Neoceptor Concept Based on Molecular Complementarity in GPCRs: A Mutant Adenosine A_3 Receptor with Selectively Enhanced Affinity for Amine-Modified Nucleosides

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Received May 24, 2001

Adenosine A_3 receptors are of interest in the treatment of cardiac ischemia, inflammation, and neurodegenerative diseases. In an effort to create a unique receptor mutant that would be activated by tailor-made synthetic ligands, we mutated the human A_3 receptor at the site of a critical His residue in TM7, previously proposed to be involved in ligand recognition through interaction with the ribose moiety. The H272E mutant receptor displayed reduced affinity for most of the uncharged A_3 receptor agonists and antagonists examined. For example, the nonselective agonist **1a** was 19-fold less potent at the mutant receptor than at the wild-type receptor. The introduction of an amino group on the ribose moiety of adenosine resulted in either equipotency or enhanced binding affinity at the H272E mutant relative to wild-type A_3 receptors, depending on the position of the amino group. 3′-Amino-3′-deoxyadenosine proved to be 7-fold more potent at the H272E mutant receptor than at the wild-type receptor, while the corresponding 2′- and 5′-amino analogues did not display significantly enhanced affinities. An 3′-amino-N^6-iodobenzyl analogue showed only a small enhancement at the mutant (K_i = 320 nM) vs wild-type receptors. The 3′-amino group was intended for a direct electrostatic interaction with the negatively charged ribose-binding region of the mutant receptor, yet molecular modeling did not support this notion. This design approach is an example of engineering the structure of mutant receptors to recognize synthetic ligands for which they are selectively matched on the basis of molecular complementarity between the mutant receptor and the ligand. We have termed such engineered receptors "neoceptors", since the ligand recognition profile of such mutant receptors need not correspond to the profile of the parent, native receptor.

Introduction

Therapeutic intervention using agonists is subject to side effects related in part to the widespread occurrence of the corresponding receptor throughout the body.[1,2] Currently, the specificity of a given drug for a target organ is usually achieved through manipulation of its pharmacokinetic properties. Past attempts to overcome this problem included generation of prodrugs that were to be preferentially activated at the target organ, either through enhanced metabolic processes or through a unique enzymatic system characteristic to the organ.[3] Our goal was to introduce and investigate a novel concept for therapeutic intervention at a specific anatomical and/or physiological locus, through a combination of receptor engineering, agonist design, and gene therapy. Elements of this potentially general approach include (a) engineering of a receptor protein to recognize synthetic ligands, for which it was selectively modified according to the molecular complementarity of the respective binding elements, while retaining its capacity for signal transduction (neoceptor); (b) synthesis of novel agonists that are not effective at the native receptor but do activate the engineered receptor (neoligand); and (c) a delivery vector to provide for selective expression of the neoceptor in the target area.

One of the numerous cases where agonist therapy has been problematic due to the widespread occurrence of receptors is the adenosine receptor family. For example, the hypotensive and bradycardiac side effects of adenosine agonists have been in part responsible for the difficulty of developing adenosine-based therapeutics for cardio- and cerebropotection.[1,3−6] The only adenosine agonist approved so far for clinical use has been adenosine itself, based on its short duration of action in the treatment of supraventricular tachycardia and in radionuclide imaging.[7] Thus, engineering of novel receptor−

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10.1021/jm010232o CCC: $20.00
Published on Web 10/16/2001

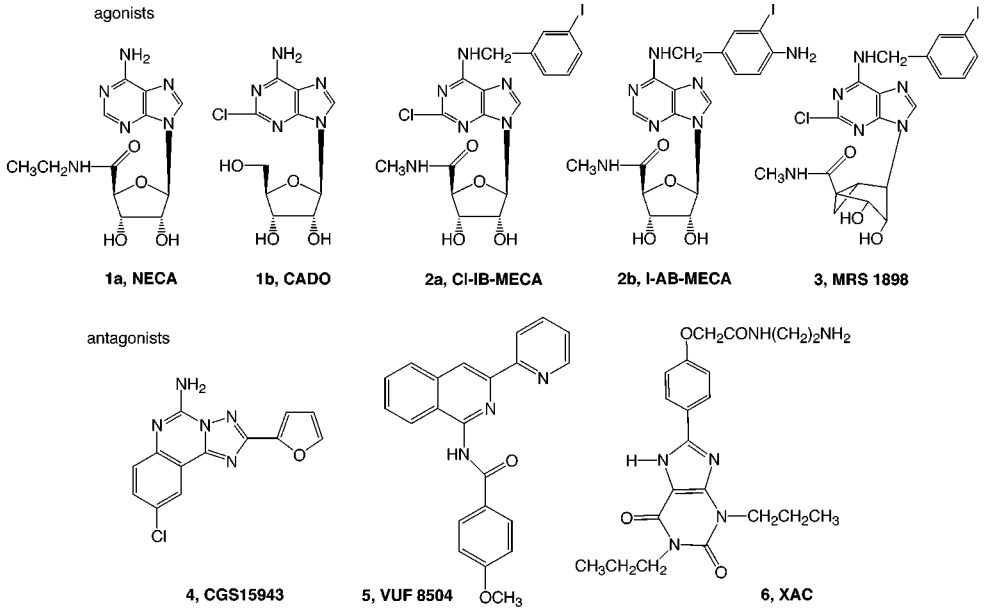

Figure 1. A_3 adenosine receptor agonists and antagonists. Wild-type and mutant receptor affinities appear in Table 1.

ligand interactions in the adenosine receptor family, through specific tailoring of both the receptors and the ligands, would provide a suitable and relevant system for investigation of the neoceptor−neoligand concept.

Adenosine is released in large amounts during ischemia and has been shown to be protective in the heart,[8] brain,[5,9] and other organs. Adenosine, when elevated prior to ischemia in cardiac tissue,[10,11] "preconditioned" the heart and protected it against injury during a subsequent period of prolonged ischemia. Synthetic agonists selective for either A_1 or A_3 adenosine receptor simulated this preconditioning effect.[12] The beneficial effects were seen following acute activation of A_1 or chronic activation of A_3 receptors. In addition to cardioprotection, adenosine A_3 receptor ligands have been proposed for the treatment of stroke,[13] inflammation,[14] and glaucoma.[15] In a model of global brain ischemia in gerbils, both A_1 and A_3 adenosine receptor-selective agonists had protective effects,[5] as judged by the histochemical and behavioral outcome following recovery. Thus, there has been much interest in the development of new therapeutic agents acting at adenosine A_3 receptors.

Ligand recognition in adenosine receptors, principally A_1 and A_{2A} receptors, has been extensively investigated using mutagenesis and molecular modeling.[16−20] The putative nucleoside binding site, which is highly homologous among subtypes of the adenosine receptors, is proposed to involve transmembrane helices (TMs) 3, 5, 6, and 7. Two conserved His residues (6.52 and 7.43, by the notation of van Rhee and Jacobson[21]) in TMs 6 and 7 of A_1 and A_{2A} receptors are considered to be among the most important amino acids involved in binding to adenosine. An assembly of aromatic amino acid side chains in the human A_{2A} receptor, principally in TMs 5 and 6, is proposed to recognize the adenine moiety. The ribose moiety is likely coordinated to hydrophilic residues in TMs 3 and 7.[19,22,23] In fact, several hydroxyl-containing residues, Thr88 (3.36) and Ser277 (7.42) of the human A_{2A} receptor, have been shown to be associated exclusively with agonist, but not antagonist, recognition.[19,22]

In the present study, we have utilized this hypothetical bound orientation of adenosine to identify a site on TM7 thought to be in proximity to the ribose moiety and amenable to introduction of a charged group intended for electrostatic interaction with the ligand. The site chosen was the conserved His (H272), present also in the A_3 receptor, which we have mutated to Glu. According to molecular modeling, this mutation would be expected to decrease the affinity of simple adenosine analogues,[19,23,24] except when strategically modified by the introduction of an amino group on the ribose moiety. Since alteration of the ribose moiety substitution pattern was also known to greatly diminish the affinity at adenosine receptors,[25] such substitution would ensure that the neoligand would not activate endogenous receptors at an effective concentration for the neoceptor. Our results suggested both the viability of the neoceptor−neoligand concept and the need for further optimization of the A_3 neoceptor−neoligand interactions.

Results

Design of a Neoceptor: Creation of a Mutant A_3 Receptor That Is Activated Selectively by Novel Agonist Derivatives. On the basis of previous studies of ligand recognition in adenosine receptors,[16,19,23,24] a His residue in TM7 of the A_3 adenosine receptor was selected as the site for mutagenesis, i.e., the introduction of a negative charge to be complementary to an amine-derivatized ligand. We examined the recognition of both known adenosine ligands (Figure 1) and synthetic agonist analogues (Figure 2) designed as neoligands, for selective recognition by the H272E mutant receptor (see below).

Mutant vs Wild-Type A_3 Receptors Using Known Adenosine Receptor Ligands. The receptor binding affinities of various adenosine agonist and antagonist derivatives were measured in standard binding assays using wild-type and H272E mutant human A_3 receptors. The high affinity of the radioligand [^{125}I]I-AB-MECA ([^{125}I]N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine),[26] **2b**, was retained in the mutant receptor,

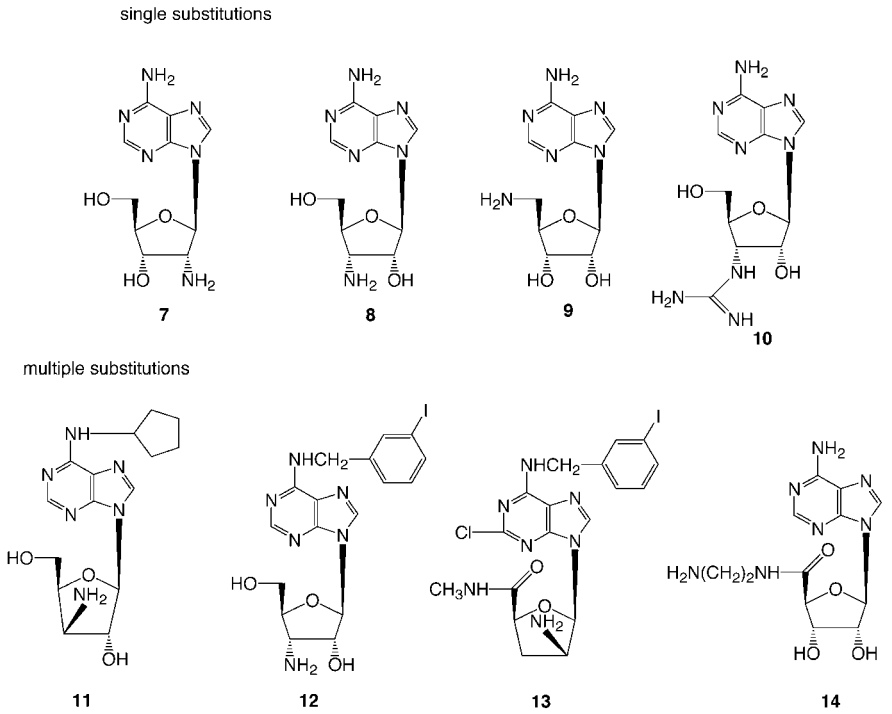

Figure 2. Derivatives of adenosine tested as novel receptor agonists. Wild-type and mutant receptor affinities appear in Table 2.

Table 1. Binding Affinity of Known Adenosine Agonists and Antagonists at Wild-type and Mutant (H272E) Human A_3 Adenosine Receptors[a,b]

	K_i (nM)			K_i (nM)	
agonists	WT	H272E	antagonists	WT	H272E
2b [^{125}I]AB-MECA (K_d)	1.2 ± 0.2	2.1 ± 0.3	**4**	253 ± 87	3260 ± 994
1a	169 ± 32	3200 ± 770	**5**	61.7 ± 23.4	1690 ± 343
1b	650 ± 130	11 600 ± 4200	**6**	110 ± 45	20 100 ± 4500
2a	4.3 ± 1.6	20.1 ± 3.6			
3	1.6 ± 0.3	9.9 ± 2.6			

[a] Structures provided in Figure 1. [b] Results are from three independent experiments performed in duplicate, using [^{125}I]AB-MECA at a concentration of 1 nM.

thus enabling the determination of the affinities of a wide range of competing ligands. K_i values for these ligands are shown in Table 1, and representative binding curves are shown in Figure 3. The closest mimic of the affinity of adenosine itself[27] was 2-chloroadenosine, **1b**, which was 18-fold less potent in binding at the H272E mutant receptor than at the wild-type receptor.

In most other cases, the affinity of competing ligands, **1**−**7**, was significantly reduced in the mutant receptor. For example, compound **1a** was 19-fold less potent at the mutant receptor than the wild-type receptor. The potent antagonist, xanthine amine congener **6**,[19] which contains a distal amino group, was 180-fold less potent at the mutant than the wild-type receptor. Other ligands, such as potent A_3 receptor agonists **2a** and the rigid analogue **3**,[13,28] were shifted to lower affinity in binding to the mutant receptor by smaller factors, i.e., 5- and 6-fold, respectively.

Synthesis of Neoligands. The amino derivatives of adenosine (**7**−**9**), a N^6-cyclopentyladenosine (**11**), and a 5′-uronamidoadenosine (**14**) were prepared as described.[29−31] A 3′-guanidino derivative of adenosine (**10**) was obtained in the protected form (**15**) from the corresponding amine using *di*-Boc-triflylguanidine,[32] followed by deprotection (Figure 4a). A sugar-modified analogue of **2,** i.e. **13**, was available from a previous study.[25] A 3′-amino derivative (**12**), which also contained an N^6-substituent favorable for A_3 receptor affinity and selectivity, was prepared as shown in Figure 4b. 3-Azido-1,2-di-*O*-acetyl-5-*O*-(4-methylbenzoyl)-3-deoxy-β-D-ribofuranose, **16**, was prepared in seven steps from xylose following a slightly altered literature procedure.[33] Coupling with N^6-(3-iodobenzyl)adenine by the method of Vorbrüggen et al.,[34] followed by alkaline deprotection, yielded 18% of the β-anomer **17**. Reduction of the azido moiety with triphenylphosphine led to smooth conversion of **17** to the desired amine **12** without detectable loss of the benzylic N^6-substituent.

Binding to Mutant vs Wild-Type A_3 Receptors. The receptor binding affinities of amine-functionalized adenosine derivatives were measured in standard binding assays using wild-type and H272E mutant human A_3 receptors expressed in COS-7 cells, rat A_3 receptors expressed endogenously in RBL-2H3 cells, and rat brain A_1 and A_{2A} receptors.[6,26,35,36] K_i values for these ligands are shown in Table 2, and representative binding curves are shown in Figure 5.

The introduction of an amino group on the ribose moiety of adenosine resulted in either equipotency or enhanced binding affinity at the H272E mutant relative to wild-type A_3 receptors, depending on the position of the amino group. 3′-Amino-3′-deoxyadenosine, **8**, proved

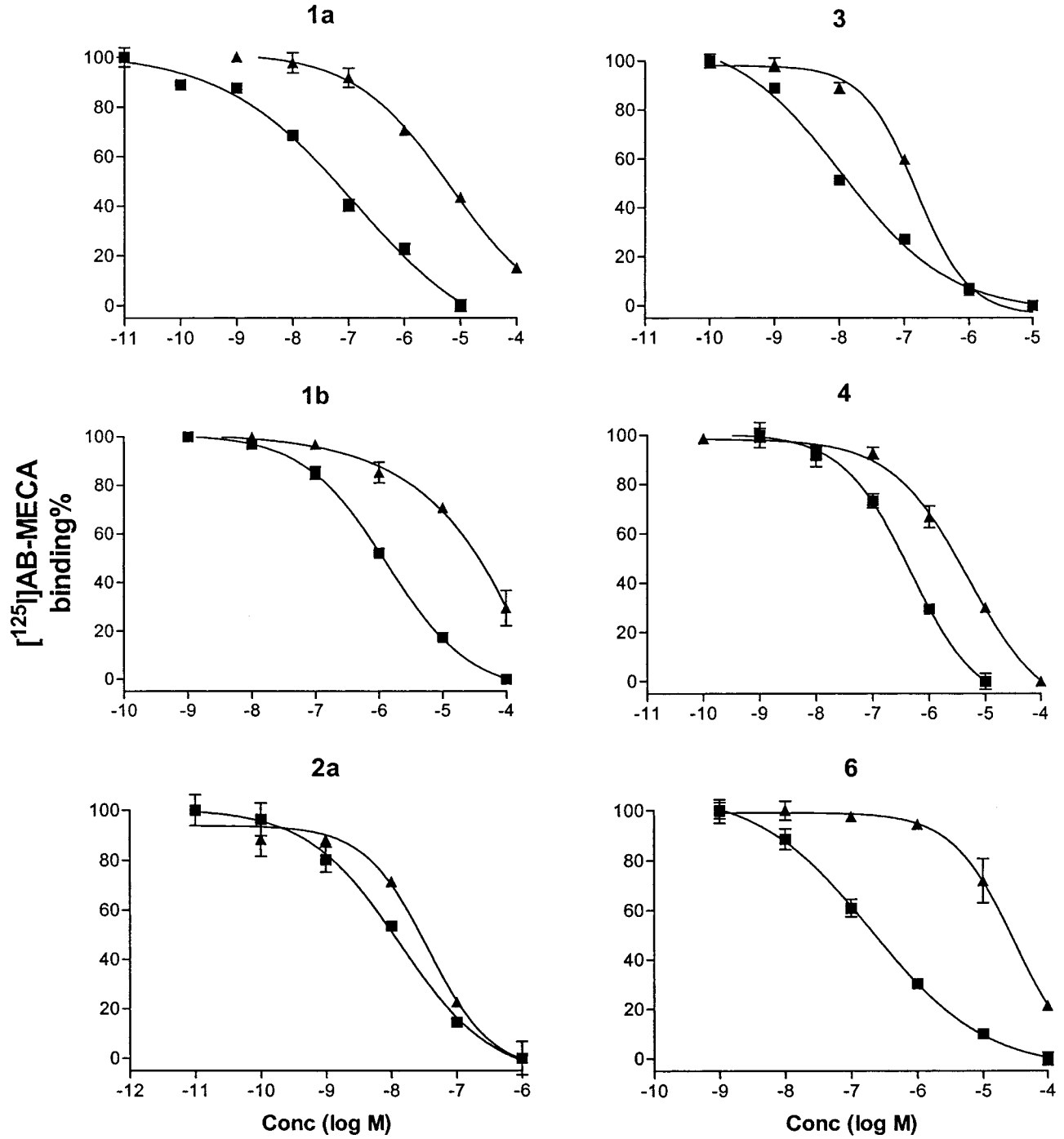

Figure 3. Binding of known A_3 adenosine receptor agonists and antagonists to wild type and mutant receptors (■), wild type; (▲), H272E mutant receptor).

to be 7-fold more potent at the H272E mutant than the wild-type receptor. Two other isomers, 2′-amino-2′-deoxyadenosine, **7**, and 5′-amino-5′-deoxyadenosine, **9**, an inhibitor of adenosine kinase,[37] did not display significantly enhanced affinity. The affinity of a 3′-guanidino analogue, **10**, was enhanced by 4-fold in the mutant versus wild-type receptors. A 2-aminoethyl analogue of **1a**, i.e., **14**,[38] was only 2-fold more potent at the mutant receptor. A 3′-amino-N^6-iodobenzyl analogue, **12**, showed a 3-fold enhancement at the mutant vs wild-type receptors, with a K_i value of 320 nM.

The ability of both mutant and wild-type receptors to activate second messengers was demonstrated. Both compounds **1b** and **11** at 10 μM inhibited cyclic AMP production[39] stimulated by forskolin in COS-7 cells expressing either the wild-type or H272E mutant receptor (Figure 6). Furthermore, both the wild-type and H272E mutant receptors in the presence of 10 μM of compound **2a** were shown to fully activate phospholipase C (data not shown), as determined by the method reported.[40]

Molecular Modeling. A model of the human A_3 receptor was built in homology to the recently published X-ray structure of bovine rhodopsin.[41] The model included the seven TMs and the second extracellular loop (EL2). In this model, residue His272 was within interaction distance from Glu19 (1.39) in TM1 (Nπ−Oϵ distance, 2.40 Å; Figure 7a). An analogous interaction

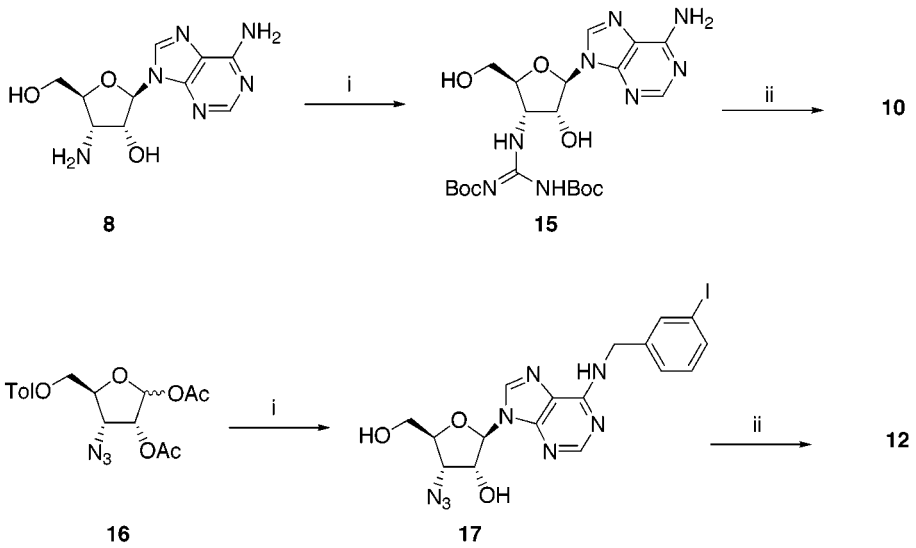

Figure 4. Synthesis of novel adenosine derivatives **10** and **12** containing 3′-guanidino (a, top) and 3′-amino (b, bottom) groups. Reagents: (a) (i) di-Boc-triflylguanidine, DMF (ii) TFA; (b) (i-1) N^6-(3-iodobenzyl)adenine, HMDS, TMSCl, TMSOTf, pyridine, (i-2) 0.1 N NaOMe, MeOH (18% combined yield); (ii) Ph_3P, pyridine, NH_4OH.

Table 2. Binding Affinity of Amine-Derivatized Adenosine Analogues at Wild-Type and Mutant (H272E) Human A_3 and Rat Adenosine Receptors[a,b]

		K_i [b] (μM)			
compd[a]	WT	H272E	rat A_3	rat A_1	rat A_{2A}
7	*c*	301 ± 142	*d* (10^{-4})	*d* (10^{-4})[f]	*d* (10^{-4})
8	442 ± 121	75 ± 32	84 ± 26	*d* (10^{-4})[f]	*d* (10^{-4})
9	*c*	425 ± 217	*d* (10^{-4})	*d* (10^{-4})[f]	*d* (10^{-4})
10	130 ± 34	33.3 ± 7.1	ND	*d* (10^{-4})	*d* (10^{-4})
11	0.54 ± 0.13	0.19 ± 0.08	*d* (10^{-4})	*d* (10^{-4})[f]	*d* (10^{-4})
12	0.87 ± 0.18	0.32 ± 0.10	ND	8.1 ± 0.47	28 ± 7.5
13	4.6 ± 1.3	2.3 ± 0.57	3.40 ± 0.79[e]	6.69 ± 0.74[e]	*d* (10^{-4})[e]
14	19.6 ± 3.1	9.6 ± 3.5	14.7 ± 2.5[e]	*d* (10^{-4})[e]	*d* (10^{-4})[e]

[a] Structures provided in Figure 2. [b] Results are from three independent experiments performed in duplicate, using [^{125}I]AB-MECA at a concentration of 1 nM at human A_3 receptors expressed in COS-7 cells or at rat A_3 receptors in RBL-2H3 cells, unless noted. Rat A_1 and A_{2A} receptors were in rat brain. [c] IC_{50} values of compounds **7** and **9** at the wild type receptor were estimated to be approximately 1 mM. The inhibition of [^{125}I]AB-MECA binding by **7** and **9** at the highest concentration examined (1 mM) was 51 and 54%, respectively. [d] <10% displacement of binding at the indicated concentration (M). [e] From Gallo-Rodriguez et al.[38] and Jacobson et al.[25] using recombinant rat A_3 receptors expressed in CHO cells. [f] IC_{50} value of **9** in the absence of adenosine deaminase estimated to be approximately 100 μM. Compounds **7**, **8**, and **11** gave identical results in the absence of adenosine deaminase.

had already been proposed for the A_{2A} receptor,[23,24] in order to explain the observed involvement of both residues in agonist binding. In the present model, the carboxylate group of Glu19 appeared to interact also with Tyr265 (7.36) and Ser73 (2.65), resulting in a relatively rigid juxtaposition of the imidazole moiety of His272 relative to other elements of the ligand binding environment (Figure 7b).

Examination of the optimized model of the **1b**−A_3 receptor complex showed that the N^6-amine nitrogen was located within H-bonding distances of the amide oxygen of Trp243 (6.48) and Oγ of Ser247 (6.52) of 2.76 and 2.51 Å, respectively (Figure 7b). The 2′-hydroxy substituent of the ribose ring was adjacent to both the Oϵ of Gln167 and Nζ of Lys152 (3.16 and 3.75 Å, respectively). The corresponding 3′-hydroxy substituent was within H-bonding distance from His272, and the terminal oxygen interacted with Ser271 (7.42) and Asn274 (7.45) in TM7. Thus, **1b** seemed to be accommodated by interactions with residues of TMs 6 and 7 and of EL2, with both 2′- and 3′-ribose hydroxy substituents proximal to basic residues.

In the optimized model of the **2a**−A_3 receptor complex (Figure 7c) the N^6-benzyl substituent appeared to be wedged between TM5 and TM6, interacting with residues Phe182 (5.43), Ile186 (5.47), and Phe187 (5.48). Consequently, the whole ligand was displaced away from TM7, compared to the corresponding complex of **1b**. The N^6 was still within interaction distance from Ser247 but was over 5 Å away from the amide oxygen of Trp243. Furthermore, the 3′-hydroxy substituent did not seem to interact with His272 (the 3′-O−Nτ distance was >4.5 Å). Reorientation of the latter was prevented by interaction with Glu19 (see above).

We used modeling to test the hypothesis that an electrostatic interaction between the positively charged ligand and the now negatively charged ribose-binding region of the receptor led to the affinity enhancement of **8** at the H272E mutant receptor. Replacement of His272 by glutamate resulted in a structure with two adjacent carboxylates, one of which was most likely protonated. Since the mobility of Glu19 was restricted by interactions with Tyr265 and Ser73, the carboxylate group of Glu272 was held in an orientation that prevented a direct H-bond interaction with the 3′-hydroxy ribose substituent (Figure 7a). This was consistent with the 20-fold lower affinity of the H272E mutant toward agonists, such as **1a** or **1b**, while the corresponding

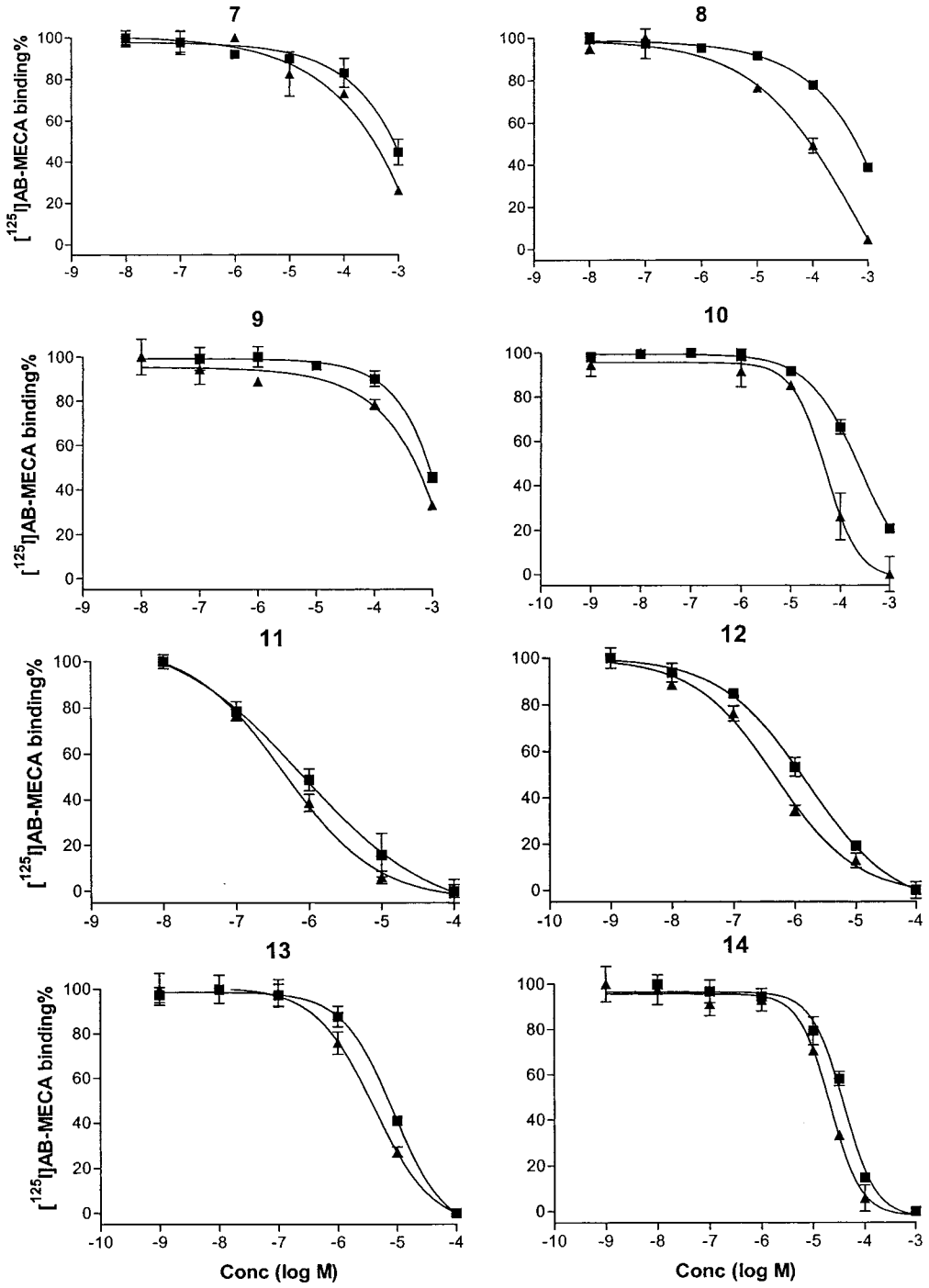

Figure 5. Binding of novel adenosine derivatives at wild-type and H272E mutant adenosine A_3 receptors (■), wild type; (▲), H272E mutant receptor).

effect on the agonists related to **2a** was much smaller. The notion that, due to the H272E mutation, a polar interaction was lost was also consistent with the 15−20-fold affinity decrease of the mutant receptor toward the three antagonists examined (Table 1). In all of those cases, modeling suggested that Glu272 could not replace His in accommodating the A_3 ligands through H-bonding interactions.

Discussion

We have investigated an approach to target agonist therapy to a specific organ or tissue based on selective activation of mutant receptors by synthetic ligands. Toward this goal we have both mutated the A_3 receptor and chemically modified the corresponding agonists, aiming to preserve the structural complementarity required for agonist function. On one hand, adenosine analogues carrying modifications of the ribose 2′- and 3′-substituents were known to be mostly inactive as agonists.[25] Thus, we concentrated on aminoadenosines in order to avoid activation of the wild-type A_3 receptors. On the other hand, simple aminoadenosines were nearly isosteric with the corresponding adenosines, implying that the former could act as agonists, provided a proper juxtaposition of its binding elements with those of an appropriate receptor could be achieved. For instance,

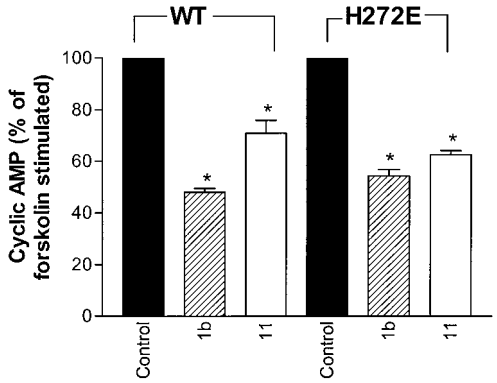

Figure 6. Cyclic AMP production by COS-7 cells expressing wild-type and H272E mutant human A_3 receptors after addition of compound **1b** (10 μM) or compound **11** (10 μM). Values are means ± SEM of three independent experiments performed in duplicate. For values marked with an asterisk, $P < 0.05$ compared with control.

introduction by mutagenesis of an acidic residue within the ligand binding site could have resulted in an electrostatic interaction with the amine substituent of the ligand.

Position 272 on TM7 of the human A_3 receptor (7.43), selected for such mutagenesis, since it appeared to play an important role across the GPCR family. In rhodopsin, this is the location of the Lys residue that forms a Schiff base with retinal. In all four of the adenosine receptors this site is occupied by His, which has been proposed to be critical for recognition of the ribose or ribose-like moiety common to all adenosine agonists thus far reported. At the A_3 receptor, His at this site was proposed as the basis for enhanced affinity of xanthine-7-ribosides relative to the parent xanthines.[27] In the A_1 receptor, mutation of this His to Ala resulted in decreased affinity of both agonists and antagonists. In the A_{2A} receptor, this site has been mutated to Ala with the loss of high-affinity binding of both agonists and antagonists,[19] while mutation to Tyr preserved the ability to bind ligands.[24] Thus, substitution that preserved H-bonding capability was allowed at this critical site. Substitution of His272 with Glu was the first example of a nonaromatic residue at this position in adenosine receptors that still allowed ligand recognition. It was especially surprising in light of the proposal that in the human A_{2A} receptor this His appeared to be coupled spatially to a Glu in TM1 through the formation of a H-bond.[24]

The affinities of the aminoadenosine analogues **7**−**14**, for the H272E mutant A_3 receptor were higher than for the corresponding wild type, demonstrating that in principle A_3 receptors could be engineered for selective interaction with synthetic agonists. In particular this was evident from the 7-fold affinity enhancement of **8** toward the mutated receptor. Thus, the notion that the H272E mutant A_3 receptor could be selectively activated in the presence of wild-type A_3 receptors appeared feasible.

The role of residue His272 in accommodating A_3 agonists and antagonists, as well as the consequences of its replacement by glutamate were investigated by molecular modeling[19,23] of the ligand−receptor complexes. The 3′-amino group was intended for a direct electrostatic interaction with the negatively charged

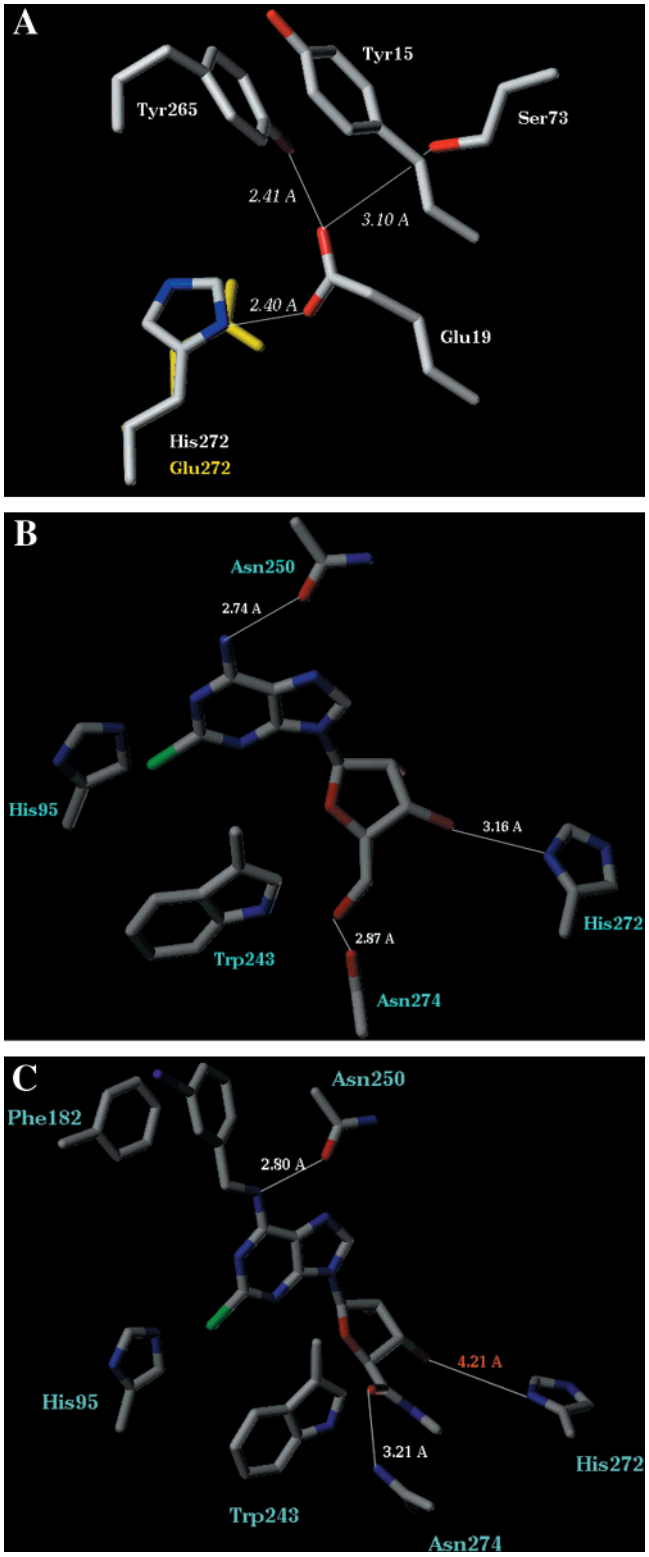

Figure 7. (a, top) Details of the putative H-bonding interactions among residues in proximity to the binding site of the human A_3 receptor either in the native receptor (His in blue and white) or for the H272E mutant (Glu in yellow). H-bonding distances to the carboxylate group of Glu19 are shown. (b, middle) Docked conformation of the nonselective adenosine agonist **1b** showing its position with respect to critical residues in the putative binding site of the wild-type human A_3 receptor. (c, bottom) Docked conformation of the A_3 selective adenosine agonist **2a** showing its position with respect to critical residues in the putative binding site of the wild-type human A_3 receptor.

ribose-binding region of the mutant receptor, yet molecular modeling did not support this notion. In brief, the higher affinity of 3′-amino-3′-deoxyadenosine at the engineered A_3 receptor appeared to result from the lack of a repulsion (positively charged ligand and a positively charged His272 side chain in the WT receptor), rather than the attractive force of opposite charges. It is also possible that a water molecule fills the space between the 3′-amino group and Glu272, thus allowing water-mediated H-bonding.

Previous models of an interaction between His278 of A_{2A} (position equivalent to 272 in the A_3 receptor)[23] and adenosine suggested the involvement of both of the ribose hydroxy substituents on binding. Yet substitution of glutamate at position 272 had a larger effect on **8** than on **7**, indicating that His/Glu272 interacted predominantly with the 3′-ribose substituent. Conversely, for aminoadenosines where N^6 was substituted by a cycloalkyl (**11**) or iodobenzyl (**12**, **13**), the effect of Glu272 was small, irrespective of the position of the amino ribose substituent (see Table 2). In addition, while the affinity of **8** toward the rat A_3 receptor resembled that of the human H272E A_3 receptor, the corresponding affinity of **13** was similar to that of the human wild-type A_3 receptor. Rationalization of these differences by means of molecular modeling of the respective complexes has provided a more detailed insight into the specific ligand−A_3 receptor interactions, allowing for further suggested modification of the A_3 receptor binding environment.

From molecular models of the human A_3 receptor complexed with **8** (Figure 8) it appears that the nearly 1000-fold lower affinity, relative to **1b,** results from a loss of a H-bond interaction of the 3′-hydroxy substituent and the electrostatic repulsion of the 3′-amine substituent and His272. Replacement at position 272 by glutamate did not restore binding with the 3′-substituent. As already mentioned Glu272 was not available for direct contact with the ligand due to an interaction with Glu19. The gain of affinity for the aminoadenosines seemed therefore to depend on relief of electrostatic repulsion, being more pronounced for **8** than for **11** and **12,** where the ligands would be displaced toward TM5 (including the 3′-amine substituent). In this respect, the finding that affinity of the wild-type rat A_3 receptor toward **8** was similar to that of the human H272E mutant A_3 receptor was particularly interesting. As already mentioned, the model of the human A_3 receptor indicated that the mobility of Glu19 was restricted by H-bond interactions with Tyr265 and Ser73, which immobilized the Glu19 carboxylate between TM7 and TM2. In the rat A_3 receptor, cysteine occurs at the position corresponding to 265, thus probably allowing for motion of the His272-Glu19 assembly in the direction of TM2 and away from the 3′-amino substituent (model not shown). This interpretation was consistent with the notion that replacement of His272 resulted mostly in removal of electrostatic repulsion but did not restore binding interaction with the 3′-substituent of the adenosine derivatives. In the case of **13** where such repulsive interaction was less pronounced (see above), the affinity at the rat A_3 receptor resembled those of both the human wild-type and the H272E A_3 receptors.

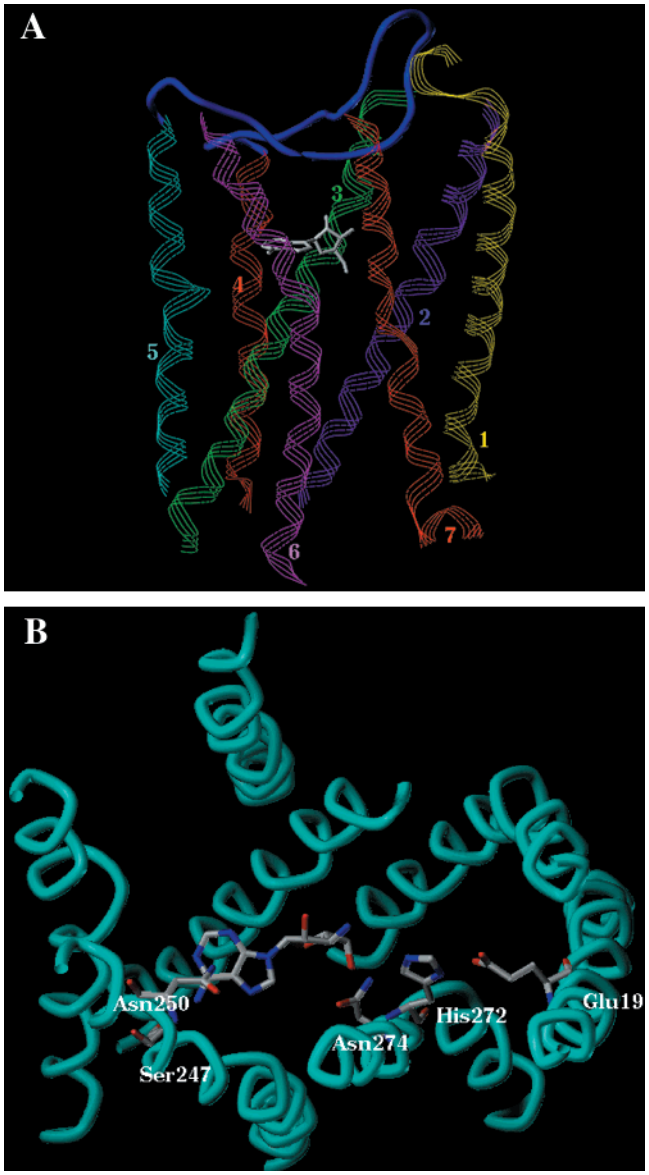

Figure 8. Docked conformation of the neoligand **8** in relation to the helical bundle and EL2 of the H272E mutant human A_3 receptor. Views from the plane of the membrane (a, top: TMs are indicated and EL2 is highlighted) or from the extracellular region (b, bottom: showing TM side chains mentioned in the text) are shown.

The notion that Glu272 did not interact directly with the ligands was consistent with the finding that the A_3 receptor affinity toward *N*-benzyl-substituted analogues was insensitive to the nature of the residue at position 272. The corresponding affinity toward **1b** was 20-fold lower (see Table 1). Furthermore, the affinity of the H272E mutant A_3 receptor toward **1b** was only 7-fold higher than that toward the 3′-amino derivative **8**, suggesting that the binding environments for the two ligands were similar. In fact, the lower affinity toward **8** was likely due to repulsive interactions with Lys152, which, according to our model, was vicinal to the 2′-hydroxy substituent. This proximity may be responsible for the low affinity of both human and rat A_3 receptors toward the aminoadenosine **7**. As already suggested, the lack of Glu272 participation in ligand accommodation, and in particular in interaction with 3-aminoadenosines, was due to its interaction with

Glu19. Consequently, replacement of Glu19 by a residue that would not restrict the mobility of Glu272 may result in a receptor with considerably higher affinity toward aminoadenosines such as **8**, and also a more wild-type-like affinity toward the endogenous ligand adenosine. Alternatively, the replacement of Ser73 by a nonpolar residue such as Ala may allow enhanced mobility of the Glu272-Glu19 assembly toward TM7 (see above), bringing the Glu272 carboxylate within interaction distance of the ligands. Thus, the doubly mutated H272E/E19X and H272E/S73X mutant A_3 receptors may be more suitable as neoceptors for the aminoadenosine neoligands.

The notion that GPCRs can be engineered to accommodate unnatural ligands has been investigated in other cases. For example, Schwartz and co-workers[42] have engineered GPCRs to have the ability to bind zinc ions through complexation with multiple His residues. In those cases, the zinc ion bound as an antagonist or partial agonist.[43] More recently Conklin and co-workers[44] engineered the κ opioid receptor to respond exclusively to synthetic small molecule ligands and not to the receptor's natural ligand. In this elegant study, impressive selectivity toward bremazocine was achieved, demonstrating the extent to which receptor properties could be modified without compromising functionality. Strader and co-workers[45] also studied the microscopic complementarity of functionality in receptor binding. However, no attempt was made to modify both the receptors and the ligands in the manner proposed in the present study.

The feasibility of a tailor-made agonist (neoligand) to interact selectively a mutant receptor (neoceptor) indicates that this novel therapeutic approach, in which such a neoceptor would be introduced specifically into a target organ through gene transfer,[46,47] may be possible. Gene transfer to the heart has been demonstrated,[48−50] and gene therapy toward the goal of cardioprotection has already been proposed.[4] The transfection of the adenosine A_1 or A_3 into a cardiac myocyte culture enhances the protective effect of either endogenous adenosine or an exogenously added, synthetic agonist of the appropriate receptor. By this approach, a neoceptor could be genetically delivered to a target organ, followed by the administration of a selective, tailor-made ligand, as needed. This agent could be administered in a dose range in which only the neoceptor, not the endogenous parent receptor, would be activated.

Experimental Section

Materials. Compounds **1a** (5′-*N*-ethylcarboxamidoadenosine), **1b** (2-chloroadenosine), **2** (N^6-(3-iodobenzyl)-2-chloroadenosine-5′-*N*-methyluronamide), **4** (*N*-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine), **6** (8-[4-[[[[(2-aminoethyl)amino]carbonyl]methyl]oxy]phenyl]-1,3-dipropylxanthine), and **9** (5′-amino-5′-deoxyadenosine) were from Sigma Chemical Co. (St. Louis, MO). Compound **3** ((1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4-{2-chloro-6-[(3-iodophenylmethyl)amino]purin-9-yl}-1-(methylaminocarbonyl)bicyclo[3.1.0]hexane-2,3-diol) was synthesized as reported.[28] Compound **6** (4-methoxy-*N*-[3-(2-pyridinyl)-1-isoquinolinyl]benzamide)[51] was the gift of Prof. Ad IJzerman of the LACDR, Leiden, The Netherlands. CHN analyses were carried out by Prof. W. Pfleiderer (Konstanz, Germany).

5′-Amino-5′-deoxyadenosine tosylate was obtained from Sigma (St. Louis, MO). 2′-Amino-2′-deoxyadenosine (**7**) was prepared as reported.[30] Other amino derivatives, 3′-amino-3′-deoxyadenosine (**8**),[29] 9-(3-amino-3-deoxy-β-D-xylofuranosyl)-N^6-cyclopentyladenine (**11**),[31] and **14**[38] were prepared according to known procedures.

Full-length cDNA encoding the human adenosine A_3 receptor was kindly provided by M. Atkinson, A. Townsend-Nicholson, and P. R. Schofield (Garvan Medical Institute, Sydney, Australia) and was subcloned in pcDNA3 as pcDNA3/hA_1R, and pcDNA3/hA_3R. The vector pcDNA3 was obtained from Invitrogen (Carlsbad, CA).

Preparation of Mutant Receptors. Procedures for the construction of the mutant receptor are provided elsewhere.[40] The plasmids expressing mutant A_3 adenosine receptor were constructed as described in the instruction manual of the QuikChange TM Site-Directed Mutagenesis Kit (La Jolla, CA). The plasmid pcDNA3/hA3-HA[40] with cDNA of wild-type A_3 adenosine receptor was used as a template for PCR. The pair of primers that contained the desired mutation (histamine to glutamic acid) was from Biosources Co. (Laurel, MD). Their sequences were 5′-atcctgctgtccgaggccaactccatg-3′; 5′-catggagttggcctcggacagcaggat-3′. Following PCR, the PCR products were digested with Dpn I and transformed into *E. coli*. The mutant plasmid was identified by sequencing, transfected in Cos-7 cells and expressed transiently.

Synthesis. ^{1}H NMR spectra were obtained with a Bruker DRX 500 spectrometer. The solvent signal of DMSO-d_6 was used as a secondary reference. All signals assigned to amino and hydroxyl groups were exchangeable with D_2O. Exact mass measurements were performed on a quadrupole/orthogonal-acceleration time-of-flight (Q/oaTOF) tandem mass spectrometer (qTOF 2, Micromass, Manchester, U.K.) equipped with a standard electrospray ionization (ESI) interface. Samples were infused in a 2-propanol:water (1:1) mixture at 3 μL/min.

Preparation of 3′-Deoxy-3′-guanidinoadenosine, Trifluoroacetic Acid Salt (10). 3′-Amino-3′-deoxyadenosine (**8**, 5 mg, 0.019 mmol) was added to a solution of *di*-Boc-triflylguanidine[32] (10 mg, 0.026 mmol) and triethylamine (10 μL, 0.07 mmol) in DMF (1 mL), and the mixture was heated to 60 °C for 12 h. The solvent was removed by nitrogen stream, and the residue was purified by preparative thin-layer chromatography (silica gel, chloroform: methanol = 3:1) to give *di*-Boc-protected 3′-guanidino-3′-deoxyadenosine (**15**, 5 mg, 53%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9H), 1.52 (s, 9H), 3.86(d, 1H, J = 12.4 Hz), 4.07 (d, 1H, J = 13.5 Hz), 4.55−4.66 (m, 2H), 5.77 (s, 2H), 6.09 (d, 1H, J = 1.4 Hz), 8.33 (s, 1H), 8.47 (s, 1H), 9.05 (d, 1H, J = 6.6 Hz), 11.36 (s, 1H); high-resolution MS (positive-ion FAB) calcd for $C_{21}H_{33}N_8O_7$ $[M + H^+]^+$: 509.2472, found 509.2475.

A solution of **15** (3 mg, 0.0059 mmol) in CH_2Cl_2 /10% trifluoroacetic acid (1 mL) was stirred overnight at 4 °C. The solvent was removed under a nitrogen stream, and the residue was purified by washing with ethyl ether (1 mL × 3) to give 3′-guanidino-3′-deoxyadenosine (**10**, 2.2 mg, 88%), as a trifluoroacetic acid salt: ^{1}H NMR (CD_3OD) δ 3.75 (dd, 1H, J = 2.5, 12.6 Hz), 4.06 (dd, 1H, J = 2.2, 12.6 Hz), 4.18−4.24 (m, 1H), 4.46−4.53 (m,1H), 4.66 (d, 1H, J = 5.2 Hz), 6.15 (d, 1H, J = 1.4 Hz), 8.38 (s, 1H), 8.64 (s, 1H); high-resolution MS (positive-ion FAB) calcd for $C_{11}H_{17}N_8O_3$ $[M + H^+]^+$: 309.1424, found 309.1427.

9-(3-Azido-3-deoxy-β-D-ribofuranosyl)-N^6-(3-iodobenzyl)adenine (17). A 725 mg (1.92 mmol) amount of **16** in 35 mL of dry 1,2-dichloroethane was added to 810 mg (2.31 mmol) of N^6-(3-iodobenzyl)adenine silylated residue[24] (417 μL, 2.31 mmol). $(CH_3)_3SiOSO_2CF_3$ was added dropwise under N_2 and continuous stirring to give a clear solution after approximately 30 min. The temperature was kept at 83 °C, and after 6 h 50 mL of CH_2Cl_2 and 100 mL of a 7% $NaHCO_3$ solution were added to the reaction mixture. The organic phase was extracted, dried with $MgSO_4$, filtered, and the filtrate evaporated to dryness. The residue was dissolved in 100 mL of 0.1 N $NaOCH_3$ in CH_3OH, stirred for 2 h, neutralized with a 9:1 H_2O-CH_3COOH solution, evaporated in vacuo, purified by column chromatography (CH_2Cl_2, then 97:3 CH_2Cl_2–MeOH) and crystallized from CH_3OH to yield 176 mg (18%) of the title compound as a white solid: ^{1}H NMR (DMSO-d_6) δ 3.58 (app

dd, $J = 3.6$ Hz and $J_{5A',5B'} = -12.3$ Hz, H-5B′), 3.69 (app dd, $J = 3.5$ Hz, H-5A′), 4.00 (app q, $J = 3.5$ and 7.0 Hz, H-4′), 4.33 (q, $J = 3.8$ and 5.2 Hz, H-3′), 4.67 (s, benzylic H), 5.02 (t, $J = 5.6$ Hz, H-2′), 5.54 (br s, 5′-OH), 5.92 (d, $J = 6.0$ Hz, H-1′), 6.24 (br s, 2′-OH), 7.11 (t, $J = 7.8$ Hz), 7.36 (d, $J = 7.6$ Hz), 7.59 (d, $J = 7.8$ Hz), 7.73 (s, aromatic H), 8.23 (s, H-2), 8.42 (s, H-8), 8.55 (br s, H−N^6); exact mass (ESI-MS) calcd for $C_{17}H_{17}I_1N_8O_3$ $[M+H]^+$: 509.0548, found 509.0547.

9-(3-Amino-3-deoxy-*β*-D-ribofuranosyl)-*N*^{6}-(3-iodobenzyl)aden ine (12). A 100 mg (0.197 mmol) amount of **17** was dissolved in 10 mL of pyridine, and 100 mg (0.381 mmol) of Ph_3P was added to the solution. After stirring for 1 h at room temperature, 5 mL of concentrated NH_4OH was added. The reaction mixture was stirred for another 2 h, evaporated to dryness, and purified by column chromatography (9:1 CH_2Cl_2–MeOH) to yield 67 mg (70%) of **12** as a white solid: ^{1}H NMR (DMSO-d_6) δ 1.69 (br s, 3′-NH_2), 3.47 (app t, $J = 6.1$ Hz, H-3′), 3.57 (app dd, $J = 4.3$ Hz and −12.6 Hz, H-5B′), 3.73 (app d, $J = 7.9$ Hz, H-5A′ and 4′), 4.29 (m, H-2′), 4.65 (s, benzylic H), 5.14 (br s, 5′-OH), 5.77 (br s, 2′-OH), 5.93 (d, $J = 2.8$ Hz, H-1′), 7.11 (t, $J = 7.8$ Hz), 7.35 (d, $J = 7.6$ Hz), 7.58 (d, $J = 7.8$ Hz), 7.71 (s, aromatic H), 8.21 (s, H-2), 8.44 (s, H-8), 8.48 (br s, H−N^6); exact mass (ESI-MS) calcd for $C_{17}H_{19}I_1N_6O_3$ $[M+H]^+$: 483.0643, found 483.0631. CHN analysis.

Radioligand Binding Studies. Binding of [^{3}H]*R*-*N*^{6}-phenylisopropyladenosine ([^{3}H]*R*-PIA) to A_1 receptors from rat cerebral cortex membranes and of [^{3}H]-2-[4-[(2-carboxyethyl)-phenyl]ethylamino]-5′-*N*-ethylcarbamoyladenosine ([^{3}H]CGS 21680) to A_{2A} receptors from rat striatal membranes was performed as previously described.[6,35] Adenosine deaminase (3 units/mL) was present during the preparation of the brain membranes, in a preincubation of 30 min at 30 °C, and during the incubation with the radioligands. Binding of [^{125}I]AB-MECA, **2b** (Amersham, Chicago, IL), to membranes prepared from CHO cells stably expressing the human A_3 receptor was performed as described.[26] The assay medium consisted of a buffer containing 10 mM Mg^{2+}, 50 mM Tris, and 1 mM EDTA, at pH 8.0. The glass incubation tubes contained 100 μL of the membrane suspension (0.3 mg of protein/mL, stored at −80 °C in the same buffer), 50 μL of [^{125}I]AB-MECA (final concentration 0.3 nM), and 50 μL of a solution of the proposed antagonist. Nonspecific binding was determined in the presence of 100 μM *N*^{6}-phenylisopropyladenosine (*R*-PIA).

All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 2%.

Incubations were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester (Brandell, Gaithersburg, MD). The tubes were rinsed three times with 3 mL of buffer each.

At least five different concentrations of competitor, spanning 3 orders of magnitude adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, calculated with the nonlinear regression method implemented in the InPlot program (Graph-PAD, San Diego, CA), were converted to apparent K_i values using the Cheng−Prusoff equation,[52] and K_i values were 1.0 nM ([^{3}H]*R*-PIA), 14 nM ([^{3}H]CGS 21680), and 0.59 and 1.46 nM ([^{125}I]AB-MECA at human and rat A_3 receptors, respectively.

Cyclic AMP Assay. COS-7 cells expressing either the mutant or wold-type receptor were harvested by trypsinization. After centrifugation and resupension in medium, cells were plated in 24-well plates in 400 μL of medium. After 24 h, the medium was removed and cells were washed three times with 500 μL of DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with rolipram (10 μM) and adenosine deaminase (3 U/mL) and the agonist to be tested. After 45 min, forskolin (10 μM) was added to the medium and incubation was continued for an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed upon the addition of 200 μL of 0.1 M cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production,[39] protein kinase A (PKA, 50 μg of protein/well) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), and either a mixture of 20 μL of the cell lysate and 30 μL of 0.1 M HCl or 50 μL of cyclic AMP solution (0−16 pmol/200 mL for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters, which were washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

Molecular Modeling. A molecular model of the human A_3 receptor was built and optimized using the Sybyl 6.6[53] modeling package, following the homology modeling approach described in our previous study[54] using the recently reported X-ray structure of bovine rhodopsin[41] as a structural template. All calculations were performed on a Silicon Graphics Octane R12000 workstation. Briefly, sequences of the A_3 transmembrane domains, identified in our previously published model,[55] were amended by comparison to the corresponding domains of rhodopsin, according to a published sequence alignment.[56] Transmembrane A_3 helices were built from these sequences, in homology to the corresponding helices of rhodopsin, and minimized individually. The minimized helices were then grouped by adding one at a time until a helical bundle (TM region), matching the overall characteristics of the crystallographic structure of rhodopsin, had been obtained. The TM region was further modified by the addition of residues 148−173, comprising the second extracellular loop (EL2). The conformation of EL2 was initially modeled according to the corresponding domain in rhodopsin including the Cys88-Cys166 disulfide bond. At each step the structures were minimized using the Tripos force field with Amber[57] all-atom force parameters until the root mean square value of the conjugate gradient (CG) was <0.1 kcal/mol/Å. A fixed dielectric constant = 4.0 was used throughout these calculations.

Models of adenosine and other A_3 receptor ligands used in this study were constructed using the "Sketch Molecule" module of Sybyl. The ligands were minimized in Sybyl (using MOPAC calculated partial atomic charges) and were rigidly docked into the helical bundle using graphical manipulation coupled to continuous energy monitoring (Dock module of Sybyl). Whenever a final position was reached, consistent with a local energy minimum, the complexes of receptor and ligand were subjected to an additional CG minimization run of up to 1500 steps.

Acknowledgment. This work was supported by RO1-HL48225 and an Established Investigatorship Award (to B.T.L.). Z.-G.G. and D.B. thank Gilead Sciences (Foster City, CA) for support. We thank Dr. Neli Melman for carrying out binding assays.

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JM010232O