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Orthogonal activation of the reengineered A₃ adenosine receptor (neoeceptor) using tailored nucleoside agonists

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Abstract

An alternative approach to overcome the inherent lack of specificity of conventional agonist therapy can be the reengineering of the GPCRs and their agonists. A reengineered receptor (neoeceptor) could be selectively activated by a modified agonist, but not by the endogenous agonist. Assisted by rhodopsin-based molecular modeling, we pinpointed mutations of the A₃ adenosine receptor (AR) for selective affinity enhancement following complementary modifications of adenosine. Ribose modifications examined included, at 3': amino, aminomethyl, azido, guanidino, ureido; and at 5': uronamido, azidodeoxy. *N*⁶-variations included: 3-iodobenzyl, 5-chloro-2-methoxybenzyl, and methyl. An *N*⁶-3-iodobenzyl-3'-ureido adenosine derivative **10** activated phospholipase C in COS-7 cells (EC₅₀=0.18 μM) or phospholipase D in chick primary cardiomyocytes mediated by a mutant (H272E), but not the wild-type, A₃AR. The affinity enhancements for **10** and the corresponding 3'-acetamidomethyl analogue **6** were >100-fold and >20-fold, respectively. **10** concentration-dependently protected cardiomyocytes transfected with the neoeceptor against hypoxia. Unlike **10**, adenosine activated the wild-type A₃AR (EC₅₀ of 1.0 μM), but had no effect on the H272E mutant A₃AR (100 μM). Compound **10** was inactive at human A₁, A_{2A}, and A_{2B}ARs. The orthogonal pair comprising an engineered receptor and a modified agonist should be useful for elucidating signaling pathways and could be therapeutically applied to diseases following organ-targeted delivery of the neoeceptor gene.

Introduction

The approach of chemical genetics has been widely used to control various protein functions and cellular processes.¹ For example, this approach has been used in studying GTP regulatory proteins to create nucleotide specificity.² Gene-directed enzyme prodrug therapy

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Supporting Information Available. Elemental analysis data for unknown compounds and 3D coordinates of two modes of docking compound **10** in homology models of the H272E mutant human A₃ receptors in PDB format (as represented in Fig. 3).

has been proposed to be an effective means against cancer.³ Tailor-made ligands for thyroid hormone nuclear receptors may have potential to restore mutation-caused genetic diseases.⁴ It has been suggested that the chemical genetic approach is superior to the traditional genetic approach, such as gene knockout, in studying protein functions,⁵ and has advantage for developing drugs against genetic diseases.⁶ Functional orthogonal ligand-receptor pairs have been used for regulation of estrogen receptors,^{7,8} Src family protein kinases,⁹ and protein methyltransferases.¹⁰

In the GPCR field, engineering of orthogonal ligand-receptor pairs was initiated shortly after the cloning of the first non-rhodopsin GPCR.^{11,12} The ligand binding domain of the β -adrenergic receptor has been genetically engineered to respond to specific classes of compounds which do not interact with the WT receptor. Targeting critical positions in the ligand binding domains with specific functional groups on ligands was proposed to provide a novel avenue for design of therapeutic agents.¹² Indeed, the Gs-coupled β -adrenergic receptor-ligand pairs were further engineered and proposed for gene therapy, which should be therapeutically useful as they could potentially rescue the loss of function caused by SNPs (single nucleotide polymorphisms) which happened naturally at this receptor.¹³ In the meantime, Coward et al.¹⁴ by using a chimeric approach, engineered Gi-coupled κ -opioid receptors, and designated them as RASSLs (receptors solely activated by synthetic ligand). A RASSL showed dramatically decreased affinity for the endogenous agonist dynorphin and a wide range of other opioid peptides, but with a lesser extent of affinity decrease for the synthetic agonist spiradoline, and slowed heart rate in transgenic mice.^{14,15} The RASSL approach is useful for unraveling the GPCR signaling mechanisms.¹⁴⁻¹⁹ However, an ideal ligand-receptor pair for this purpose should be truly orthogonal, i.e. the endogenous agonist should not activate the engineered receptor and the synthetic ligand should not activate the wild-type receptor, and this goal has not actually been achieved. More recently, Bruysters et al.²⁰ engineered the Gq-coupled histamine H₁ receptor for enhanced affinity of a synthetic agonist, with decreased affinity for the endogenous agonist, but orthogonality was not complete.

Our initial work on engineering the receptors and ligands for orthogonal activation started with the A₃ adenosine receptor (AR),²¹ the activation of which is both cerebro- and cardio-protective^{22,23} and has an anticancer effect.²⁴ An integrated approach of engineering both ligands and receptors combined with molecular modeling was used. A H272E mutant receptor (designated as neoceptor) was found to have decreased affinity for classical ligands (20-50-fold), such as NECA (5'-N-ethylcarboxamidoadenosine) and Cl-IB-MECA (2-chloro-N⁶-(3-iodobenzyl)-5'-N-methylcarbamoyladenosine), and with a modest affinity increase for a 3'-amine-derivatized adenosine (7-fold), but orthogonality has not been complete.²¹ Further application of the neoceptor approach to 5'-uronamide derivatives that bound orthogonally at strategically mutated A_{2A}ARs suggested that, in addition to the charge, the chain length of the interacting moiety (e.g., amine) was also critical for the affinity enhancement.^{25,26} This observation was instructive for additional optimization of A₃ neoceptor-neoligand interactions.

The neoceptor approach could be particularly suitable for application to ARs to achieve the receptor subtype-selectivity and tissue selectivity, which has proved difficult to achieve due to the ubiquitous presence of ARs. Here, with the assistance of molecular modeling, we systematically studied the interactions between the 3'-position of the nucleoside with negatively charged residues in place of the His residue. The charge, chain length, and H-bonding properties of the 3'-substitution were optimized in combination with substitutions at other positions of the adenosine molecule. We found that a derivative (**10**, N⁶-(3-iodobenzyl)-3'-ureidoadenosine) bearing both 3'-ureido and N⁶-3-iodobenzyl (IB) modifications displayed high selectivity for the H272E mutant receptor expressed in COS-7

cells in comparison to wild-type in binding and phospholipase C (PLC) assays. In contrast, the endogenous agonist adenosine (100 μ M) had no effect at the H272E mutant receptor. The orthogonality of the neoceptor-neoagonist pair was functionally tested in a cardiomyocyte model showing activation of the cardioprotective phospholipase D (PLD) pathway and an anti-ischemic effect.^{27,28} Additionally, it was found that, T94E, but not T94A or Q167E, showed a similar pattern of affinity enhancement for a series of 3'-amine derivatized adenosine analogs. This selective gain of function, achieved by matching attractive substituents on the ligand with those on the receptor at two spatially distinct sites, could be explained by rhodopsin-based molecular modeling on the basis of multiple binding modes of the nucleoside.

Results

Mutagenesis

Single amino acid replacements of the hA₃AR were made at strategic locations of the putative ribose binding region, as defined by rhodopsin-based molecular modeling.^{31,35} Thr94 (3.36) and His272 (7.43) were selected for mutation and individually replaced with Ala, Asp, or Glu. Both of these hydrophilic residues are predicted to be involved in coordination of the ribose moiety. The Gln167 residue of EL2, predicted to be in proximity to A₃AR-bound nucleosides,³⁶ also served as a mutation site. His272, predicted to be in proximity to the 3'-OH group, was used successfully in the first study of neoceptors.²¹

Nucleoside structure

A variety of adenine nucleoside derivatives (**1** – **18**, Table 1) were examined in this study as potential neoligands with respect to the above receptor mutations. Most of the modified adenosine derivatives were reported previously. Compounds **1**, **3**, **4**, **7**, **17**, and **18** were prepared as described.^{21,26} The 3'-amino derivative **1** was identified previously as a suitable neoligand when paired with the H272E A₃AR neoceptor, although the degree of enhancement was low (Table 1). The 3'-aminomethyl derivatives **2** and **5–9** and 3'-azide **15** were reported recently.³⁷ The 3'-ureido moiety, found to result in inactivity the wild-type ARs, was included in analogues **10–14** and **16**.^{38,39} Compounds **17** and **18** were identified previously as suitable neoligands when paired with A_{2A}AR neoceptors.^{25,26} The variations at the N⁶-position included unsubstituted, IB, 5-chloro-2-methyloxybenzyl (CMB), and methyl. We previously reported that the CMB group increased affinity and selectivity at the human (h) A₃AR in comparison to IB.³⁶ The 5'-position mainly consisted of either the ribose-like CH₂OH or the NECA-like 5'-uronamide. To enhance selectivity for the hA₃AR, the optimally substituted 5'-methyluronamide moiety was included. The 3'-position appeared to be the most critical for achieving selective affinity enhancement at neoceptors derived from the hA₃AR.²¹ Thus, a variety of polar substituents (amino, guanidine, aminomethyl, acetamidomethyl, and ureido) were compared at this position. Only one compound, the 3'-ureido derivative **11**, was also substituted at the adenine 2-position.

Chemical synthesis

N⁶-Substituted-3'-ureidoadenosine derivatives **10**, **11**, and **13** were synthesized starting from 1,2:5,6-di-*O*-isopropylidene- β -glucose (**19**), as shown in Scheme 1. Reaction of **19** with triflic anhydride followed by treating the resulting triflate **20** with sodium azide afforded 3-azido derivative **21**. Selective hydrolysis of 5,6-isopropylidene group of **21** followed by oxidative cleavage and reduction gave **22** in 85% yield. Treatment of **22** with acetic anhydride gave **23** which was converted to the glycosyl donor **24** in two steps (hydrolysis and acetylation). Condensation of **24** with silylated 6-chloropurine and 2,6-dichloropurine in the presence of TMSOTf afforded the protected nucleosides **25a** and **25b**,

respectively. 6-Chloropurine derivative **25a** was treated with methylamine and 3-iodobenzylamine followed by treating with sodium methoxide to give the *N*⁶-methyl derivative **26a** and *N*⁶-3-iodobenzyl derivative **26b**, respectively. 2,6-Dichloropurine derivative **25b** was also converted to 2-chloro-*N*⁶-3-iodobenzyl derivative **26c**. Compounds **26a-26c** were re-protected as TBS-ethers **27a-27c**, respectively. The reason that the acetyl protecting groups were replaced with TBS protecting groups was because of the facile migration of a 2'-acetyl group to the 3'-ureido group. Reduction of azido group of **27a-27c** using triphenylphosphine and ammonium hydroxide in aqueous solution yielded amino derivatives, **28a-28c**, respectively. For the conversion of 3'-amino group into 3'-ureido group, the 3'-amino derivatives **28a-28c** were treated with chloroacetyl isocyanate in DMF to yield 3'-chloroacetyl urea derivatives **29a-29c**, which were smoothly converted to the 3'-ureido derivatives **30a-30c**, respectively, after treating with sodium methoxide. As mentioned above, migration of a 2'-acetyl or benzoyl group to the 3'-ureido group was observed upon treating 3'-chloroacetyl urea derivatives with sodium methoxide, giving *N*-acetyl- or *N*-benzoylureido derivative as a sole product. This migration was prevented by using a TBS group as a protecting group. Removal of the TBS group of **30a-30c** with TBAF in THF afforded the final *N*⁶-substituted-3'-ureidoadenosine derivatives **13**, **10**, and **11**, respectively.

*N*⁶-Substituted-3'-ureidoadenosine-5'-methyluronamides **14** and **12** were also synthesized starting from the same intermediate **21**, as illustrated in Scheme 2.³⁸ Selective hydrolysis of 5,6-isopropylidene group of **21** using 75% aqueous acetic acid followed by the treatment of the resulting diol with NaIO₄/RuCl₃ in CCl₄/CH₃CN/H₂O (2/2/3) produced acid derivative **32**. Treatment of **32** with oxalyl chloride gave the activated ester, which was converted to methyl amide **33** by treating with methylamine. Compound **33** was converted to the key intermediate **34** using the same conditions described in Scheme 1. The key intermediate **34** was transformed to the final nucleosides **12** and **14** according to the same procedure used in the preparation of **10**, **11**, and **13** in Scheme 1.

Ligand binding properties at the wild-type and mutant receptors

The affinities of the nucleoside derivatives were initially evaluated at wild-type and H272E mutant A₃ARs expressed in COS-7 cells (Table 1). Only the substituted *N*⁶-benzyl derivatives **4**, **5**, **9**, and **16** displayed a *K*_i value at the wild-type A₃AR of <1 μM.^{26,38} All of the other compounds were weak or inactive at all four subtypes of ARs.^{37,38}

Adenosine bound to the wild-type A₃AR with a *K*_i value of 1 μM, while at the mutant H272E A₃AR adenosine at 100 μM failed to inhibit radioligand binding (Fig. 1). Considerable enhancement of affinity was observed for several nucleoside analogues. Following the introduction of a carboxylic acid side chain at position 7.43, the 3'-acetamidomethyl derivative **6** and the 3'-aminomethyl derivatives **7** and **8** were 10- to 20-fold enhanced in affinity. The enhancement of the 3'-aminomethyl derivatives was dependent on the presence of an *N*⁶-benzyl-type group (either IB in **7** or CMB in **8**). The corresponding simple 6-NH₂ derivative **2** was not enhanced in affinity. The greatest degree of enhancement (>100-fold) was observed for the 3'-ureido derivative **10** (Fig. 1A), which also contained the IB group. The corresponding 2-Cl analogue **11** showed an approx. 50-fold affinity increase. Compounds **15** and **16** both had azido groups on the ribose moiety, while only the 3'-azido derivative **15**, not 5'-azido derivative **16**, displayed enhanced affinity at the H272E mutant A₃AR, further supporting a specific interaction between the 3'-position and His272. Other two 5'-substituted derivatives, **17** and **18**, also did not show any enhancement at the H272E neoceptor.

The affinity enhancement at the neoceptors, for nucleosides substituted at the 3'-position with amino, aminomethyl, and ureido moieties, was greatly attenuated when a 5'-uronamide group was also present, i.e., **5**, **9**, and **12**. Although the affinity at the wild-type A₃AR was increased by this structural change—for example, by 22-fold in the case of 5'-methyluronamide **9**—there was no affinity enhancement at the H272E mutant receptor, as was observed for the corresponding **8**. The affinity of 5'-methyluronamide **12** was decreased in comparison to the corresponding 5'-OH derivative **10** at the H272E mutant A₃AR.

Table 2 shows a comparison of affinities of selected nucleosides at various mutant A₃ARs. Negatively charged side chains were introduced at positions 3.36 and 7.43. Thr94 (3.36) is analogous to Thr88 of the hA_{2A}AR, which was the site of mutagenesis used successfully to construct neoceptors of that subtype.²⁶ The Gln167 residue of EL2, predicted to be in proximity to A₃AR-bound nucleosides,³⁶ also served as a mutation site. However, no enhancement of affinity of any modified nucleoside in the present study was observed at T94A, T94E, and Q167E mutant receptors. The affinity at the H272D mutant receptor closely paralleled the affinity at the H272E mutant receptor. This indicates that there is sufficient steric freedom within the binding site to preserve the effect after adding or subtracting one methylene unit. The T94A and T94E mutant receptors were similar to wild-type.

Binding experiments were carried out at human A₁ and A_{2A}ARs expressed in CHO (Chinese hamster ovary) cells using standard radioligand binding assays, as described.³⁷ Compounds **1** – **3**, **5** – **9** and **15** were previously determined to have K_i values >1 μM at the A₁AR and >10 μM at the A_{2A}AR.³⁷ Compound **4** was previously determined to have K_i values of 8.1 μM and 28 μM at the rat A₁ and A_{2A}ARs, respectively.²¹ 3'-Ureido derivatives **10** – **14** were assayed in binding to human A₁ and A_{2A}ARs. In all cases, <10% inhibition of binding was observed at 10 μM, except for **11** at the A₁AR (34% inhibition). Thus, all of the nucleosides that displayed affinity enhancement at the H272E mutant receptor only weakly bound, if at all, to two other AR subtypes. Compound **10** at 10 μM was inactive in the stimulation of cyclic AMP formation mediated by the human A_{2B}AR expressed in CHO cells.³⁶

Functional effects of nucleosides at wild-type and mutant A₃ARs

An assay of PLC was used in the determination of functional coupling of the wild-type and mutant A₃ARs expressed in COS-7 cells in response to known AR agonists and modified nucleosides. As illustrated in Fig. 1B, **10** induced accumulation of inositol phosphates in COS-7 cells expressing the H272E mutant receptor with an EC₅₀ of 0.18 ± 0.098 μM (n=3), while it had no effect on the WT ARs at concentrations as high as 10 μM. In contrast, the known agonist NECA (Fig. 1E) activated the WT A₃AR with an EC₅₀ of 0.078 ± 0.020 μM (n=3), and H272E with an EC₅₀ of 10.4 ± 3.3 μM (n=3). Similar to the loss of affinity for NECA, the endogenous agonist, adenosine, activated the WT ARs with an EC₅₀ of 1.04 μM while it had no effect on the H272E mutant receptor at 100 μM (Fig. 1F). No enhancement in basal PLC activity was observed for the mutant receptor in the absence of nucleoside. A 3'-azido substituted derivative **15**, and a 5'-azido derivative **16**, failed to activate PLC in either the wild-type A₃AR or the H272E neoceptor (data not shown). The functional effects of other derivatives at the wild-type A₃AR expressed in CHO cells were probed previously.³⁷

Cardiomyocyte model

In a known model of cardioprotection from 90 min of simulated ischemia,^{27,28,33,40} endogenous adenosine significantly protected cardiomyocytes transfected with the hA₃AR (10 ± 7% cells killed in hA₃R-transfected cells, n = 9, compared with 28 ± 6.5%, n = 8, in

vector-transfected overexpressing myocytes; one-way ANOVA, $F = 14.7$, $P < 0.0001$, followed by posttest comparison, $P < 0.001$). Unlike the wild-type A₃AR, cells expressing the neoceptor H272E cDNA only showed a slight change. In cells expressing the H272E mutant receptor, the neoligand **10** induced potent cardioprotection against hypoxia and activated the known cardioprotective PLD pathway (Fig. 2). Adenosine (100 μ M) did not activate the PLD pathway in myocytes expressing the H272E mutant receptor.

Molecular Modeling and Nucleoside Docking

Recently a hA₃AR model, including the seven TMs and loop regions, was constructed^{31,41,42} by homology to the X-ray structure of bovine rhodopsin.³⁵

For the side-chain refinement of the neoceptors, the H272E mutant receptor was optimized through a molecular dynamics procedure after the mutation of His to Glu. In the wild-type A₃AR, there was a TM H-bonding network between the highly conserved His272 (7.43) and Glu19 (1.39), as previously described.²¹ The mutant H272E hA₃AR showed the same preference of the side-chain χ_1 angle at position 7.43 as did wild-type, but it had a different intramolecular H-bonding pattern. The H272E mutant receptor lost H-bonding between position 7.43 and Glu19, due to the electronic repulsion between two ionized Glu side chains, showing a distance of 4.88 Å between two C_δ atoms of Glu19 and Glu272.

A conformational search of the isolated 3'-ureido derivative **10**, carried out with a MOPAC PM3 calculation,⁴³ showed that the lowest-energy conformer displayed intramolecular H-bonding between the 3'-carbonyl oxygen of the ureido group and the 5'-hydroxyl group and between the 3'-amino and 2'-hydroxyl groups. The lowest-energy conformer of **12**, having a 5'-uronamide, formed H-bonding between the 3'-CO and 2'-OH groups and between the 5'-NH and the O of the ribose ring. However, this conformer of **12** showed no interaction between 3'- and 5'-substituents. Thus, the thermodynamic stability of the various intramolecular H-bonds, depending on the substitution pattern, might affect the binding affinity.

A complex of the A₃-selective agonist Cl-IB-MECA docked in the hA₃AR was constructed with only minor modifications from the previous model.^{31,41} This model featured putative H-bonds between the exocyclic NH and the side chain nitrogen (lone pair) of N250 (6.55) and between the carboxamide NH of Gln167 (EL2) and the purine N³ atom. This docked ligand showed an *anti*-conformation of the adenine ring. In the putative binding site of the ribose ring, intermolecular H-bonds formed between the 2'-OH group and the carbonyl O atom of Ile268 (7.39) and among the 3'-OH group, the backbone O of Ser271 (7.42), and the imidazole ring of His272 (7.43). The 5'-amide NH also formed a H-bond with Thr94 (3.36), and the 5'-carbonyl group displayed intramolecular H-bonding with the 3'-hydroxyl group. However, the docked conformation of N⁶-methyl-3'-ureidoadenosine **14**³⁸ showed no H-bonding interaction of the 3'- and 5'-substituents with Ser271 or His272, consistent with its loss of binding affinity at all subtypes of ARs. The lack of H-bonding was expected from the observed electronic and steric repulsion of the starting geometry of 3'-ureidoadenosine derivatives.³⁸

While these 3'-ureidoadenosine analogues lost binding affinity at all subtypes of ARs, they exhibited highly selective enhancement of binding affinity at the T94D, T94E, H272D or H272E mutant A₃ARs, suggesting a favorable interaction between 3'-ureido group and mutated acidic residues. While Cl-IB-MECA, a selective A₃ agonist, displayed a single favorable binding mode, the docking result of the 3'-ureido analogue **10** from the combination of the FlexiDock and FlexX⁵⁷ automatic docking programs had two energetically favorable binding modes. The docking results showing two putative complexes of the hA₃ H272E AR with compound **10** are shown in Fig. 3. As illustrated in Fig. 3A, one

binding mode similar to that of Cl-IB-MECA, gained energetically through H-bonding of the 3'-ureido substituent and of the 2'-hydroxyl group with the γ -carboxylate group of Glu272 (7.43), but lost hydrophilic interactions, i.e. H-bonding, at the N^6 and the N^3 atoms in the adenine ring. According to the model, the oxygens of the Glu272 side chain were each H-bonded to one of the urea NH groups in a bidentate fashion. The 5'-hydroxyl group H-bonded with the backbone O atom of Ser271 (7.42).

Fig. 3B shows the other binding mode, in which the 3'-ureido group interacted with the Thr94 (3.36) side chain through H-bonding and the 5'-hydroxyl group formed a H-bond with the carboxylate group of Glu272 (7.43) and the terminal 3'-NH₂ group was close to Ser97 (3.39). The interaction of the 3'-substituent and Thr94 would contribute to the selective enhancement at the T94D/E receptors. The N^6 amino group interacted through H-bonding with Gln167 (EL2), a residue previously predicted to be in proximity to the nucleoside.³⁶ In comparison to Fig. 3A, this docking mode required a different orientation of the N^6 -benzyl ring toward Phe182 (5.43). Molecular modeling indicates that the reason for the enhanced binding affinity of N^6 -benzyl compared with N^6 -methyl derivatives may be additional hydrophobic interaction at the N^6 -benzyl group.

Discussion

In this study, we used an integrated approach of mutagenesis, radioligand binding and functional assays combined with molecular modeling to identify matched pairs of neoligands and neoreceptors. We tested a wide range of synthetic amine-bearing nucleoside analogues and modulated the 3'-substituent chain length in concert with N^6 -, 5'-, and 2-modifications to identify engineered agonists that orthogonally activate mutant but not wild-type receptors. Mutation of His272, to Ala, Asp, and Glu, was compared with the wild-type A₃AR to optimize the electrostatic or H-bonding interaction between the 3'-amino moiety and the carboxylate group of the mutation site. The extended and multiply H-bonding 3'-deoxy-3'-ureido derivative **10**, containing an N^6 -3-iodobenzyl substituent, had no significant effect on the wild-type A₃AR but displayed a dramatically enhanced affinity of 0.22 μ M at the H272E neoreceptor. In a previous study,²¹ it was demonstrated that the H272E neoreceptor showed decreased affinity for NECA and Cl-IB-MECA, here we further demonstrated this neoreceptor is completely insensitive to the endogenous agonist, adenosine, as demonstrated in both binding and functional assays. Thus, truly orthogonal ligand-receptor pairs have been identified, which should be useful in elucidating signaling transduction mechanisms and should provide insights into the therapeutics of genetically-related diseases.

Mutations in genes encoding G protein-coupled receptors (GPCRs) are an important cause of human disease.⁴⁴ Study of the high-resolution SNP maps of 23 genes encoding GPCRs in the Japanese population identified 300 SNPs, including 83 in adenosine receptor family genes.⁴⁵ The A_{2A} adenosine receptor 1976T>C genetic variant has been shown to confer susceptibility to panic disorder and caffeine-induced anxiety.^{46,47} Specific GPCR polymorphisms in adrenergic genes have already been shown to confer susceptibility to congestive heart failure.¹³ Development of small molecules for GPCRs to rescue mutation induced functional loss or to inhibit mutation-caused constitutive activation will be novel forms of treatment for such diseases.

The reengineering of the interface of small molecules and proteins, such that the mutated protein will interact exclusively with chemically modified ligand in an orthogonal manner, has been explored for a variety of targets including receptors, enzymes, etc., to elucidate the role of signal transduction pathways in living systems.⁴⁸⁻⁵¹ The neoreceptor approach, which focuses on cell-surface GPCRs, is intended for therapeutic application that employs a

targeted vector to deliver the neoceptor gene to a target organ. In addition to the potential therapeutic applications, the neoceptor/neoligand pairs may be used for specific mechanistic probing of pharmacological effects, for example, in cases where the application of a normal receptor agonist might cause complicating effects. In several aspects, the neoceptor approach described in this study may be distinguished from the RASSL approach as described.¹⁴ Firstly, the neoceptor approach concerns the reengineering of both the receptor and the ligands using molecular modeling. Secondly, the RASSL described earlier¹⁴ showed somewhat parallel affinity decrease for both endogenous and synthetic ligands, which is not truly orthogonal. The synthetic agonist may activate the endogenous receptor and the engineered receptor simultaneously. Thirdly, the RASSL was achieved by engineering a chimeric receptor, while the neoceptor is achieved by reengineering the TM binding site for a specific group of the small-molecule agonist as predicted by using molecular modeling.

The neoceptor approach could be particularly useful for studying ARs to achieve the receptor-subtype-selectivity and tissue selectivity due to the ubiquitous presence of ARs.⁵² In the present study, the functionality of the neoagonist/neoceptor concept was further tested in a cardiac myocyte model of simulated ischemia and cardioprotection. Cardiomyocytes overexpressing the wild-type hA₃AR, activated by endogenous adenosine released by ischemia, showed potent anti-ischemic resistance. In contrast, cells expressing the H272E mutant receptor showed only a slight change. Activation of the overexpressed neoceptor by the neoligand resulted in a full anti-ischemic protective effect. The demonstration that the neoligand was able to induce a potent activation of cardioprotective PLD pathway^{27,28} provides additional support for this concept.

We used site-directed mutagenesis to probe the recognition elements responsible for the selective affinity enhancement. The neoligand **10** was found to bind with higher affinity to carboxylic acid mutations at a spatially distinct region of the A₃AR, i.e., receptors containing H272E (or D) in TM7 mutations, expressed in COS-7 cells, while other mutations (T94A, T94E or Q167E in EL2) did not result in this gain in affinity. This was consistent with the docking mode of **10** that was similar to that of the high affinity agonist Cl-IB-MECA (Figure 3A), but not the alternate binding mode (Figure 3B).

A carboxylic acid introduced in the sequence of the human A₃AR was found to pair with several functional groups on the ligand in an energetically favorable manner. Pairing of an Asp or Glu residue with an amino or aminomethyl group could be explained on the basis of an electrostatic attraction. The enhancement of the 3'-azido derivative **15** may also result from an electrostatic interaction with the middle nitrogen of the N₃ group, which would be positively charged. Finally, the successful pairing of a 3'-urea group with the carboxylate could be explained on the basis of the ability of both groups to form multiple H-bonds.

Conclusions

We have identified an engineered agonist that activates exclusively neoceptors and have shown that the orthogonality of binding and activation results in cardioprotective effects. This suggests that the 3'-urea derivatives described in this study might be useful therapeutically in combination with targeted gene delivery of a carboxylic acid mutant A₃AR such as H272E. The neoceptor, in general, could be an important therapeutic approach for tissue-specific GPCR activation, given successful targeted delivery (without side effects) of the neoceptor gene to a specific organ or tissue.

Experimental Procedures

Chemical synthesis

General—¹H NMR spectra were recorded in a 300 MHz apparatus using tetramethylsilane (TMS) as an internal standard, and the chemical shifts are reported in ppm (δ). Coupling constants are reported in hertz (Hz). Optical rotations were determined on a Jasco polarimeter in methanol of DMF. Infrared spectra were recorded in a Perkin-Elmer 1710 FTIR spectrophotometer. Mass spectra recorded by FAB (Fast atom bombardment) on a VG Tro-2, GC-MS. TLC were carried out on Merck silica gel 60 F₂₅₄ precoated plates, and silica gel column chromatography was performed on silica gel 60, 230~400 mesh, Merck. All anhydrous solvents were distilled over CaH₂ or Na/benzophenone prior to use.

3-Azido-3-deoxy-1,2;5,6-di-O-isopropylidene-β-D-allofuranose (21)—To a stirred solution of 1,2;5,6-di-O-isopropylidene-β-D-glucofuranose (3.0 g, 11.53 mmol) and pyridine (2.8 mL, 34.62 mmol) in dichloromethane (30 mL) was added trifluoromethanesulfonic anhydride (2.9 mL, 17.24 mmol) at 0 °C. After being stirred for 1 h at 0 °C, the reaction mixture was extracted with dichloromethane and water. The organic layer was washed with brine, dried (MgSO₄), filtered and evaporated to give **20**. To a solution of **20** in anhydrous DMF (20 mL) was added sodium azide (2.25 g, 34.61 mmol) and the mixture was stirred at rt for 48 h. The reaction mixture was poured into water (200 mL) and extracted with ethyl acetate (×3). The combined organic layers were dried (MgSO₄), filtrated and evaporated. The residue was purified by silica gel column chromatography (Hexanes/EtOAc = 4/1) to give **21** (1.45 g, 44%) as an oil: ¹H NMR (CDCl₃) δ 5.76 (d, 1 H, *J* = 3.7 Hz), 4.70 (t, 1 H, *J* = 4.1 Hz), 3.95–4.21 (m, 4 H), 3.48 (dd, 1 H, *J* = 4.9, 9.0 Hz), 1.56 (s, 3 H), 1.46 (s, 3 H), 1.36 (s, 3 H), 1.34 (s, 3 H); IR (KBr): 2109 (N₃) cm⁻¹; FAB-MS *m/z* 286 [M+H]⁺. Anal. (C₁₂H₁₉N₃O₅) C, H, N.

3-Azido-3-deoxy-1,2-O-isopropylidene-β-D-ribofuranose (22)—A mixture of **21** (2.63 g, 9.22 mmol) in 75% AcOH (30 mL) was stirred at 55 °C for 1.5 h. The reaction mixture was evaporated and coevaporated with toluene. The residue was dissolved in EtOH (30 mL) and a solution of NaIO₄ (2.37 g, 11.09 mmol) in H₂O (15 mL) was added dropwise at 0 °C to the reaction mixture. After the mixture was stirred at 0 °C for 20 min, NaBH₄ (1.05 g, 27.76 mmol) was added and the reaction mixture was stirred at 0 °C for 1 h. The reaction mixture was filtered and the filtrate was evaporated. The residue was purified by silica gel column chromatography (Hexanes/EtOAc = 1/1) to give **22** (1.69 g, 85%) as an oil: ¹H NMR (CDCl₃) δ 5.79 (d, 1 H, *J* = 3.4 Hz), 4.72 (t, 1 H, *J* = 4.1 Hz), 4.09 (m, 1 H), 3.96 (dd, 1 H, *J* = 2.4, 12.5 Hz), 3.66 (dd, 1 H, *J* = 2.9, 12.6 Hz), 3.56 (dd, 1 H, *J* = 4.6, 9.5 Hz), 1.56 (s, 3 H), 1.35 (s, 3 H); IR (KBr): 2108 (N₃) cm⁻¹; FAB-MS *m/z* 216 [M+H]⁺. Anal. (C₈H₁₃N₃O₄) C, H, N.

5-O-Acetyl-3-azido-3-deoxy-1,2-O-isopropylidene-β-D-ribofuranose (23)—To a stirred solution of **22** (1.5 g, 6.97 mmol) in anhydrous pyridine (15 mL) was added Ac₂O (1.32 mL, 13.99 mmol). The reaction mixture was stirred at rt for 3 h and evaporated. The residue was partitioned between EtOAc and water. The organic layer was washed with brine, dried (MgSO₄), filtered and evaporated. The residue was purified by silica gel column chromatography (Hexanes/EtOAc = 2/1) to give **23** (1.76 g, 98%) as an oil: ¹H NMR (CDCl₃) δ 5.78 (d, 1 H, *J* = 3.7 Hz), 4.71 (dd, 1 H, *J* = 3.6, 4.8 Hz), 4.34 (m, 1 H), 4.15–4.26 (m, 2 H), 3.31 (dd, 1 H, *J* = 4.6, 9.5 Hz), 2.08 (s, 3 H), 1.56 (s, 3 H), 1.34 (s, 3 H); IR (KBr): 2109 (N₃) cm⁻¹; FAB-MS *m/z* 258 [M+H]⁺. Anal. (C₁₀H₁₅N₃O₅) C, H, N.

1,2,5-Tri-O-acetyl-3-azido-3-deoxy-D-ribofuranose (24)—A solution of **23** (1.76 g, 6.84 mmol) in 85% formic acid (30 mL) was stirred for 1.5 h at 60 °C and evaporated. The

residue was dissolved in pyridine (20 mL) and Ac₂O (8.39 mL, 88.9 mmol) was added to the reaction mixture. The mixture was stirred at rt for 16 h and evaporated. The residue was partitioned between EtOAc and water. The organic layer was washed with brine, dried (MgSO₄), filtered and evaporated. The residue was purified by silica gel column chromatography (Hexanes/EtOAc = 2/1) to give **24** (2 g, 97%) as an oil: ¹H NMR (CDCl₃) δ 6.15 (m, 1 H), 5.33 (dd, 1 H, *J* = 4.9, 9.7 Hz), 4.05–4.38 (m, 5 H), 2.09 (m, 9 H); IR (KBr): 2116 (N₃) cm⁻¹; FAB-MS *m/z* 324 [M+Na]⁺. Anal. (C₁₁H₁₅N₃O₇) C, H, N.

3-Azido-3-deoxy-1,2-O-isopropylidene-β-D-ribofuranuronic acid methyl amide (33)—

A solution of **21** (2.08 g, 7.29 mmol) in 75% AcOH (30 mL) was stirred at 55 °C for 1.5 h. The reaction mixture was evaporated and the residue was dissolved in CCl₄ (14 mL), CH₃CN (14 mL) and H₂O (20 mL). To this solution were added NaIO₄ (6.55 g, 30.6 mmol) and ruthenium trichloride hydrate (33 mg, 0.16 mmol) at rt. The reaction mixture was stirred at rt for 4 h and filtrated through a Celite pad. The filtrate was extracted with CH₂Cl₂ (×3). The Combined organic layers were dried (MgSO₄), filtrated and evaporated to give **32**.

Oxalyl chloride (1.5 mL) was added to a solution of **32** in anhydrous CH₂Cl₂ (15 mL). DMF (0.5 mL) was added and the reaction mixture was stirred at rt for 16 h and evaporated. The residue was dissolved in anhydrous CH₂Cl₂ (15 mL) and cooled to 0 °C. A solution of 2 M NH₂CH₃ in THF (0.37 mL) was added dropwise. After being stirred for 3 h, the mixture was diluted with water and extracted with CH₂Cl₂ (×3). The combined organic layers were dried (MgSO₄), filtrated and evaporated. The residue was purified by silica gel column chromatography (Hexanes/EtOAc = 1/2) to give **33** (772 mg, 44%) as a solid: ¹H NMR (CDCl₃) δ 6.43 (brs, 1 H), 5.84 (d, 1 H, *J* = 3.3 Hz), 4.71 (dd, 1 H, *J* = 3.3, 4.6 Hz), 4.48 (d, 1 H, *J* = 9.6 Hz), 3.63 (dd, 1 H, *J* = 4.5, 9.5 Hz), 2.86 (d, 1 H, *J* = 5.0 Hz), 1.58 (s, 3 H), 1.38 (s, 3 H); FAB-MS *m/z* 265 [M+Na]⁺. Anal. (C₉H₁₄N₄O₄) C, H, N.

1,2-Di-O-acetyl-3-azido-3-deoxy-D-ribofuranuronic acid methyl amide (34)—

Compound **33** (772 mg, 3.19 mmol) was dissolved in a solution of AcOH (20 mL) and Ac₂O (2.4 mL). The mixture was cooled to 0 °C and c-H₂SO₄ (0.014 mL) was added to this solution. The reaction mixture was stirred at rt for 18 h and poured into the saturated NaHCO₃ solution. The mixture was extracted with CH₂Cl₂ (×3). The combined organic layers were washed with brine, dried (MgSO₄), filtered and evaporated. The residue was purified by silica gel column chromatography (Hexanes/EtOAc = 1/2) to give **34** (750 mg, 82%) as an oil: ¹H NMR (CDCl₃) δ 6.44 (brs, 1 H), 6.15 (s, 1 H), 5.30 (d, 1 H, *J* = 4.8 Hz), 4.48 (d, 1 H, *J* = 7.2 Hz), 4.39 (dd, 1 H, *J* = 4.8, 7.2 Hz), 2.86 (d, 1 H, *J* = 5.0 Hz), 2.18 (s, 3 H), 2.08 (s, 3 H); IR (KBr): 2120 (N₃), 1752 (C=O), 1674 (C=O) cm⁻¹; FAB-MS *m/z* 287 [M+H]⁺. Anal. (C₁₀H₁₄N₄O₆) C, H, N.

General Procedure for the Synthesis of 25a, 25b, and 35—A mixture of 6-chloropurine or 2,6-dichloropurine (2.0 equiv) and ammonium sulfate (catalytic amount) in anhydrous HMDS (30 mL) was refluxed under nitrogen atmosphere for 16 h and concentrated under anhydrous conditions. The residue was dissolved in anhydrous 1,2-dichloroethane (20 mL) and a solution of **24** and **34** in 1,2-dichloroethane (20 mL) was added to this solution followed by addition of TMSOTf (2.0 equiv) at 0 °C. The reaction mixture was stirred at rt for 20 min and then at 60 °C for 2 h. The mixture was quenched by addition of saturated NaHCO₃ solution, filtered through a Celite pad. The filtrate was extracted with CH₂Cl₂ (×3). The combined organic layers were dried (MgSO₄), filtrated and evaporated. The residue was purified by silica gel column chromatography to give **25a**, **25b**, and **35**.

9-(2,3-Di-O-acetyl-3-azido-3-deoxy-β-D-ribofuranosyl)-6-chloropurine (25a)—67% yield; white foam; ¹H NMR (CDCl₃) δ 8.78 (s, 1 H), 8.25 (s, 1 H), 6.12 (d, 1 H, *J* = 3.5 Hz), 5.98 (dd, 1 H, *J* = 5.9, 3.5 Hz), 4.71 (t, 1 H, *J* = 6.3), 4.29-4.40 (m, 3 H), 2.21 (s, 3 H), 2.09 (s, 3 H); IR (KBr): 2115 (N₃), 1746 (C=O) cm⁻¹; FAB-MS *m/z* 396 [M+H]⁺. Anal. (C₁₄H₁₄ClN₇O₅) C, H, N.

9-(2,3-Di-O-acetyl-3-azido-3-deoxy-β-D-ribofuranosyl)-2,6-dichloropurine (25b)—51% yield; white foam; ¹H NMR (CDCl₃) δ 8.24 (s, 1 H), 6.10 (d, 1 H, *J* = 3.9 Hz), 5.84 (dd, 1 H, *J* = 3.9, 5.7 Hz), 4.61 (m, 1 H), 4.29-4.50 (m, 3 H), 2.22 (s, 3 H), 2.12 (s, 3 H); IR (KBr): 2116 (N₃), 1745 (C=O) cm⁻¹; FAB-MS *m/z* 431 [M+H]⁺. Anal. (C₁₄H₁₃Cl₂N₇O₅) C, H, N.

3-Azido-5-(6-chloro-purin-9-yl)-4-acetoxy-tetrahydro-furan-2-carboxylic acid methylamide (35)—73% yield; white foam; ¹H NMR (CDCl₃) δ 8.78 (s, 1 H), 8.25 (s, 1 H), 7.62 (brs, 1 H), 6.15 (d, 1 H, *J* = 7.0 Hz), 5.87 (dd, 1 H, *J* = 5.7, 7.0 Hz), 4.86 (dd, 1 H, *J* = 3.1, 5.7 Hz), 4.58 (d, 1 H, *J* = 3.1 Hz), 2.92 (d, 1 H, *J* = 5.0 Hz), 2.05 (s, 3 H); IR (KBr): 2117 (N₃), 1751 (C=O), 1673 (C=O) cm⁻¹; FAB-MS *m/z* 381 [M+H]⁺. Anal. (C₁₃H₁₃ClN₈O₄) C, H, N.

General Procedure for the Synthesis of 26a and 36—A mixture of **25a** and **35** and 40% methylamine in water (4 mL) in 1,4-dioxane (10 mL) was stirred at rt for 4 h. The reaction mixture was evaporated and the residue was purified by silica gel column chromatography to give **26a** and **36**, respectively.

N⁶-Methyl-9-(3-azido-3-deoxy-β-D-ribofuranosyl)adenine (26a)—90% yield; white solid; ¹H NMR (DMSO-*d*₆) δ 8.31 (s, 1 H), 8.20 (s, 1 H), 7.83 (brs, 1 H), 6.18 (d, 1 H, *J* = 5.7 Hz), 5.86 (d, 1 H, *J* = 6.1 Hz), 5.56 (dd, 1 H, *J* = 4.5, 7.6 Hz), 4.95 (dd, 1 H, *J* = 5.7, 11.4 Hz), 4.27 (dd, 1 H, *J* = 3.5, 5.4 Hz), 3.93 (dd, 1 H, *J* = 3.3, 6.8 Hz), 3.50-3.69 (m, 2 H), 2.92 (s, 3 H); IR (KBr): 3430 (OH), 2104 (N₃), 1634 (C=O) cm⁻¹; FAB-MS *m/z* 307 [M+H]⁺. Anal. (C₁₁H₁₄N₈O₃) C, H, N.

3-Azido-5-(6-methylaminopurin-9-yl)-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide (36)—87% yield; white solid; ¹H NMR (DMSO-*d*₆) δ 8.69 (d, 1 H, *J* = 4.4 Hz), 8.38 (s, 1 H), 8.27 (s, 1 H), 7.87 (brs, 1 H), 6.58 (d, 1 H, *J* = 5.5 Hz), 5.95 (d, 1 H, *J* = 6.4 Hz), 4.90 (dd, 1 H, *J* = 5.6, 11.4 Hz), 4.45 (dd, 1 H, *J* = 3.0, 5.0 Hz), 4.29 (d, 1 H, *J* = 2.9 Hz), 2.93 (s, 3 H), 2.66 (d, 3 H, *J* = 4.6 Hz); IR (KBr): 3379 (OH), 2123 (N₃), 1662 (C=O) cm⁻¹; FAB-MS *m/z* 334 [M+H]⁺. Anal. (C₁₂H₁₅N₉O₃) C, H, N.

General Procedure for the Synthesis of 26b, 26c, and 15—A mixture of **25a**, **25b** and **15**, 3-iodobenzylamine hydrochloride (1.1 equiv) and Et₃N (3.0 equiv) in EtOH (10 ml) was stirred at 50 °C for 18 h. The reaction mixture was evaporated and the residue was partitioned between CH₂Cl₂ and water. The organic layer was washed with brine, dried (MgSO₄), filtered and evaporated. The residue was dissolved in MeOH (10 ml) and 28% NaOMe (1 ml) was added to this solution. The reaction mixture was stirred at rt for 2 h and evaporated. The residue was purified by silica gel column chromatography to give **26b**, **26c** and **15**, respectively.

N⁶-(3-Iodobenzyl)-9-(3-azido-3-deoxy-β-D-ribofuranosyl)adenine (26b)—86% yield; solid; ¹H NMR (DMSO-*d*₆) δ 8.60 (s, 1 H), 8.47 (s, 1 H), 8.29 (s, 1 H), 7.78 (s, 1 H), 7.63 (d, 1 H, *J* = 7.9 Hz), 7.40 (d, 1 H, *J* = 7.4 Hz), 7.14 (t, 1 H, *J* = 7.9 Hz), 6.28 (d, 1 H, *J* = 5.3 Hz), 5.97 (d, 1 H, *J* = 5.8 Hz), 5.57 (dd, 1 H, *J* = 4.6, 7.1 Hz), 5.05 (m, 1 H), 4.73 (m, 2

H), 4.37 (m, 1 H), 4.05 (m, 1 H), 3.72 (m, 1 H), 3.62 (m, 1 H); IR (KBr): 3426 (OH), 2106 (N_3), 1622 (C=O) cm^{-1} ; FAB-MS m/z 590 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{17}\text{H}_{17}\text{IN}_8\text{O}_3$) C, H, N.

2-Chloro-N⁶-(3-iodobenzyl)-9-(3-azido-3-deoxy- β -D-ribofuranosyl)adenine (26c)—95% yield; solid; ^1H NMR ($\text{DMSO}-d_6$) δ 7.71 (s, 1 H), 7.69 (s, 1 H), 7.59 (d, 1 H, $J = 7.9$ Hz), 7.26 (m, 1 H), 7.02 (t, 1 H, $J = 7.7$ Hz), 6.55 (s, 1 H), 5.97 (d, 1 H, $J = 10.1$ Hz), 5.70 (d, 1 H, $J = 7.3$ Hz), 5.24 (m, 1 H), 4.64–4.77 (m, 3 H), 4.31 (d, 1 H, $J = 5.1$ Hz), 4.19 (s, 1 H), 3.94 (d, 1 H, $J = 12.81$ Hz), 3.68 (m, 1 H); IR (KBr): 3305 (OH), 2112 (N_3), 1621 (C=O) cm^{-1} ; FAB-MS m/z 543 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{17}\text{H}_{16}\text{ClIN}_8\text{O}_3$) C, H, N.

3-Azido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide (15)—89% yield; white solid; ^1H NMR (CDCl_3) δ 8.61 (d, 1 H, $J = 4.4$ Hz), 8.28 (s, 1 H), 7.71 (s, 1 H), 7.69 (s, 1 H), 7.60 (d, 1 H, $J = 7.8$ Hz), 7.30 (d, 1 H, $J = 7.7$ Hz), 7.04 (t, 1 H, $J = 7.7$ Hz), 6.48 (brs, 1 H), 5.79 (d, 1 H, $J = 7.1$ Hz), 5.07 (m, 2 H), 4.77 (m, 2 H), 4.48 (m, 2 H), 2.85 (d, 1 H, $J = 4.9$ Hz); FAB-MS m/z 536 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{18}\text{H}_{18}\text{IN}_9\text{O}_3$) C, H, N.

General Procedure for the Synthesis of 27a-27c and 37a-37b—A mixture of **26a-26c**, **36**, and **15**, imidazole (5 equiv), and TBSCl (2.5 equiv) in anhydrous DMF (15 mL) was stirred at rt for 24 h. The reaction mixture was poured into water (200 mL) and extracted with ethyl acetate ($\times 3$). The combined organic layers were dried (MgSO_4), filtrated and evaporated. The residue was purified by silica gel column chromatography to give **27a-27c** and **37a-37b**, respectively.

N⁶-Methyl-9-(3-azido-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (27a)—76% yield; oil; ^1H NMR (CDCl_3) δ 8.24 (s, 1 H), 7.94 (s, 1 H), 5.87 (d, 1 H, $J = 4.0$ Hz), 5.72 (brs, 1 H), 4.73 (dd, 1 H, $J = 4.0, 5.0$ Hz), 4.06 (m, 1 H), 3.91–3.97 (m, 2 H), 3.67 (dd, 1 H, $J = 2.8, 11.6$ Hz), 3.05 (d, 3 H, $J = 4.6$ Hz), 0.82 (s, 9 H), 0.73 (s, 9 H), 0.11 (s, 3 H), 0.00 (s, 3 H), -0.08 (s, 3 H), -0.19 (s, 3 H); IR (KBr): 2107 (N_3) cm^{-1} ; FAB-MS m/z 535 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{23}\text{H}_{42}\text{N}_8\text{O}_3\text{Si}_2$) C, H, N.

N⁶-(3-Iodobenzyl)-9-(3-azido-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (27b)—84% yield; oil; ^1H NMR (CDCl_3) δ 8.24 (s, 1 H), 7.98 (s, 1 H), 7.59 (s, 1 H), 7.46 (d, 1 H, $J = 7.8$ Hz), 7.19 (d, 1 H, $J = 7.7$ Hz), 6.90 (t, 1 H, $J = 7.7$ Hz), 5.99 (brs, 1 H), 5.88 (d, 1 H, $J = 3.9$ Hz), 4.70–4.75 (m, 3 H), 4.07 (m, 1 H), 3.91–3.97 (m, 2 H), 3.68 (dd, 1 H, $J = 2.6, 11.6$ Hz), 0.81 (s, 9 H), 0.74 (s, 9 H), 0.01 (s, 3 H), 0.00 (s, 3 H), -0.06 (s, 3 H), -0.16 (s, 3 H); IR (KBr): 2106 (N_3) cm^{-1} ; FAB-MS m/z 737 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{29}\text{H}_{45}\text{IN}_8\text{O}_3\text{Si}_2$) C, H, N.

2-Chloro-N⁶-(3-iodobenzyl)-9-(3-azido-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (27c)—83% yield; oil; ^1H NMR (CDCl_3) δ 8.24 (s, 1 H), 7.99 (s, 1 H), 7.57 (s, 1 H), 7.47 (d, 1 H, $J = 7.9$ Hz), 7.18 (d, 1 H, $J = 7.7$ Hz), 6.90 (t, 1 H, $J = 7.9$ Hz), 6.05 (brs, 1 H), 5.82 (d, 1 H, $J = 3.3$ Hz), 4.62 (m, 3 H), 4.08 (m, 1 H), 3.93 (dd, 1 H, $J = 2.9, 11.7$ Hz), 3.82 (dd, 1 H, $J = 4.8, 6.2$ Hz), 3.67 (dd, 1 H, $J = 2.4, 11.6$ Hz), 0.80 (s, 9 H), 0.76 (s, 9 H), 0.02 (s, 3 H), 0.00 (s, 3 H), -0.03 (s, 3 H), -0.10 (s, 3 H); IR (KBr): 2106 (N_3) cm^{-1} ; FAB-MS m/z 771 $[\text{M}]^+$. Anal. ($\text{C}_{29}\text{H}_{44}\text{ClIN}_8\text{O}_3\text{Si}_2$) C, H, N.

3-Azido-5-(6-methylaminopurin-9-yl)-4-t-butylidimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (37a)—83% yield; foam; ^1H NMR (CDCl_3) δ 9.31 (brs, 1 H), 8.49 (s, 1 H), 7.88 (s, 1 H), 6.10 (d, 1 H, $J = 4.6$ Hz), 5.89 (d, 1 H, $J = 8.1$ Hz), 5.19 (dd, 1 H, $J = 5.1, 8.1$ Hz), 4.59 (s, 1 H), 4.40 (d, 1 H, $J = 5.1$ Hz), 3.33 (brs, 3 H),

3.06 (d, 1 H, $J = 4.8$ Hz), 0.87 (s, 9 H), 0.00 (s, 3 H), -0.36 (s, 3 H); IR (KBr): 2105 (N_3) cm^{-1} ; FAB-MS m/z 448 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{18}\text{H}_{29}\text{N}_9\text{O}_3\text{Si}$) C, H, N.

3-Azido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-t-butylidimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (37b)—71% yield; foam; ^1H NMR (CDCl_3) δ 9.22 (d, 1 H, $J = 4.2$ Hz), 8.48 (s, 1 H), 7.88 (s, 1 H), 7.82 (s, 1 H), 7.72 (d, 1 H, $J = 7.9$ Hz), 7.43 (d, 1 H, $J = 7.7$ Hz), 7.16 (t, 1 H, $J = 7.7$ Hz), 6.37 (t, 1 H, $J = 6.0$ Hz), 5.88 (d, 1 H, $J = 7.9$ Hz), 5.18 (dd, 1 H, $J = 5.2, 7.9$ Hz), 4.90 (brs, 2 H), 4.59 (s, 1 H), 4.40 (d, 1 H, $J = 5.1$ Hz), 3.05 (d, 1 H, $J = 5.8$ Hz), 0.87 (s, 9 H), 0.00 (3, 3 H), -0.36 (s, 3 H); IR (KBr): 2105 (N_3) cm^{-1} ; FAB-MS m/z 672 $[\text{M}+\text{Na}]^+$. Anal. ($\text{C}_{24}\text{H}_{32}\text{IN}_9\text{O}_3\text{Si}$) C, H, N.

General Procedure for the Synthesis of 28a-28c and 38a-38b—To a stirred solution of **27a-27c** and **37a-37b** in THF (15 mL) was added triphenylphosphine (1.5 equiv) at 0 °C. After being stirred for 30 min, NH_4OH (1.8 mL) and H_2O (0.3 mL) were added to the reaction mixture. The mixture was stirred overnight at rt and evaporated. The residue was purified by silica gel column chromatography to give **28a-28c** and **38a-38b**, respectively.

N⁶-Methyl-9-(3-amino-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (28a)—88% yield; foam; ^1H NMR (CDCl_3) δ 8.24 (s, 1 H), 8.13 (s, 1 H), 5.89 (d, 1 H, $J = 1.7$ Hz), 5.75 (dd, 1 H, $J = 5.9, 3.5$ Hz), 4.24 (dd, 1 H, $J = 1.7, 4.8$ Hz), 3.93 (m, 1 H), 3.74-3.82 (m, 2 H), 3.45 (dd, 1 H, $J = 4.8, 8.3$ Hz), 3.03 (d, 3 H, $J = 5.0$ Hz), 0.81 (s, 9 H), 0.80 (s, 9 H), 0.07 (s, 3 H), 0.01 (s, 3 H), 0.00 (s, 3 H), -0.02 (s, 3 H); IR (KBr): 3290, 2931, 1624, 1119, 838 cm^{-1} ; FAB-MS m/z 509 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{23}\text{H}_{44}\text{N}_6\text{O}_3\text{Si}_2$) C, H, N.

N⁶-(3-Iodobenzyl)-9-(3-amino-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (28b)—96% yield; foam; ^1H NMR (CDCl_3) δ 8.24 (s, 1 H), 8.17 (s, 1 H), 7.60 (s, 1 H), 7.45 (d, 1 H, $J = 7.0$ Hz), 7.20 (d, 1 H, $J = 7.7$ Hz), 6.90 (t, 1 H, $J = 7.7$ Hz), 6.00 (brs, 1 H), 5.90 (d, 1 H, $J = 1.7$ Hz), 4.68 (brs, 2 H), 4.23 (dd, 1 H, $J = 1.5, 4.6$ Hz), 3.94 (m, 1 H), 3.74-3.82 (m, 2 H), 3.45 (dd, 1 H, $J = 4.7, 8.4$ Hz), 0.81 (s, 9 H), 0.80 (s, 9 H), 0.08 (s, 3 H), 0.02 (s, 3 H), 0.01 (s, 3 H), 0.00 (s, 3 H); IR (KBr): 2931, 1618, 1469, 1120, 839, 780 cm^{-1} ; FAB-MS m/z 711 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{29}\text{H}_{47}\text{IN}_6\text{O}_3\text{Si}_2$) C, H, N.

2-Chloro-N⁶-(3-iodobenzyl)-9-(3-amino-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (28c)—94% yield; foam; ^1H NMR (CDCl_3) δ 8.18 (s, 1 H), 7.59 (s, 1 H), 7.47 (d, 1 H, $J = 7.9$ Hz), 7.19 (d, 1 H, $J = 7.7$ Hz), 6.91 (t, 1 H, $J = 7.7$ Hz), 6.08 (brs, 1 H), 5.82 (d, 1 H, $J = 1.1$ Hz), 4.63 (s, 2 H), 4.17 (d, 1 H, $J = 4.7$ Hz), 3.96 (dd, 1 H, $J = 2.4, 11.5$ Hz), 3.74-3.80 (m, 2 H), 3.38 (dd, 1 H, $J = 4.4, 8.8$ Hz), 0.82 (s, 9 H), 0.81 (s, 9 H), 0.15 (s, 3 H), 0.03 (s, 3 H), 0.02 (s, 3 H), 0.00 (s, 3 H); IR (KBr): 2930, 1618, 1466, 1314, 1121, 838, 780 cm^{-1} ; FAB-MS m/z 746 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{29}\text{H}_{46}\text{ClIN}_6\text{O}_3\text{Si}_2$) C, H, N.

3-Amino-5-(6-methylaminopurin-9-yl)-4-t-butylidimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (38a)—95% yield; oil; ^1H NMR (CD_3OD) δ 8.37 (s, 1 H), 8.35 (s, 1 H), 6.09 (d, 1 H, $J = 5.5$ Hz), 4.85 (dd, 1 H, $J = 5.5, 11.0$ Hz), 4.39 (d, 1 H, $J = 3.8$ Hz), 3.72 (m, 1 H), 3.15 (brs, 3 H), 2.90 (s, 3 H), 0.87 (s, 9 H), 0.00 (s, 3 H), -0.16 (s, 3 H); IR (KBr): 3431, 2930, 1629, 1054, 833, 643 cm^{-1} ; FAB-MS m/z 422 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{18}\text{H}_{31}\text{N}_7\text{O}_3\text{Si}$) C, H, N.

3-Amino-5-(6-(3-iodobenzylamino)purin-9-yl)-4-t-butylidimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (38b)—94% yield oil; ^1H NMR (CDCl_3) δ 8.99 (brs, 1 H), 8.50 (s, 1 H), 7.91 (s, 1 H), 7.85 (s, 1 H), 7.74 (d, 1 H, $J = 7.8$ Hz), 7.45 (d, 1 H, $J = 7.7$ Hz), 7.17 (t, 1 H, $J = 7.9$ Hz), 6.35 (brs, 1 H), 6.11 (d, 1 H, $J = 6.6$

Hz), 4.91 (m, 3 H), 4.56 (s, 1 H), 3.93 (dd, 1 H, $J = 2.6, 5.5$ Hz), 3.05 (d, 1 H, $J = 4.7$ Hz), 0.92 (s, 9 H), 0.00 (s, 3 H), -0.21 (s, 3 H); IR (KBr): 3272, 2931, 1670, 1619, 1473, 1338, 1253, 1151, 1059, 839, 754 cm^{-1} ; FAB-MS m/z 624 $[\text{M}+\text{H}]^+$. Anal. ($\text{C}_{24}\text{H}_{34}\text{IN}_7\text{O}_3\text{Si}$) C, H, N.

General Procedure for the Synthesis of 29a-29c and 39a-39b—To a stirred solution of **28a-28c** and **38a-38b** in anhydrous DMF (10 mL) was added chloroacetyl isocyanate (1.1 equiv) at 0 °C. After being stirred for 2 h at 0 °C, the reaction mixture was evaporated and the residue was purified by silica gel column chromatography to give **29a-29c** and **38a-38b**.

N⁶-Methyl-9-(3-chloroacetylureido-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl) adenine (29a)—87% yield; oil; ^1H NMR (CDCl_3) δ 8.41 (m, 2 H), 8.25 (s, 1 H), 8.10 (s, 1 H), 5.94 (d, 1 H, $J = 2.6$ Hz), 5.72 (brs, 1 H), 4.47-4.55 (m, 2 H), 4.12 (m, 1 H), 3.99 (s, 2 H), 3.90 (dd, 1 H, $J = 2.0, 11.5$ Hz), 3.72 (dd, 1 H, $J = 3.0, 11.5$ Hz), 3.06 (d, 3 H, $J = 4.7$ Hz), 0.81 (s, 9 H), 0.75 (s, 9 H), 0.00 (s, 6 H), -0.07 (s, 3 H), -0.12 (s, 3 H); IR (KBr): 3303, 2953, 1702, 1623, 1536, 1254, 1229, 1127, 837 cm^{-1} ; FAB-MS m/z 629 $[\text{M}+\text{H}]^+$.

N⁶-(3-Iodobenzyl)-9-(3-chloroacetylureido-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (29b)—77% yield; foam; ^1H NMR (CDCl_3) δ 8.39 (m, 2 H), 8.25 (s, 1 H), 8.15 (s, 1 H), 7.60 (s, 1 H), 7.46 (d, 1 H, $J = 8.0$ Hz), 7.20 (d, 1 H, $J = 7.7$ Hz), 6.90 (t, 1 H, $J = 7.9$ Hz), 6.02 (brs, 1 H), 5.95 (d, 1 H, $J = 2.2$ Hz), 4.69 (brs, 2 H), 4.48-4.55 (m, 2 H), 4.12 (m, 1 H), 3.99 (s, 2 H), 3.91 (dd, 1 H, $J = 2.2, 11.7$ Hz), 3.72 (dd, 1 H, $J = 2.7, 11.7$ Hz), 0.82 (s, 9 H), 0.75 (s, 9 H), 0.00 (s, 6 H), -0.04 (s, 3 H), -0.10 (s, 3 H); IR (KBr): 3299, 2932, 1701, 1617, 1473, 1254, 1126, 838, 781 cm^{-1} ; FAB-MS m/z 831 $[\text{M}+\text{H}]^+$.

2-Chloro-N⁶-(3-iodobenzyl)-9-(3-chloroacetylureido-2,5-di-O-t-butylidimethylsilyl-3-deoxy- β -D-ribofuranosyl)adenine (29c)—81% % yield; foam; ^1H NMR (CDCl_3) δ 8.38 (s, 1 H), 8.31 (s, 1 H), 8.19 (s, 1 H), 7.59 (s, 1 H), 7.47 (d, 1 H, $J = 7.7$ Hz), 7.19 (d, 1 H, $J = 7.9$ Hz), 6.91 (t, 1 H, $J = 7.9$ Hz), 6.08 (brs, 1 H), 5.88 (d, 1 H, $J = 1.8$ Hz), 4.63 (s, 2 H), 4.42-4.51 (m, 2 H), 4.11 (m, 1 H), 3.99 (s, 2 H), 3.93 (dd, 1 H, $J = 2.2, 11.9$ Hz), 3.69 (dd, 1H, $J = 2.4, 11.7$ Hz), 0.81 (s, 9 H), 0.78 (s, 9 H), 0.04 (s, 3 H), 0.00 (s, 6 H), -0.04 (s, 3 H); IR (KBr): 3296, 2952, 1700, 1618, 1537, 1470, 1314, 1223, 1127, 837, 781 cm^{-1} ; FAB-MS m/z 863 $[\text{M}+\text{H}]^+$.

3-Chloroacetylureido-5-(6-methylaminopurin-9-yl)-4-t-butylidimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (39a)—94% yield; oil; ^1H NMR (CDCl_3) δ 8.92 (d, 1 H, $J = 5.1$ Hz), 8.53 (d, 1 H, $J = 4.6$ Hz), 8.47 (s, 1 H), 7.96 (s, 1 H), 6.71 (brs, 1 H), 6.51 (brs, 1 H), 6.26 (d, 1 H, $J = 4.2$ Hz), 5.96 (d, 1 H, $J = 5.7$ Hz), 5.01 (t, 1 H, $J = 7.9$ Hz), 4.74-4.81 (m, 2 H), 4.13 (s, 2 H), 3.29 (brs, 3 H), 2.98 (d, 1 H, $J = 4.7$ Hz), 0.86 (s, 9 H), 0.00 (s, 3 H), -0.20 (s, 3 H); IR (KBr): 3296, 2953, 1703, 1626, 1536, 1237, 1155, 840, 756 cm^{-1} ; FAB-MS m/z 542 $[\text{M}+\text{H}]^+$.

3-Chloroacetylureido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-t-butylidimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (39b)—88% yield; oil; ^1H NMR (CDCl_3) δ 8.87 (s, 1 H), 8.46 (s, 1 H), 8.31 (s, 1 H), 7.97 (s, 1 H), 7.80 (s, 1 H), 7.63 (d, 1 H, $J = 7.8$ Hz), 7.39 (d, 1 H, $J = 7.7$ Hz), 7.11 (t, 1 H, $J = 7.7$ Hz), 6.42 (m, 2 H), 6.00 (s, 1 H), 5.96 (d, 1 H, $J = 5.5$ Hz), 4.76-5.00 (m, 5 H), 4.22 (s, 2 H), 2.97 (d, 3 H, $J = 4.8$ Hz), 0.85 (s, 9 H), 0.00 (s, 3 H), -0.18 (s, 3 H); IR (KBr): 3290, 2952, 1705, 1619, 1535, 1225, 841, 755 cm^{-1} ; FAB-MS m/z 744 $[\text{M}+\text{H}]^+$.

General Procedure for the Synthesis of 30a-30c and 40a-40b—To a stirred solution of **29a-29c** and **39a-39b** in MeOH (10 mL) was added 28% NaOMe (0.27 mL) at rt. The reaction mixture was stirred for 18 h at rt and evaporated. The residue was purified by silica gel column chromatography to give **30a-30c** and **40a-40b**.

N⁶-Methyl-9-(2,5-di-O-*t*-butyldimethylsilyl-3-deoxy-3-ureido-β-D-ribofuranosyl)adenine (30a)—75% yield; foam; ¹H NMR (CDCl₃) δ 8.25 (s, 1 H), 8.01 (s, 1 H), 5.90 (d, 1 H, *J* = 3.5 Hz), 5.79 (brs, 1 H), 4.96 (d, 1 H, *J* = 6.1 Hz), 4.66 (s, 2 H), 4.55 (m, 1 H), 4.24 (dd, 1 H, *J* = 6.1, 12.3 Hz), 4.05 (m, 1 H), 3.89 (dd, 1 H, *J* = 2.2, 11.5 Hz), 3.76 (dd, 1 H, *J* = 2.6, 11.7 Hz), 3.08 (d, 3 H, *J* = 4.4 Hz), 0.81 (s, 9 H), 0.75 (s, 9 H), 0.00 (s, 3 H), -0.01 (s, 3 H), -0.10 (s, 3 H), -0.11 (s, 3 H); IR (KBr): 3328, 2932, 1624, 1256, 1125, 837, 782 cm⁻¹; FAB-MS *m/z* 552 [M+H]⁺. Anal. (C₂₄H₄₅N₇O₄Si₂) C, H, N.

N⁶-(3-Iodobenzyl)-9-(2,5-di-O-*t*-butyldimethylsilyl-3-deoxy-3-ureido-β-D-ribofuranosyl)adenine (30b)—89% yield; foam; ¹H NMR (CDCl₃) δ 8.25 (s, 1 H), 8.05 (s, 1 H), 7.61 (s, 1 H), 7.48 (d, 1 H, *J* = 7.7 Hz), 7.21 (d, 1 H, *J* = 7.7 Hz), 6.92 (t, 1 H, *J* = 7.7 Hz), 6.00 (brs, 1 H), 5.90 (d, 1 H, *J* = 3.1 Hz), 4.86 (d, 1 H, *J* = 6.9 Hz), 4.72 (brs, 2 H), 4.57 (m, 3 H), 4.25 (dd, 1 H, *J* = 6.8, 12.4 Hz), 4.04 (m, 1 H), 3.90 (dd, 1 H, *J* = 2.2, 11.7 Hz), 3.75 (dd, 1 H, *J* = 2.4, 11.5 Hz), 0.81 (s, 9 H), 0.76 (s, 9 H), 0.00 (s, 3 H), -0.01 (s, 3 H), -0.05 (s, 3 H), -0.08 (s, 3 H); IR (KBr): 3306, 2931, 1671, 1617, 1471, 1336, 1256, 1125, 837, 781 cm⁻¹; FAB-MS *m/z* 754 [M+H]⁺. Anal. (C₃₀H₄₈IN₇O₄Si₂) C, H, N.

2-Chloro-N⁶-(3-iodobenzyl)-9-(2,5-di-O-*t*-butyldimethylsilyl-3-deoxy-3-urido-β-D-ribofuranosyl)adenine (30c)—91% yield; foam; ¹H NMR (CDCl₃) δ 8.06 (s, 1 H), 7.60 (s, 1 H), 7.49 (d, 1 H, *J* = 7.9 Hz), 7.20 (d, 1 H, *J* = 7.7 Hz), 6.92 (t, 1 H, *J* = 7.7 Hz), 6.19 (brs, 1 H), 5.85 (d, 1 H, *J* = 3.1 Hz), 4.89 (d, 1 H, *J* = 6.9 Hz), 4.66 (s, 4 H), 4.46 (m, 1 H), 4.21 (dd, 1 H, *J* = 6.8, 12.5 Hz), 4.04 (m, 1 H), 3.90 (dd, 1 H, *J* = 2.2, 11.7 Hz), 3.73 (dd, 1 H, *J* = 2.2, 11.7 Hz), 0.80 (s, 9 H), 0.77 (s, 9 H), 0.00 (s, 6 H), -0.02 (s, 3 H), -0.05 (s, 3 H); IR (KBr): 3307, 2931, 1671, 1617, 1468, 1311, 1256, 1125, 837, 781 cm⁻¹; FAB-MS *m/z* 788 [M+H]⁺. Anal. (C₃₀H₄₇ClIN₇O₄Si₂) C, H, N.

3-Ureido-5-(6-methylaminopurin-9-yl)-4-*t*-butyldimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (40a)—72% yield; oil; ¹H NMR (CDCl₃) δ 8.69 (brs, 1 H), 8.37 (s, 1 H), 7.79 (s, 1 H), 5.95 (brs, 1 H), 5.78 (d, 1 H, *J* = 7.2 Hz), 5.37 (brs, 1 H), 4.95 (t, 1 H, *J* = 7.4 Hz), 4.74 (d, 1 H, *J* = 2.1 Hz), 4.05 (m, 1 H), 3.23 (brs, 3 H), 2.95 (d, 1 H, *J* = 5.0 Hz), 0.78 (s, 9 H), -0.13 (s, 3 H), -0.32 (s, 3 H); IR (KBr): 3301, 2933, 1668, 1625, 1377, 1252, 1116, 840, 755 cm⁻¹; FAB-MS *m/z* 465 [M+H]⁺. Anal. (C₁₉H₃₂N₈O₄Si) C, H, N.

3-Ureido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-*t*-butyldimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (40b)—72% yield; oil; ¹H NMR (CDCl₃) δ 8.58 (s, 1 H), 8.37 (s, 1 H), 7.83 (s, 1 H), 7.63 (d, 1 H, *J* = 7.9 Hz), 7.30 (d, 1 H, *J* = 7.8 Hz), 7.05 (t, 1 H, *J* = 7.7 Hz), 6.33 (s, 1 H), 5.81 (d, 1 H, *J* = 7.0 Hz), 5.52 (s, 2 H), 4.72-4.95 (m, 4 H), 4.36 (d, 1 H, *J* = 5.9 Hz), 2.94 (d, 3 H, *J* = 5.5 Hz), 0.78 (s, 9 H), -0.11 (s, 3 H), -0.39 (s, 3 H); IR (KBr): 3276, 2930, 1680, 1617, 1435, 1180, 1119, 723, 694 cm⁻¹; FAB-MS *m/z* 667 [M+H]⁺. Anal. (C₂₅H₃₅IN₈O₄Si) C, H, N.

General Procedure for the Synthesis of 13, 10, 11, 14, and 12—To a stirred solution of **30a-30c** and **40a-40b** in THF (10 mL) was added 1M TBAF in THF (4 equiv) at rt. The reaction mixture was stirred for 4 h at rt and evaporated. The residue was purified by silica gel column chromatography to give **13, 10, 11, 14** and **12**.

N⁶-Methyl-9-(3-deoxy-3-ureido-β-D-ribofuranosyl)adenine (13)—88% yield; white solid; mp 191-194 °C; $[\alpha]^{25}_{\text{D}} -42.3^{\circ}$ (*c* 0.13, DMF); ¹H NMR (DMSO-*d*₆) δ 8.37 (s, 1 H), 8.25 (s, 1 H), 7.79 (brs, 1 H), 6.24 (s, 1 H), 6.15 (d, 1 H, *J* = 7.5 Hz), 5.91 (d, 1 H, *J* = 2.6 Hz), 5.74 (brs, 2 H), 5.13 (t, 1 H, *J* = 5.1 Hz), 4.41 (dd, 1 H, *J* = 2.6, 5.7 Hz), 4.27 (m, 1 H), 3.86 (m, 1 H), 3.68 (m, 1 H), 3.49 (dd, 1 H, *J* = 4.6, 12.5 Hz), 2.94 (s, 3 H); ¹³C NMR (DMSO-*d*₆) δ 22.6, 50.4, 60.8, 73.1, 83.1, 89.4, 119.6, 139.0, 147.9, 153.0, 155.0, 170.0; IR (KBr): 3422, 1633, 1543, 1382, 1335, 1221, 1102, 1065, 521 cm⁻¹; FAB-MS *m/z* 324 [M + H]⁺. Anal. (C₁₂H₁₇N₇O₄) C, H, N.

N⁶-(3-Iodobenzyl)-9-(3-deoxy-3-ureido-β-D-ribofuranosyl)adenine (10)—70% yield; white solid; mp 165-168 °C; $[\alpha]^{25}_{\text{D}} -43.3^{\circ}$ (*c* 0.12, DMF); ¹H NMR (DMSO-*d*₆) δ 8.44 (brs, 1 H), 8.43 (s, 1 H), 8.22 (s, 1 H), 7.71 (s, 1 H), 7.55 (d, 1 H, *J* = 7.7 Hz), 7.33 (d, 1 H, *J* = 7.9 Hz), 7.06 (t, 1 H, *J* = 7.7 Hz), 6.22 (d, 1 H, *J* = 5.0 Hz), 6.15 (d, 1 H, *J* = 7.7 Hz), 5.93 (d, 1 H, *J* = 2.3 Hz), 5.78 (brs, 2 H), 5.11 (t, 1 H, *J* = 5.7 Hz), 4.66 (brs, 2 H), 4.42 (m, 1 H), 4.25 (m, 1 H), 3.87 (m, 1 H), 3.69 (m, 1 H), 3.50 (m, 1 H); ¹³C NMR (DMSO-*d*₆) δ 42.2, 50.9, 61.0, 73.4, 84.2, 89.5, 94.7, 119.5, 126.7, 130.5, 135.4, 135.7, 139.2, 142.9, 148.3, 152.5, 154.3, 158.7; IR (KBr): 3398, 1621, 1538, 1477, 1338, 1221, 1103, 822 cm⁻¹; FAB-MS *m/z* 526 [M + H]⁺. Anal. (C₁₈H₂₀IN₇O₄) C, H, N.

{5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-4-hydroxy-2-hydroxymethyl-tetrahydro-furan-3-yl}-urea (11)—82% yield; white solid; mp 132.3-135 °C; $[\alpha]^{25}_{\text{D}} -27.4^{\circ}$ (*c* 0.35, DMF); ¹H NMR (DMSO-*d*₆) δ 8.92 (brs, 1 H), 8.47 (s, 1 H), 7.73 (s, 1 H), 7.58 (d, 1 H, *J* = 7.9 Hz), 7.33 (d, 1 H, *J* = 7.5 Hz), 7.09 (t, 1 H, *J* = 7.7 Hz), 6.25 (d, 1 H, *J* = 4.7 Hz), 6.16 (d, 1 H, *J* = 7.7 Hz), 5.87 (d, 1 H, *J* = 2.0 Hz), 5.77 (s, 2 H), 5.05 (t, 1 H, *J* = 5.5 Hz), 4.58 (d, 2 H, *J* = 5.0 Hz), 4.34 (m, 1 H), 3.86 (m, 1 H), 3.39 (m, 1 H), 3.51 (m, 1 H); ¹³C NMR (DMSO-*d*₆) δ 42.5, 50.7, 60.7, 73.6, 84.1, 89.2, 94.7, 118.5, 126.8, 130.6, 135.6, 136.1, 139.4, 141.9, 149.3, 153.1, 154.8, 158.6; IR (KBr): 3405, 1619, 1346, 1312, 1221, 1105, 781, 633 cm⁻¹; FAB-MS *m/z* 560 [M + H]⁺. Anal. (C₁₈H₁₉ClIN₇O₄) C, H, N.

3-Ureido-5-(6-methylaminopurin-9-yl)-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide (14)—66% yield; white solid; mp 118.2-120.0 °C; $[\alpha]^{25}_{\text{D}} -13.3^{\circ}$ (*c* 0.15, MeOH); ¹H NMR (CD₃OD): δ 8.48 (s, 1 H), 8.29 (s, 1 H), 6.07 (d, 1 H, *J* = 3.1 Hz), 4.61 (m, 2 H), 4.41 (d, 1 H, *J* = 6.0 Hz), 3.11 (s, 3 H), 2.80 (s, 3 H); ¹³C NMR (CD₃OD) δ 14.1, 20.9, 24.2, 24.9, 26.4, 56.7, 59.7, 74.9, 83.7, 92.2, 141.0, 154.2, 173.0; IR (KBr): 3425, 1667, 1630, 1534, 1356, 1306, 1084, 936, 636 cm⁻¹; FAB-MS *m/z* 351 [M + H]⁺. Anal. (C₁₃H₁₈N₈O₄) C, H, N.

3-Ureido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide (12)—82% yield; white solid; mp: 120.7-122.2 °C; $[\alpha]^{25}_{\text{D}} -10.0^{\circ}$ (*c* 0.10, DMF); ¹H NMR (DMSO-*d*₆): δ 8.69 (s, 1 H), 8.53 (brs, 1 H), 8.33 (d, 1 H, *J* = 4.6 Hz), 8.23 (s, 1 H), 7.72 (s, 1 H), 7.55 (d, 1 H, *J* = 7.7 Hz), 7.34 (d, 1 H, *J* = 7.7 Hz), 7.06 (t, 1 H, *J* = 7.7 Hz), 6.29 (d, 1 H, *J* = 4.4 Hz), 6.22 (d, 1 H, *J* = 6.7 Hz), 6.02 (d, 1 H, *J* = 2.2 Hz), 5.76 (s, 2 H), 4.65 (br s, 2 H), 4.41 (m, 2 H), 4.23 (d, 1 H, *J* = 6.0 Hz), 2.63 (d, 1 H, *J* = 4.6 Hz); ¹³C NMR (CD₃OD) δ 14.1, 20.9, 24.9, 26.4, 56.7, 59.7, 75.1, 83.6, 92.3, 128.0, 131.6, 137.5, 137.7, 141.3, 143.3, 154.2, 156.2, 161.7, 173.0; IR (KBr): 3424, 2932, 1619, 1476, 1338, 1056, 645 cm⁻¹; FAB-MS *m/z* 553 [M + H]⁺. Anal. (C₁₉H₂₁IN₈O₄) C, H, N.

Numbering scheme of GPCRs—For sequence alignments of selected regions of the A₃AR and other GPCRs, a standardized numbering system²⁹ was used to identify residues in the TMs of various receptors. Each residue is identified by two numbers: the first corresponds to the TM in which it is located; the second indicates its position relative to the

most conserved residue in that helix, arbitrarily assigned to 50. For example, Thr3.36 is the threonine in TM3 (Thr94), located 14 residues before the most conserved arginine Arg3.50; His7.43 corresponds to His272.

Biological Methods

Materials—The vector pcDNA3 was obtained from Invitrogen. Oligonucleotides used were synthesized by Bioserve Biotechnologies (Laurel, MD, USA). Adenosine deaminase, 2-chloroadenosine, and NECA (5'-*N*-ethylcarboxamidoadenosine) were obtained from Sigma (St. Louis, MO, USA). [¹²⁵I]I-AB-MECA (2000 Ci/mmol) was from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other compounds were obtained from standard commercial sources and were of analytical grade.

Site-directed mutagenesis—The protocols used were as described in the QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). Mutations were confirmed by DNA sequencing.

Transfection of wild-type and mutant A₃AR to COS-7 cells—Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for transfection of wild-type and mutant receptor cDNA to COS-7 cells following the manufacturer's protocol.

Membrane preparation—After 48 h of transfection, COS-7 cells were harvested and homogenized with a Polytron homogenizer. The homogenates were centrifuged at 20,000 *g* for 20 min, and the resulting pellet was resuspended in the 50 mM Tris-HCl buffer (pH 7.4) in the presence of 3 Units/mL adenosine deaminase and incubated at 37°C for 30 min, and then stored at -80°C in aliquots. The protein concentration was determined by using the method of Bradford.³⁰

Radioligand binding assays and inositol phosphate determination in COS-7 cells—The procedures for A₃AR-binding experiments using [¹²⁵I]I-AB-MECA ([¹²⁵I]N⁶-(4-aminobenzyl)-5'-*N*-methylcarboxamidoadenosine) were as previously described.⁴¹ The procedures for binding at human A₁ and A_{2A}ARs and a functional assay of cyclic AMP formation mediated by the human A_{2B}AR expressed in CHO cells was as described.³⁶ The method for PLC determination in transiently transfected COS-7 cells has been previously described.³¹

Preparation of cardiac myocyte model of simulated ischemia and gene transfer into cardiac myocytes—Ventricular cells were cultured from chick embryos 14 d *in ovo* and maintained in culture as previously described.²⁷ All experiments were performed on day 3 in culture, at which time the medium was changed to a HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)-buffered medium containing 139 mM NaCl, 4.7 mM KCl, 0.5 mM MgCl₂, 0.9 mM CaCl₂, 5 mM HEPES, and 2% fetal bovine serum (pH 7.4, 37 °C). Myocytes were then exposed to simulated ischemia, which was induced by 90 min of hypoxia and glucose deprivation in a hypoxic incubator (NuAire, Plymouth, MN, USA) where O₂ was replaced by N₂ as previously described.²⁷ The extent of myocyte injury was determined at the end of the 90-min ischemia, at which time myocytes were taken out of the hypoxic incubator and reexposed to room air (normal percentage O₂), followed by quantitation of the number of viable cells. Viable cells were also determined by the ability to exclude Trypan blue.²⁷ Measurement of the basal level of cell injury was made after parallel incubation of control cells under a normal percentage of O₂. The extent of ischemia-induced injury was quantitatively determined by the percentage of cells killed, according to a previously described method.²⁷ The percentage of cells killed was calculated as the number of cells obtained from the control group (representing cells not

subjected to hypoxia or drug treatment) minus the number of cells from the treatment group divided by the number of cells in the control group multiplied by 100%.

Cells were then transfected with either the vector pcDNA₃ or cDNA encoding the wild-type human A₃AR or the neoceptor H272E, with the use of FuGENE 6 as previously described.²⁸ Forty-eight hours after the transfection, myocytes were exposed to simulated ischemia, and myocyte injury was determined in the presence or absence of the neoligand **10**. In other experiments, the PLD activity was determined 48 h after the transfection of myocytes with either the wild-type hA₃AR or the neoceptor H272E cDNA.

Measurement of PLD activity—For measurement of PLD activity, cultured ventricular myocytes labeled with [³H]myristate (49 Ci/mmol, 2 μCi/ml) for 24 h were exposed to receptor agonist in the presence of 0.5% (v/v) ethanol. Lipids were extracted by the method of Bligh and Dyer.³² The formation of [³H]PEt was an indication of PLD activity. Quantitation of PEt (phosphatidylethanol) was carried out as previously described.³³ Briefly, the formation of [³H]PEt in cells was quantitated by separation of the labeled lipids via thin-layer chromatography and scintillation counting of the ³H label. The position of PEt was determined visually by placing the thin-layer plate in an iodine chamber, and its level was expressed as a percentage of total lipids. Data were also expressed as percentage increases in the amount of PEt relative to that for unstimulated cells.

Statistical analysis—Binding and functional parameters were estimated with GraphPad Prism software (GraphPad, San Diego, CA, USA). IC₅₀ values obtained from competition curves were converted to K_i values by using the Cheng-Prusoff equation.³⁴ Data were expressed as mean ± standard error.

Computational Methods

All calculations were performed using the SYBYL program version 6.9⁵³ on a Silicon Graphics Octane workstation (300 MHz MIPS R12000 (IP30) processor).

Conformational search—The N⁶-I-benzyl-3'-ureido compound, **10**, was constructed using the "Sketch Molecule" and subjected to a random search performed for all rotatable bonds. The options of the random search consisted of 3,000 iterations, 3 kcal energy cutoff, and chirality checking. MMFF force field⁵⁴ and charge were applied using distance-dependent dielectric constants and the conjugate gradient method until the gradient reached 0.05 kcal/mol/Å. After clustering the low energy conformers from the result of the conformational search, the relative stabilities of various representative conformers from all groups were checked by semi-empirical molecular orbital calculations using the PM3 method in the MOPAC 6.0 package⁴³. During the PM3 optimization all abortive results from electrostatic collapse were removed.

Molecular Dynamics of the H272E neoceptors—The previously published hA₃ AR model (PDB code: 1o74) built by homology modeling⁵⁵ from the X-ray structure of bovine rhodopsin with 2.8 Å resolution³⁵ was used for the docking study. For the side-chain refinement of the H272E neoceptors, the optimized structures were then used as the starting point for subsequent 50-ps MD, during which the protein backbone atoms in the secondary structures were constrained as in the previous step. The options of MD at 300 K with a 0.2-ps coupling constant were a time step of 1fs and a nonbonded update every 25 fs. The SHAKE algorithm⁵⁶ was employed to fix the lengths of bonds to hydrogen atoms. The average structure from the last 10-ps trajectory of MD was optimized with backbone constraints in the secondary structure and then the unconstrained structure was minimized as described above.

FlexiDock docking—Compound **10** was docked within the hA₃ AR and H272E neoceptors. Flexible docking was facilitated through the FlexiDock utility in the Biopolymer module of SYBYL 6.9. Flexible docking allowed for flexibility of all rotatable bonds in **10**, except those of the ribose ring, and the side chains of surrounding hydrophilic residues (T94, Q167, N250, E272) in the putative binding site of CI-IB-MECA. After the hydrogen atoms were added to the receptor, atomic charges were recalculated by using Kollman All-atom for the protein and Gasteiger-Hückel for the ligand. H-bonding sites were marked for the acidic residue, E272, of the neoceptor and for the 3'-ureido groups of the neoligands, which were able to act as H-bond donor or acceptor. The lowest energy conformer of neoligand was variously pre-positioned in the putative binding cavity as a starting point for FlexiDock, based on the reported point-mutational results. FlexiDock parameters were set at 30,000-generation for genetic algorithms. To increase the binding interaction, the torsion angles of the side chains that directly interacted within 5 Å of the ligands, according to the results of FlexiDock, were manually adjusted. The atom types of all ligands were manually assigned with an Amber all-atom force field. Finally, the complex structure was minimized using an Amber force field with a fixed dielectric constant (4.0), until the conjugate gradient reached 0.1 kcal • mol⁻¹ • Å⁻¹.

FlexX Docking—FlexX 1.13⁵⁷ is a fast docking method that uses a new algorithmic approach based on a pattern recognition technique called pose clustering, allowing conformational flexibility of the ligand by MINUMBA⁵⁸ conformer library to grow ligands during the docking process. The free binding energy of complex including H-bond, ionic, aromatic, or lipophilic interactions, was estimated by the scoring function. Cscore calculations were included for scoring. A putative binding site including T94 (3.36), N250 (6.55), S271 (7.42), and H272 (7.43) was manually selected, based on the previous point-mutational results.⁴¹ Formal charges were applied to the ligands. All default parameters, as implemented in the 6.9 release of SYBYL, were used.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Buskirk AR, Liu DR. Creating small-molecule-dependent switches to modulate biological functions. *Chem Biol.* 2005; 12:151–161. [PubMed: 15734643]
2. Hwang YW, Miller DL. A mutation that alters the nucleotide specificity of elongation factor Tu, a GTP regulatory protein. *J Biol Chem.* 1987; 262:13081–13085. [PubMed: 3308869]
3. Niculescu-Duvaz I, Friedlos F, Niculescu-Duvaz D, Davies L, Springer CJ. Prodrugs for antibody- and gene-directed enzyme prodrug therapies (ADEPT and GDEPT). *Anticancer Drug Des.* 1999; 14:517–538. [PubMed: 10834273]
4. Hashimoto A, Shi Y, Drake K, Koh JT. Design and synthesis of complementing ligands for mutant thyroid hormone receptor TRbeta(R320H): a tailor-made approach toward the treatment of resistance to thyroid hormone. *Bioorg Med Chem.* 2005; 13:3627–3639. [PubMed: 15862991]
5. Strausberg RL, Schreiber SL. From knowing to controlling: a path from genomics to drugs using small molecule probes. *Sci.* 2003; 300:294–295.

6. Koh JT, Biggins JB. Ligand-receptor engineering and its application towards the complementation of genetic disease and target identification. *Curr Top Med Chem*. 2005; 5:413–420. [PubMed: 15892683]
7. Shi Y, Koh JT. Functionally orthogonal ligand-receptor pairs for the selective regulation of gene expression generated by manipulation of charged residues at the ligand-receptor interface of ER alpha and ER beta. *J Am Chem Soc*. 2002; 124:6921–6928. [PubMed: 12059215]
8. Tedesco R, Thomas JA, Katzenellenbogen BS, Katzenellenbogen JA. The estrogen receptor: a structure-based approach to the design of new specific hormone-receptor combinations. *Chem Biol*. 2001; 8:277–287. [PubMed: 11306352]
9. Liu Y, Shah K, Yang F, Witucki L, Shokat KM. Engineering Src family protein kinases with unnatural nucleotide specificity. *Chem Biol*. 1998; 5:91–101. [PubMed: 9495830]
10. Lin Q, Jiang F, Schultz PG, Gray NS. Design of allele-specific protein methyltransferase inhibitors. *J Am Chem Soc*. 2001; 123:11608–11613. [PubMed: 11716715]
11. Strader CD, Sigal IS, Register RB, Candelore MR, Rands E, Dixon RA. Identification of residues required for ligand binding to the beta-adrenergic receptor. *Proc Natl Acad Sci USA*. 1987; 84:4384–4388. [PubMed: 2885836]
12. Strader CD, Gaffney T, Sugg EE, Candelore MR, Keys R, Patchett AA, Dixon RA. Allele-specific activation of genetically engineered receptors. *J Biol Chem*. 1991; 266:5–8. [PubMed: 1670767]
13. Small KM, Wagoner LE, Levin AM, Kardia SL, Liggett SB. Synergistic polymorphisms of beta1- and alpha2C-adrenergic receptors and the risk of congestive heart failure. *N Engl J Med*. 2002; 347:1135–1142. [PubMed: 12374873]
14. Coward P, Wada HG, Falk MS, Chan SD, Meng F, Akil H, Conklin BR. Controlling signaling with a specifically designed Gi-coupled receptor. *Proc Natl Acad Sci USA*. 1998; 95:352–357. [PubMed: 9419379]
15. Redfern CH, Coward P, Degtyarev MY, Lee EK, Kwa AT, Hennighausen L, Bujard H, Fishman GI, Conklin BR. Conditional expression and signaling of a specifically designed Gi-coupled receptor in transgenic mice. *Nat Biotechnol*. 1999; 17:165–169. [PubMed: 10052353]
16. Searce-Levie K, Lieberman MD, Elliott HH, Conklin BR. Engineered G protein coupled receptors reveal independent regulation of internalization, desensitization and acute signaling. *BMC Biol*. 2005; 3:3. [PubMed: 15707483]
17. Claeysen S, Joubert L, Sebben M, Bockaert J, Dumuis A. A single mutation in the 5-HT₄ receptor (5-HT₄-R D100(3.32)A) generates a Gs-coupled receptor activated exclusively by synthetic ligands (RASSL). *J Biol Chem*. 2003; 278:699–702. [PubMed: 12441358]
18. Pauwels PJ. Unravelling multiple ligand-activation binding sites using RASSL receptors. *Trends Pharmacol Sci*. 2003; 24:504–507. [PubMed: 14559400]
19. Zhao GQ, Zhang Y, Hoon MA, Chandrashekar J, Erlenbach I, Ryba NJ, Zuker CS. The receptors for mammalian sweet and umami taste. *Cell*. 2003; 115:255–266. [PubMed: 14636554]
20. Bruysters M, Jongejan A, Akdemir A, Bakker RA, Leurs R. Mutant histamine H₁ receptor Phe435Ala: A Gq/11-coupled receptor activated solely by synthetic ligands (RASSL). *J Biol Chem*. 2005 Jul 18. Epub ahead of print.
21. Jacobson KA, Gao ZG, Chen A, Barak D, Kim SA, Lee K, Link A, Van Rompaey P, Van Calenbergh S, Liang BT. Neoreceptor concept based on molecular complementarity in GPCRs: A mutant adenosine A₃ receptor with selectively enhanced affinity for amine-modified nucleosides. *J Med Chem*. 2001; 44:4125–4136. [PubMed: 11708915]
22. Liang BT, Jacobson KA. A physiological role of the adenosine A₃ receptor: sustained cardioprotection. *Proc Natl Acad Sci USA*. 1998; 95:6995–6999. [PubMed: 9618527]
23. Fredholm BB, Chen JF, Masino SA, Vaugeois JM. Actions of adenosine at its receptors in the CNS: insights from knockouts and drugs. *Annu Rev Pharmacol Toxicol*. 2005; 45:385–412. [PubMed: 15822182]
24. Fishman P, Bar-Yehuda S, Madi L, Cohn I. A₃ adenosine receptor as a target for cancer therapy. *Anticancer Drugs*. 2002; 13:437–443. [PubMed: 12045454]
25. Kim SK, Gao ZG, Van Rompaey P, Gross AS, Chen A, Van Calenbergh S, Jacobson KA. Modeling the adenosine receptors: Comparison of binding domains of A_{2A} agonist and antagonist. *J Med Chem*. 2003; 46:4847–4859. [PubMed: 14584936]

26. Jacobson KA, Ohno M, Duong HT, Kim SK, Tchilibon S, Cesnek M, Holy A, Gao ZG. A neoceptor approach to unraveling microscopic interactions between the human A_{2A} adenosine receptor and its agonists. *Chem Biol.* 2005; 12:237–247. [PubMed: 15734651]
27. Strickler J, Jacobson KA, Liang BT. Direct preconditioning of cultured chick ventricular myocytes: novel functions of cardiac adenosine A_{2A} and A₃ receptors. *J Clin Invest.* 1996; 98:1773–1779. [PubMed: 8878427]
28. Mozzicato S, Joshi BV, Jacobson KA, Liang BT. Role of direct RhoA-phospholipase D interaction in mediating adenosine-induced protection from cardiac ischemia. *FASEB J.* 2004; 18:406–408. [PubMed: 14688204]
29. Visiers I, Ballesteros JA, Weinstein H. Three-dimensional representations of G protein-coupled receptor structures and mechanisms. *Methods Enzymol.* 2002; 343:329–371. [PubMed: 11665578]
30. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
31. Gao ZG, Chen A, Barak D, Kim SK, Müller CE, Jacobson KA. Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A₃ adenosine receptor. *J Biol Chem.* 2002; 277:19056–19063. [PubMed: 11891221]
32. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* 1959; 37:911–917. [PubMed: 13671378]
33. Lee JE, Bokoch G, Liang BT. Signaling mechanism of the adenosine A₃ receptor: Novel cardioprotective role of RhoA. *FASEB J.* 2001; 15:1886–1894. [PubMed: 11532968]
34. Cheng Y, Prusoff WH. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. *Biochem Pharmacol.* 1973; 22:3099–3108. [PubMed: 4202581]
35. Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, Le Trong I, Teller DC, Okada T, Stenkamp TE, Yamamoto M, Miyano M. Crystal structure of rhodopsin: a G protein-coupled receptor. *Sci.* 2000; 289:739–745.
36. Ohno M, Gao ZG, Van Rompaey P, Tchilibon S, Kim SK, Harris BA, Blaustein J, Gross AS, Duong HT, Van Calenbergh S, Jacobson KA. Modulation of adenosine receptor affinity and intrinsic efficacy in nucleosides substituted at the 2-position. *Bioorg Med Chem.* 2004; 12:2995–3007. [PubMed: 15142558]
37. Van Rompaey P, Jacobson KA, Gross AS, Gao ZG, Van Calenbergh S. Exploring human adenosine A₃ receptor complementarity and activity for adenosine analogues modified in the ribose and purine moiety. *Bioorg Med Chem.* 2005; 13:973–983. [PubMed: 15670905]
38. Jeong LS, Kim MJ, Kim HO, Kim SK, Gao ZG, Jacobson KA, Chun MW. Design and synthesis of 3'-ureidoadenosine-5'-uronamides: Effects of the 3'-ureido group on binding to the A₃ adenosine receptor. *Bioorg Med Chem Lett.* 2004; 14:4851–4854. [PubMed: 15341938]
39. Jeong, L.S.; Kim, M.J.; Kim, A.Y.; Lee, J.A.; Jacobson, K.A.; Gao, Z.G.; Kim, S.K.; Chun, M.W. 229th National Meeting of the Am Chem Soc; San Diego, CA. March 13-17, 2005; Abstract MEDI82
40. Dougherty C, Barucha J, Schofield P, Jacobson KA, Liang BT. Cardiac myocytes rendered ischemia resistant by overexpressing the human adenosine A₃ receptor. *FASEB J.* 1998; 12:1785–1792. [PubMed: 9837869]
41. Gao ZG, Kim SK, Gross AS, Chen A, Blaustein J, Jacobson KA. Identification of essential residues involved in the allosteric modulation of the human A₃ adenosine receptor. *Mol Pharmacol.* 2003; 63:1021–1031. [PubMed: 12695530]
42. Costanzi S, Lambertucci C, Vittori S, Volpini R, Cristalli G. 2- and 8- alkynyladenosines: conformational studies and docking to human adenosine A₃ receptor can explain their different biological behavior. *J Mol Graph Model.* 2003; 21:253–262. [PubMed: 12479925]
43. Stewart JJP. MOPAC: A Semiempirical Molecular Orbital Program. *J Comput-Aided Mol Des.* 1990; 4:1–105. [PubMed: 2197373]
44. Spiegel AM, Weinstein LS. Inherited diseases involving G proteins and G protein-coupled receptors. *Annu Rev Med.* 2004; 55:27–39. [PubMed: 14746508]
45. Iida A, Saito S, Sekine A, Kataoka Y, Tabei W, Nakamura Y. Catalog of 300 SNPs in 23 genes encoding G-protein coupled receptors. *J Hum Genet.* 2004; 49:194–208. [PubMed: 15042431]

46. Lam P, Hong CJ, Tsai SJ. Association study of A_{2A} adenosine receptor genetic polymorphism in panic disorder. *Neurosci Lett*. 2005; 378:98–101.
47. Alsene K, Deckert J, Sand P, de Wit H. Association between A_{2a} receptor gene polymorphisms and caffeine-induced anxiety. *Neuropsychopharmacology*. 2003; 28:1694–702. [PubMed: 12825092]
48. Koh JT. Designing selectivity and discrimination into re-engineered ligand-receptor interfaces; Beyond bumps and holes. *Chem Biol*. 2002; 9:17–23. [PubMed: 11841935]
49. Belshaw PJ, Schoepfer JG, Liu K-Q, Morrison KL, Schreiber SL. Rational design of orthogonal receptor ligand combinations. *Angew Chem Int Ed Engl*. 1995; 34:2129–2132.
50. Gillespie PG, Gillespie SK, Mercer JA, Shah K, Shokat KM. Engineering of the myosin-ibeta nucleotide-binding pocket to create selective sensitivity to N⁶-modified ADP analogs. *J Biol Chem*. 1999; 274:31373–31381. [PubMed: 10531338]
51. Ulrich SM, Buzko O, Shah K, Shokat KM. Towards the engineering of an orthogonal protein kinase/nucleotide triphosphate pair. *Tetrahedron*. 2000; 56:9495–9502.
52. Fredholm BB, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev*. 2001; 53:527–552. [PubMed: 11734617]
53. Sybyl Molecular Modeling System, version 6.9. Tripos Inc.; St. Louis, Missouri 63144, USA:
54. Halgren TA. MMFF VII. Characterization of MMFF94, MMFF94s, and Other Widely Available Force Fields for Conformational Energies and for Intermolecular-Interaction Energies and Geometries. *J Comput Chem*. 1999; 20:730–748.
55. Gao Z-G, Kim S-K, Biadatti T, Chen W, Lee K, Barak D, Kim SG, Johnson CR, Jacobson KA. Structural Determination of A₃ Adenosine Receptor Activation: Nucleoside Ligands at the Agonist/Antagonist Boundary. *J Med Chem*. 2002; 45:4471–4484. [PubMed: 12238926]
56. Ryckaert JP, Ciccotti G, Berendsen HJC. Numerical Integration of the Cartesian Equations of Motion for a System with Constraints: Molecular Dynamics of n-alkanes. *J Comput Phys*. 1977; 23:327–341.
57. Rarey M, Kramer B, Lengauer T, Klebe G. A fast flexible docking method using an incremental construction algorithm. *J Mol Biol*. 1996; 261:470–489. [PubMed: 8780787]
58. Klebe G, Mietzner T. A fast and efficient method to generate biologically relevant conformations. *J Comput-Aided Mol Des*. 1994; 8:583–606. [PubMed: 7876902]

Fig. 1. Binding inhibition and functional activation in wild-type and H272E mutant A_3AR s. The receptors were expressed transiently in COS-7 cells as described in Experimental Procedures. The binding affinity (K_i) was determined by using the agonist radioligand $[^{125}I]$ -AB-MECA (0.5 nM). The structure of compound **10** is given in Table 1.

Fig. 2.

Activation of the neoceptor by the neoagonist **10** protects heart cells from ischemia-induced injury. A) Activation of PLD by **10** in chick cardiomyocytes expressing the mutant human H272E A₃AR. B) Effect of **10** on anti-ischemic cardioprotection in neoceptor-transfected cardiac myocytes is shown. Cardiac ventricular myocytes were transfected with cDNA encoding the neoceptor H272E, and the percentage of cells killed was determined in the absence or the presence of **10** during the 90-min simulated ischemia, as described in Experimental Procedures. Data were plotted as the percentage of cells killed during the prolonged simulated ischemia. [#] $P < 0.01$ compared with control (ANOVA). ANOVA (all four groups), $F = 15.6$, $P < 0.0001$; all posttest comparisons were significant at $P < 0.01$ except for the percentage of cells killed at 300 nM **10** compared with 1,000 nM and that for 100 nM compared with 300 nM.

A

B

Fig. 3.

Two energetically favorable binding modes of the N^6 -(3-I-benzyl)-3'-ureidoadenosine **10** in the binding site of the mutant H272E hA₃AR. The binding mode similar to the hA₃/Cl-IB-MECA complex (A) and another binding mode (B), which was energetically unfavorable in the hA₃/Cl-IB-MECA complex, are shown. All ligands are displayed as ball-and-stick models in the atom-by-atom color, and the side chains of the hA₃AR are shown as stick models. The H-bonding between each ligand and the mutant hA₃AR is displayed in yellow. The A₃AR is represented by a tube model with a different color for each TM (TM2 in orange, TM3 in yellow, TM4 in green, TM5 in cyan, TM6 in blue, TM7 in purple).

Scheme 1.

Reagents and conditions: (a) TiCl_4 , pyridine, 0 °C, 1 h; (b) NaN_3 , DMF, rt, 48 h; (c) i) 75% AcOH , 55 °C, 1.5 h; ii) $\text{NaIO}_4/\text{H}_2\text{O}$, EtOH, 0 °C, 20 min then NaBH_4 ; (d) Ac_2O , pyridine, rt, 3 h; (e) i) 85% HCO_2H , 60 °C, 1.5 h; ii) Ac_2O , pyridine, rt, 16 h; (f) Silylated 6-chloropurine or 2,6-dichloropurine, TMSOTf , $\text{C}_2\text{H}_4\text{Cl}_2$, 0 °C to 60 °C, 2 h; (g) MeNH_2 , 1,4-dioxane, rt, 4 h or 3-iodobenzylamine hydrochloride, Et_3N , EtOH, 50 °C, 18 h then NaOMe , MeOH, rt, 2 h; (h) TBSCl , imidazole, DMF, rt, 24 h; (i) Ph_3P , $\text{NH}_4\text{OH}/\text{H}_2\text{O}$, THF, rt, 18 h; (j) Chloroacetyl isocyanate, DMF, 0 °C, 3 h; (k) NaOMe , MeOH, rt, 18 h; (l) TBAF , THF, rt, 4 h. IB = 3-iodobenzyl.

Scheme 2.

Reagents and Conditions: (a) i) 75% AcOH, 55 °C, 1.5 h; ii) NaIO₄, RuCl₃.H₂O, CCl₄/CH₃CN/H₂O, rt, 4h; (b) i) (COCl)₂, DMF, CH₂Cl₂, rt, 16 h; ii) 2M NH₂CH₃, CH₂Cl₂, 0 °C, 3 h; (c) AcOH/Ac₂O/c-H₂SO₄, rt, 16 h; (d) Silylated 6-chloropurine, TMSOTf, C₂H₄Cl₂, 0 °C to 60 °C, 2 h; (e) MeNH₂, 1,4-dioxane, rt, 4 h or 3-iodobenzylamine hydrochloride, Et₃N, EtOH, 50 °C, 18 h then NaOMe, MeOH, rt, 2 h; (f) TBSCl, imidazole, DMF, rt, 24 h; (g) Ph₃P, NH₄OH/H₂O, THF, rt, 18 h; (h) Chloroacetyl isocyanate, DMF, 0 °C, 3 h; (i) NaOMe, MeOH, rt, 18 h; (j) TBAF, THF, rt, 4 h or Et₃N.3HF, THF, 50 °C, 16 h. IB = 3-iodobenzyl.

Table 1

Affinities of various 3'-amine-derivatized adenosine analogues in binding experiments at wild-type and H272E mutant human A₃ARs. X = O and R₂ = H, unless noted.

	Adenine substituent ^d	Ribose substituents		K _i (μM) or percentage inhibition at 10 μM		Affinity Enhancement
		R ₃ =	R ₅ =	wild-type	H272E	
1^a	H	NH ₂	CH ₂ OH	442 ± 121	75 ± 32	6-fold
2^b	H	CH ₂ NH ₂	CH ₂ OH	3 ± 2%	2 ± 2%	NE
3^a	H	NHC≡NHNH ₂	CH ₂ OH	130 ± 34	33.3 ± 7.1	4-fold
4^a	IB	NH ₂	CH ₂ OH	0.87 ± 0.18	0.32 ± 0.10	3-fold
5	IB	CH ₂ NH ₂	CONHCH ₃	0.137 ± 0.041	0.185 ± 0.027	NE
6	IB	CH ₂ NHCOCH ₃	CH ₂ OH	15%	0.700 ± 0.099	>20-fold
7	IB	CH ₂ NH ₂	CH ₂ OH	9.5 ± 1.7	0.61 ± 0.23	15-fold
8	CMB	CH ₂ NH ₂	CH ₂ OH	13.8 ± 1.4	0.709 ± 0.163	20-fold
9	CMB	CH ₂ NH ₂	CONHCH ₃	0.557 ± 0.164	0.592 ± 0.081	NE
10^c	IB	NHCONH ₂	CH ₂ OH	9 ± 1%	0.22 ± 0.04	>100-fold
11	IB, R ₂ = Cl	NHCONH ₂	CH ₂ OH	47 ± 9%	0.20 ± 0.03	~50-fold
12^b	IB	NHCONH ₂	CONHCH ₃	5 ± 1%	2.7 ± 0.4	>10-fold
13	Me	NHCONH ₂	CH ₂ OH	3 ± 1%	30 ± 6%	NE
14^b	Me	NHCONH ₂	CONHCH ₃	7 ± 2%	7 ± 1%	NE
15^b	IB, X = O	N ₃	CONHCH ₃	2.26 ± 0.48	0.186 ± 0.034	12-fold

	Adenine substituent ^d	Ribose substituents		K_i (μ M) or percentage inhibition at 10 μ M		Affinity Enhancement
16	IB, X = S	OH	CH ₂ N ₃	0.022 $\pm$ 0.004	0.23 $\pm$ 0.04	NE
17	H	OH	CONH-(CH ₂) ₂ NH ₂	>100	>100	NE
18 ^e	H	OH	CONHNH ₂	1.5 $\pm$ 0.6	>10	NE

Binding parameters were measured in transiently transfected COS-7 cells as described in Experimental Procedures. The binding affinity was determined by using the agonist radioligand [¹²⁵I]-AB-MECA (0.5 nM). Values represent the mean $\pm$ SE of at least three independent determinations.

^a Affinity at wild-type and mutant A₃ARs previously reported.²¹

^b Affinity at wild-type A₃AR stably expressed in CHO cells previously reported.^{37,38}

^c MRS3481, LJ720.

^d IB = 3-iodobenzyl, CMB = 5-chloro-2-methoxybenzyl, Me = methyl, NE = no enhancement or insignificant enhancement.

^e MRS3412.

Table 2

Comparison of the effects of 3'-aminomethyl- and 3'-ureido-derivatized adenosine analogues in binding experiments at wild-type and mutant human A₃ARs.

Compound	K _i values (μM)					
	Wild-type	T94A	T94E	H272D	H272E	Q167E
5	0.14 ± 0.04	0.12 ± 0.01	0.11 ± 0.03	0.12 ± 0.03	0.18 ± 0.03	0.57
6	>10	>10	>10	1.0 ± 0.3	0.70 ± 0.10	>10
7	9.5 ± 1.7	10.7 ± 2.9	10.0 ± 1.4	0.85 ± 0.12	0.61 ± 0.23	>10
8	13.8 ± 1.4	9.8 ± 4.2	>10	2.0 ± 0.4	0.71 ± 0.16	>10
9	0.56 ± 0.16	ND	0.85 ± 0.19	0.39 ± 0.20	0.59 ± 0.08	7.2
10	>10	>10	>10	0.47 ± 0.07	0.22 ± 0.04	>100
15	2.26 ± 0.48	1.37 ± 0.13	2.0 ± 0.1	0.14 ± 0.00	0.19 ± 0.03	ND ^b

Binding parameters (K_i values) were measured in transiently transfected COS-7 cells as described in Experimental Procedures by using the agonist radioligand [¹²⁵I]-AB-MECA (0.5 nM). The K_D values for the WT and T94A mutant were determined to be 1.8 ± 0.8 and 2.6 ± 1.2 nM, respectively, while over 10 nM for other mutants. Values represent the mean ± SE of at least three independent determinations.

^aThe H272A mutant receptor lost high-affinity binding to both agonist and antagonist radioligands; therefore, competitive binding experiments could not be performed.

^bND = not determined.