Bioorganic & Medicinal Chemistry 16 (2008) 8546–8556

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Selective A_3 adenosine receptor antagonists derived from nucleosides containing a bicyclo[3.1.0]hexane ring system

Artem Melman [a,†], Ben Wang [a,†], Bhalchandra V. Joshi [a,†], Zhan-Guo Gao [a], Sonia de Castro [a], Cara L. Heller [a], Soo-Kyung Kim [a,c], Lak Shin Jeong [b], Kenneth A. Jacobson [a,*]

[a] Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 8A, Room B1A-19, Bethesda, MD 20892, USA
[b] Laboratory of Medicinal Chemistry, College of Pharmacy, Ewha Womans University, Seoul 120-750, Republic of Korea
[c] Beckman Institute, Materials and Process Simulation Center, California Institute of Technology, Pasadena, CA 91125, USA

ARTICLE INFO

Article history:
Received 16 June 2008
Revised 1 August 2008
Accepted 4 August 2008
Available online 7 August 2008

Keywords:
G protein-coupled receptor
Purines
Molecular modeling
Structure–activity relationship
Radioligand binding
Adenylate cyclase

ABSTRACT

We have prepared 5′-modified derivatives of adenosine and a corresponding (N)-methanocarba nucleoside series containing a bicyclo[3.1.0]hexane ring system in place of the ribose moiety. The compounds were examined in binding assays at three subtypes of adenosine receptors (ARs) and in functional assays at the A_3 AR. The H-bonding ability of a group of 9-riboside derivatives containing a 5′-uronamide moiety was reduced by modification of the NH; however these derivatives did not display the desired activity as selective A_3 AR antagonists, as occurs with 5′-*N*,*N*-dimethyluronamides. However, truncated (N)-methanocarba analogues lacking a 4′-hydroxymethyl group were highly potent and selective antagonists of the human A_3 AR. The compounds were synthesized from D-ribose using a reductive free radical decarboxylation of a 5′-carboxy intermediate. A less efficient synthetic approach began with L-ribose, which was similar to the published synthesis of (N)-methanocarba A_3AR agonists. Compounds **33b**–**39b** (N^6-3-halobenzyl and related arylalkyl derivatives) were potent A_3AR antagonists with binding K_i values of 0.7–1.4 nM. In a functional assay of [^{35}S]GTPγS binding, **33b** (3-iodobenzyl) completely inhibited stimulation by NECA with a K_B of 8.9 nM. Thus, a highly potent and selective series of A_3AR antagonists has been described.

1. Introduction

There are four subtypes of receptors for adenosine **1**, designated A_1, A_{2A}, A_{2B}, and A_3.[1] Selective antagonists of the A_3 adenosine receptor (AR) are of potential use for clinical targets, including the treatment of cancer,[2] glaucoma,[3] and inflammation.[4] A number of competitive tricyclic[5] and nucleoside[6] antagonists of ARs have been reported recently. The structure of some of the antagonists may resemble adenosine, while other compounds possess a completely different structure. An important advantage of nucleoside-based A_3AR antagonists is their species-independent activity.[7] These antagonists typically possess high affinity at the human, rat, and mouse A_3ARs and therefore may be suitable for evaluation in small animal models or for further development as drugs. For example, A_3AR antagonists derived from adenosine have been shown to lower intraocular pressure in a mouse model of glaucoma.[7]

We have previously shown that local modifications of the potent AR agonist Cl-IB-MECA **2** (Chart 1) may result in a complete loss of agonist efficacy, while retaining a strong binding inhibition constant (K_i).[8] These modifications resulted in potent ARs antagonists of this type in compounds **3** and **4**. The most probable reason for the drastic change in activity is a disruption of H-bonding at the 5′ position either through the introduction of an additional *N*-methyl substituent, or enforcement of a conformation of the amide group at this position that is unable to activate the receptor.[9] Another approach to adenosine-derived A_3AR antagonists involves

Abbreviations used: AIBN, 2,2′-azo-*bis*-isobutyronitrile; AR, adenosine receptor; CCPA, 2-chloro-N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; DCC, dicyclohexylcarbodiimide; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; GPCR, G protein-coupled receptor; I-AB-MECA, 2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; DIPEA, diisopropylethylamine; DCM, dichloromethane; DMF, *N*,*N*-dimethylformamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRMS, high resolution mass spectroscopy; MRS1292, (2*R*,3*R*,4*S*,5*S*)-2-[N^6-3-iodobenzyl)adenos-9'-yl]-7-aza-1-oxa-6-oxospiro[4.4]-nonan-4,5-diol; PIA, R-N^6-(phenylisopropyl)adenosine; TEA, triethylamine; TLC, thin layer chromatography

* Corresponding author. Tel.: +1 301 496 9024; fax: +1 301 480 8422.
E-mail address: kajacobs@helix.nih.gov (K.A. Jacobson).
† Present addresses: Nektar Therapeutics, Huntsville, AL 35801, USA (B.V.J.), Northern Virginia Community College, VA, USA (B.W.), and Clarkson University, Potsdam, NY, USA (A.M.).

0968-0896/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2008.08.007

the complete removal of the substituent at position 4′. This approach has been successfully implemented in thionucleoside antagonists of the class represented by compound **6** (Chart 2), which was derived from **5**, the 4′-thio analogue of Cl-IB-MECA.[10]

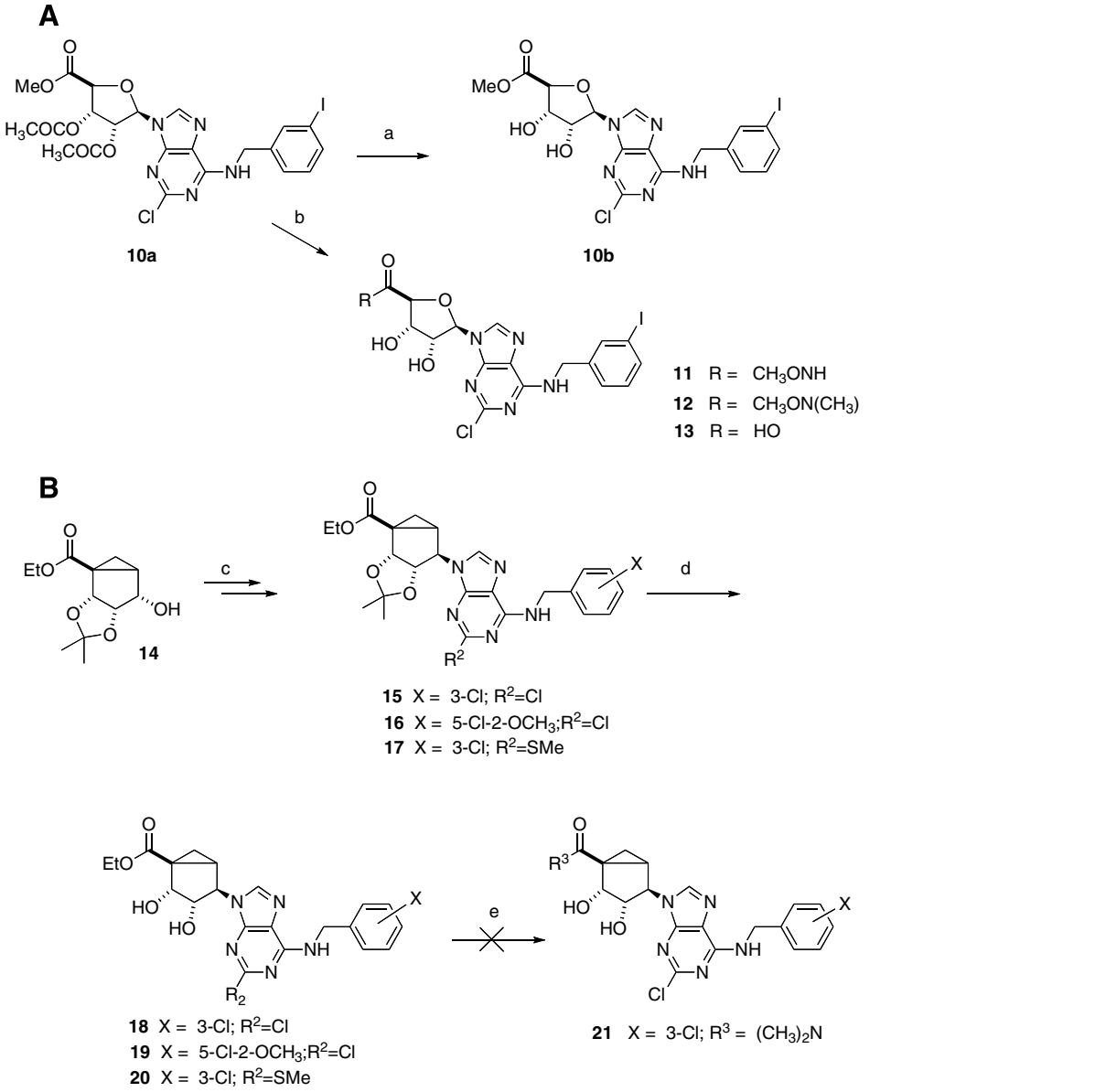
Both the binding and selectivity of prototypical A_3AR agonists can be substantially increased by replacing the flexible ribose scaffold in the series of compounds related to **2** with a rigid bicyclo[3.1.0]hexane ring system. The resulting (N)-methanocarba adenosine agonists **7**–**9** have been found to possess high potency and enhanced selectivity for the A_3 receptor,[11] and agonists of this class are currently under development as anti-arthritis drug candidates.

This study reports on a new series of selective A_3AR antagonists of the (N)-methanocarba family. Design of these compounds involved the disruption of H-bonding at the 4′ position through removal of the *N*-methylcarboxamide function, while retaining the conformationally restricted (N)-methanocarba scaffold bearing 6-(arylalkylamino)-2-chloropurine moieties, as in agonists **7**–**9**.

2. Results

2.1. Chemical synthesis

Prior to probing the structure–activity relationship (SAR) with the more synthetically challenging (N)-methanocarba system, we explored alternatives to the *N*,*N*-dimethyl group as a means of obtaining A_3AR antagonists in the ribose series similar to compound **3**. The corresponding methyl ester **10b** (Scheme 1A, prepared from the protected intermediate **10a**)[10,12] was prepared using a previously reported synthetic approach. This ester group was directly aminolyzed using several hydroxylamino nucleophiles to provide **11** and **12**. Alternately, the ester was hydrolyzed to yield the carboxylic acid **13.** All of these derivatives were intended for testing at ARs, because they have altered H-bonding ability in relation to **1**. Attempts to prepare an active ester from **13** as an intermediate for further amide synthesis were unsuccessful.



Scheme 1. Reagents: (a) K_2CO_3, MeOH, H_2O; (b) K_2CO_3, MeOH, H_2O, MeONH, MeONHMe or LiOH, NaOH, H_2O; (c) two steps[11]; (d) TFA, EtOH–water; (e) R^3H (amine), EtOH.

In the (N)-methanocarba series, the intermediate 2′,3′-isopropylidene protected derivatives **15**–**17** were prepared from the key intermediate **14** as described in the literature (Scheme 1B).[11,13] The key intermediate **14** was in turn prepared according to our recent modified procedure.[13b] The isopropylidene group in **15**–**17** was conveniently removed by using aqueous TFA in EtOH to afford the requisite 4′-ester methanocarba derivatives **18**–**20** in good yields. Attempts to react the ester group of either **15**–**17** or **18**–**20** with dimethylamine to prepare dimethylamides similar to **3** in the (N)-methanocarba series, that is, **21**, were unsuccessful.

Our initial synthetic approach (Scheme 2) toward (N)-methanocarba derivatives lacking the 4′ substituent was based on our previously published synthesis of (N)-methanocarba agonists from L-ribose **22**.[13] It involved protection of a key alcohol in **14** as a benzyl ether followed by hydrolysis of the ester group in **24** with a subsequent free-radical reductive decarboxylation of **25** using the methodology of Barton,[14] leading to conversion into the desired compounds by previously described methodology.[13] The decarboxylation of **25** to yield **26** was performed using a one-pot modification,[15] which involved reaction with a mixture of tributyltin hydride, 2,2′-dithiopyridine-1,1′-dioxide, tributylphosphine, and a radical initiator. We found that under these conditions preparation of benzyl ether **26** could be achieved, although the reaction yield was variable and difficult to reproduce. Another problem was the difficulty of deprotection of benzyl ether **26** by hydrogenation, which gave alcohol **27** in poor yield.

These synthetic problems prompted us to evaluate an alternative synthetic approach (Scheme 3). It involved the synthesis of alcohol **29** from inexpensive D-ribose using the same methodology.[13] Alcohol **29** was protected with TBDPS-Cl followed by alkaline hydrolysis, thus providing acid **30**.

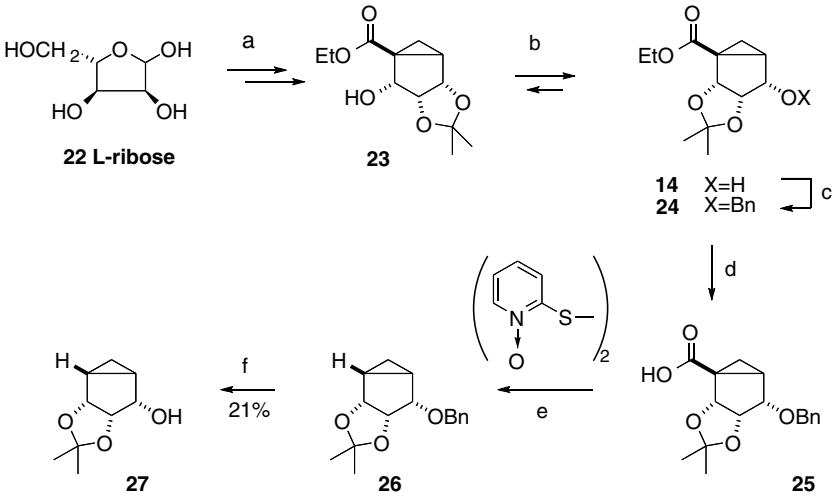
Reductive decarboxylation of acid **30** was initially carried out through a sequential one-pot procedure involving a DCC-mediated coupling of the acid with 2-mercaptopyridine *N*-oxide at room temperature followed by reductive free radical decarboxylation with tributyltin hydride at 80 °C.[14a] However, the resultant ether **31** was difficult to separate from toxic and foul-smelling tributyltin-containing byproducts. A modified approach for the reductive decarboxylation used non-toxic tris(trimethylsilyl)silane as a hydrogen donor[16] and produced the silyl ether **31** in 40% yield. To the best of our knowledge, this is the first application of tris(trimethylsilyl)silane for reductive decarboxylation, and this reaction may possess a considerable preparative potential.

In contrast to the low-yielding hydrogenation of **26**, silyl ether **31** was smoothly deprotected with TBAF. The resultant alcohol **27** was converted into a key dichloropurine derivative **32** through a Mitsonobu reaction (Scheme 3).[13] The dichloropurine derivative **32** reacted with an excess of the corresponding primary amine to give the N^6 substituted and 2′,3′-isopropylidene protected derivatives **33a**–**39a**, followed by acid catalyzed deprotection to give the N^6-3-halobenzyl and related arylmethyl derivatives **33b**–**39b**.

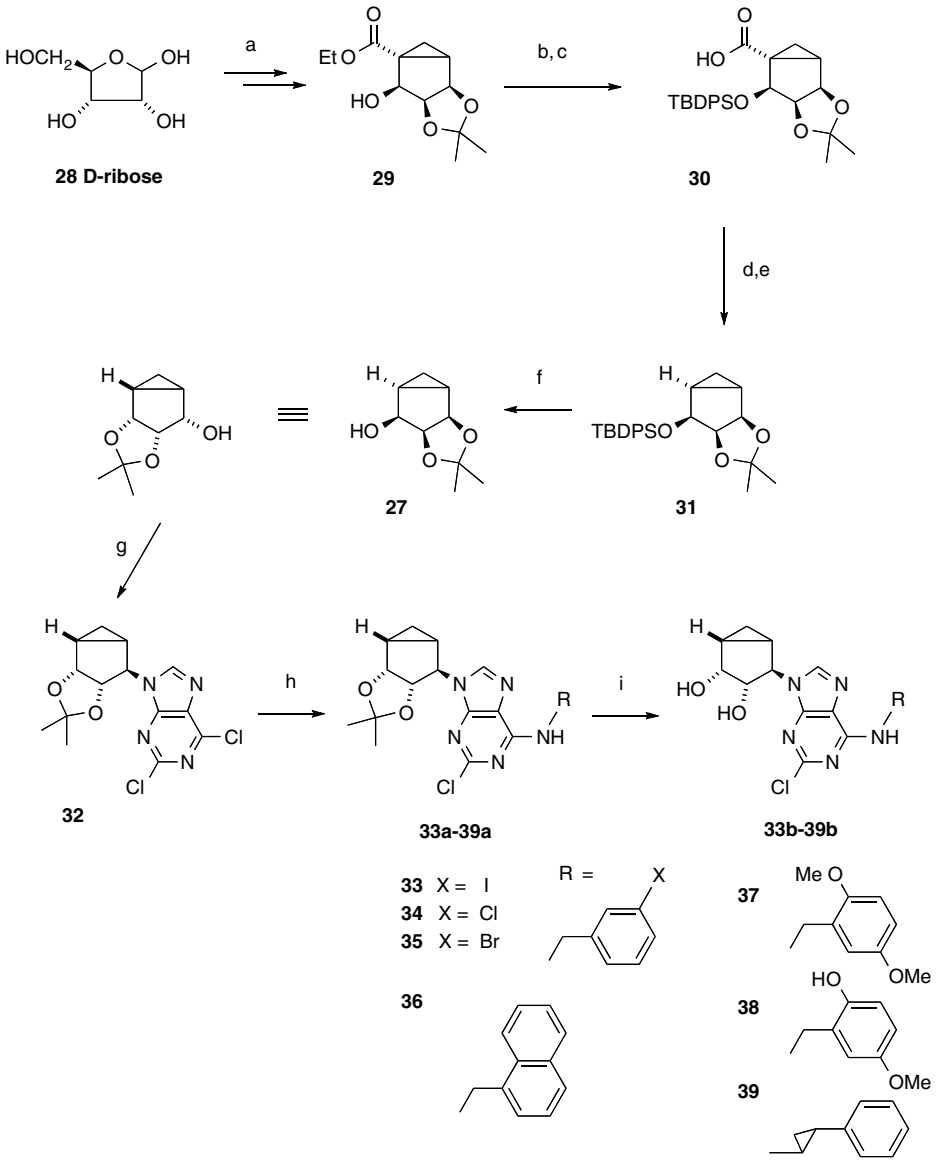
3. Pharmacological activity

The 3-chloro- and 5-chloro-2-methoxy derivatives, **8** and **9**, respectively, of compound **7** were used for comparison in the biological assays (Table 1). Binding assays were carried out using standard radioligands and membrane preparations from Chinese hamster ovary (CHO) cells (A_1 and A_3) or HEK293 cells (A_{2A}) stably expressing a hAR subtype (Table 1). Functional effects at the A_3AR were determined in assays of adenylate cyclase or guanine nucleotide binding.[22,23] Replacement of the amide NH of **2** with an isosteric, but non-H-bond donating, ester oxygen in **10b** resulted in a one-order of magnitude loss of affinity at the A_1 and A_3ARs, and no change at the A_{2A}AR. Insertion of an O between the NH and the methyl group in **11** reduced the A_3AR affinity by 62-fold. Both **10b** and **11** lost nearly all efficacy as A_3AR agonists in relation to full agonist **2**. This confirmed previous findings that the efficacy of adenosine derivatives in activating the A_3AR was highly dependent on structural factors in the region of the 4′ carbon. The *N*-methylation of derivative **12** had no effect on the A_3AR affinity, while completely abolishing activation of the receptor. Unfortunately, compound **12** was not a suitable lead as an A_3AR antagonist, because it was only 4-fold selective in comparison to the A_1AR. However, this compound could be a useful pharmacological probe of mixed selectivity for the A_1AR and A_3AR. Compound **12** was tested in a functional assay at the A_1AR ([^{35}S]GTPγS binding in A_1AR-expressing CHO cells) and found to lack the ability to activate the receptor (data not shown). Therefore, compound **12** appears to be a mixed A_1/A_3 AR antagonist. Compound **13**, derived by saponification of **10a**, was nearly inactive at the A_1 and A_{2A}ARs and a very weak agonist at the A_3AR. Thus, a negative charge in the region of the 4′ carbon appears to be highly detrimental toward interaction with ARs.

The (N)-methanocarba agonists **7**–**9** were already reported to be potent and selective at the A_3AR and fully efficacious.[11] Replacement of the amide NH of **8** or **9** with an ester oxygen resulted in



Scheme 2. Reagents and condition: (a) Seven steps[13]; (b) CF_3SO_3H (cat.), DCM; (c) BnCl, NaH, THF; d) NaOH/H_2O/MeOH, reflux; (e) PBu_3, AIBN, Bu_3SnH, toluene; (f) H_2/Pd, MeOH.



Scheme 3. Reagents (a) Seven steps[13]; (b) TBDPS-Cl, imidazole, DMF; (c) NaOH, H_2O, MeOH, reflux; (d) 2-mercaptopyridine *N*-oxide, DCC, toluene; (e) $(Me_3Si)_3SiH$, AIBN, toluene; (f) Bu_4NF, THF; (g) 2,6-dichloropurine, PPh_3, DIAD, THF; (h) RNH_2, EtOH; (i) TFA/H_2O/MeOH.

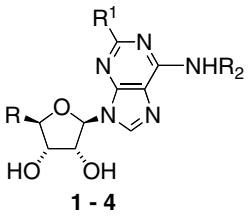
greatly reduced affinity at the three AR subtypes, and only partial activation of the A_3AR was observed. Thus, this modification is unlikely to provide an entry into selective A_3AR antagonists. Replacement of the 2-chloro of **18** with methylthio, which was tolerated in the ribose series of A_3AR agonists,[6b] resulted in **20** and failed to increase the affinity. However, compound **20** completely lacked the ability to activate the A_3AR and thus appears to be a weak antagonist.

Compounds **33b**–**35b** (3-halobenzyl) in the (N)-methanocarba series were potent A_3 AR ligands with binding K_i values of 0.7–1.4 nM. Compound **35b** (3-bromobenzyl analogue) proved to be the most potent A_3AR ligand of this series in binding with a K_i value of 0.73 nM, and it displayed high selectivity (2400-fold and 2190-fold in comparison to the A_1 and A_{2A}AR, respectively). The most A_3AR selective compound was the 3-chloro analogue **34b** with 2900-fold and 4250-fold selectivity in comparison to the A_1 and A_{2A}AR, respectively. The SAR of substitution of the N^6-benzyl group further showed that dimethoxy substitution (**37b**), fusion of the phenyl ring to a second ring (**36b**), and extension by one carbon (i.e., in the rotationally constrained 2-phenylcyclopropyl analogue, **39b**) were all tolerated with nanomolar binding affinity at the A_3AR. Compound **38b**, a demethylated analogue of **37b**, was slightly less potent in binding to the A_3AR.

Compounds **33b**–**39b** were examined for the ability to stimulate [^{35}S]GTPγS binding in membranes of CHO cells expressing the human A_3AR. None of the truncated (N)-methanocarba derivatives induced substantial (>10%) activation of the A_3AR. In the same functional assay applied to detect antagonism, a representative analogue **33b** completely inhibited stimulation by 1 μM NECA (5′-*N*-ethylcarboxamidoadenosine) with an IC_{50} of 29.8 nM (Fig. 1). Schild analysis of the right shifts by **33b** of the response curves in the inhibition of adenylate cyclase by NECA provided a K_B value of 8.9 nM. Thus, truncated (N)-methanocarba derivatives displayed A_3AR antagonist properties.

3.1. A_3AR molecular modeling: Docking of nucleoside agonist and antagonists

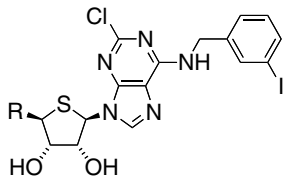
The previous docking study showed that the binding site of a full agonist (Cl-IB-MECA **2**) in the A_3AR was located in the upper transmembrane domain (TM) near EL2 (second extracellular loop) shown in Figure 2A.[17] In the present study, the nucleoside antago-



1 - 4

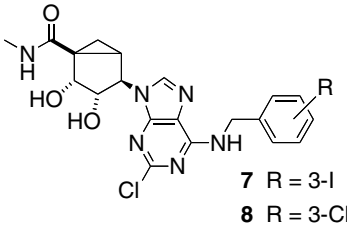
		R	R^1, R^2	Activity at A_3AR
1	adenosine	CH_2OH	H, H	agonist
2	Cl-IB-MECA	CONHMe	Cl, 3-iodobenzyl	agonist
3	MRS3771	$CONMe_2$	Cl, 3-iodobenzyl	antagonist
4	MRS542	CH_2OH	Cl, 3-iodobenzyl	antagonist

Chart 1. Adenine 9-riboside derivatives as prototypical ligands for the A_3AR.



5 R=CONHMe A_3 agonist

6 R=H A_3 antagonist



7 R = 3-I MRS1898

8 R = 3-Cl MRS3558

9 R = 5-Cl-2-OCH_3

Chart 2. 4′-Thio and (N)-methanocarba derivatives of adenosine as later generation ligands for the A_3AR.

nist **33b** shown in Figure 2B was initially docked to the A_3AR model based on the structure with Cl-IB-MECA **2** bound, followed by minimization and molecular dynamics, and the complexes were compared. These two A_3AR selective ligands were exactly overlapped in the binding site with respect to the phenyl ring of the N^6 substituent and the ribose ring, as depicted in Figure 2C. Common binding sites were conserved: (1) The NH of the side-chain of Q167 in EL2 formed H-bonds with the 2′-hydroxyl group; (2) The 3′-hydroxyl group interacted with the side chain of $H272^{7.43}$ and the backbone of $S271^{7.42}$. However, different additional interactions were evident for agonist **2** and antagonist **33b**. In the agonist binding domains, additional interactions were present at the site of helical bending of TM6 (due to a Pro residue) in proximity to TM7. The additional interactions were shown in the interaction of the ribose 4′O with $T94^{3.36}$, the 5′-carbonyl group with $S272^{7.42}$, and the 5′-terminal methyl group with the hydrophobic side-chain of $F239^{6.44}$ in the conserved FxxxWxP motif of Class A GPCRs.

The docking complex of compound **33b** shown in Figure 2B indicated the importance for agonism of binding at the helical bending region of TM6 in proximity to TM7. Compound **33b**, an (N)-methanocarba analogue of Cl-IB-MECA **2** lacking the 5′-group, bound to the A_3AR with a similar binding affinity to **2** but did not induce activation. The docking result showed a loss of the typical interaction of the 5′-position with T3.36, S7.42, and F6.44. The analysis of binding energy showed losses of 1.3 kcal/mol, 3.6 kcal/mol, and 2.8 kcal/mol related to binding in TM3, TM6, and TM7, respectively, compared to the agonist **2**. However, the N^6-(3-iodobenzyl) moiety formed the same hydrophobic interaction with F168 in EL2 with the similar binding energy in TM5 (0.04 kcal/mol energy difference). The EL2, which interacted with the common 2′-hydroxyl group, revealed a more favorable energy of interaction, by 0.5 kcal/mol, for the antagonist **33b**. Since this docking result is based on the structure of the *meta*-I state of rhodopsin, in which a different rotamer of W6.48 was revealed following an anti-clockwise rotation from the extracellular perspective, the docking with the ground-state structure, the inverse-agonist preferred form, was more energetically favorable in the antagonist **33b**-bound structure. However, the agonist **2** displayed a severe steric clash at W6.48 and failed to form an optimum interaction in the ground state.

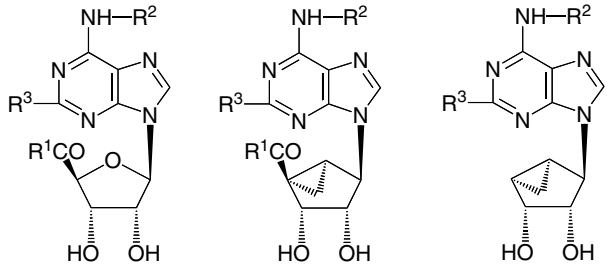
As reported previously,[8,9] the flexibility and the additional H-bonding of the 5′-substituent correlated with putative conformational changes of the receptor associated with the movement of W6.48 upon activation. Selective A_3AR agonists, such as Cl-IB-MECA **2** and its 4′-thio analogue **5**, have been successfully transformed into antagonists selective for the A_3AR by appending an additional *N*-methyl group on the 5′-uronamide position or by reducing the flexibility through cyclization of the 5′ substituent.[8] The 5′-CO group of those antagonists was oriented perpendicular to the ribose ring and could form a H-bond with W6.48, thus blocking the shift of W6.48 side-chain during the conformational change. Thus, the 5′-cyclized uronamide MRS1292 and the 5′-*N*,*N*-dimethyluronamide **3** did not adopt the typical receptor-bound agonist conformation because of a locked conformation and a different energetically favored geometry, respectively.[8,17] The current mode of docking of a new nucleoside antagonist **33b** also supports the view that the additional binding domains present in the full agonist **2** but absent in **33b** are associated with receptor activation. Thus, nucleoside ligands have spatially distinct regions to fulfill separate roles in binding and activation processes.

4. Discussion

The truncation of the 5′-$CONHCH_3$ of A_3AR-selective agonists proved to be the most useful means of converting agonists into pure antagonists. This reduces both the H-bond donating and accepting abilities of the ribose or ribose-like ring. Other means of diminishing the H-bonding ability through methylation, etc. tended to reduce the potency and or selectivity, or allow residual efficacy to remain, which was evident in assays of adenylate cyclase.

The AR binding affinities of the five agonists (i.e., 5′-$CONHCH_3$ derivatives) reported by Tchilibon et al.[11] were compared to those of the corresponding truncated compounds **33b**–**35b**, **37b**, and **39b**. The mean affinities (nM) at the human A_{2A} and A_3ARs were comparable in the two series (A_{2A}AR: 3800 for the agonists, 2400 for the truncated analogues; A_3AR: 1.22 for the agonists, 0.87 for

Table 1
Potency of a series of (*N*)-methanocarba adenosine derivatives at three subtypes of human ARs and the functional efficacy at the A_3AR



Compound	Structure			Affinity (K_i, nM) or % inhibition[a]			% Efficacy[b]
	R^1	R^3	R^2	A_1	A_{2A}	A_3	A_3
Series A							
2[c]	CH_3NH	Cl	3-I-Phenyl-CH_2	222 ± 22	5360 ± 2470	1.4 ± 0.3	100
3[d,e]	$(CH_3)_2N$	Cl	3-I-Phenyl-CH_2	5870 ± 930	>10,000	29.0 ± 4.9	0
10b	CH_3O	Cl	3-I-Phenyl-CH_2	1600 ± 130	4770 ± 240	12.4 ± 2.4	11
11	CH_3ONH	Cl	3-I-Phenyl-CH_2	870 ± 180	1670 ± 350	86.2 ± 8.4	4
12	$CH_3O(CH_3)N$	Cl	3-I-Phenyl-CH_2	252 ± 8	(52 ± 3%)	70.2 ± 6.8	0
13	HO	Cl	3-I-Phenyl-CH_2	(18 ± 5%)	(4 ± 4%)	(58 ± 3%)	42
Series B							
7	CH_3NH	Cl	3-I-Phenyl-CH_2	136 ± 22[d]	784 ± 97[d]	1.5 ± 0.2[c]	100[c]
8[c]	CH_3NH	Cl	3-Cl-Phenyl-CH_2	260 ± 60	2300 ± 100	0.29 ± 0.04	103 ± 7
9[c]	CH_3NH	Cl	5-Cl-2-MeOPh-CH_2	240 ± 50	1200 ± 100	1.5 ± 0.0	107 ± 15
18	C_2H_5O	Cl	3-Cl-Phenyl-CH_2	4820 ± 680	(19%)	310 ± 66	54
19	C_2H_5O	Cl	5-Cl-2-MeOPh-CH_2	1010 ± 120	(37%)	196 ± 33	39
20	C_2H_5O	SCH_3	3-Cl-Phenyl-CH_2	6630 ± 120	(20%)	1010 ± 130	0
Series C							
33b[e]	—	Cl	3-I-Phenyl-CH_2	3040 ± 610	1080 ± 310	1.44 ± 0.60	1.0 ± 3.2[f]
34b	—	Cl	3-Cl-Phenyl-CH_2	3070 ± 1500	4510 ± 910	1.06 ± 0.36	2.9 ± 3.7[f]
35b[e]	—	Cl	3-Br-Phenyl-CH_2	1760 ± 1010	1600 ± 480	0.73 ± 0.30	5.8 ± 0.8[f]
36b	—	Cl	1-Naphthyl-CH_2	1120 ± 640	1530 ± 350	1.42 ± 0.12	3.1 ± 0.3[f]
37b	—	Cl	2,5-diMeO-Ph-CH_2	3000 ± 1260	2620 ± 730	1.58 ± 0.56	4.6 ± 3.8[f]
38b	—	Cl	2-OH-5-MeO-Ph-CH_2	1110 ± 300	6870 ± 1440	4.06 ± 0.35	0.4 ± 1.3[f]
39b	—	Cl	*trans*-2-Ph-cyclopropyl	1790 ± 1430	2010 ± 890	1.30 ± 0.39	9.7 ± 4.1[f]

[a] All experiments were done on CHO or HEK (A_{2A} only) cells stably expressing one of four subtypes of human ARs. The binding affinity for A_1, A_{2A}, and A_3ARs was expressed as K_i values (n = 3–5), and was determined by using agonist radioligands ([^{3}H]CCPA or [^{3}H]R-PIA; [^{3}H]CGS21680; or [^{125}I]I-AB-MECA; respectively). A percent in parentheses refers to inhibition of radioligand binding at 10 μM.

[b] Unless noted, the efficacy at the human A_3AR was determined by inhibition of forskolin-stimulated cyclic AMP production in AR-transfected CHO cells, as described in the text. At a concentration of 10 μM, in comparison to the maximal effect of a full agonist NECA at 10 μM. Data are expressed as mean ± standard error (n = 3).

[c] Values from Ref. 11.

[d] Values from Ref. 8.

[e] Compounds **3**, MRS3771; **33b**, MRS5127; **35b**, MRS5147.

[f] A_3AR functional assay consisted of stimulation of [^{35}S]GTPγS binding at 10 μM, expressed as a percentage of the full effect induced by 10 μM NECA (100 ± 5%).

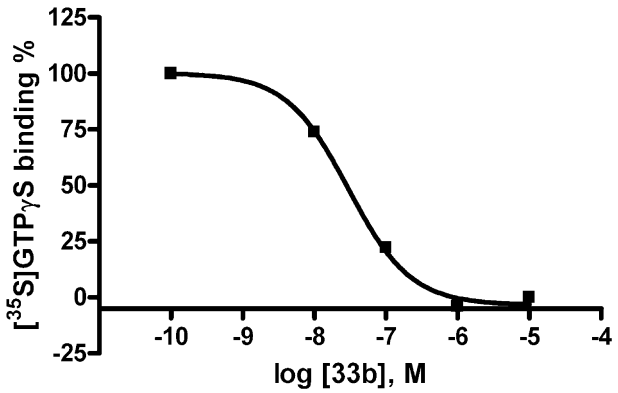


Figure 1. Functional antagonism by the 3-iodobenzyl analogue **33b** tested in an assay of guanine nucleotide binding ([^{35}S]GTPγS) in membranes of CHO cells expressing the human A_3AR.

the truncated analogues). However, the mean affinities (nM) at the human A_1AR were higher in the agonist series (610) in comparison to the truncated analogues (2500). Thus, the A_3AR selectivity in comparison to the A_1AR tended to be higher in the truncated series. The relatively close correspondence of the AR binding affinities in the agonist and truncated antagonist series is consistent with the mode of overlay of the representative ligands docked in the receptor, that is, the riboside agonist **2** and the truncated (N)-methanocarba antagonist **33b**. The moderate hydrophobicity of the truncated (N)-methanocarba analogues was similar to the corresponding agonist series (e.g., **7**–**9**) and in a favorable range for in vivo administration. For example, the *c*log*P* value of the 3-bromo truncated derivative **35b** is 1.96 in comparison to 2.17 for the 3-bromo agonist analogue.

In conclusion, a highly potent and selective series of A_3AR antagonists in the truncated (N)-methanocarba series has been described. These analogues, particularly the 3-bromobenzyl **35b** and 3-chlorobenzyl **34b** and other analogues, can now be examined in models of glaucoma and other diseases for which modulation of the A_3AR has been proposed to be useful.

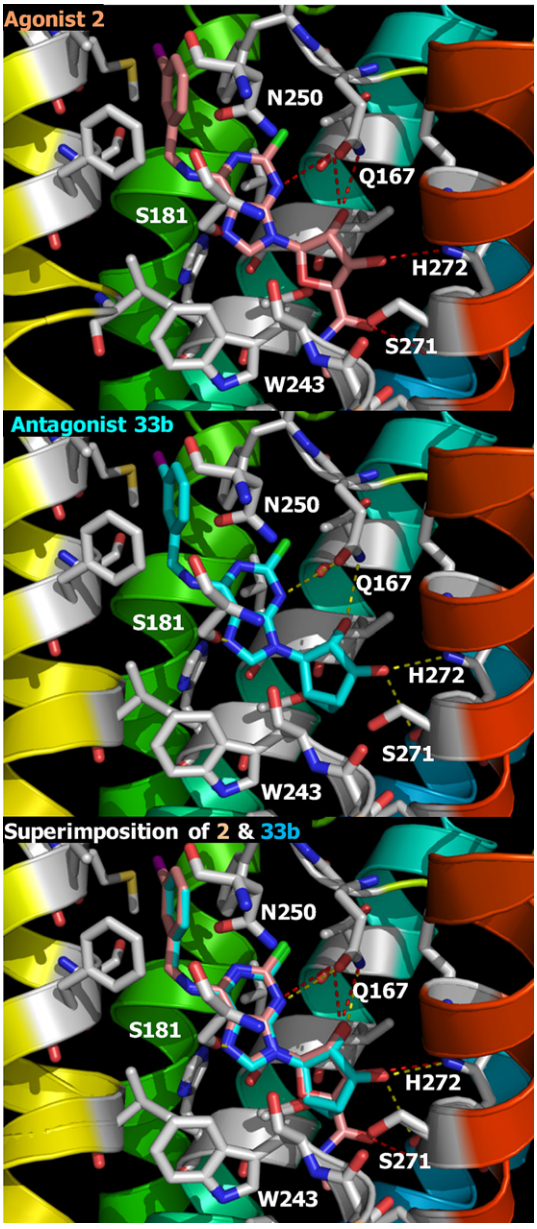


Figure 2. Docking complexes of A_3AR ligands. (A) nucleoside agonist **2**, Cl-IB-MECA in light pink color, (B) nucleoside antagonist **33b** in cyan color, and (C) the superimposition of agonist **2** and antagonist **33b**. Intermolecular H-bonding is indicated with a dotted line. Using Pymol program, all ligands are represented by stick models and the A_3AR are shown in ribbon model with different colors for each TM (TM1: purple, TM2: blue, TM3: light blue, TM4: green, TM5: yellow, TM6: orange, TM7: red).

5. Experimental

5.1. Chemical synthesis

5.1.1. Materials and instrumentation

D-ribose, L-ribose, and other reagents and solvents were purchased from Sigma–Aldrich (St. Louis, MO). Alcohol derivative **14** was prepared as reported.[10] ^{1}H NMR spectra were obtained with a Varian Gemini 300 spectrometer using $CDCl_3$ and CD_3OD as solvents. Chemical shifts are expressed in δ values (ppm) using as standards tetramethylsilane (δ 0.00) in $CDCl_3$ and CHD_2OD (δ 3.30) in CD_3OD. TLC analysis was carried out on aluminum sheets precoated with silica gel F_{254} (0.2 mm) from Aldrich. HPLC mobile phases consisted of System A: linear gradient solvent system: CH_3CN/triethyl ammonium acetate from 5:95 to 60:40 in 20 min, flow rate 1.0 mL/min; System B: linear gradient solvent system: CH_3CN/tetrabutyl ammonium phosphate from 20:80 to 60:40 in 20 min, flow rate 1.0 mL/min. Low-resolution mass spectrometry was performed with a JEOL SX102 spectrometer with 6-kV Xe atoms following desorption from a glycerol matrix or on an Agilent LC/MS 1100 MSD, with a Waters (Milford, MA) Atlantis C18 column. High resolution mass spectroscopic (HRMS) measurements were performed on a proteomics optimized Q-TOF-2 (Micromass-Waters) using external calibration with polyalanine, unless noted. Observed mass accuracies are those expected based on known performance of the instrument as well as trends in masses of standard compounds observed at intervals during the series of measurements. Reported masses are observed masses uncorrected for this time-dependent drift in mass accuracy.

5.1.2. 5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid methyl ester (10b)

The methyl ester **10a** (0.062 g, 0.1 mmol) was dissolved in MeOH (5 mL), potassium carbonate (0.028 g, 0.2 mmol) was added, and the mixture was stirred at room temperature for 10 min. Acetic acid (0.2 mL) was added to neutralize the base, the reaction mixture was concentrated under reduced pressure and subjected to preparative thin layer chromatography by using chloroform/methanol (9:1) as solvent to afford the diol **10b** as a colorless solid (0.023 g, 43%). 1H NMR ($CDCl_3$) δ 8.20 (s, 1H), 7.73 (s, 1H), 7.66 (d, 1H, 7.5 Hz), 7.29 (d, 1H, 6.5 Hz), 7.12 (t, 1H, 4.0, 2.4 Hz), 6.05 (d, 1H, 5.1 Hz), 4.70–4.82 (m, 2H),4.55–4.67 (m, 2H), 3.79 (s, 3H), HRMS calculated for $C_{18}H_{18}ClIN_5O_5^+$ $(M+H)^+$: Exact Mass: 546.0041; found, 546.0042. HPLC: RT 21.5 min (98%) in solvent system A, 10.5 min (99%) in system B.

5.1.3. 5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid methoxy-amide (11)

The methyl ester **10a** (0.031 g, 0.05 mmol) was dissolved in MeOH (5 mL), potassium carbonate (0.054 g, 0.4 mmol) was added, and the mixture was stirred at room temperature for 10 min. Methoxyamine hydrochloride (0.017 g, 0.2 mmol) was added and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was treated with acetic acid (0.3 mL) and concentrated under reduced pressure. The residue was subjected to preparative thin layer chromatography by using chloroform/methanol (8:2) as solvent to afford the diol **11** as a colorless solid (0.004 g, 16%). ^{1}H NMR (CD_3OD) δ 8.26 (s, 1H), 7.79 (s, 1H), 7.61 (d, 1H, 6.5 Hz), 7.40 (d, 1H, 6.5 Hz), 7.10 (t, 1H, 4.2, 2.4 Hz), 5.98 (d, 1H, 6.6 Hz), 4.72–4.82 (m, 2H), 4.45–4.67 (m, 2H), 3.78 (s, 3H), HRMS calculated for $C_{18}H_{19}ClIN_6O_5^+$ $(M+H)^+$: 561.0156; found 561.0150. HPLC: RT 19.4 min (98%) in solvent system A, 11.1 min (98%) in system B.

5.1.4. 5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid methoxy-methyl-amide (12)

The methyl ester **10a** (0.031 g, 0.05 mmol) was dissolved in MeOH (5 mL), potassium carbonate (0.054 g, 0.4 mmol) was added, and the mixture was stirred at room temperature for 10 min. *N,O*-Dimethylhydroxylamine hydrochloride (0.019 g, 0.2 mmol) was added and the reaction mixture was stirred at room temperature for 30 min. The reaction mixture was treated with acetic acid (0.3 mL) and concentrated under reduced pressure. The residue was subjected to preparative thin layer chromatography by using chloroform/methanol (8:2) as solvent to afford the diol **12** as a colorless solid (0.004 g, 14%). ^{1}H NMR (CD_3OD) δ 8.29 (s, 1H), 7.74 (s, 1H), 7.52 (d, 1H, 5.5 Hz), 7.36 (d, 1H, 8.5 Hz), 7.14 (t, 1H, 4.1,

2.5 Hz), 6.04 (d, 1H, 6.4 Hz), 4.62–4.80 (m, 2H), 4.42–4.62 (m, 2H), 3.58 (s, 3H), 2.68 (s, 3H) HRMS calculated for $C_{19}H_{21}ClIN_6O_5^+$ $(M+H)^+$: 575.0312; found, 575.0310. HPLC: RT 15.2 min (98%) in solvent system A, 18.5 min (99%) in system B.

5.1.5. 5-[2-Chloro-6-(3-iodo-benzylamino)-purin-9-yl]-3,4-dihydroxy-tetrahydro-furan-2-carboxylic acid (13)

The methyl ester **10a** (0.062 g, 0.1 mmol) was dissolved in EtOH (3 mL), lithium hydroxide (0.012 g, 0.5 mmol) was added, and the mixture was stirred at room temperature for 10 h. Acetic acid (0.2 mL) was added to neutralize the base, and the reaction mixture was concentrated under reduced pressure. The resulting residue was triturated with ethyl acetate (10 mL). The solid obtained was filtered and dried to afford acid **13** as a colorless solid (0.027 g, 53%). ^{1}H NMR (CD_3OD) δ 8.91 (s, 1H), 7.79 (s, 1H), 7.61 (d, 1H, 7.2 Hz), 7.39 (d, 1H, 7.2 Hz), 7.10 (t, 1H, 5.8 Hz), 6.15 (d, 1H, 6.9 Hz), 4.62–4.80 (m, 2H), 4.35–4.42 (m, 2H). HRMS calculated for $C1_{17}H_{16}ClIN_5O_5^+$ $(M+H)^+$: 531.9879; found, 531.9910. HPLC: RT 15.2 min (98%) in solvent system A, 18.0 min (95%) in system B.

5.1.6. (1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[2-Chloro-6-(4-chloro-benzylamino)-purin-9-yl]-2′,3′-dihydroxy-bicyclo[3.1.0]hexane-1-carboxylic acid ethyl ester (18)

Compound **15** (10 mg, 0.02 mmol) was treated with a solution of trifluoroacetic acid in ethanol (10% TFA, 1.0 mL) and H_2O (0.1 mL). The mixture was heated to 70 °C for 3 h. The solution was then cooled to room temperature and the solvent was removed under vacuum. The residue obtained was subjected to preparative silica gel column chromatography ($CHCl_3$/MeOH 85:15) to afford diol **(18).** (7 mg, 74%). ^{1}H NMR ($CDCl_3$) δ 7.70 (s, 1H), 7.83 (d, 1H, 8.0 Hz), 7.26 (d, 1H, 8.5 Hz), 7.14 (t, 1H, 4.2, 2.8 Hz), 5.40–5.48 (m, 1H), 4.95 (s, 2H), 4.1–4.4 (m, 3H), 3.61 (s, 1H), 2.20–2.12 (m, 1H), 1.67–1.63 (m, 1H), 1.26 (t, 3H, 8.5, 6.5 Hz), 0.97–0.93 (m, 1H). HRMS calculated for $C_{21}H_{22}Cl_2N_5O_4^+$ $(M+H)^+$: 478.1043; found 478.1042. HPLC: RT 22.1 min (98%) in solvent system A, 17.5 min (99%) in system B.

5.1.7. (1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[2-Chloro-6-(2-chloro-5-methoxy-benzylamino)-purin-9-yl]-2,3-dihydroxy-bicyclo[3.1.0]hexane-1-carboxylic acid ethyl ester (19)

This was prepared following the same procedure as for compound **(18)** starting from the isopropylidene derivative **16** to give **19** in 71% yield. ^{1}H NMR ($CDCl_3$) δ 7.63 (s, 1H), 7.28 (s, 1H), 7.12 (d, 1H, 2.4 Hz), 6.72(d, 1H, 8.7 Hz), 6.32 (bs, 1H), 5.27 (s, 1H), 4.72 (d, 2H, 27 Hz), 4.0–4.2 (m, 3H), 3.78 (s, 3H), 2.08–2.10 (m, 1H), 1.74–1.81 (m, 1H), 1.51–1.59 (m, 1H), 1.22 (t, 3H, 1.2, 6.9 Hz). HRMS calculated for $C_{22}H_{24}Cl_2N_5O_5^+$ $(M+H)^+$: 508.1154; found 508.1163. HPLC: RT 22.6 min (99%) in solvent system A, 21.2 min (100%) in system B.

5.1.8. (1′*S*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[6-(4-Chloro-benzylamino)-2-methylsulfanyl-purin-9-yl]-2,3-dihydroxy-bicyclo[3.1.0]hexane-1-carboxylic acid ethyl ester (20)

This was prepared following the same procedure as for compound **(18)** starting from the isopropylidene derivative **17** to afford **20** in 68% yield. ^{1}H NMR ($CDCl_3$) δ 7.60 (s, 1H), 7.35 (s, 1H), 7.18–7.29 (m, 3H), 6.26 (bs, 1H), 5.31–5.36 (m, 1H), 4.78–4.85 (m, 2H), 4.21–4.29 (m, 3H), 2.52 (s, 3H), 2.20–2.39 (m, 1H), 1.64–1.71 (m, 1H), 1.51–1.59 (m, 1H), 1.26–1.31 (m, 4H). HRMS calculated for $C_{22}H_{25}ClN_5O_4S^+$ $(M+H)^+$: 490.1316; found 490.1299. HPLC: RT 23.45 min (97%) in solvent system A, 21.2 min (98%) in system B.

5.1.9. Ethyl (1*S*,2*R*,3*S*,4*S*,5*S*)-2,3-*O*-(isopropylidene)-4-*O*-benzyl-2,3,4-trihydroxybicyclo[3.1.0]hexanecarboxylate (24)

Alcohol **14** (240 mg, 1.00 mmol) was dissolved in 5.0 mL of a DMF/THF (4:1) solution. The solution was cooled to −70 °C under nitrogen, and 60% NaH in mineral oil (45.0 mg, 1.10 mmol) was added. The reaction mixture was stirred at −70 °C for 5 min and then allowed to warm to 0 °C. Benzyl bromide (130 μl, 1.10 mmol) was added to the mixture. The mixture was stirred for 5 min at 0 °C, allowed to warm to room temperature, and then stirred under nitrogen gas for an additional 16 h. The solvent was removed under vacuum. The resulting orange oil was dissolved in 50 mL ethyl acetate, washed with 3× 50 mL water, and dried over Na_2SO_4. The extract was concentrated to a pale yellow-orange oil and purified by column chromatography (silica gel, ethyl acetate/hexanes 1:9) to provide **24** (220 mg, 66%) as a colorless oil. ^{1}H NMR ($CDCl_3$): 7.42–7.27 (m, 5H), 5.26 (d, 1H, 6.7 Hz), 4.69 (s, 2H), 4.56 (t, 1H, 6.7 Hz), 4.25–4.05 (m, 4H), 2.31–2.29 (m, 1H), 1.85 (t, 1H, 4.5 Hz), 1.59 (s, 3H), 1.54–1.50 (m, 1H), 1.31 (s, 3H), 1.28–1.22 (m, 4H). HRMS calculated for $C_{19}H_{24}NaO_5^+$ $(M+Na)^+$: 355.1521; found, 355.1482.

5.1.10. (1*S*,2*R*,3*S*,4*S*,5*S*)-2,3-*O*-(Isopropylidene)-4-*O*-benzyl-2,3,4-trihydroxybicyclo[3.1.0]-hexanecarboxylic acid (25)

A mixture of 1.50 mL of 6 N KOH(aq) and **24** (220 mg, 0.66 mmol) in a sealed vial was heated to 80 °C and stirred for 6 h. The mixture was cooled to 0 °C and diluted with 10 mL of ice water. The mixture was neutralized upon the dropwise addition of 0.50 mL glacial acidic acid. The neutralized solution was extracted with 3 × 10 mL ethyl acetate. The extracts were combined and dried over Na_2SO_4. The solvent was removed under vacuum and the resulting carboxylic acid **25** was used without further purification (200 mg, 99%). ^{1}H NMR ($CDCl_3$) 7.42–7.27 (m, 5H), 5.21 (d, 1H, 6.7 Hz), 4.69 (s, 2H), 4.56 (t, 1H, 6.7 Hz), 4.23 (t, 1H, 5.0 Hz) 2.31–2.40 (m, 1H), 1.91 (t, 1H, 4.5 Hz), 1.58 (s, 3H), 1.54–1.50 (m, 1H), 1.31 (s, 3H). HRMS calculated for $C_{18}H_{21}O_7^-$ $(M+HCO_2)^-$: 349.1287; found 349.1287.

5.1.11. (1*R*,2*R*,3*S*,4*S*,5*S*)-2,3-*O*-(Isopropylidene)-4-*O*-benzyl-2,3,4-trihydroxybicyclo-[3.1.0]hexane (26)

Carboxylic acid **25** (200 mg, 0.66 mmol) was dissolved in 15.0 mL of dry toluene. The solution was purged of oxygen by 3 cycles of freeze and thaw under vacuum. 2,2′-Dithiopyridine-1,1′-dioxide (199 mg, 0.79 mmol), Bu_3SnH (956 mg, 3.30 mmol), and Bu_3P (332 mg, 1.65 mmol) were added to the solution. The reaction mixture was covered with foil and stirred at room temperature under nitrogen gas for 3 h. Over the course of 3 h, the reaction mixture turned slightly yellow. At the end of 3 h, AIBN (54.0 mg, 0.33 mmol) was added to the mixture. The reaction mixture was then heated to 90 °C for an additional hour and the yellow color disappeared. The reaction mixture was cooled to room temperature. The reaction mixture was diluted with 30 mL H_2O and extracted with 3 × 20 mL ethyl acetate. The extract was dried over Na_2SO_4 and the solvent was removed under vacuum. The crude oil was purified with silica preparative TLC using 20% ethyl acetate/hexanes to give **26** (85.0 mg, 49%). ^{1}H NMR ($CDCl_3$) 7.42–7.27 (m, 5H), 4.82 (t, 1H, 6.0 Hz), 4.69 (s, 2H), 4.50 (t, 1H, 6.7 Hz), 4.30 (t, 1H, 5.0 Hz), 1.70–7.75 (m, 1H), 1.60 (s, 3H), 1.31–1.26 (m, 4H), 0.65–0.60 (m, 1H). HRMS calculated for $C_{16}H_{24}NO_3^+$ $(M+NH_4)^+$: 278.1756; found 278.1752.

5.1.12. (1*R*,2*R*,3*S*,4*S*,5*S*)-2,3-*O*-(Isopropylidene)-2,3,4-trihydroxybicyclo[3.1.0]hexane (27), Method A

Benzyl ether **26** (50.0 mg, 0.33 mmol) was dissolved in a mixture of 2.0 mL anhydrous methanol and 0.10 mL formic acid. The solution was stirred in an ice bath and palladium black (8.50 mg, 10% w/w) was added. The solution was carefully purged with H_2(g) and fitted with a balloon filled with H_2(g). The reaction mixture was heated to 50 °C and stirred for 16 h. The H_2(g) was discharged and the reaction mixture filtered through a nylon syringe filter. The filter was washed with 10 mL of methanol and

the filtrate was dried under vacuum. The resulting oil was purified on a silica preparative TLC plate with 30% ethyl acetate/hexanes as eluant to give **27** as a homogeneous product (15.0 mg, 27%). ^{1}H NMR ($CDCl_3$) 4.88 (t, 1H, 4.2 Hz), 4.53 (bs, 1H), 4.48 (t, 1H, 6.7 Hz), 4.30 (t, 1H, 5.0 Hz), 2.40 (br s, 1H), 1.87–1.83 (m, 1H), 1.67–1.63 (m, 1H), 1.55 (s, 3H), 1.29 (s, 3H), 0.99–0.95 (m, 1H), 0.68–0.60 (m, 1H). HRMS calculated for $C_9H_{14}O_3$ $(M)^+$: 170.0943; found, 170.0942.

5.1.13. (1′*R*,2′*R*,3′*S*,4′*S*,5′*S*)-4′-[(2,6-Dichloropurine)-2′,3′-*O*-(isopropylidene)-2′,3′-dihydroxybicyclo[3.1.0]hexane (32)

A mixture of triphenyl phosphine (46 mg, 0.176 mmol) and 2,6-dichloropurine (33 mg, 0.176 mmol) in dry THF (2.0 mL) was treated with diisopropylazodicarboxylate (36 mg, 0.176 mmol). The mixture was stirred at room temperature for 20 min. The mixture was then added to a stirring solution of **27** (15 mg, 0.088 mmol) dissolved in dry THF (0.50 mL). The mixture was stirred for an additional 8 h at room temperature. The solvent was evaporated, and the crude oil was purified with silica preparative TLC in 30% ethyl acetate/hexanes solution to give **32** (15 mg, 50%). ^{1}H NMR ($CDCl_3$) 8.15 (s, 1H), 5.38 (t, 1H, 4.2 Hz), 5.06 (s, 1H), 4.67 (d, 1H, 6.7 Hz), 2.20–2.15 (m, 1H), 1.67–1.63 (m, 3H), 1.56 (s, 3H), 1.26 (s, 3H), 1.03–0.97 (m, 3H). HRMS calculated for $C_{14}H_{14}Cl_2N_4O_2$ $(M)^+$: 340.0494; found, 340.0495.

5.1.14. (1*R*,2*S*,3*R*,4*R*,5*R*)-3,4-*O*-(Isopropylidene)-2-*O*-(*tert*-butyldiphenylsilyl)-2,3,4-trihydroxybicyclo[3.1.0]hexane-1-carboxylic acid (30)

tert-Butyldiphenylsilyl chloride (2.70 g, 10 mmol) and triethylamine (2.0 g, 20 mmol) were added to a solution of alcohol **29** (prepared from D-ribose **28** following the standard procedure,[13] 1.22 g, 5 mmol) and imidazole (140 mg, 2 mmol) in DMF (3 mL) while stirring at room temperature. The solution was stirred at 60 °C for 16 h. The reaction mixture was cooled to room temperature and diluted with a 4:1 ethyl acetate/hexane mixture (50 mL), washed with water, dried, and solvent was evaporated. The residue was purified by flash chromatography (0–10% ethyl acetate–hexane) to give ethyl (1*R*,2*S*,3*R*,4*R*,5*R*)-2,3-*O*-(isopropylidene)-4-*O*-(*tert*-butyldiphenylsilyl)-2,3,4-trihydroxybicyclo[3.1.0]-hexane-1-carboxylate. The compound was dissolved in MeOH (5 mL) and 2 N aq NaOH (5 mL) was added, and the reaction mixture was refluxed for 2 h. The reaction mixture was neutralized with NaH_2PO_4, and extracted with DCM. The combined DCM solutions were dried and evaporated, and the residue was purified by flash chromatography to give title compound **30** (1.65 g, 73%). ^{1}H NMR ($CDCl_3$), δ 7.72 (d, 4H, J = 7.8 Hz), 7.39 (m, 6H), 5.05 (d, 1H, J = 6.3 Hz), 4.43 (t, 1H, J = 6.0 Hz), 4.08 (t, 1H, J = 6.6 Hz), 2.26 (m, 1H), 1.97 (s, 3H), 1.56 (s, 3H), 1.52 (m, 1H), 1.21 (s, 3H), 1.08 (s, 9H).

5.1.15. (1*S*,2*S*,3*R*,4*R*,5R)-3,4-*O*-(Isopropylidene)-2-*O*-(*tert*-butyldiphenylsilyl)-2,3,4-trihydroxybicyclo[3.1.0]hexane (31)

A 1M solution of DCC in oxygen-free toluene (0.96 mL) was added to a solution of acid **30** (363 mg, 0.80 mmol), 2-mercaptopyridine *N*-oxide (112 mg, 0.88 mmol), and AIBN (40 mg, 0.24 mmol) in dry oxygen-free toluene (4 mL). The reaction mixture was stirred for 4 h at 25 °C, tris(trimethylsilyl)silane (0.50 mL, 1.6 mmol) was added, and the reaction mixture was heated at 85 °C for 4 h. The reaction mixture was evaporated, and the residue was separated by flash chromatography (0–10% ethyl acetate–hexane mixture) to afford the title compound **31** (121 mg, 40%). ^{1}H NMR ($CDCl_3$), δ 7.76 (d, 4H, J = 7.8 Hz), 7.39 (m, 6H), 4.66 (t, 1H, J = 6.0 Hz), 4.44 (t, 1H, J = 6.6 Hz), 4.03 (t, 1H, J = 6.6 Hz), 1.6 (m, 1H), 1.57 (s, 3H), 1.45 (m, 1H), 1.33 (s, 1H), 1.20 (s, 3H), 1.09 (s, 9H), 0.58 (m, 1H).

5.1.16. (1*R*,2*R*,3*S*,4*S*,5S)-2.3-*O*-(Isopropylidene)-2,3,4-trihydroxy-bicyclo[3.1.0]hexane (27), Method B

A 1M solution of *tert*-butylammonium fluoride in THF (1 mL) was added to a solution of silyl ether **31** (102 mg, 0.25 mmol) in THF (1 mL). The reaction mixture was left at 20 °C for 16 h and evaporated. The residue was diluted with ethyl acetate (20 mL) and washed with a small amount of brine. The ethyl acetate solution was dried and evaporated, and the residue was purified by flash chromatography to afford the title compound **27** (33 mg, 84%). ^{1} H NMR and MS are provided under Method A.

5.1.17. General procedure for preparation of compounds 33b–39b

An amine (RNH_2 in Scheme 3 and 0.5 mmol) was added to a solution of **32** (20 mg, 0.06 mmol) in DCM (0.1 mL). The reaction mixture was stirred at room temperature for 16 h. The solvent was removed under vacuum, and the residue was separated by flash chromatography (30–100% ethyl acetate–hexane) to afford the corresponding 6-alkylaminopurine derivative that was dissolved in a mixture of MeOH (4 mL), TFA (0.2 mL), and water (2 mL). The reaction mixture was stirred at 70 °C for 16 h, and then evaporated. The residue was evaporated twice with water, and the residue was purified by flash chromatography (50–100% ethyl acetate).

5.1.18. (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-Chloro-6-(3-iodobenzylamino)-purine]-2′,3′-*O*-dihydroxybicyclo-[3.1.0]hexane (33b)

Yield 15 mg (51% ^{1}H NMR (CD_3OD), δ 8.16 (s, 1H), 7.49 (s, 1H), 7.60 (d, 1H, 8.5 Hz), 7.40 (d, 1H, 8.5 Hz), 7.10 (t, 1H, 8.5 Hz), 4.71 (s, 2H), 3.90 (d, 3.3 Hz, 1H), 3.65 (s, 1H), 2.05–1.95 (m, 1H), 1.67–1.63 (m, 1H), 1.36 (s, 1H), 1.31–1.27 (m, 1H), 0.95–0.87 (m, 1H), 0.77–0.75 (m, 1H). HRMS calculated for $C_{18}H_{18}ClIN_5O_2^+$ $(M+H)^+$: 498.0194; found, 498.0194. HPLC: RT 21.6 min (98%) in solvent system A, 17.0 min (98%) in system B.

5.1.19. (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-Chloro-6-(3-chlorobenzylamino)purine]-2′,3′-*O*-dihydroxybicyclo-[3.1.0]hexane (34b)

Yield 58%. ^{1}H NMR (CD_3OD), δ 8.16 (br s, 1H), 7.41 (s, 1H), 7.29 (m, 3H), 4.79 (s, 1H), 4.75 (br s, 2H), 4.70 (br t, 1H, J = 5.4 Hz), 3.86 (d, 1H, J = 6.6 Hz), 1.97 (m, 1H), 1.65 (m, 1H), 1.30 (m, 1H), 0.75 (m, 1H). HRMS (ESI MS m/z): calculated for $C_{18}H_{18}Cl_2N_5O_2^+$ $(M+H)^+$, 406.0832; found, 406.0825. HPLC RT 20.3 min (98%) in solvent system A, 15.6 min (98%) in system B.

5.1.20. (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-Chloro-6-(3-bromobenzylamino)-purine]-2′,3′-*O*-dihydroxybicyclo-[3.1.0]hexane (35b)

Yield 65%. ^{1}H NMR (CD_3OD): 8.03 (s, 1H), 7.45 (s, 1H), 7.29 (m, 2H), 7.12 (t, 1H, J = 7.8 Hz), 4.68 (s, 1H), 4.63 (br s, 2H), 4.59 (br t, 1H, J = 5.4 Hz), 3.79 (d, 1H, J = 6.6 Hz), 1.86 (m, 1H), 1.55 (m, 1H), 1.20 (m, 1H), 0.64 (m, 1H). HRMS (ESI MS m/z) calculated for $C_{18}H_{18}BrClN_5O_2^+$ $(M+H)^+$, 450.0327; found 450.0315. HPLC RT 20.74 min (98%) in solvent system A, 16.1 min (99%) in system B.

5.1.21. (1′*R*,2′*R*,3′*S*,4′*R*, 5′*S*)-4′-[2-Chloro-6-(1-naphthylamino)purine]-2′,3′-*O*-dihydroxybicyclo[3.1.0]hexane (36b)

Yield 48%. ^{1}H NMR (CD_3OD): 8.13 (br d, 2H, J = 7.8 Hz), 7.84 (m, 2H), 7.49 (m, 4 H), 5.21 (s, 1H), 4.79 (br s, 1H), 4.78 (br s, 2H), 4.67 (br t., 1H, J = 5.1 Hz), 3.88 (d, 1H, J = 6.6 Hz), 1.93 (m, 1H), 1.62 (m, 1H), 1.25 (m, 1H), 0.73 (m, 1H). HRMS (ESI MS *m/z*) calculated for $C_{22}H_{21}ClN_5O_2^+$ $(M+H)^+$, 422.1378; found 422.1385. HPLC RT 21.5 min (97%) in solvent system A, 17.0 min (98%) in system B.

5.1.22. (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-Chloro-6-(2,5-dimethoxybenzylamino)purine]-2′,3′-*O*-dihydroxybicyclo-[3.1.0]hexane (37b)

Yield 44%. ^{1}H NMR (CD_3OD): 8.4 (very br s, 1H), 6.95 (s, 1H, J = 2.7 Hz), 6.89 (d, 1H, J = 9.3 Hz), 6.78 (dd, 1H, J = 2.7, 9.0 Hz), 4.80 (s, 1H), 4.75 (br m, 3H), 3.87 (d, 1H, J = 6.3 Hz), 3.83 (s, 3H), 3.71 (s, 3H), 1.95 (m, 1H), 1.64 (m, 1H), 1.29 (m, 1H), 0.74 (m, 1H). HRMS (ESI MS m/z) calculated for $C_{20}H_{23}ClN_5O_4^+$ $(M+H)^+$, 432.1433; found 432.1439. HPLC RT 18.7 min (98%) in solvent system A, 16.6 min (98%) in system B.

5.1.23. (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-Chloro-6-(2-hydroxy-5-methoxybenzylamino)purine]-2′,3′-*O*-dihydroxybicyclo-[3.1.0]hexane (38b)

Yield 39%. ^{1}H NMR (CD_3OD): 8.07 (s, 1H), 6.60–6.82 (m, 3H), 4.69 (s, 1H), 4.59 (br t., 1H, J = 6.0 Hz), 4.56 (br s, 2H), 3.79 (d, 1H, J = 6.6 Hz), 3.61 (s, 3H) 1.86 (m, 1H), 1.55 (m, 1H), 1.20 (m, 1H), 0.65 (m, 1H). HRMS (ESI MS m/z) calculated for $C_{19}H_{21}ClN_5O_4^+$ $(M+H)^+$, 418.1277; found, 418.1277. HPLC RT 16.0 min (100%) in solvent system A, 11.0 min (98%) in system B.

5.1.24. (1′*R*,2′*R*,3′*S*,4′*R*,5′*S*)-4′-[2-Chloro-6-(*trans*-2-phenylcyclopropylamino)purine]-2′,3′-*O*-dihydroxybicyclo-[3.1.0]hexane (39b)

Yield 52%. ^{1}H NMR (CD_3OD): 8.16 (very br s, 1H), 7.0–7.48 (m, 5H), 4.79 (s, 1H), 4.68 (br s, 2H), 3.88 (d, 1H, J = 5.7 Hz), 2.17 (m, 1H) 1.97 (m, 1H), 1.65 (m, 1H), 1.29 (m, 2H), 0.74 (m, 1H). HRMS (ESI MS m/z) calculated for $C_{20}H_{21}ClN_5O_2^+$ $(M+H)^+$, 398.1378; found, 398.1372. HPLC RT 20.3 min (99%) in solvent system A, 15.6 min (98%) in system B.

6. Molecular modeling

6.1. Docking study

The homology model of the hA_3AR (PDB id: 1OEA) derived using the X-ray structure of bovine rhodopsin with a 2.8 Å resolution (PDB id: 1F88) as a template was used for the docking study. The previously reported A_3AR conformation,[17] which was bound to Cl-IB-MECA, **2**, was used for the antagonist docking. For the docking study of compound **33b**, a pharmacophore match among hydrophilic interaction sites, for the 2′-OH, 3′-OH, and N^6H groups, was performed to obtain an initial geometry. The refinement of the side chain in the binding site was followed by binding site minimization with fixed backbone atoms, complex minimization, and 200 ps molecular dynamics.

Atomic charges were recalculated by Mullekin charge using the DFT/6311G** basis set for ligands **2** and **33b**. Finally, the complex structure was minimized using an Amber 7 force field 99 with a fixed dielectric constant (4.0), until the conjugate gradient reached 0.1 kcal mol^{-1} Å^{-1}. Values of clog P were calculated using ChemDraw Ultra 11.0.

6.2. Receptor binding and functional assays

[^{125}I]N^6-(4-Amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide (I-AB-MECA; 2000 Ci/mmol), [^{3}H]cyclic AMP (40 Ci/mmol), and other radioligands were purchased from Perkin–Elmer Life and Analytical Science (Boston, MA). [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) was a custom synthesis product (Perkin-Elmer). Test compounds were prepared as 5 mM stock solutions in DMSO and stored frozen.

Cell culture and membrane preparation: CHO (Chinese hamster ovary) cells expressing the recombinant human A_3AR were cultured in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 μmol/mL glutamine, and 800 μg/mL geneticin. The CHO cells expressing rat A_3ARs were cultured in DMEM and F12 (1:1). Cells were harvested by trypsinization. After homogenization and suspension, cell membranes were centrifuged at 500 g for 10 min, and the pellet was re-suspended in 50 mM Tris HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 1 mM EDTA, and 0.1 mg/mL CHAPS (3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid). The suspension was homogenized with an electric homogenizer for 10 s, and was then re-centrifuged at 20,000*g* for 20 min at 4 °C. The resultant pellets were resuspended in buffer in the presence of adenosine deaminase (3 U/mL), and the suspension was stored at −80 °C until the binding experiments. The protein concentration was measured using the Bradford assay.[18]

6.2.1. Binding assays at the A_1 and A_{2A} receptors

For binding to human A_1 receptors,[19] [^{3}H]R-PIA (N^6-[(R)-phenylisopropyl]adenosine, 2 nM) or [^{3}H]CCPA (0.5 nM) was incubated with membranes (40 μg/tube) from CHO cells stably expressing human A_1 receptors at 25 °C for 60 min in 50 mM Tris–HCl buffer (pH 7.4; $MgCl_2$, 10 mM) and increasing concentrations of the test ligand in a total assay volume of 200 μl. Nonspecific binding was determined using 10 μM of CPA (N^6-cyclopentyladenosine). For human A_{2A} receptor binding,[20] membranes (20 μg/tube) from HEK-293 cells stably expressing human A_{2A} receptors were incubated with [^{3}H]CGS21680 (2-[*p*-(2-carboxyethyl)phenyl-ethylamino]-5′-*N*-ethylcarboxamido-adenosine, 15 nM) and increasing concentrations of the test ligand at 25 °C for 60 min in 200 μl 50 mM Tri–HCl, pH 7.4, containing 10 mM $MgCl_2$. NECA (10 μM) was used to define nonspecific binding. The reaction was terminated by filtration with GF/B filters.

6.2.2. Binding assay at the human A_3 receptor

For the competitive binding assay, each tube contained 50 μL membrane suspension (20 μg protein), 25 μL of [^{125}I]I-AB-MECA (1.0 nM),[21] and 25 μL of increasing concentrations of the test ligands in Tris–HCl buffer (50 mM, pH 8.0) containing 10 mM $MgCl_2$, and 1 mM EDTA. Nonspecific binding was determined using 10 μM of Cl-IB-MECA in the buffer. The mixtures were incubated at 37 °C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using a MT-24 cell harvester (Brandell, Gaithersburgh, MD, USA). Filters were washed three times with 9 mL ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. IC_{50} values were converted to K_i values as described.[25]

6.2.3. Cyclic AMP accumulation assay

Intracellular cyclic AMP levels were measured with a competitive protein binding method.[22,23] CHO cells that expressed the recombinant human or rat A_3AR or the human A_1 or $A_{2B}AR$ were harvested by trypsinization. After centrifugation and resuspended in medium, cells were planted in 24-well plates in 1.0 mL medium. After 24 h, the medium was removed, and cells were washed three times with 1 mL DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated with the agonist NECA and/or test compound (e.g., 33b) in the presence of rolipram (10 μM) and adenosine deaminase (3 U/mL). 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 ice-cold HCl. The cell lysate was resuspended and stored at −20 °C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl or 50 μL of cyclic AMP solution (0–16 pmol/200 μL for standard curve). Bound radioactivity was separated by

rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation spectrometry.

6.2.4. [^{35}S]GTPγS binding assay

[^{35}S]GTPγS binding was measured by a variation of the method described.[24] Each assay tube consisted of 200 μL buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 μM GDP, 1 mM dithiothreitol, 100 mM NaCl, 3 U/ml ADA, 0.2 nM [^{35}S]GTPγS, 0.004% 3-[(3-cholamidopropyl) dimethylammonio]propanesulfonate (CHAPS), and 0.5% bovine serum albumin. Incubations were started upon addition of the membrane suspension (CHO cells stably expressing either the native human A_1AR or A_3AR, 5 μg protein/tube) to the test tubes, and they were carried out in duplicate for 30 min at 25 °C. The reaction was stopped by rapid filtration through Whatman GF/B filters, pre-soaked in 50 mM Tris–HCl, 5 mM $MgCl_2$ (pH 7.4) containing 0.02% CHAPS. The filters were washed twice with 3 mL of the same buffer, and retained radioactivity was measured using liquid scintillation counting. Nonspecific binding of [^{35}S]GTPγS was measured in the presence of 10 μM unlabeled GTPγS.

Acknowledgments

We thank Dr. John Lloyd and Dr. Noel Whittaker (NIDDK) for mass spectral determinations. This research was supported by the Intramural Research Program of the NIH, National Institute of Diabetes and Digestive and Kidney Diseases.

References and notes

1. Jacobson, K. A.; Gao, Z. G. *Nat. Rev. Drug Disc.* **2006**, *5*, 247.
2. Fishman, P.; Jacobson, K. A.; Ochaion, A.; Cohen, S.; Bar-Yehuda, S. *Immunol. Endocr. Metabol. Agents Med. Chem.* **2007**, *7*, 298.
3. Avila, M. Y.; Stone, R. A.; Civan, M. M. *Invest. Ophthalmol. Vis. Sci.* **2002**, *43*, 3021.
4. Young, H. W.; Molina, J. G.; Dimina, D.; Zhong, H.; Jacobson, M.; Chan, L. N.; Chan, T. S.; Lee, J. J.; Blackburn, M. R. *J. Immunol.* **2004**, *173*, 1380.
5. Moro, S.; Deflorian, F.; Bacilieri, M.; Spalluto, G. *Curr. Med. Chem.* **2006**, *13*, 639.
6. (a) Joshi, B. V.; Jacobson, K. A. *Curr. Topics Med. Chem.* **2005**, *5*, 1275; (b) Kim, H. O.; Ji, X.-d.; Siddiqi, S. M.; Olah, M. E.; Stiles, G. L.; Jacobson, K. A. *J. Med. Chem.* **1994**, *37*, 3614; (c) Gao, Z. G.; Mamedova, L. K.; Chen, P.; Jacobson, K. A. *Biochem. Pharmacol.* **2004**, *68*, 1985.
7. Yang, H.; Avila, M. Y.; Peterson-Yantorno, K.; Coca-Prados, M.; Stone, R. A.; Jacobson, K. A.; Civan, M. M. *Curr. Eye Res.* **2005**, *30*, 747.
8. (a) Gao, Z. G.; Kim, S. K.; Biadatti, T.; Chen, W.; Lee, K.; Barak, D.; Kim, S. G.; Johnson, C. R.; Jacobson, K. A. *J. Med. Chem.* **2002**, *45*, 4471; (b) Gao, Z.-G.; Joshi, B. V.; Klutz, A. M.; Kim, S.-K.; Lee, H. W.; Kim, H. O.; Jeong, L. S.; Jacobson, K. A. *Bioorg. Med. Chem. Lett.* **2006**, *16*, 596.
9. Kim, S. K.; Jacobson, K. A. *J. Chem. Inf. Model.* **2007**, *47*, 1225.
10. Jeong, L. S.; Choe, S. A.; Gunaga, P.; Kim, H. O.; Lee, H. W.; Lee, S. K.; Tosh, D.; Patel, A.; Palaniappan, K. K.; Gao, Z. G.; Jacobson, K. A.; Moon, H. R. *J. Med. Chem.* **2007**, *50*, 3159.
11. (a) Tchilibon, S.; Joshi, B. V.; Kim, S. K.; Duong, H. T.; Gao, Z. G.; Jacobson, K. A. *J. Med. Chem.* **2005**, *48*, 1745; (b) Lee, K.; Ravi, R. G.; Ji, X.-d.; Marquez, V. E.; Jacobson, K. A. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 1333.
12. Kim, H. O.; Hawes, C.; Towers, P.; Jacobson, K. A. *J. Label. Comp. Radiopharm.* **1996**, *38*, 547.
13. (a) Joshi, B. V.; Melman, A.; Mackman, R. L.; Jacobson, K. A. *Nucleosides Nucleotides Nucleic Acids* **2008**, *27*, 279; (b) Joshi, B. V.; Moon, H. R.; Fettinger, J. C.; Marquez, V. E.; Jacobson, K. A. *J. Org. Chem.* **2005**, *70*, 439.
14. (a) Barton, D. H. R.; Samadi, M. *Tetrahedron* **1992**, *48*, 7083; (b) Gawronska, K.; Gawronski, J.; Walborsky, H. M. *J. Org. Chem.* **1991**, *56*, 2193.
15. Yamaguchi, K.; Kazuta, Y.; Abe, H.; Matsuda, A.; Shuto, S. *J. Org. Chem.* **2003**, *68*, 9255.
16. Chatgilialoglu, C. *Acc. Chem. Res.* **1992**, *25*, 188–194.
17. (a) Kim, S.-K.; Gao, Z.-G.; Van Rompaey, P.; Gross, A. S.; Chen, A.; Van Calenbergh, S.; Jacobson, K. A. *J. Med. Chem.* **2003**, *46*, 4847; (b) Kim, S.-K.; Gao, Z.-G.; Jeong, L. S.; Jacobson, K. A. *J. Mol. Graphics Model* **2006**, *25*, 562.
18. Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
19. (a) Schwabe, U.; Trost, T. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1980**, *313*, 179; (b) Perreira, M.; Jiang, J. K.; Klutz, A. M.; Gao, Z. G.; Shainberg, A.; Lu, C.; Thomas, C. J.; Jacobson, KA. *J. Med. Chem.* **2005**, *48*, 4910.
20. Jarvis, M. F.; Schutz, R.; Hutchison, A. J.; Do, E.; Sills, M. A.; Williams, M. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 888–893.
21. Olah, M. E.; Gallo-Rodriguez, C.; Jacobson, K. A.; Stiles, G. L. *Mol. Pharmacol.* **1994**, *45*, 978.
22. Nordstedt, C.; Fredholm, B. B. *Anal. Biochem.* **1990**, *189*, 231.
23. Post, S. R.; Ostrom, R. S.; Insel, P. A. *Methods Mol. Biol.* **2000**, *126*, 363.
24. (a) Lorenzen, A.; Lang, H.; Schwabe, U. *Biochem. Pharmacol.* **1998**, *56*, 1287; (b) Jacobson, K. A.; Ji, X.-d.; Li, A. H.; Melman, N.; Siddiqui, M. A.; Shin, K. J.; Marquez, V. E.; Ravi, R. G. *J. Med. Chem.* **2000**, *43*, 2196.
25. Cheng, Y.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099.