Orthogonal Activation of the Reengineered A3 Adenosine Receptor (Neoceptor) Using Tailored Nucleoside Agonists

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Orthogonal activation of the reengineered A$_3$ adenosine receptor (neoceptor) 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 (neoceptor) 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 A$_3$ 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-methyloxybenzyl, and methyl. An $N^6$-3-iodobenzyl-3′-ureido adenosine derivative 10 activated phospholipase C in COS-7 cells (EC$_{50}$=0.18 μM) or phospholipase D in chick primary cardiomyocytes mediated by a mutant (H272E), but not the wild-type, A$_3$AR. The affinity enhancements for 10 and the corresponding 3′-acetamidomethyl analogue 6 were $>$100-fold and $>$20-fold, respectively. 10 concentration-dependently protected cardiomyocytes transfected with the neoceptor against hypoxia. Unlike 10, adenosine activated the wild-type A$_3$AR (EC$_{50}$ of 1.0 μM), but had no effect on the H272E mutant A$_3$AR (100 μM). Compound 10 was inactive at human A$_1$, 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 neoceptor 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
has been proposed to be an effective means against cancer. Tailor-made ligands for thyroid hormone nuclear receptors may have potential to restore mutation-caused genetic diseases. It has been suggested that the chemical genetic approach is superior to the traditional genetic approach, such as gene knockout, in studying protein functions, and has advantage for developing drugs against genetic diseases. Functional orthogonal ligand-receptor pairs have been used for regulation of estrogen receptors, Src family protein kinases, and protein methyltransferases.

In the GPCR field, engineering of orthogonal ligand-receptor pairs was initiated shortly after the cloning of the first non-rhodopsin GPCR. 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. 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. In the meantime, Coward et al. 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. The RASSL approach is useful for unraveling the GPCR signaling mechanisms. 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. engineered the Gq-coupled histamine H$_1$ receptor for enhanced affinity of a synthetic agonist, but orthogonality was not complete.

Our initial work on engineering the receptors and ligands for orthogonal activation started with the A$_3$ adenosine receptor (AR), the activation of which is both cerebro- and cardio-protective and has an anticancer effect. 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-N$^6$-(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. Further application of the neoceptor approach to 5’-uronamide derivatives that bound orthogonally at strategically mutated A$_2$A 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. This observation was instructive for additional optimization of A$_3$ 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 bearing both 3’-ureido and N$^6$-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 μM) 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 A2AR 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-D-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.
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. All of the other compounds were weak or inactive at all four subtypes of ARs.

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-NH_{2} 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′-methylyuronamide 9—there was no affinity enhancement at the H272E mutant receptor, as was observed for the corresponding 8. The affinity of 5′-methylyuronamide 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 hA2AAR, 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 A2AARs 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 Ki values >1 μM at the A1AR and >10 μM at the A2AAR.37 Compound 4 was previously determined to have Ki values of 8.1 μM and 28 μM at the rat A1 and A2AARs, respectively.21 3′-Ureido derivatives 10 – 14 were assayed in binding to human A1 and A2AARs. 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 A2BAR 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 A\(_3\)AR, cells expressing the neorceptor 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 \( \mu \)M) did not activate the PLD pathway in myocytes expressing the H272E mutant receptor.

### Molecular Modeling and Nucleoside Docking

Recently a hA\(_3\)AR 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 A\(_3\)AR, 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 hA\(_3\)AR showed the same preference of the side-chain \( \chi_1 \) 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\( \delta \) 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 A\(_3\)-selective agonist CI-IB-MECA docked in the hA\(_3\)AR 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 N\( ^3 \) 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 N\(^6\)-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 A\(_3\)ARs, suggesting a favorable interaction between 3′-ureido group and mutated acidic residues. While CI-IB-MECA, a selective A\(_3\) agonist, displayed a single favorable binding mode, the docking result of the 3′-ureido analogue 10 from the combination of the FlexiDock and FlexX\(^{57} \) automatic docking programs had two energetically favorable binding modes. The docking results showing two putative complexes of the hA\(_3\) 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 N6 and the N3 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 N6 amino group interacted through H-bonding with Gln167 (EL2), a residue previously predicted to be in proximity to the nucleoside. In comparison to Fig. 3A, this docking mode required a different orientation of the N6-benzyl ring toward Phe182 (5.43). Molecular modeling indicates that the reason for the enhanced binding affinity of N6-benzyl compared with N6-methyl derivatives may be additional hydrophobic interaction at the N6-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 neoceptors. We tested a wide range of synthetic amine-bearing nucleoside analogues and modulated the 3′-substituent chain length in concert with N6-, 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 N6-3-iodobenzyl substituent, had no significant effect on the wild-type A3AR but displayed a dramatically enhanced affinity of 0.22 μM at the H272E neoceptor. In a previous study, it was demonstrated that the H272E neoceptor showed decreased affinity for NECA and Cl-IB-MECA, here we further demonstrated this neoceptor 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. 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. The A2A adenosine receptor 1976T>C genetic variant has been shown to confer susceptibility to panic disorder and caffeine-induced anxiety. Specific GPCR polymorphisms in adrenergic genes have already been shown to confer susceptibility to congestive heart failure. 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. The neoceptor 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. Firstly, the neoceptor approach concerns the reengineering of both the receptor and the ligands using molecular modeling. Secondly, the RASSL described earlier 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. In the present study, the functionality of the neagonist/neceptor 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 provides additional support for this concept.

We used site-directed mutagenesis to probe the recognition elements responsible for the selective affinity enhancement. The neoligand 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 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 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—^{1}H 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 F_{254} precoated plates, and silica gel column chromatography was performed on silica gel 60, 230–400 mesh, Merck. All anhydrous solvents were distilled over CaH_{2} 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-isopropyldiene-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 mL) 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 (MgSO_{4}), 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 (MgSO_{4}), 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: {^{1}H NMR (CDCl_{3}) 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 (N\textsubscript{3}) cm\textsuperscript{-1}; FAB-MS m/z 286 [M+H]+. Anal. (C_{12}H_{19}N_{3}O_{5}) 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 NaIO_{4} (2.37 g, 11.09 mmol) in H_{2}O (15 mL) was added dropwise at 0 °C. After the mixture was stirred at 0 °C for 20 min, NaBH_{4} (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: {^{1}H NMR (CDCl_{3}) 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), 1.46 (s, 3 H), 1.36 (s, 3 H), 1.34 (s, 3 H); IR (KBr): 2108 (N\textsubscript{3}) cm\textsuperscript{-1}; FAB-MS m/z 216 [M+H]+. Anal. (C_{8}H_{13}N_{3}O_{4}) 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 Ac_{2}O (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 (MgSO_{4}), 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: {^{1}H NMR (CDCl_{3}) δ 5.78 (d, 1 H, J = 3.7 Hz), 4.71 (dd, 1 H, J = 3.6, 4.8 Hz), 3.34 (m, 1 H), 4.15-4.26 (m, 2 H), 3.31 (dd, 1 H, J = 4.6, 9.5 Hz), 2.08 (dd, 1 H, J = 4.6, 9.5 Hz), 1.56 (s, 3 H), 1.34 (s, 3 H); IR (KBr): 2109 (N\textsubscript{3}) cm\textsuperscript{-1}; FAB-MS m/z 258 [M+H]+. Anal. (C_{10}H_{15}N_{3}O_{5}) 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 CH₂Cl₂ (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 = 7.2 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), 6.15 (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); 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-d₆) 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-d₆) 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), 1634 (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-d₆) δ 8.60 (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.
89% yield; white solid; carboxylic acid methylamide (15)—3-Azido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-hydroxy-tetrahydro-furan-2-
2-Chloro-N(C=O) cm
83% yield; foam; furan-2-carboxylic acid methylamide (37a)—3-Azido-5-(6-methylaminopurin-9-yl)-4-t-butyldimethylsiloxy-tetrahydro-
N76% yield; oil; 
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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 [M+H]\(^+\). Anal. (C\(_{18}H_{29}N_9O_6Si\)) C, H, N.

3-Azido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-t-butyldimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (37b)—71% yield; foam; \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 8.42 (s, 1 H), 7.78 (s, 1 H), 7.12 (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 [M+Na]\(^+\). Anal. (C\(_{24}H_{32}I_3N_9O_6Si\)) 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\(_2\)OH (1.8 mL) and H\(_2\)O (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.

N\(^6\)-Methyl-9-(3-amino-2,5-di-O-t-butyldimethylsilanyloxy-3-deoxy-\(\beta\)-D-ribofuranosyl) adenine (28a)—88% yield; foam; \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 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): 2930, 1624, 1191, 838 cm\(^{-1}\); FAB-MS \( m/z \) 509 [M+H]\(^+\). Anal. (C\(_{23}H_{44}N_9O_3Si\)) C, H, N.

N\(^6\)-(3-Iodobenzyl)-9-(3-amino-2,5-di-O-t-butyldimethylsilanyloxy-3-deoxy-\(\beta\)-D-ribofuranosyl) adenine (28b)—96% yield; foam; \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 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): 2930, 1618, 1469, 1120, 839, 780 cm\(^{-1}\); FAB-MS \( m/z \) 711 [M+H]\(^+\). Anal. (C\(_{29}H_{47}I_2N_9O_3Si\)) C, H, N.

2-Chloro-N\(^6\)-(3-Iodobenzyl)-9-(3-amino-2,5-di-O-t-butyldimethylsilanyloxy-3-deoxy-\(\beta\)-D-ribofuranosyl) adenine (28c)—94% yield; foam; \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 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 [M+H]\(^+\). Anal. (C\(_{29}H_{46}ClIN_9O_3Si\)) C, H, N.

3-Amino-5-(6-methylaminopurin-9-yl)-4-t-butyldimethylsiloxo-tetrahydrofuran-2-carboxylic acid methylamide (38a)—95% yield; oil; \(^1\)H NMR (CD\(_3\)OD) \( \delta \) 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, 1624, 1054, 833, 643 cm\(^{-1}\); FAB-MS \( m/z \) 422 [M + H]\(^+\). Anal. (C\(_{18}H_{33}N_7O_3Si\)) C, H, N.

3-Amino-5-(6-(3-iodobenzylamino)purin-9-yl)-4-t-butyldimethylsiloxo-tetrahydrofuran-2-carboxylic acid methylamide (38b)—94% yield oil; \(^1\)H NMR (CDCl\(_3\)) \( \delta \) 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).
Hz), 4.91 (m, 3 H), 4.56 (dd, 1 H, J = 2.6, 5.5 Hz), 3.05 (d, 1 H, J = 4.7 Hz), 0.92 (s, 3 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 [M+H]⁺. Anal. (C₂₄H₃₄IN₇O₃Si) 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-butyldimethylsilyl-3-deoxy-β-D-ribofuranosyl) adenine (29a)—87% yield; oil; ¹H NMR (CDCl₃) 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 [M+H]⁺.

N⁶-(3-Iodobenzyl)-9-(3-chloroacetylureido-2,5-di-O-t-butyldimethylsilyl-3-deoxy-β-D-ribofuranosyl)adenine (29b)—77% yield; foam; ¹H NMR (CDCl₃) 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), 6.02 (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), 3.72 (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 [M+H]⁺.

2-Chloro-N⁶-(3-iodobenzyl)-9-(3-chloroacetylureido-2,5-di-O-t-butyldimethylsilyl-3-deoxy-β-D-ribofuranosyl)adenine (29c)—81% yield; foam; ¹H NMR (CDCl₃) 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), 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 [M+H]⁺.

3-Chloroacetylureido-5-(6-methylaminopurin-9-yl)-4-t-butyldimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (39a)—94% yield; oil; ¹H NMR (CDCl₃) 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 [M+H]⁺.

3-Chloroacetylureido-5-(6-(3-iodobenzylamino)purin-9-yl)-4-t-butyldimethylsiloxy-tetrahydro-furan-2-carboxylic acid methylamide (39b)—88% yield; oil; ¹H NMR (CDCl₃) 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 [M+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), 7.49 (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), 6.00 (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), 3.73 (dd, 1 H, J = 2.2, 11.7 Hz), 0.80 (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), 6.19 (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.

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-urido-β-D-ribofuranosyl)adenine (13)—88% yield; white solid; mp 191-194 °C; [α]D²⁵ +23.0° (c 0.13, DMF); ¹H NMR (DMSO-d₆) 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-d₆) 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]⁺.  

**N⁶-(3-Iodobenzyl)-9-(3-deoxy-3-urido-β-D-ribofuranosyl)adenine (10)—70% yield; white solid; mp 165-168 °C; [α]D²⁵ +43.3° (c 0.12, DMF); ¹H NMR (DMSO-d₆) 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-d₆) δ 42.2, 50.9, 61.0, 73.4, 84.2, 89.5, 94.7, 119.5, 126.7, 130.5, 135.4, 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]⁺.  

**{[2-Chloro-6-(3-iodobenzylamino)-purin-9-yl]-4-hydroxy-2-hydroxymethyl-tetrahydro-furan-3-yl]urea (11)—82% yield; white solid; mp 132.3-135 °C; [α]D²⁵ +27.4° (c 0.35, DMF); ¹H NMR (DMSO-d₆) 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-d₆) 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]⁺.  

**3-Ureido-5-(6-methylaminopurin-9-yl)-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide (14)—66% yield; white solid; mp 120.7-122.2 °C; [α]D²⁵ -10.0° (c 0.10, DMF); ¹H NMR (DMSO-d₆) 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 (CD₂OD) 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 553 [M+H]⁺.  

**3-Ureido-5-(6-iodobenzylamino)purin-9-yl)-4-hydroxy-tetrahydro-furan-2-carboxylic acid methylamide (12)—82% yield; white solid; mp 120.7-122.2 °C; [α]D²⁵ -10.0° (c 0.10, DMF); ¹H NMR (DMSO-d₆) 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 (CD₂OD) δ 14.1, 20.9, 24.2, 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]⁺.  

**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). [125]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.

Radioligand binding assays and inositol phosphate determination in COS-7 cells—The procedures for A3AR-binding experiments using [125]I-AB-MECA ([125]I)N6-(4-aminobenzyl)-5′-N-methylcarboxamidoadenosine) were as previously described. The procedures for binding at human A1 and A2AARs and a functional assay of cyclic AMP formation mediated by the human A2BAR 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. 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. 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 neoeceptor H272E, with the use of FuGENE 6 as previously described. 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 neoeceptor 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. The formation of [3H]PEt was an indication of PLD activity. Quantitation of PEt (phosphatidylethanol) was carried out as previously described. 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 Ki values by using the Cheng-Prusoff equation. Data were expressed as mean ± standard error.

**Computational Methods**

All calculations were performed using the SYBYL program version 6.9 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 field 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 package. 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 modeling from the X-ray structure of bovine rhodopsin with 2.8 Å resolution 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 algorithm 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 hA$_3$ 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 Cl-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 1.1$^{57}$ 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 MINUMBA$^{58}$ 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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Fig. 1.
Binding inhibition and functional activation in wild-type and H272E mutant A3ARs. 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 $[^{125}\text{I}]$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.
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 hA$_3$AR. The binding mode similar to the hA$_3$/Cl-IB-MECA complex (A) and another binding mode (B), which was energetically unfavorable in the hA$_3$/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 hA$_3$AR are shown as stick models. The H-bonding between each ligand and the mutant hA$_3$AR is displayed in yellow. The A$_3$AR 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) Tf₂O, pyridine, 0 °C, 1 h; (b) NaN₃, DMF, rt, 48 h; (c) i) 75% AcOH, 55 °C, 1.5 h; ii) NaIO₄/H₂O, EtOH, 0 °C, 20 min then NaBH₄; (d) Ac₂O, pyridine, rt, 3 h; (e) i) 85% HCO₂H, 60 °C, 1.5 h; ii) Ac₂O, pyridine, rt, 16 h; (f) Silylated 6-chloropurine or 2,6-dichloropurine, TMSOTf, C₂H₄Cl₂, 0 °C to 60 °C, 2 h; (g) MeNH₂, 1,4-dioxane, rt, 4 h or 3-iodobenzylamine hydrochloride, Et₃N, EtOH, 50 °C, 18 h then NaOMe, MeOH, rt, 2 h; (h) TBSCI, imidazole, DMF, rt, 24 h; (i) Ph₃P, NH₄OH/H₂O, 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/CH₂CN/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.

<table>
<thead>
<tr>
<th>Adenine substituent&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Ribose substituents</th>
<th>$K_i$ (µM) or percentage inhibition at 10 µM</th>
<th>Affinity Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td>R₆ =</td>
<td>R₃ =</td>
<td>R₅ =</td>
<td>wild-type</td>
</tr>
<tr>
<td>1&lt;sup&gt;a&lt;/sup&gt; R₆ = H</td>
<td>NH₂</td>
<td>CH₂OH</td>
<td>442 ± 121</td>
</tr>
<tr>
<td>2&lt;sup&gt;b&lt;/sup&gt; R₆ = H</td>
<td>CH₂NH₂</td>
<td>CH₂OH</td>
<td>3 ± 2%</td>
</tr>
<tr>
<td>3&lt;sup&gt;a&lt;/sup&gt; R₆ = H</td>
<td>NH≡NHNH₃</td>
<td>CH₂OH</td>
<td>130 ± 34</td>
</tr>
<tr>
<td>4&lt;sup&gt;a&lt;/sup&gt; R₆ = IB</td>
<td>NH₂</td>
<td>CH₂OH</td>
<td>0.87 ± 0.18</td>
</tr>
<tr>
<td>5&lt;sup&gt;a&lt;/sup&gt; R₆ = IB</td>
<td>CH₂NH₂</td>
<td>CONHCH₃</td>
<td>0.137 ± 0.041</td>
</tr>
<tr>
<td>6&lt;sup&gt;a&lt;/sup&gt; R₆ = IB</td>
<td>CH₂NHCOCH₃</td>
<td>CH₂OH</td>
<td>15%</td>
</tr>
<tr>
<td>7&lt;sup&gt;a&lt;/sup&gt; R₆ = IB</td>
<td>CH₂NH₂</td>
<td>CH₂OH</td>
<td>9.5 ± 1.7</td>
</tr>
<tr>
<td>8&lt;sup&gt;a&lt;/sup&gt; R₆ = CMB</td>
<td>CH₂NH₂</td>
<td>CH₂OH</td>
<td>13.8 ± 1.4</td>
</tr>
<tr>
<td>9&lt;sup&gt;a&lt;/sup&gt; R₆ = CMB</td>
<td>CH₂NH₂</td>
<td>CONHCH₃</td>
<td>0.557 ± 0.164</td>
</tr>
<tr>
<td>10&lt;sup&gt;c&lt;/sup&gt; R₆ = IB</td>
<td>NHCONH₂</td>
<td>CH₂OH</td>
<td>9 ± 1%</td>
</tr>
<tr>
<td>11&lt;sup&gt;a&lt;/sup&gt; R₆ = IB, R₂ = Cl</td>
<td>NHCONH₂</td>
<td>CH₂OH</td>
<td>47 ± 9%</td>
</tr>
<tr>
<td>12&lt;sup&gt;b&lt;/sup&gt; R₆ = IB</td>
<td>NHCONH₂</td>
<td>CONHCH₃</td>
<td>5 ± 1%</td>
</tr>
<tr>
<td>13&lt;sup&gt;a&lt;/sup&gt; R₆ = IB</td>
<td>NHCONH₂</td>
<td>CH₂OH</td>
<td>3 ± 1%</td>
</tr>
<tr>
<td>14&lt;sup&gt;b&lt;/sup&gt; R₆ = IB</td>
<td>NHCONH₂</td>
<td>CONHCH₃</td>
<td>7 ± 2%</td>
</tr>
<tr>
<td>15&lt;sup&gt;b&lt;/sup&gt; R₆ = IB, X = O</td>
<td>N₃</td>
<td>CONHCH₃</td>
<td>2.26 ± 0.48</td>
</tr>
<tr>
<td>Adenine substituent&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Ribose substituents</td>
<td>(K_i) (µM) or percentage inhibition at 10 µM</td>
<td>Affinity Enhancement</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>16</td>
<td>IB, X = S</td>
<td>OH</td>
<td>(0.022\pm0.004)</td>
</tr>
<tr>
<td>17</td>
<td>H</td>
<td>OH</td>
<td>CONH-(CH(_2))(_3)NH(_2)</td>
</tr>
<tr>
<td>18&lt;sup&gt;c&lt;/sup&gt;</td>
<td>H</td>
<td>OH</td>
<td>CONHNH(_2)</td>
</tr>
</tbody>
</table>

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 \([^{125}\text{I}]\text{I-AB-MECA}\) (0.5 nM). Values represent the mean ± SE of at least three independent determinations.

<sup>a</sup>Affinity at wild-type and mutant A\(_3\)ARs previously reported.<sup>21</sup>

<sup>b</sup>Affinity at wild-type A\(_3\)AR stably expressed in CHO cells previously reported.<sup>37,38</sup>

<sup>c</sup>MRS3481, LJ720.

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

<sup>e</sup>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.

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

Binding parameters (Kᵢ values) were measured in transiently transfected COS-7 cells as described in Experimental Procedures by using the agonist radioligand [125I]I-AB-MECA (0.5 nM). The Kᵢ 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.

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

b ND = not determined.