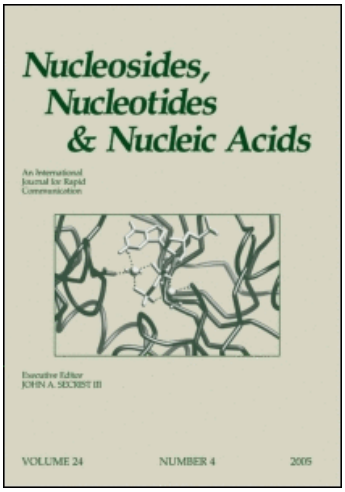

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PURINE-SUBSTITUTED ADENOSINE DERIVATIVES WITH SMALL N^6-SUBSTITUENTS AS ADENOSINE RECEPTOR AGONISTS

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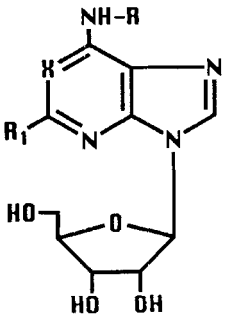
Abstract. Adenosine, 6-hydroxylaminopurineriboside (HAPR), N^6-methyladenosine and various derivatives of which the synthesis is described, were evaluated as adenosine receptor ligands in radioligand binding studies to probe the relative importance for affinity of small N^6-substituents. The findings were incorporated in a recently developed three-dimensional model for the adenosine A_1 receptor, rationalising the more or less equal contribution to affinity of such different substituents as -OH and -CH_3.

INTRODUCTION

Adenosine (FIG. 1) is the endogenous ligand for so-called P_1-purinoceptors, or adenosine receptors. A further subdivision of this receptor class has been made. At least three subtypes exist, *viz.* A_1, A_2, and A_3. Structure-activity relationships of adenosine derivatives for A_1 and A_2 receptors have been studied extensively. They show that bulky, hydrophobic N^6-substituents can enhance affinity and selectivity dramatically.[1] This information has been used to generate a computer graphics model for the so-called N^6-region of the adenosine receptor.[2] In the latter study it was concluded that the region closest to N^6 itself



No	X	R	R_1
1	N	H	H
2	N	H	Cl
3	CH	H	H
4	CH	H	Cl
5	N	OH	H
6	N	OH	Cl
7	CH	OH	H
8	CH	OH	Cl
9	N	CH_3	H

FIG. 1. Chemical structures of adenosine and derivatives examined in radioligand binding studies in this study

could not be properly evaluated due to a lack of adenosine derivatives with small substituents.

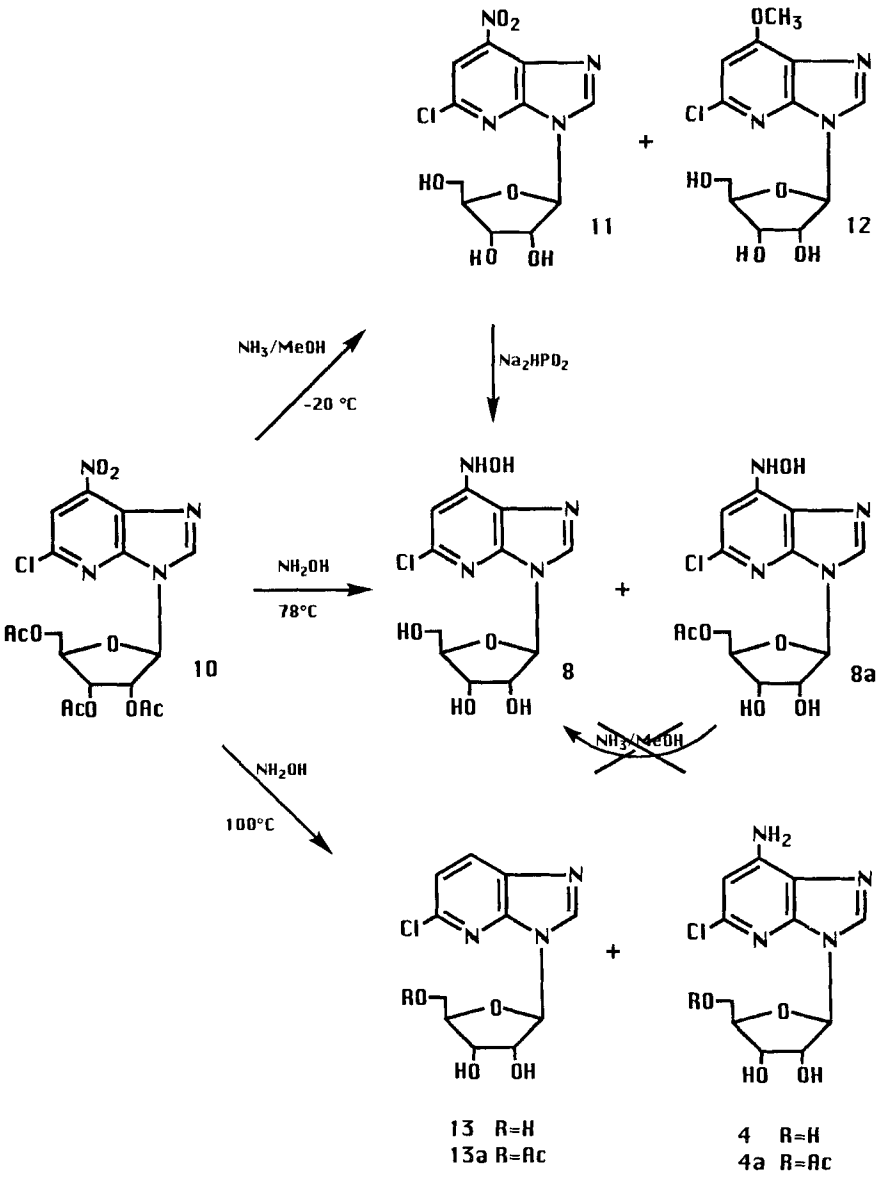
In the present study we analysed the interaction of adenosine (**1**, FIG. 1) and derivatives of adenosine and 1-deazaadenosine (**2-9**, FIG. 1), bearing a hydroxyl or methyl group on N^6, with both A_1 and A_2 receptors. First, an alternative synthetic route for one of the compounds, 5-chloro-7-hydroxylamino-3-ß-D-ribofuranosyl-3H-imidazo-[4,5-b]pyridine (2-chloro-1-deaza-HAPR, **8**) was developed. Second, data from radioligand binding studies were integrated with a recently developed three-dimensional model for the adenosine A_1 receptor,[3] rationalising the effects of small substituents. An interesting finding in this study was that HAPR, an antitumor drug, and its derivatives possess significant affinity for adenosine receptors.

CHEMISTRY

The synthesis of 5-chloro-7-hydroxylamino-3-β-D-ribofuranosyl-3H--imidazo[4,5-b]pyridine (**8**)[4] was previously accomplished by reduction of

5-chloro-7-nitro-3-β-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine (**11**)[5] with sodium hypophosphite and 5% Pd/C in THF. Compound **11** was obtained by deacetylation of the blocked nucleoside **10** in methanolic ammonia at -20 °C for 4 h. In order to avoid the formation of the corresponding 7-methoxy derivative **12** during the deprotection reaction, an alternative route was carried out by substituting directly the nitro group of compound **10** with an excess of ethanolic hydroxylamine at 78 °C for 4 hour (Scheme I). Purification of the reaction mixture by flash chromatography afforded the desired compound **8** in a 52% total yield and the monoacetylated derivative **8a**. Attempts to remove the 5'- acetyl blocking group of **8a**, even at -20 °C, were unsuccessful, leading to a mixture of products. Moreover, another attempt to fully transform **8a** in **8** during the reaction, by heating the mixture in a steel bomb at 100 °C for 48 h, gave an unexpected result. Purification of the reaction mixture by preparative thin layer chromatography afforded four nucleosides which were identified by their ^{1}H NMR spectra as 5-chloro-3-ß-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine (**13**), 7-amino-5-chloro-3-ß-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine (**4**),[5] and the corresponding 5'-acetylated derivatives **13a** and **4a**. A possible explanation is that, under the conditions of temperature and pressure described, the ethanolic hydroxylamine behaved as a reductive system by substituting the nitro group of **10** with a hydride to give compounds **13** and **13a**. The formation of **4** and **4a** can be explained by a reduction of a nitro to an amino group or, more likely, by reduction of the intermediate hydroxylamine derivatives **8** and **8a**, which were detected in the mixture when the reaction was stopped after 20 h.

The structure of **13**, and consequently that of **13a**, was assigned by its ^{1}H NMR spectrum which showed two doublets at δ 7.43 and 8.22 and a singlet at δ 8.79 ppm, indicating monosubstitution in the pyridine ring. Catalytic dechlorination gave the expected 3-ß-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine.[6] The position of the chlorine atom was attributed by comparison of **13** with an authentic sample of 7-chloro-3-ß-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine.[6] Thin layer chromatography in $CHCl_3$-nC_6H_{14}-MeOH (70:20:10) clearly discriminated the two



Scheme I

TABLE 1. UV spectra of **13** and 7-chloro-3-ß-D-ribofuranosyl-3H-imidazo-[4,5-b]pyridine.

	UV λ_{max} nm (ε)	
Compound	**pH 1**	**pH10**
5-chloro-3-(ß-D-ribo-furanosyl)-3-H-imidazo-[4,5-b]pyridine[9] (**13**)	284 (8300)	287 (8200) 253 (4000)
7-chloro-3-(ß-D-ribo-furanosyl)-3-H-imidazo-[4,5-b]pyridine	272 (6700) 249 (5900)	277 (6200) 256 (5900)

TABLE 2. N.O.E.-data % of compound **11** upon irradiation of H-1' (DMSO-d_6, 25 °C, 300 MHz)

	H-2'	H-3'	H-4'	H-2
11	2.0	a	1.7	3.0

a: no detectable intensity enhancement (< 0.5%)

compounds. Moreover, the UV spectrum of compound **13** showed a spectral profile different from that observed for the 7-chloro derivative (TABLE 1). The ^{1}H NMR spectrum of **13** presented a doublet at higher field (δ 8.22 vs. δ 8.33 ppm) with a larger coupling constant ($J_{7,6}$ = 8.4 Hz vs. $J_{5,6}$ = 5.3 Hz), according to that reported by Itoh et al. in the case of 5-chloro-3-methyl-3H-imidazo[4,5-b]-pyridine in comparison with the corresponding 7-chloro derivative.[7]

The anomeric configuration and the ribosylation site of compound **10** and, as a consequence, that of all the nucleosides described in this paper, was assigned applying n.O.e. difference spectroscopy to compound **11**. Saturation of H-1' resulted in n.O.e.s of the H-2' and H-4' signals (2.0 % and 1.7 %,

respectively), establishing the ß-D-configuration (TABLE 2). Furthermore a strong n.O.e. effect was observed on H-2 when H-1' was irradiated, confirming N^3-glycosylation (corresponding to N^9 in the purine ring system).
The structure of compound **4** was confirmed by comparison of its physical data with those of an authentic sample previously synthesized.[5]

RESULTS

Radioligand binding studies

The affinities of all compounds were determined in radioligand binding studies. Adenosine A_1 receptor affinities were determined on rat cortical membranes with [^{3}H]DPCPX as the radioligand, both in the absence and presence of 1 mM GTP (TABLE 3). On the A_1 receptor adenosine (**1**) proved more potent than HAPR (**5**) and N^6-methyladenosine (**9**). The other compounds in the adenosine and 1-deazaadenosine series (**2-4**) displayed affinities similar to or slightly higher than the corresponding HAPR analogs (**6-8**). The values for adenosine (**1**) and HAPR (**5**) are approximate, due to the following methodological paradox. Routinely, adenosine deaminase (ADA) is present in the adenosine receptor membrane preparation in order to remove/metabolise endogenous adenosine and to allow the proper estimation of ligands' affinities. As a consequence, the affinities of adenosine and HAPR, both substrates for ADA, cannot be measured, unless pentostatin, a very potent ADA inhibitor with little or no A_1 receptor affinity (data not shown) is present. In that case, however, it is conceivable that some endogenous adenosine is momentarily being formed.

The GTP shifts, expressed as the ratio $IC_{50,+GTP}$ / $IC_{50,-GTP}$ range between 3.3 and 8.3, comparable to the value obtained for the reference A_1 receptor agonist N^6-cyclopentyladenosine (6.0; results not shown). In the absence of GTP the displacement curves had a rather shallow appearance with pseudo-Hill coëfficients less than unity. In that case the data were best analysed according to

TABLE 3. Adenosine A_1 and A_2 receptor affinities (μM) of tested compounds as determined on rat tissues (ado = adenosine).

	compound	IC_{50} (A_1) - GTP	IC_{50} (A_1) + GTP	GTP shift	IC_{50} (A_2)
1	Ado	0.41 ± 0.10	2.4 ± 0.2	5.9	-
2	2-chloro-ado	0.30 ± 0.10	1.2 ± 0.4	4.0	0.08 ± 0.03
3	1-deaza-ado	8.9 ± 3.1	49 ± 5	5.5	0.97 ± 0.06
4	2-chloro-1-deaza-ado	1.8 ± 0.9	15 ± 2	8.3	0.43 ± 0.09
5	HAPR	3.0 ± 0.8	10 ± 2	3.3	-
6	2-chloro-HAPR	1.1 ± 0.5	4.8 ± 0.1	4.4	0.52 ± 0.14
7	1-deaza-HAPR	9.4 ± 2.8	44 ± 2	4.7	10 ± 3
8	2-chloro-1-deaza-HAPR	2.5 ± 1.5	15 ± 2	6.0	3.1 ± 2.1
9	N^6-methylado	1.1 ± 0.1	8.6 ± 0.5	7.8	10 ± 2

a two binding-states model ($P<0.05$). Thus, N^6-methyladenosine (**9**), as an example, interacted with the A_1 receptor in a dual way, i.e. one state with higher affinity (IC_{50} = 0.25 μM) and another with lower affinity (IC_{50} = 6.3 μM) could be discriminated.

Affinities for the A_2 receptor were established with the agonist radioligand [^{3}H]CGS 21680. Since pentostatin (see above) interfered with [^{3}H]CGS 21680 binding no approximate IC_{50} values for adenosine and HAPR could be obtained. Similarly, GTP interfered, and thus IC_{50} values in the presence of GTP could also not be determined. Of all remaining compounds tested 2-chloroadenosine (**2**) was the most active material (IC_{50} = 0.08 μM). All HAPR analogs (**6-8**) were approximately 10 times less active than their adenosine counterparts (**2-4**).

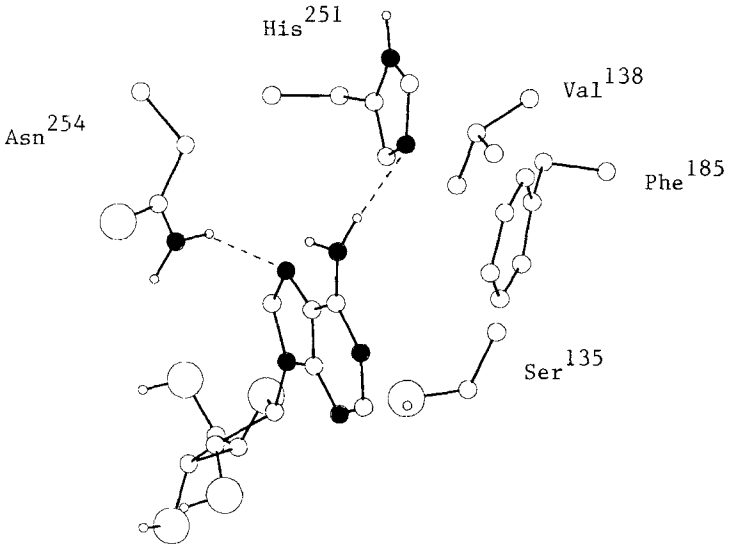


FIG. 2. Molecular model of adenosine bound to the adenosine A_1 receptor, surrounded by the five amino acids that make up the lining of the N^6-region. Representation: small circles: hydrogen atoms connected to either oxygen or nitrogen (other hydrogens are not represented for reasons of clarity); intermediate circles: carbon atoms (white), nitrogen atoms (black); large circles: oxygen atoms; ----: putative hydrogen bonds.

Molecular modeling studies

In FIG. 2 adenosine in its receptor bound conformation is shown. The N^6- region is surrounded by five amino acids, *viz.* Ser^{135}, Val^{138}, Phe^{185}, His^{251} and Asn^{254}. Two hydrogen bonds are conceivable, i) between His^{251} and N^6-H, and ii) between Asn^{254} and N7 of the purine ring system. There is enough space to accommodate an N^6-OH substituent (as in HAPR, **5**) or an N^6-CH_3 substituent (as in N^6-methyladenosine, **9**). The purine and ribose moieties are also surrounded by amino acids, but for reasons of clarity they are not shown in FIG. 2.

DISCUSSION

Adenosine (**1**), HAPR (**5**) and N^6-methyladenosine (**9**) were all moderately potent in radioligand binding studies on A_1 receptors. The IC_{50} value of adenosine in the absence of GTP (0.41 μM) is in good agreement with adenosine's affinity (K_i = 0.22 μM) obtained under comparable conditions by Lohse and coworkers.[8] Substitution of one N^6-hydrogen by -OH, as in HAPR (**5**), or $-CH_3$, as in N^6-methyladenosine (**9**), yielded compounds that were slightly less active than adenosine. Daly *et al.* have reported an IC_{50} value for N^6-methyladenosine of 0.12 μM, employing a radiolabelled agonist in their binding studies.[9] This value is in good agreement with the value we found for the high affinity state as mentioned above (IC_{50} = 0.25 μM). To our knowledge binding studies with HAPR have not been performed. Interestingly, HAPR, an antitumor drug, and its derivatives have significant adenosine receptor affinity. This micromolar potency equals or surpasses their in vitro antitumor activity.[4]

Apparently, hydrophilic (-OH) or hydrophobic ($-CH_3$) substitution is acceptable in the receptor environment. This finding can be rationalised by inspection of FIG. 2. There is only limited space in the immediate environment of adenosine's exocyclic amino group, probably causing some steric hindrance, even for small substituents as -OH and $-CH_3$. Both hydrophobic (Val^{138} and Phe^{185}) and hydrophilic residues (Ser^{135}, His^{251} and Asn^{254}) surround the N^6-region of adenosine in the A_1 receptor model. Therefore, either Van der Waals interactions (hydrophobicity) or hydrogen bonds (hydrophilicity) will influence the eventual affinity of an N^6-substituted adenosine derivative. Compounds such as HAPR and N^6-methyladenosine, however, do not benefit very much, since their substituents only marginally fill the N^6-region. In contrast, a cyclopentyl substituent as in N^6-cyclopentyladenosine, inducing high affinity on A_1 receptors, appeared to interact favourably with the most distant residue Val^{138}.[3]

Changes on the purine ring system yielded compounds that were all resistant to ADA activity.[10] The IC_{50} values determined in the absence and

presence of pentostatin (0.1 μM) were identical (data not shown). Compounds **2-4** have been studied earlier, again in an agonist radioligand binding assay on rat A_1 receptors. IC_{50} values were 0.012 μM (**2**), 1.4 μM (**3**) and 0.56 μM (**4**), a potency order also observed in the present study.[5] Except for N^6-methyl-adenosine, all compounds were not very selective for either one adenosine receptor subtype. For example, the IC_{50} value of 2-chloroadenosine for the high affinity state of the A_1 receptor was 0.05 μM, comparable to the IC_{50} value found on the A_2 receptor. This latter value of 0.08 μM also corresponds to a high affinity state, since it was determined with a radiolabelled agonist.

The similar potency order for compounds **1-4** and compounds **5-8** suggests that the binding mode for the purine riboside part of the molecules is identical. Thus, the nature of the N^6-substituent may not influence the interaction of the rest of the molecule with the receptor. The agonistic activity of all compounds, as identified by their GTP shifts, may be attributed to their common structural characteristic, *viz.* the ribose group. An intact ribose group has been shown to be a prerequisite for full agonistic behaviour.[11]

In conclusion, small substituents on the exocyclic amino group of adenosine slightly diminish adenosine receptor affinity. This is probably caused by some steric restraints imposed by the immediate receptor environment, while the favourable N^6-region is hardly occupied.

EXPERIMENTAL SECTION

Chemistry

Adenosine was from Janssen Chimica (Beerse, Belgium). 6-Hydroxylamino-purine riboside (HAPR) was purchased from Sigma (St. Louis, MO, USA). GTP was obtained from Aldrich Chemie (Brussels, Belgium). All adenosine and HAPR derivatives were synthesized according to procedures described elsewhere,[4] except for 2-chloro-1-deaza-HAPR (**8**), which synthesis is described

below. [^{3}H]DPCPX (1,3-dipropyl-8-cyclopentylxanthine; 108 Ci/mmol) and [^{3}H]CGS 21680 (2-[*p*-(2-carbonylethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine; 38.3 Ci/mmol) were purchased from DuPont NEN ('s-Hertogenbosch, The Netherlands). All other chemicals were of reagent grade. ^{1}H NMR spectra were obtained with a Varian Gemini 200 MHz and a Varian VXR 300 MHz spectrometer. UV spectra were recorded on a Varian Cary 13 spectrophotometer. TLC was carried out on pre-coated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. Elemental analyses were determined on a Carlo Erba model 1106 analyser.

5-Chloro-7-hydroxylamino-3-β-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine (8) and 5-chloro-7-hydroxylamino-3-(5-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (8a)

A solution of 0.50 g (1.11 mmol) of 5-chloro-7-nitro-3-(2,3,5-tri-O-acetyl--β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (**10**)[5] in 5 mL of ethanol was added to 0.6 g (18 mmol) of NH_2OH in 15 mL of ethanol and the reaction mixture was heated at reflux for 4 h. The filtrate was concentrated in vacuo and the residue chromatographed on a flash silica gel column eluting with a gradient of AcOEt-MeOH (from 94:6 to 90:10) to provide 0.182 g (52%) of **8** and 0.09 g (23%) of **8a** as chromatographically pure solids. All analytical data of compound **8** were consistent with that of an authentic sample synthesized previously.[4]

8a: ^{1}H NMR (Me_2SO-d_6) δ 2.04 (s, 3H, $COCH_3$), 4.12 (m, 1H, H-3'), 4.27 (m, 3H, CH_2-5' and H-4'), 4.63 (m, 1H, H-2'), 5.93 (d, 1H, J = 5.1 Hz, H-1'), 6.64 (s, 1H, H-6), 8.37 (s, 1H, H-2), 9.13 (s, 1H, NHOH), 10.18 (s, 1H, NHOH). Anal. ($C_{13}H_{15}ClN_4O_6$) C, H, N.

5-Chloro-3-β-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine (13), and 5-chloro--3-(5-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4,5-b] pyridine (13a); 7-amino-5-chloro-3-β-D-ribofuranosyl-3H-imidazo[4,5-b]pyridine (4) and

7-amino-5-chloro-3-(5-O-acetyl-β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (4a)

A solution of 0.14 g (0.31 mmol) of 5-chloro-7-nitro-3-(2,3,5-tri-O-acetyl--β-D-ribofuranosyl)-3H-imidazo[4,5-b]pyridine (**10**)[5] in 5 mL of ethanol was added to 0.6 g (18 mmol) of NH_2OH in 15 mL of ethanol and the reaction mixture was heated in a steel bomb at 100 °C for 48 h. The filtrate was concentrated in vacuo and the residue chromatographed on a preparative thin layer chromatography eluting with $CHCl_3$-MeOH (90:10) to provide 15 mg (14%) of **4**[5], 7 mg (8%) of **4a**, 27 mg (27%) of **13** and 9 mg (11%) of **13a** as chromatographically pure solids:

4a: ^{1}H NMR (Me_2SO-d_6) δ 2.04 (s, 3H, $COCH_3$), 4.22 (m, 4H, CH_2-5', H-4', and H-3'), 4.62 (m, 1H, H-2'), 5.89 (d, 1H, J = 5.1 Hz, H-1'), 6.41 (s, 1H, H-6), 6,85 (bs, 2H, NH_2), 8.32 (s, 1H, H-2). Anal. ($C_{13}H_{15}ClN_4O_5$) C, H, N.

13: ^{1}H NMR (Me_2SO-d_6) δ 3.64 (m, 2H, CH_2-5'), 3.98 (m, 1H, H-4'), 4.19 (m, 1H, H-3'), 4.60 (m, 1H, H-2'), 6.01 (d, 1H, J = 5.9 Hz, H-1'), 7.43 (d, 1H, $J_{6,7}$ = 8.4 Hz, H-6), 8.22 (d, 1H, $J_{7,6}$ = 8.4 Hz, H-7), 8.79 (s, 1H, H-2). Anal. ($C_{11}H_{12}ClN_3O_4$) C, H, N.

13a: ^{1}H NMR (Me_2SO-d_6) δ 2.03 (s, 3H, $COCH_3$), 4.23 (m, 4H, CH_2-5', H-4', and H-3'), 4.70 (m, 1H, H-2'), 6.03 (d, 1H, J = 5.1 Hz, H-1'), 7.44 (d, 1H, $J_{6,7}$ = 8.4 Hz, H-6), 8.23 (d, 1H, $J_{7,6}$ = 8.4 Hz, H-7), 8.74 (s, 1H, H-2). Anal. ($C_{13}H_{14}ClN_3O_5$) C, H, N.

Radioligand binding studies

Adenosine A_1 receptor affinities were determined on rat cortical membranes with [^{3}H]DPCPX as the radioligand according to a protocol published previously.[12] Measurements with [^{3}H]DPCPX were performed in the presence and absence of 1 mM GTP. When adenosine and HAPR were tested, pentostatin (0.1 μM) was added simultaneously to the incubation mixture. Adenosine A_2 receptor affinities were determined on rat striatial membranes with [^{3}H]CGS 21680 as the

radioligand.[13,14] Data were analysed with InPlot 4.0 (GraphPad Software, Inc., San Diego, CA, USA).

Molecular modelling

Adenosine, HAPR and N^6-methyladenosine were docked into the ligand binding site on the A_1 receptor, according to a receptor model published recently.[3] Briefly, the amino acid sequence of the canine A_1 receptor and the atomic coördinates of a structurally related protein, bacteriorhodopsin, were combined to generate a three-dimensional model for the adenosine A_1 receptor. This model consists of seven amphipathic alpha-helices, forming a pore. The highly potent and selective ligand, N^6-cyclopentyladenosine, was docked into this cavity according to the conformational characteristics of the ligand, obtained from indirect modeling studies by the 'active analogue approach'.[2] Adenosine, HAPR and N^6-methyladenosine were placed in this binding site. The three resulting structures of ligand - receptor complexes were energy minimized, exactly as described.[3]

All modelling studies were carried out using the software package BIOGRAF version 3.1 (Molecular Simulations, Waltham, USA) implemented on a Silicon Graphics 4D/25GT workstation.

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Analytical appendix I. Elemental analyses of the new compounds

No	Anal. for	calculated			found		
		C	H	N	C	H	N
4a	$C_{13}H_{15}ClN_4O_5$	45.56	4.41	16.35	45.22	4.18	16.68
8a	$C_{13}H_{15}ClN_4O_6$	43.53	4.21	15.62	43.24	4.02	15.96
13	$C_{11}H_{12}ClN_3O_4$	46.25	4.23	14.71	45.94	4.07	15.07
13a	$C_{13}H_{14}ClN_3O_5$	47.64	4.31	12.82	47.31	4.13	13.09

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