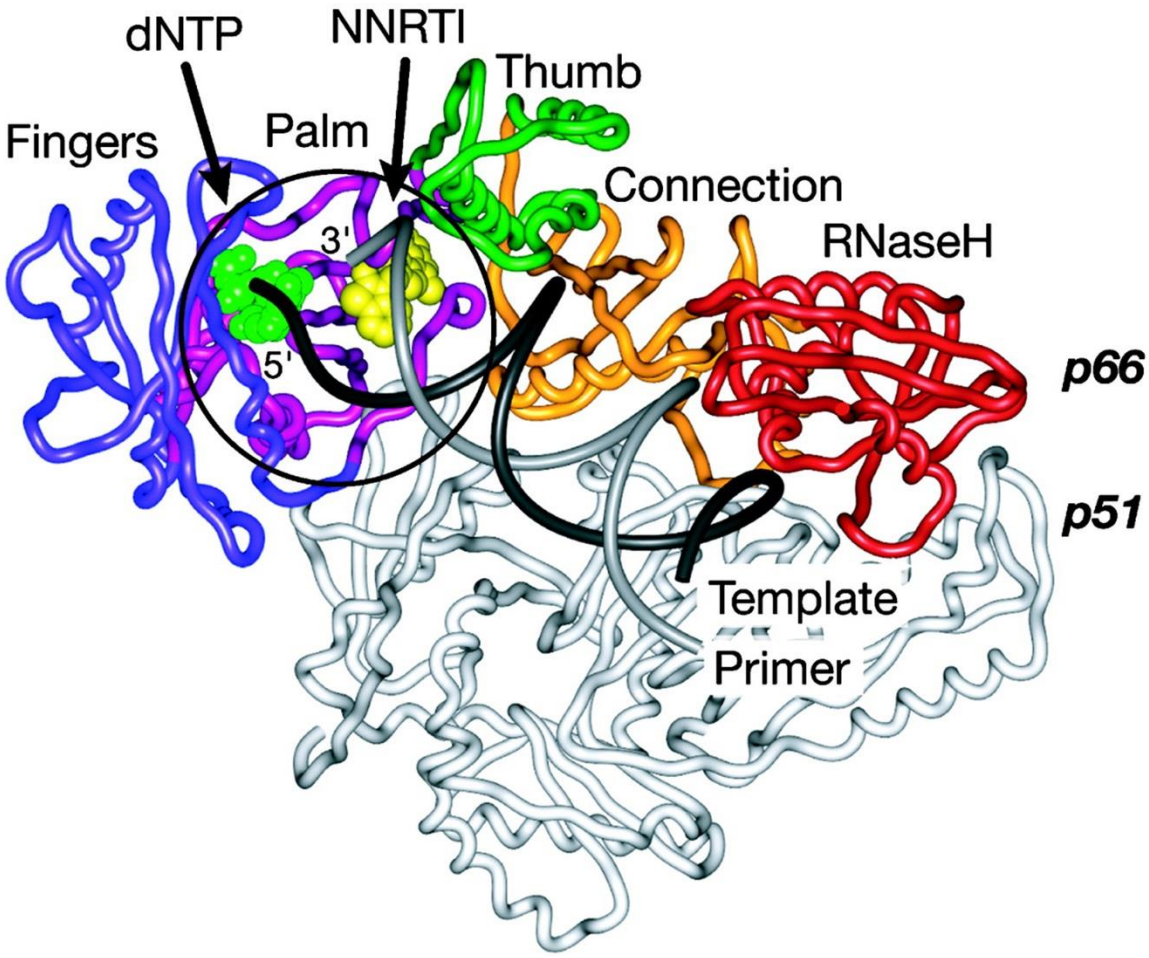
dNTP
NNRTI
Thumb
Fingers
Palm
Connection
RNaseH
3'
5'
p66
p51
Template
Primer

Figure 5: The allosteric NNRTI binding site is modeled to be structurally distinct from the RT active site. Figure from (186). Associated, original figure legend (with minor editorial changes): Model of HIV-1 RT with NNRTI, DNA primer/template, and incoming dNTP. The NNRTI from the structure described here (CP-94,707) is shown superimposed on the ternary complex of HIV-1 RT bound to DNA substrates, Protein Data Bank ID code 1RTD (191). The incoming dNTP (green) and CP-94,707 (yellow) are in space-filling representation. The DNA primer (light gray) and template (dark gray); fingers (blue), palm (purple), thumb (green), connection (yellow), and RNaseH (red) subdomains of the p66 subunit of HIV-1 RT; and p51 subunit (white) are in ribbons representation. The region circled includes the polymerase active site and NNRTI-binding pocket. The structure of this region in complex with CP-94,707 is shown in more detail in subsequent figures. Figures prepared with the program SPOCK (192).

The first approved GPCR-targeting allosteric modulator is cinacalcet. Cinacalcet is a calcium mimetic, acting as a PAM on the calcium-sensing receptor, a class C GPCR. As the endogenous ligand is the Ca^{2+} ion, chemically targeting the orthosteric binding pocket of the calcium sensing receptor is very difficult, causing allosteric targeting to be the optimal method of intervention. Cinacalcet has been proposed to bind in the center of the GPCR at the level of the plasma membrane, with chemical contacts in TM3 and TM7 identified by mutagenesis (4).

Recently, a phase II clinical study (www.clinicaltrials.gov ID: NCT00809679) evaluating the analgesic efficacy and safety of AE T-62 for subjects with postherpetic neuralgia was conducted, marking the first clinical trial of an allosteric modulator targeting ARs. The study was terminated as some subjects experienced asymptomatic, transient elevation of liver transaminases. Notably, transaminase elevation is frequently specific to chemical compounds, not drug classes. For example, nonsteroidal anti-inflammatory drug (NSAID) sulindac accounts for most NSAID transaminase elevation (54). The T-62 trial did not result in cardiovascular complications, retaining the potential for future AE therapeutics.

The pharmacokinetic profile of allosteric modulators is substantially different than ligands targeting the orthosteric site. For example, many allosteric modulators require endogenous ligand for activity and therefore target specific sites. Thus, when transient mediators, such as adenosine, are released (for example, during injury), AEs amplify AR signaling only in the site of release. The effects of AEs terminates when adenosine is metabolized. After adenosine is metabolized, the AE still resides in the

body, but does not alter signaling or A_1R activation and is "silently" metabolized over time. This contrasts with orthosteric-targeting agents that activate receptors throughout the body, following a sigmoidal pharmacological concentration-response profile (21).

A_1R Pharmacology

Agonists and Partial Agonists: A_1R agonists have generally been evaluated in clinical trials investigating the effects of activating A_1R expressed in the AV node of the heart. A_1R agonists targeting this location act as potent mediators of heart rate and pacing. However, A_1R expressed in the AV node also serve as a primary concern of compounds targeting A_1R for other indications; the potential for cardiac contraindications (such as heart block) is a very serious concern.

Chemical compounds selectively acting as agonists and antagonists have long been known for A_1R (55). Generally, A_1R agonists are adenosine-derived, but in 2004 capadenoson, a 2-aminodicyanopyridine was discovered as a non-nucleoside agonist. Generally, A_1R agonist selectivity over other ARs is chemically conferred by cycloalkyl or aromatic group (phenylisopropyl or phenyl) at the exocyclic amino group (55). Currently, at least four clinical trials are evaluating or have evaluated A_1R agonists capadenoson (BAY 68-4986) for treatment of atrial fibrillation (www.clinicaltrials.gov ID: NCT00568945), GW493838 for treatment of peripheral neuropathic pain (www.clinicaltrials.gov ID: NCT00376454), tecadenoson for atrial fibrillation (www.clinicaltrials.gov ID: NCT00713401), GS9667/CVT-3619 for treatment of hypertriglyceridemia associated with diabetes, GR79236 and DTI-0009

(www.clinicaltrials.gov ID: NCT00040001) for the study of slowing heart rate during atrial fibrillation. As A_1R agonists, these compounds result in a decrease in intracellular cAMP. Several agonist radioligands are commonly used to study A_1R, including tritiated (^3H) versions of: R-PIA, N^6-cyclohexyladenosine (CHA), and 2-chloro-N^6-cyclopentyladenosine.

Antagonists: A_1R antagonists are also employed for cardiac indications as they affect the cardiovascular system as profoundly as agonists. Rolofylline, SLV320 and BG9928/tonapofylline are all currently being developed for treatment of heart failure and volume overload. Rolofylline was evaluated in PROTECT-2 (www.clinicaltrials.gov ID: NCT00354458), a Merck and NovaCardia, Inc.-designed study to assess its effect on renal function and heart failure-associated congestion. The study did not demonstrate clinical efficacy of rolofylline, but did improve signs and symptoms of heart failure. A_1R antagonists are also being evaluated preclinically as diuretics, as the preglomerular arterioles constrict upon A_1R activation (55).

While not an A_1R specific, non-selective AR antagonist caffeine (with highest AR potency in A_1R), is currently being clinically evaluated for effects on ischemic preconditioning (IPC), an A_1R-attributed effect where repeated periods of transient ischemia protect tissues from subsequent, prolonged ischemia (www.clinicaltrials.gov IDs: NCT00184912 and NCT00184847).

Predicted Mechanisms of GPCR Ligand Binding

As clinical candidates, the mechanisms by which orthosteric ligands bind have been of great interest. The precise molecular mechanisms by which orthosteric binding occurs can inform our understanding of AE activity, as they influence agonist association and dissociation. A very informative recent study uses molecular dynamics simulations to predict the precise mechanism and energy barriers associated with orthosteric ligand entry and exit (40). The simulations in this study predict that several agonists and antagonists follow the same pathway – through several anticipated metastable states – to bind in the orthosteric binding pocket of the β_2 adrenergic receptor. The energy limiting step of the binding process is the first binding of ligand to a predocking vestibule between ECL2 and ECL3. From there, the ligand enters the orthosteric pocket via a crevice between ECL2, TM5, TM6 and TM7. About 50% of ligands that bind in the predocking vestibule enter the binding pocket, and the rest diffuse back into solution. The discovery of this site was proposed as a potential location for allosteric modulation. Currently there are no known allosteric modulators for adrenergic receptors. Our results demonstrate that AEs occupy an A_1R homolog of this vestibule.

Once in the orthosteric binding pocket, structural studies demonstrate that adenosine has an interaction in the $A_{2A}R$ with phenylalanine residue 168. This residue is conserved throughout the AR subfamily (F171 in A_1R), and is thought to participate in π-stacking interactions with ligands of all ARs (97). The location of this ECL2 residue underlies its importance. From the ECL2 backbone, it sticks down to form a side of the orthosteric pocket. It is also two residues removed from the GPCR-conserved disulfide

bond (C169-C80 in A_1R), which may function to hold this phenylalanine residue in place (along with the rest of ECL2) (97).

Recently, the connections between agonist and G protein binding have been further probed. Using NMR on β_2 adrenergic receptors containing mutations designed to evaluate receptor flexibility and movement, agonist binding was demonstrated to have a substantial effect stabilizing the extracellular half of the receptor transmembrane domains. While this did not explain how agonist binding results in $G_{\alpha s}$ binding, follow up experiments demonstrate that when G_{as} was also bound, the entire receptor was stable, with reduced transmembrane domain movement (43).

The circumstantial case for an AE binding pocket in ECL2 is further bolstered by data suggesting ECL2 is an important mediator of ligand binding in the glucagon-like peptide-1 receptor (GLP-1R). These authors demonstrated that ECL2 regulates orthosteric, but not allosteric binding (46). The results of this experiment suggest that the computationally predicted predocking vestibule (40) may be conserved between several GPCRs. However, the vestibule may have slightly different positions and functions – as a predocking site for orthosteric ligand between ECL2 and ECL3 in adrenergic receptors, but defined more by ECL2 in GLP-1R. As demonstrated in successive chapters, ECL2 residue S150 forms a key hydrogen bond with the AE 2-amino group, resulting in this pocket forming an allosteric binding site.

Reactive Oxygen Species (ROS) are Mediators of Cellular Signaling

Chapter 3 discusses the influence of ROS on A_1R signaling. This section intends to provide additional background and briefly summarize the influence of ROS on intracellular signaling and the mechanisms by which ROS carry out these activities. ROS is the name given to small, unstable, oxygen-containing molecules. ROS are generally derived from incomplete oxygen reduction, and refer to several chemicals, including: superoxide anion, peroxide, hydrogen peroxide (H_2O_2), hydroxyl radical or hydroxyl ion. All of these molecules have unstable electron configurations, allowing them to chemically function as oxidants. Their small molecular size allows them to function with limited specificity, which results in a short $t_{½}$ (193).

In cells, ROS are generated from at least two sources: mitochondria and nicotinamide adenine dinucleotide phosphate (NADPH) oxidases. Cells and mitochondria also have several enzymes designed to specifically remove reactive oxygen, including peroxidases, superoxide dismutases, catalase and glutathione peroxidases. Once generated, and if not degraded, ROS will oxidize several substrates. Specific examples of this oxidation include the peroxidation of lipids and oxidation of DNA and amino acids by hydroxyl radical and oxidation of metallo-enzyme complexes and amino acid residues cysteine and methionine by H_2O_2 (193). As a result of these disruptive chemical reactions, eukaryotic cells have evolved a two-step response to combat high intracellular concentrations of ROS: first is activation of antioxidant transcription programs by transcription factors, such as peroxisome proliferator-activated receptor-γ (PPARγ) coactivator-1α (PGC1α), which is specifically activated by H_2O_2. The second mechanism

is apoptosis, which can be triggered directly by enzymes such as mitochondrial peroxidase PRX3, or indirectly by factors sensing (ROS-induced) DNA damage, such as the p53 tumor suppressor (193).

ROS also serve to protect the body. As part of the innate immune response, polymorphonuclear cells (PMNs) and macrophages, upon sensing a microbe or other foreign entity, actively upregulate NADPH oxidase subunits that associate and convert molecular oxygen to ROS on the cell surface. These ROS serve several purposes. First, they are bactericidal, aseptic agents. Second, they recruit additional immune cells to the site of infection. Finally, however, they can cause tissue damage in surrounding tissues (and frequently cause the death of the PMNs themselves). The combination of the second and third factors results in tissue inflammation around septic tissues and necrotic cells. Cell necrosis can be triggered by surgical intervention or pathophysiological situations such as those that occur during ischemia or ischemia-reperfusion injury, underscoring the importance of ROS in clinical outcomes.

Ischemia-reperfusion injury is an inflammatory injury resulting from the restoration of blood flow after a period of hypoxia. One common site of ischemic and reperfusion injury is the heart. Through multiple mechanisms, cardiac injury and stress result in the generation and extracellular release of ROS from cardiac myocytes (104) and infiltrating immune cells. IPC (ischemic preconditioning), characterized by repeated short periods of ischemia followed by a prolonged ischemic event, is a specific ischemia-reperfusion injury and protects cardiac tissues through ROS and A_1R signaling. AEs exert an additional protective effect during IPC in the heart (53) and kidney (61).

While there is still much to learn about ROS biology, what is known indicates ROS act via two contradictory mechanisms. While ROS kill invading cells, they are also a suspected or proven component of several diseases, including Parkinson's disease, Alzheimer's disease, atherosclerosis, sickle cell disease, diabetes, heart failure and myocardial infarction. ROS are also suspected of inducing mutations that can result in the formation of cancer cells (194).

New mechanisms of ROS activity have recently been identified. In addition to being an injury mediator, ROS act as positive allosteric modulators of several ligand- and voltage-gated ion channels, including the purinergic $P2X_2$ channel (195). The specific mechanisms and molecular determinants of this modulation are unknown. ROS are also indicated in altering protein expression, including increasing A_1R expression over 24 hours (196). Recently, H_2O_2 was demonstrated to participate with adenosine A_{2A} receptors and smooth muscle cell K_{ATP} channels to promote coronary vasodilation, although the exact nature of the participation is unknown (197). Herein, we characterize ROS acting as an AE on A_1R (**Chapter 3**). This is the first example of ROS acting directly on a GPCR to alter signaling, demonstrating a unique synergy between the adenosine and ROS injury response pathways. This finding may promote a future role of A_1R-targeting therapeutics in the treatment of oxidation-implicated disease. Additionally, this finding may open new avenues of research into ROS modulation of GPCRs, and the impacts such modulation may have in disease states and homeostatic human health.

To better delineate the role of ROS in A_1R-injury signaling and to characterize this mechanism of AEs, we evaluated the roles ROS, AEs and the GPCR-conserved

disulfide bond play in A_1R activation. This disulfide bond, bridging ECL1 and ECL2, is highly conserved in the GPCR superfamily, shared by 78.9% of GPCRs (96), and is essential for ligand binding in several GPCR subfamilies, including ARs (as described in the immediately preceding section). Our experiments identify that H_2O_2 stabilizes A_1R in the agonist-bound, G protein-coupled state of A_1R, similar to the mechanism of AEs. H_2O_2 is present in ischemic heart tissues (198,199). With further experimentation, we identified that mutations designed to occlude the A_1R pocket (bordered by TM2, ECL1 and TM3) that exposes the GPCR-conserved disulfide bond decreased activity of H_2O_2 and all three chemical classes of AEs. These results signify that the disulfide bond pocket and GPCR-conserved disulfide bond are universal components of AE activity and potential targets for pharmacological oxidation. These intriguing results indicate that the ROS generated during cardiac ischemia and reperfusion injury may actually facilitate A_1R activation, resulting in physiological protection as A_1R activation promotes negative chromotropic and dromotropic effects on the heart, decreasing cardiac oxygen demand. The impact of these results may be to alter the way clinicians treat injury and oxidative diseases.

Abbreviations

Adenosine	(2*R*,3*R*,4*S*,5*R*)-2-(6-amino-9*H*-purin-9-yl)-5-(hydroxymethyl) oxolane-3,4-diol
A_1R	adenosine A_1 receptor
ARs	adenosine receptors
^{125}I-ABA	[^{125}I]N^6-(3-iodo-4-aminobenzyl)adenosine
AE	Allosteric enhancer
ATL	Adenosine Therapeutics, LLC
ATP	adenosine triphosphate
cAMP	cyclic AMP
CHA	N^6-cyclohexyladenosine
CPA	N^6-cyclopentyladenosine
CVT	CV Therapeutics
dNTP	deoxynucleoside triphosphate molecules
ECL	Extracellular loop
GABA	γ-aminobutyric acid
GLP-1R	glucagon-like peptide-1 receptor
GPCR	G protein-coupled receptor
GTPγS	guanosine 5-[γ-thio]triphosphate
H_2O_2	hydrogen peroxide
HIV	human immunodeficiency virus
ICL3	third intracellular loop

IPC	ischemic preconditioning
NADPH	nicotinamide adenine dinucleotide phosphate
NAM	negative allosteric modulator
NNRTIs	non-nucleoside reverse transcriptase inhibitors
PAM	positive allosteric modulator
PMNs	polymorphonuclear leukocytes
PGC1α	PPARγ coactivator-1α
PPARγ	peroxisome proliferator-activated receptor-γ
PSVT	paroxysmal supraventricular tachycardia
R-G complex	receptor-G protein complex
ROS	reactive oxygen species
RT	reverse transcriptase
R-PIA	(-)[^{125}I]-hydroxyphenylisopropyladenosine
$t_{½}$	half-life

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