

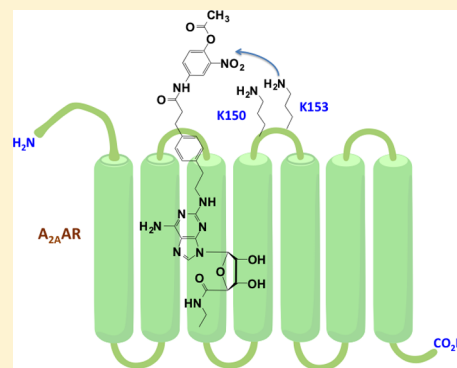
# Structure-Based Design of Reactive Nucleosides for Site-Specific Modification of the A<sub>2A</sub> Adenosine Receptor

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**S** Supporting Information

**ABSTRACT:** Adenosine receptors (ARs) are members of the G protein-coupled receptor (GPCR) superfamily and have shown much promise as therapeutic targets. We have used an agonist-bound A<sub>2A</sub>AR X-ray crystallographic structure to design a chemically reactive agonist for site-specific chemical modification of the receptor. To further explore and chemically engineer its binding cavity, a 2-nitrophenyl active ester was attached through an elongated chain at adenine C2 position. This general structure was designed for irreversible transfer of a terminal acyl group to a nucleophilic amino group on the A<sub>2A</sub>AR. Preincubation with several O-acyl derivatives prevented radioligand binding that was not regenerated upon extensive washing. In silico receptor docking suggested two lysine residues (second extracellular loop) as potential target sites for an O-acetyl derivative (MRSS854, **3a**), and site-directed mutagenesis indicated that K153 but not K150 is essential. Similarly, a butyl azide for click reaction was incorporated in the active ester moiety (**3b**). These promising results indicate a stable, covalent modification of the receptor by several reactive adenosine derivatives, which could be chemical tools for future imaging, structural probing, and drug discovery. Thus, structure-based ligand design has guided the site-specific modification of a GPCR.



**KEYWORDS:** G protein-coupled receptor, nucleoside, adenosine receptor, covalent modification, affinity labeling

The covalent modification of proteins is a useful research and diagnostic technique that benefits from a wide range of possible chemical attachments, including fluorescent and radiolabeled prosthetic groups to assist in both qualitative and quantitative characterization of a specific protein.<sup>1,2</sup> Covalent modification of receptors and enzymes, such as formation of a disulfide bond with the P2Y<sub>12</sub> receptor by thienopyridine drugs<sup>3</sup> or acetylation of a specific serine on cyclooxygenase by acetylsalicylic acid,<sup>4</sup> also has therapeutic utility. Previously, affinity labeling reagents for receptors were designed empirically,<sup>5</sup> but recent advances in the structural elucidation of cell surface receptors make rational design a possible process.<sup>6,7</sup>

Adenosine receptors (ARs) are a 4-member subfamily of the large family of G protein-coupled receptors (GPCRs), which is of particular interest to biomedical research,<sup>8</sup> consistent with the estimate that more than a third of all pharmaceuticals on the market target GPCRs.<sup>6,7</sup> The A<sub>2A</sub>AR, which is the only AR subtype structurally elucidated with X-ray crystallography, has medical relevancy in various diseases including agonists for respiratory disorders and Huntington's disease and antagonists for Parkinson's disease, attention deficit disorder, cancer, and many others.<sup>9</sup> Over the past several years, much work has been done toward structural characterization of the A<sub>2A</sub>AR, including the elucidation of its X-ray crystal structure in both agonist- and antagonist-bound conformations.<sup>10–12</sup> Despite the large research effort devoted to understanding the structure and

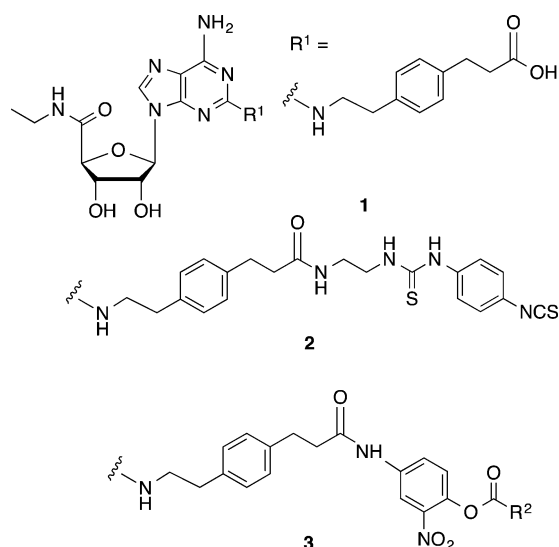
function of the A<sub>2A</sub>AR, the role of the flexible second extracellular loop (EL2), which makes contact with bound ligands, in recognition of orthosteric and allosteric ligands and in receptor activation, is still only partially understood.<sup>13–15</sup> The EL2 of the A<sub>2A</sub>AR plays an important role in ligand recognition as indicated by the replacement of the EL2 of A<sub>2B</sub>AR with corresponding segment of the A<sub>2A</sub>-EL2.<sup>16</sup> The chimeric receptor showed affinity for the prototypical selective A<sub>2A</sub>AR agonist 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5'-*N*-ethylcarboxamidoadenosine (CGS21680, **1**, Chart 1). Therefore, we focused on the A<sub>2A</sub>AR-EL2 and attempted to target specific nucleophilic amino acids on the receptor protein with high affinity ligands that incorporate chemically reactive, electrophilic moieties. We have used an agonist-bound A<sub>2A</sub>AR X-ray crystallographic structure<sup>12</sup> to design chemically reactive agonists for site-specific chemical modification of the receptor. The covalent inhibition of GPCRs and enzymes is used therapeutically, for example, with the antithrombotic drug clopidogrel.<sup>3</sup> Thus, the ability to modify the A<sub>2A</sub>AR protein effectively and permanently by targeting EL2 has implications for receptor characterization, imaging, and drug design.

Our experimental strategy aimed to covalently modify the A<sub>2A</sub>AR using reactive A<sub>2A</sub>AR selective agonist ligands (active

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**Chart 1. Prototypical Competitive  $A_{2A}$ AR Agonist (1) and Irreversibly Binding  $A_{2A}$ AR Agonist (2)<sup>a</sup>**

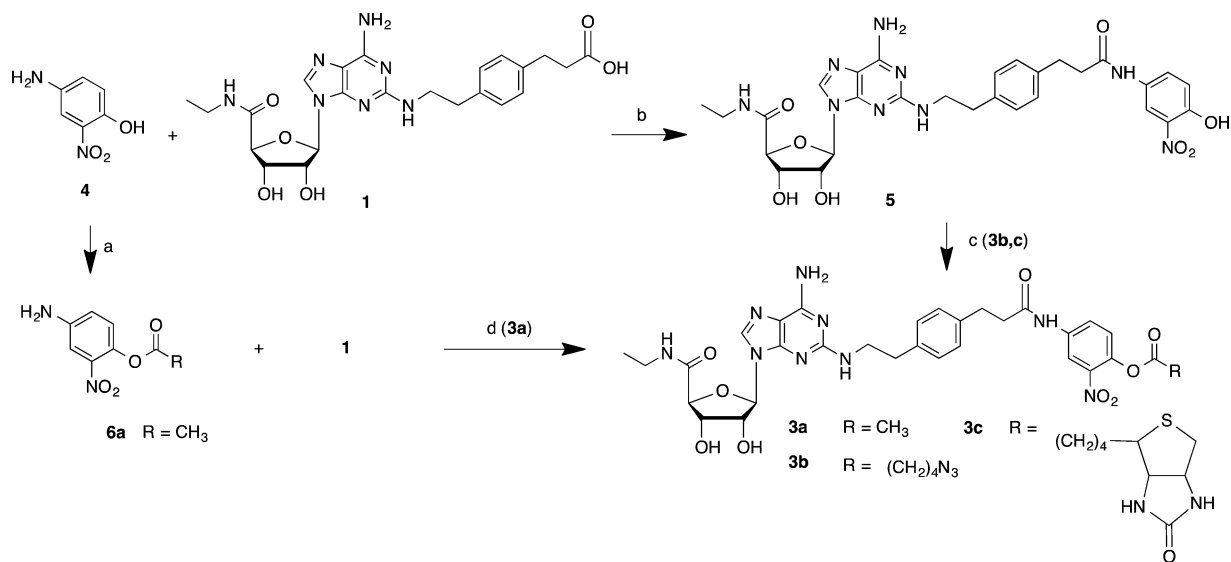
<sup>a</sup>The target compounds for transfer of an acyl moiety to the receptor are active esters of the general formula 3. R<sup>2</sup> is an alkyl group or reporter group.

esters 3, Chart 1) derived from 1. A set of high affinity acylating nucleosides was introduced with the aim of binding reversibly to the  $A_{2A}$ AR and subsequently transferring an acyl group covalently to the receptor protein. There is a precedent for the design of acylating nucleoside ligands in our previous report of irreversible binding to the  $A_{2A}$ AR of isothiocyanate derivatives,<sup>17</sup> in which this reactive electrophilic group was attached through a long chain at the C2 position (R<sup>1</sup>). A potent ethylenediamine adduct of 1 was coupled to a single isothiocyanate group of a symmetric cross-linking reagent, *p*-phenylene diisothiocyanate, to form 2.<sup>17</sup> This reactive nucleoside persisted in the binding site to produce long-lasting vasodilation of the coronary artery in the isolated guinea pig

heart, which could not be reversed by the application of an AR antagonist, consistent with covalent reaction with the receptor. Additionally, various other derivatives containing reactive isothiocyanates at the extended C2 position were able to bind irreversibly to the rabbit  $A_{2A}$ AR.<sup>5</sup> Irreversible inactivation of the receptor was dependent on the presence of an aryl-NCS group.

In the design of acylating ligands applied here, unlike the isothiocyanate derivatives, these reactive nucleosides are intended to deliver a chemically functionalized handle to a nucleophilic amino group on the receptor. The nucleoside portion would serve as a leaving group, and the portion covalently bound to the  $A_{2A}$ AR, i.e., detached from the nucleoside, would be used as a reporter group for receptor characterization.

**Results and Discussion. Chemistry and Modeling.** Our scheme for targeted covalent modification used as starting material a known selective  $A_{2A}$ AR agonist 1 (Scheme 1), through modification of its terminal carboxylic acid. This functional group was condensed with a primary aniline 4 that has an *o*-nitrophenol group to serve as a good leaving group, consistent with the known reactivity of *o*-nitrophenyl esters.<sup>18</sup> When acylated with a variety of acyl (RCO) species, this phenolic spacer group was converted into an electrophilic moiety, specifically an active nitrophenyl ester 3. Compound 3a was prepared by an alternate route, i.e., via 6a. This ester was intended to react with the nucleophilic groups in the vicinity of the terminal position of the C2 functionalized chain of the ligand (R<sup>1</sup>, Chart 1) when bound to the receptor. Thus, during acylation the nucleoside portion of the active ester would be detached along with the *o*-nitrophenol. In this manner, a R<sup>2</sup>CO group would be targeted for covalent transfer to a specific nucleophilic group on an EL region of the receptor, but the pharmacophore might not remain anchored to the receptor, unlike standard affinity labeling techniques. We varied R<sup>2</sup> from the simplest acetyl group 3a to an alkyl azide 3b and biotin 3c.<sup>19</sup> An alkyl azide is a particularly versatile group for site-specific attachment to GPCRs, due to its potential reactivity

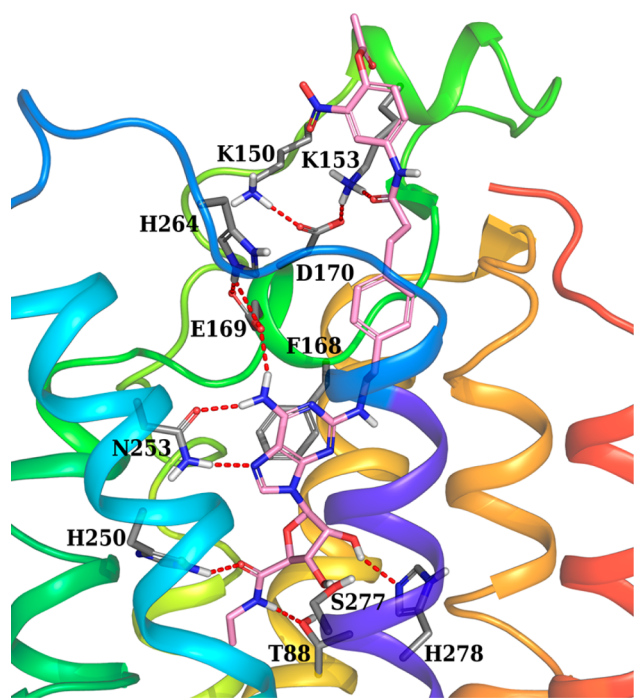
**Scheme 1. Synthesis of Reactive Agonist Ligands (3a–c) for Acyl Transfer to the  $A_{2A}$ AR<sup>a</sup>**

<sup>a</sup>Reagents and yields: a. acyl anhydride, pyridine, 30–40%; b. EDC·HCl, DMF, 60%; c. RCO<sub>2</sub>H, DCC, DMAP, DMF, 30–50%, for 3b and 3c. d. PyBOP/DIEA, DMF, 70%, for 3a.

toward alkynes through a biocompatible Cu-free click reaction.<sup>20</sup>

From the X-ray structure of an agonist-bound A<sub>2A</sub>AR and subsequent molecular modeling (docking of various known adenosine ligands),<sup>11,21</sup> it was evident that the distal portions of C2-extended chains in potent A<sub>2A</sub>AR agonists were in close proximity to EL2 and EL3.<sup>21</sup> The only nucleophilic residues that were available for this interaction in the vicinity of the docked C2 chain were the flexible side chains of Lys150 and Lys153, being the only lysines present in the extracellular portion of the receptor. Thus, we predicted that a primary amine on one of the two lysine residues in EL2 (K150 or K153) would be in proximity to react with the active ester attachment. The same residues were predicted in our previous docking study of C2-extended A<sub>2A</sub>AR agonists to be proximal to the reactive isothiocyanate group of **2**.<sup>17</sup> Interestingly, hA<sub>1</sub>AR and hA<sub>3</sub>AR sequences lack lysine residues in this region of EL2.

We determined more precisely the docking of compound **3a** in an agonist-bound structure of the A<sub>2A</sub>AR (Figure 1). This



**Figure 1.** Docking pose of compound **3a** (pink carbon sticks) at the hA<sub>2A</sub>AR (2YDV crystal structure<sup>12</sup>). Some residues important for ligand recognition are shown in sticks (gray carbons), and H-bonds are indicated by red dashed lines.

compound shows all the interactions important for agonist binding of A<sub>2A</sub>AR, highlighted by crystallographic complexes.<sup>11,12</sup> In particular, the ribose moiety and the adenine core participate in H-bonds with key residues of the binding site, such as Thr88 (3.36), Glu169 (EL2), His250 (6.52), Asn253 (6.55), Ser277 (7.42), and His278 (7.43) (numbers in parentheses follow the Ballesteros–Weinstein notation).<sup>22</sup> Moreover, the adenine core forms a  $\pi$ – $\pi$  stacking interaction with Phe168 in EL2. The C2 substituent extends toward the extracellular environment in proximity of EL2 with the carbonyl of the amide group forming an H-bond with Lys153. Therefore, the active ester group, even though not in direct contact with the amino group of Lys153, is in close proximity to this residue

making it the first possible site for covalent modification. In fact, even though during docking residues of the binding site were kept fixed, Lys153 can be expected to easily interact with the active ester moiety of compound **3a** due to the flexibility of its side chain. Moreover, other low energy docking conformations (not shown) display the flexible C2 chain bending toward Lys150 and positioning the active ester favorably to interact with it as a possible second site for covalent modification. It is worth noting that in the A<sub>2A</sub>AR crystal structure both of these lysine residues are forming electrostatic interactions with Asp170 in EL2 (Figure 1). These interactions can be crucial in preserving the conformation of the loop, and consequently, acylation of the lysine residues could result in an altered loop conformation affecting ligand binding.

The molecular modeling predictions also place the active ester **3c** in a similar proximity to the two lysine residues on EL2 when this A<sub>2A</sub>AR-specific ligand is docked to the receptor. Probably due to the bulkiness of biotin active ester **3c**, steric effects precluded its ability to bind unhindered to the A<sub>2A</sub>AR and irreversibly modify the receptor (see below), possibly through transfer of the reactive acyl group. The ability to effectively react with the receptor would be related to the residence time on the receptor,<sup>23</sup> which was not evaluated here. Moreover, click reactions through the azide handle on the receptor protein, derived from **3b**, could be used to tether many different kinds of groups, including reporter groups such as fluorescent labels, even in live cells and tissues. Other potential chemical reactions between **3a** and Lys153 could include nucleophilic aromatic substitution or aminolysis of the arylamide, but both of these are much less likely than reaction at the highly activated *o*-nitrophenyl ester.

**Pharmacology.** Three active esters **3a**–**3c** were initially tested in competitive radioligand binding assays at three human (h) AR subtypes (Table 1) and compared to nonreactive

**Table 1. Competitive Binding Results for Nucleoside Derivatives at Three AR Subtypes<sup>a</sup>**

compd	A <sub>1</sub> AR, % inhibition or K <sub>i</sub> (nM)	A <sub>2A</sub> AR, K <sub>i</sub> (nM)	A <sub>3</sub> AR, K <sub>i</sub> (nM)
<b>1</b>	380	70	570
<b>3a</b>	500 ± 72	23.0 ± 6.7	207 ± 47
<b>3b</b>	(30 ± 5%)	4360 ± 760	1810 ± 470
<b>3c</b>	(37 ± 5%)	3280 ± 680	1960 ± 100
<b>5</b>	1890 ± 670	267 ± 66	171 ± 6

<sup>a</sup>Binding in membranes of CHO or HEK293 (A<sub>2A</sub> only) cells stably expressing one of three hAR subtypes. The binding affinity for hA<sub>1</sub>, A<sub>2A</sub>, and A<sub>3</sub>ARs was expressed as K<sub>i</sub> values (mean of 3–4 determinations ± SEM) using agonists [<sup>3</sup>H]N<sup>6</sup>-R-phenylisopropyladenosine **8** ([<sup>3</sup>H]R-PIA), [<sup>3</sup>H]2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5'-N-ethylcarboxamidoadenosine **1**, or [<sup>125</sup>I]N<sup>6</sup>-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide **9** ([<sup>125</sup>I]I-AB-MECA), respectively. A percent in parentheses refers to inhibition of binding at 10  $\mu$ M. Competition was performed at a concentration range of 1 nM to 10  $\mu$ M against the selective radioligand agonist for each subtype. Nonspecific binding was determined using 10  $\mu$ M 5'-N-ethylcarboxamidoadenosine **10**. Values for **1** are from ref 21.

intermediates **1** and **5**. Compound **3a**, which contained the simple acetyl active ester moiety, had an 11-fold higher binding affinity at the A<sub>2A</sub>AR than compound **5**, which contains the same *o*-nitrophenol spacer but lacks an active ester. Additionally, compound **3a** displayed moderate selectivity for the A<sub>2A</sub>AR over the A<sub>1</sub>AR (22-fold) and A<sub>3</sub>AR (9-fold) that was not present in compound **5**. Compound **3a** also fully stimulated



cAMP formation in CHO cells stably expressing the A<sub>2A</sub>AR with an EC<sub>50</sub> value of  $2.12 \pm 0.81$  nM, compared to  $5.86 \pm 1.29$  nM for **1** (Supporting Information, Figure S1). Thus, highly potent full agonism is maintained in the acetyl active ester derivative.

With the high potency and selectivity of compound **3a** in mind, we tested the irreversibility of its A<sub>2A</sub>AR binding, which would be indicative of covalent receptor modification. Following preincubation with compound **3a** at a 1  $\mu$ M concentration for 30 min, membranes containing the A<sub>2A</sub>AR lost most of the ability to bind either agonist ([<sup>3</sup>H]**1**, 19% binding remaining) or antagonist ([<sup>3</sup>H]**4**-[2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5-yl-amino]ethylphenol **7**, 11% binding remaining) radioligand. This is in contrast to preincubation of the A<sub>2A</sub>AR-expressing membranes with reversible agonist ligands **1** or **5** (1  $\mu$ M), in which binding function was largely restored following several washing steps (Figure 2). Furthermore, after repeated washing, the **3a**-pretreated membranes were incubated with 50  $\mu$ M theophylline for 1 h and again washed by cycles of centrifugation in an attempt to use this weak antagonist to regenerate binding

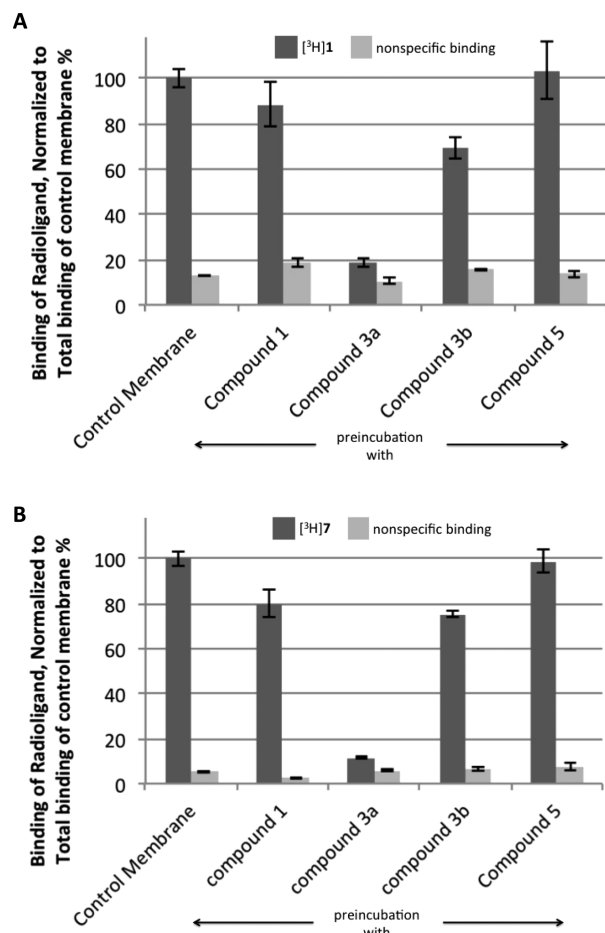
ability. However, treatment with the antagonist failed to restore A<sub>2A</sub>AR binding of either [<sup>3</sup>H]**1** or [<sup>3</sup>H]**7**, indicating that the modification was irreversible and not simply a function of slow dissociation kinetics. Additionally, binding saturation studies were performed with the antagonist radioligand [<sup>3</sup>H]**7** following the same membrane preincubation procedure. The results showed similar K<sub>d</sub> values for all compounds ranging from 3 to 5 nM and a 13-fold greater B<sub>max</sub> value for the control saturation curve at  $9.90 \pm 0.95$  pmol/mg compared to membranes incubated with compound **3a** at  $0.79 \pm 0.26$  pmol/mg (Table 2A and Figure S2, Supporting Information).

**Table 2. Results of Antagonist ([<sup>3</sup>H]**7**) Radioligand Saturation Binding Experiments: (A) With HEK-293 Cells Stably Overexpressing the WT A<sub>2A</sub>AR Showing K<sub>d</sub> and B<sub>max</sub> Values Following Preincubation with Various Nucleosides; or (B) with WT, K150A, and K153A A<sub>2A</sub>AR Mutant Receptors, Transiently Transfected in COS-7 Cells; Nonspecific Binding Was Determined Using 10 (10  $\mu$ M)**

A			
incubated with	K <sub>d</sub> (nM)	B <sub>max</sub> (pmol/mg protein)	
control	$5.28 \pm 2.21$	$9.90 \pm 0.95$	
<b>3a</b>	$5.04 \pm 1.53$	$0.79 \pm 0.26$	
<b>1</b>	$4.36 \pm 1.93$	$7.76 \pm 1.38$	
<b>3b</b>	$3.15 \pm 1.35$	$4.80 \pm 0.60$	

B			
A <sub>2A</sub> AR construct	preincubated with <b>3a</b>	K <sub>d</sub> (nM)	B <sub>max</sub> (pmol/mg protein)
A <sub>2A</sub> -WT	—	$1.81 \pm 0.76$	$2.21 \pm 0.19$
A <sub>2A</sub> -WT	+	$2.24 \pm 0.46$	$0.29 \pm 0.08$
A <sub>2A</sub> -K150A	—	$1.99 \pm 0.77$	$2.18 \pm 0.16$
A <sub>2A</sub> -K150A	+	$3.17 \pm 1.21$	$0.20 \pm 0.07$
A <sub>2A</sub> -K153A	—	$1.47 \pm 0.77$	$2.36 \pm 0.13$
A <sub>2A</sub> -K153A	+	$1.03 \pm 0.39$	$1.65 \pm 0.18$



**Figure 2.** Level of residual binding after preincubation of HEK-293 cell membranes expressing the hA<sub>2A</sub>AR with compounds **1**, **3a**, and **5** (1  $\mu$ M) and **3b** (10  $\mu$ M), as well as controls in the absence of added nucleoside and with added **1**. (A) Using an agonist radioligand, [<sup>3</sup>H]**1** (10 nM). (B) Using an antagonist radioligand, [<sup>3</sup>H]**7** (1.0 nM). The receptor was incubated with the compound indicated and then washed several times in order to restore binding to any A<sub>2A</sub>AR present that was intact and not chemically modified.

The preincubation step of this experiment was done alternatively with either membrane extracts or whole cells (Figure S3, Supporting Information), and nearly identical loss of binding was observed.

To further probe the structural basis for the irreversible inhibition, we mutated the two target lysine residues in EL2 to determine if we were able to restore binding after treatment with compound **3a**. Mutant A<sub>2A</sub>AR-K150A showed results similar to the A<sub>2A</sub>AR WT control in which binding was almost completely abolished. However, mutant A<sub>2A</sub>AR-K153A showed a restoration of binding after being treated with compound **3a** of about 70% based on the respective B<sub>max</sub> values (Table 2B and Figure S4, Supporting Information). While the binding was not completely restored, these results indicate a covalent interaction between Lys153 and the active ester derivative.

Because of the ability of compound **3a** to irreversibly modify the receptor, we attempted to further functionalize the acyl group of the *o*-nitrophenyl ester. A biotinylated derivative **3c** showed poor binding affinity, selectivity, and an inability to permanently inhibit binding of the A<sub>2A</sub>AR. To overcome this limitation, a smaller (alkyl azide) group was attached through the acyl group in active ester **3b**. The azide group, if covalently anchored to the receptor, would be subjected to a well-established method of Cu-free click reaction with a cyclo-octyne-derivatized fluorophore or chromophore.<sup>24</sup> Compound **3b** displayed an A<sub>2A</sub>AR affinity similar to the biotinylated compound **3c**; however, there was partial irreversible inhibition

of binding. Following treatment with **3b** and washing to remove all traces of the noncovalently bound ligand, approximately 50% of the initial receptor binding remained inhibited, as is demonstrated with the saturation curves that show a  $B_{\max}$  value of  $4.80 \pm 0.60$  pmol/mg versus the control that showed a  $B_{\max}$  of  $9.90 \pm 0.95$  pmol/mg (Table 2A and Figure S2, Supporting Information).

Initially, dibenzocyclooctyne (DIBO) with an AlexaFluor-488 fluorophore (Figure S5, Supporting Information) was tested for the ability to perform a Cu-free click reaction of the **3b**-pretreated  $A_{2A}AR$ .<sup>25,26</sup> A high level of background fluorescence was observed in membrane extracts of control  $A_{2A}AR$ -expressing cells not exposed to **3b**, and specifically, post-**3b** labeling was not discernible. A similar experiment was performed using difluorooctyne (DIFO) with a rhodamine-based fluorophore (TAMRA), but there was no observable increase in fluorescence. Despite these results, there is still a potential for the site-specific functionalization of the  $A_{2A}AR$  with the alkyl azide.

The irreversible inhibition of ligand binding was consistent with a covalent modification of an amino acid residue that is integral to the binding pocket of the  $A_{2A}AR$ . *A priori*, we were uncertain if the modification of a residue on EL2 would impede subsequent radioligand binding. Evidently, the modification of the receptor by **3a** completely blocks subsequent binding of reversible agonist or antagonist radioligand. We are uncertain if the residual nucleoside moiety of the affinity reagent is trapped in the binding site after covalent modification, or if it dissociates from the binding site. The nature of the covalently modified  $A_{2A}AR$  and its functional pharmacological properties will be the subject of further investigations of this reagent family. We do not know if a prolonged activation of the receptor is present following **3a** pretreatment, as was observed with isothiocyanate derivative **2** in the guinea pig heart.<sup>17</sup>

The question of why the chemically altered  $A_{2A}AR$  loses its ability to bind radioligands remains. It is conceivable that blocking the entry of small molecule ligands to the pharmacophore binding site greatly impedes their high affinity binding. In principle, a reporter group could be directed toward the receptor and deposited there for detection by various methods.

**Conclusions.** In conclusion, a selective agonist ligand was modified as a selective agonist ligand containing an active ester intended for transferring various acyl moieties covalently to a GPCR protein. Covalent modification of the  $A_{2A}AR$  was previously accomplished empirically, but this is the first structure-based, targeted covalent modification of the  $A_{2A}AR$ . A strategically designed reactive *O*-acetyl derivative (**3a**) and a corresponding butyl azide for click reaction (**3b**) both caused irreversible loss of binding function. We have confirmed, using site-directed mutagenesis, our prediction from molecular modeling that a specific lysine residue (K153) on EL2 is the principal site of covalent modification. Determining if the receptor modification is by acyl transfer, as designed, and if the nucleoside portion of the agonist dissociates following covalent reaction will be topics for future study. Assuming a mechanism of transfer of a reactive acyl moiety, the agonist derivative was designed to contain a wide range of functionality for receptor characterization and quantification. Thus, structure-based ligand design of chemical tools has led to the site-specific modification of a GPCR, with the potential for future imaging, structural probing, and drug discovery.

## ■ ASSOCIATED CONTENT

### ■ Supporting Information

NMR and HRMS of nucleosides, structures of the cyclooctane reagents, and the coordinates of the model of the  $A_{2A}AR$  complex with compound **3a**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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## ■ ABBREVIATIONS

AR, adenosine receptor; cAMP, adenosine 3',5'-cyclic monophosphate; CHO, Chinese hamster ovary; EL, extracellular loop; GPCR, G protein-coupled receptor; HEK, human embryonic kidney; I-AB-MECA, *N*<sup>6</sup>-(4-amino-3-iodobenzyl)-adenosine-5'-*N*-methyl-uronamide; HRMS, high resolution mass spectroscopy; NMR, nuclear magnetic resonance; WT, wild-type

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