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2,9-Disubstituted-N^6-(arylcarbamoyl)-8-azaadenines as new selective A_3 adenosine receptor antagonists: Synthesis, biochemical and molecular modelling studies

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Abstract—A number of N^6-(*N*-arylcarbamoyl)-2-substituted-9-benzyl-8-azaadenines, obtained by a modification of the synthetic scheme used to prepare selective A_1 ligands, by only three or two steps, are described. At first we prepared a series of 2-phenyl-9-benzyl-8-azaadenines having as N^6 substituent a variously substituted *N*-phenylcarbamoyl group. Some of these derivatives demonstrated good affinity towards the A_3 subtype but low selectivity. Compounds having *p*-CF_3, *p*-F and *p*-OCH_3, as substituents on the phenylcarbamoyl group were selected as lead compounds for the second part of this study. Without modifying the N^6 substituent, which would assure A_3 affinity, we varied the 9 and 2 positions on these molecules to enhance selectivity. Some compounds having a *p*-methyl group on the 2-phenyl substituent showed a very good affinity and selectivity for the A_3 subtype, revealing the first class of A_3 adenosine receptor selective antagonists with a bicyclic structure strictly correlated to the adenine nucleus. The molecular modelling work, carried out using the DOCK program, supplied two models which may be useful for a better understanding of the binding modes. Both models highlighted the preferred interacting tautomeric forms of the antagonists for human A_1 and A_3 receptors.

1. Introduction

Adenosine receptors mediate many physiological functions by interaction with adenosine. Four subtypes (A_1, A_{2A}, A_{2B} and A_3) are recognised; human A_3 adenosine receptors were the last to have been cloned[1,2] and intensively studied: in humans they are found not only in the lung and liver, but also in the CNS, testes, heart and the immune system.[3] Activation of A_3 adenosine receptors mediates both inflammatory and pro-inflammatory responses[4] and plays an important role in brain ischemia,[5,6] immunosuppression,[7] and bronchospasm in several animal models.[8] As for A_1 and A_{2A} subtypes, nearly all A_3 agonists are adenosine derivatives, such as N^6-substituted-phenyl-carbamoyl-adenosine-5′-uronamides, prepared in 1996 by Baraldi et al.[9], or the new compound CP-608039.[10]

Selective A_3 adenosine receptor antagonists have been indicated as potential drugs for the treatment of asthma, and despite anti-inflammatory responses to A_3-receptor activation, for the treatment of inflammatory conditions.[11] Other possible roles for these agents include the therapy of rhinitis, an antiallergenic role and the treatment of glaucoma and strokes.[3]

The research of A_3 selective antagonists started from xanthine derivatives, but these efforts largely failed;[12,13] recently a series of pyrido[2,1-*f*]purine-2,4-dione derivatives has been described as fused xanthine derivatives with high affinity for A_3 receptors.[14] Other classes of compounds have been studied, such as

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triazolonaphthyridines, pyridines, pyrans, flavonoids, triazoloquinazolines, isoquinolines, triazolopyrimidines[15] and imidazopurines,[16] and some compounds with high affinity and selectivity have been discovered.

It is known that adenine derivatives, analogous to the physiological agonist but lacking the ribose moiety, are antagonists of A_1 adenosine receptors.[17] On the contrary, until now few A_3 antagonists have a bicyclic structure correlated to the adenine nucleus. For example, a 7-deazaadenine[18] and an adenosine derivative, 8-phenylethyl-9-ethyladenosine,[19] resulted in good but not very selective A_3 antagonists.

Considering the chemical classes of A_3 agonists and antagonists one can find some different structures having a *N*-phenylcarbamoyl function (Fig. 1). This functional group appears to be an important recognition element in the interaction with A_3 receptors. In some cases, insertion of that function on A_1 or A_{2A} ligands has resulted in the discovery of very potent and selective A_3 compounds.[9,20–22] For example, a series of adenosine-5′-uronamide derivatives bearing N^6-phenylurea groups (**1**, Fig. 1) are selective A_3 agonists;[9] or a series of 5-amino-pyrazolo[4,3-*e*]-1,2,4-triazole[1,5-*c*]pyridines containing a 2-furyl substituent at the 2 position, a carbamoyl residue at the exocyclic amine and a small group for R (**2**, Fig. 1) proved to be potent A_3 receptor antagonists.[21] Small groups in the p-position of the phenyl group interacted with the A_3 receptors including F, Cl, OCH_3 and CH_3. Only hydrogen is tolerated in the m-position, and the o-position could be substituted with a chloro group.[3]

A number of isoquinoline (**3**, Fig. 1) and quinazoline derivatives (**4**, Fig. 1) were developed as A_3 receptor antagonists.[23] In these compounds, the ureido-linker can be replaced by an amide linker. A methoxy group

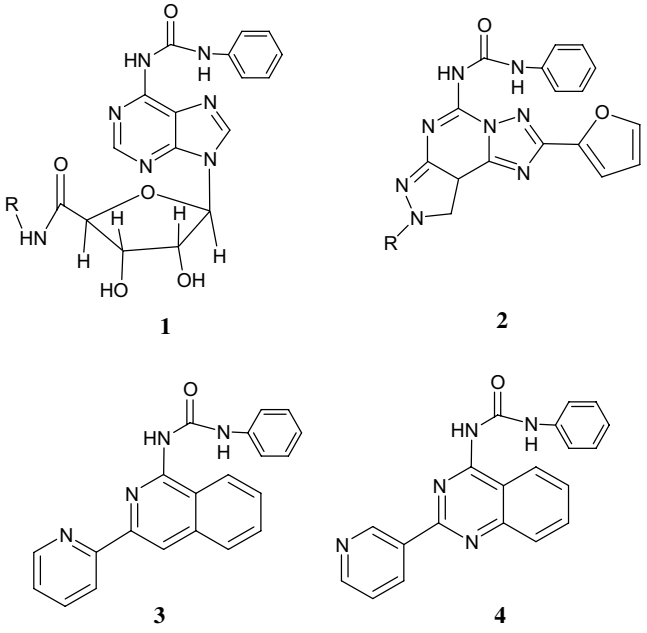


Figure 1. Phenylureido derivatives with high activity towards A_3 adenosine receptors.

in the phenyl substituent increased both affinity and selectivity.[3] The carbonyl group in these structures may function as an important H-bond acceptor.[24]

Previously, we have synthesised a number of N^6-substituted-2-phenyl-9-benzyl-8-azaadenines as very potent A_1 ligands[25–27] in which the N^6-substituent was an alkyl, cycloalkyl or aralkyl group. Our review of the literature (see above) suggested that replacement of these N^6-substituents with a phenylcarbamoyl moiety could lead to a new class of A_3 receptor antagonists. A modification of the simple synthetic scheme used previously in our laboratories to prepare selective A_1 ligands[25] would lead to N^6-ureidosubstituted-2-phenyl-9-benzyl-8-azaadenines (see Scheme 1), whose adenine-like structure would ensure an adenosine receptor antagonist functional profile and whose phenylcarbamoyl group would ensure a biological activity directed towards the A_3 subtype.

The first lead compounds obtained (compounds **40**, **43** and **46**, Table 1) proved to have good A_3 activity but little A_1/A_3 selectivity. Then they were submitted to a number of structural changes to increase activity and selectivity on A_3 adenosine receptors.

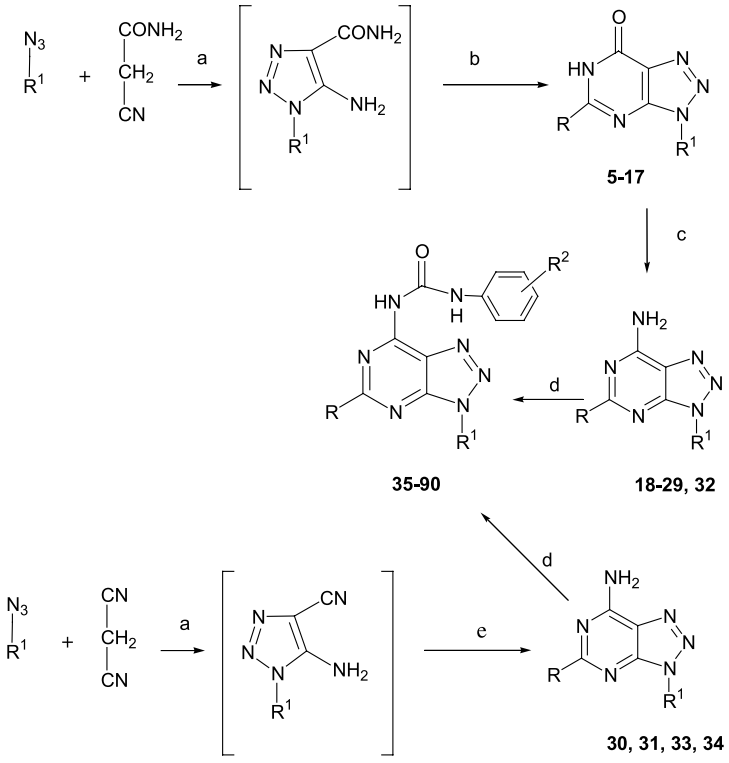
In our laboratory, theoretical models for the human A_1 adenosine receptors had been previously built, improved and validated through interaction studies with ligands.[27,28] Residues that appear to be relevant for ligand–receptor interaction and chiral selectivity in subtype A_1 had been highlighted. Here, we focus our interest on molecular features required for antagonists to show A_1/A_3 selectivity, in order to develop a predictive model for designing new selective A_3 antagonists.

1.1. Chemistry

In the scheme are depicted the simple routes to synthesise compounds **35**–**90**. Compounds **35**–**80**, **85** and **86** were obtained by a three-step procedure and compounds **81**–**84**, **87**–**90** by a method which needs only two steps. 2,9-Disubstituted-8-azaahypoxanthines **5**–**17** (see Fig. 2), obtained by following procedures described,[29] were transformed by the reaction with thionyl chloride in the corresponding chlorides, which were not characterised and reacted with NH_3 to give the corresponding 8-azaadenines **18**–**29** and **32** (see Fig. 2). Adenines **30**, **31**, **33** and **34** (see Fig. 2) were obtained by a 'one-pot' reaction[30] from the suitable azides, malononitrile and suitable nitriles. All 8-azaadenines synthesised (**18**–**34**) reacted with the proper isocyanates to give compounds **35**–**90**. Some of the intermediate compounds (**5**–**7**, **11**–**15**, **18**–**20** and **25**–**29**) have been described in the previous papers (see Fig. 2 for references).

1.2. Biochemistry

The new compounds **35**–**90** were tested in radioligand binding assays to determine their affinities towards recombinant human A_1, A_{2A} and A_3 adenosine receptors. In particular, affinities for human A_1, A_{2A} and A_3 adenosine receptors expressed in CHO cells were determined in competition assays using, respectively, [^{3}H]DPCPX,



Scheme 1. Reagents and conditions: (a) EtONa,110 °C; (b) RCOOEt, EtONa, 110 °C; (c) (1) $POCl_3$, *N*,*N*-diethylaniline, 90 °C; (2) NH_3, EtOH, 110 °C; (d) $R_2C_6H_4NCO$, CH_3CN, reflux, 60 min; (e) RCN, EtONa, 110 °C.

[^{3}H]NECA and [^{125}I]AB-MECA as radioligands and have been reported in Table 1.

With the aim to evaluate the pharmacological profile of the new synthesised compounds the agonist-induced stimulation of binding of [^{35}S]GTP-γ-S to activated G proteins was employed. This assay has been used as a functional one for a variety of receptors, including adenosine receptors.[31,32] In particular, the effects of the most potent and A_3-selective compounds (**81**, **82**, **83** and **84**) on agonist-induced stimulation of [^{35}S]GTP-γ-S binding from membranes of CHO cells expressing the human A_3 adenosine receptors were studied. Each of the new synthesised compound caused a concentration-dependent loss of binding of [^{35}S]GTP-γ-S in the presence of a constant high concentration of NECA (10 μM) (Fig. 3). IC_{50} values ranged from 83 ± 10 nM for the new synthesised compound **84** to 398 ± 40 nM for the new synthesised compound **82** (Table 2). In each case the IC_{50} value was between 4.3- and 14-fold the K_i value obtained in a radioligand binding assay at human A_3 adenosine receptors.

In conclusion, by the functional assay it was found that these compounds behave as antagonists for A_3 adenosine receptors.

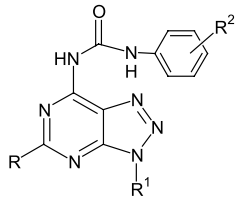
2. Molecular modelling

This work is a development of the previous modelling project started with the aim of analysing the interactions between the human A_1 receptor with some antagonists.[27,28] Based on this fact, an improved theoretical model for the human A_1 and a new model for the human A_3 receptor were built, by exploiting their homology with bovine rhodopsin, for which an accurate model had been recently released in PDB (PDB entry 1L9H).[33] This work started by aligning the sequences of human A_1 and A_3 receptors and bovine rhodopsin, using the CLUSTAL W program.[34] The alignment is shown in Figure 4. The biopolymer module of InsightII was used in order to create the model for both human A_1 and A_3 receptors. The correctness of fold was evaluated using the Homology module of InsightII. The structure was quite good even if not optimised, although a series of steric clashes needed to be resolved with a further and accurate geometry optimisation.

In order to build models for the complexes of interest we needed to accurately identify the binding site locations. With regard to the A_1 receptor subtype, we exploited previous studies already carried out in our laboratory, while for the A_3 receptor subtype, the identification of the binding site was based upon the work of Baraldi et al.[35] Then we proceeded to pre-position ligand **82**, in the binding site of both A_1 and A_3 receptors. The above compound was chosen for its high affinity and selectivity towards the A_3 receptor.

The AMBER[36] program was used to obtain a low-energy structure for both human A_1 and A_3 receptor in complex with ligand **82**. With regard to the solvation, the explicit solvent model TIP3P water was used, resembling a cap of water around the protein filled to 20 Å around the ligand. As for the minimisation parameters, the SHAKE procedure was employed to constrain all

Table 1. Binding activity at human A_1, A_{2A} and A_3 adenosine receptors



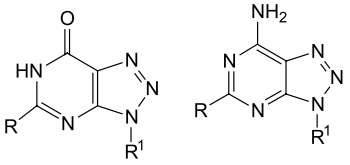
35-90

Compd	R	R^1	R^2	K_i[a] (nM) or *I* (%)			A_1/A_3[e]
				hA_1[b]	hA_{2A}[c]	hA_3[d]	
35	C_6H_5	$CH_2C_6H_5$	2-Cl	1120 ± 100	46% ± 5	333 ± 36	3.4
36	C_6H_5	$CH_2C_6H_5$	3-Cl	489 ± 50	672 ± 58	300 ± 40	1.6
37	C_6H_5	$CH_2C_6H_5$	4-Cl	304 ± 31	15% ± 1	507 ± 52	0.6
38	C_6H_5	$CH_2C_6H_5$	2-CF_3	197 ± 20	42% ± 4	50 ± 5	3.9
39	C_6H_5	$CH_2C_6H_5$	3-CF_3	190 ± 18	2260 ± 203	27 ± 3	7.0
40	C_6H_5	$CH_2C_6H_5$	4-CF_3	168 ± 15	25% ± 3	54 ± 5	3.1
41	C_6H_5	$CH_2C_6H_5$	2-F	92 ± 9	28% ± 3	36 ± 4	2.6
42	C_6H_5	$CH_2C_6H_5$	3-F	110 ± 10	18% ± 2	27 ± 3	4.1
43	C_6H_5	$CH_2C_6H_5$	4-F	91 ± 9	409 ± 51	18 ± 2	5.1
44	C_6H_5	$CH_2C_6H_5$	2-OCH_3	1180 ± 130	47% ± 5	1000 ± 90	1.2
45	C_6H_5	$CH_2C_6H_5$	3-OCH_3	500 ± 48	43% ± 4	250 ± 26	2.0
46	C_6H_5	$CH_2C_6H_5$	4-OCH_3	190 ± 21	656 ± 59	39 ± 5	4.9
47	C_6H_5	$CH_2C_6H_5$	4-I	1600 ± 150	28% ± 3	1800 ± 210	0.9
48	C_6H_5	$(CH_2)_6CH_3$	4-OCH_3	30% ± 4	35% ± 4	0%	—
49	C_6H_5	$(CH_2)_6CH_3$	4-CF_3	40% ± 4	5% ± 1	0%	—
50	C_6H_5	$(CH_2)_6CH_3$	4-F	6% ± 1	20% ± 2	22% ± 3	—
51	C_6H_5	$(CH_2)_2C_6H_5$	4-OCH_3	45% ± 4	3% ± 1	0%	—
52	C_6H_5	$(CH_2)_2C_6H_5$	4-CF_3	7% ± 1	3% ± 1	0%	—
53	C_6H_5	$(CH_2)_2C_6H_5$	4-F	0%	24% ± 3	28% ± 3	—
54	C_6H_5	*o*-$CH_2C_6H_4Cl$	4-OCH_3	42% ± 3	16% ± 2	35% ± 4	—
55	C_6H_5	*o*-$CH_2C_6H_4Cl$	4-CF_3	34% ± 4	0%	25% ± 3	—
56	C_6H_5	*o*-$CH_2C_6H_4Cl$	4-F	1900 ± 180	10% ± 2	346 ± 31	5.5
57	C_6H_5	*o*-$CH_2C_6H_4F$	4-OCH_3	400 ± 36	0%	700 ± 59	—
58	C_6H_5	*o*-$CH_2C_6H_4F$	4-CF_3	56% ± 6	0%	32% ± 3	—
59	C_6H_5	*o*-$CH_2C_6H_4F$	4-F	2200 ± 200	48% ± 5	295 ± 30	7.5
60	C_6H_5	*p*-$CH_2C_6H_4CH_3$	4-OCH_3	30% ± 3	0%	22% ± 2	—
61	C_6H_5	*p*-$CH_2C_6H_4CH_3$	4-CF_3	18% ± 2	0%	29% ± 3	—
62	C_6H_5	*p*-$CH_2C_6H_4CH_3$	4-F	800 ± 74	16% ± 1	490 ± 50	1.6
63	$CH_2C_6H_5$	$CH_2C_6H_5$	4-OCH_3	5% ± 1	45% ± 5	0%	—
64	$CH_2C_6H_5$	$CH_2C_6H_5$	4-CF_3	15% ± 2	5% ± 1	0%	—
65	$CH_2C_6H_5$	$CH_2C_6H_5$	4-F	7% ± 1	0%	22% ± 2	—
66	$(CH_2)_3CH_3$	$CH_2C_6H_5$	4-OCH_3	14% ± 2	6% ± 1	0%	—
67	$(CH_2)_3CH_3$	$CH_2C_6H_5$	4-CF_3	0%	30% ± 3	0%	—
68	$(CH_2)_3CH_3$	$CH_2C_6H_5$	4-F	7% ± 1	11% ± 1	23% ± 2	—
69	$(CH_2)_2CH_3$	$CH_2C_6H_5$	4-OCH_3	48% ± 5	10% ± 1	15% ± 1	—
70	$(CH_2)_2CH_3$	$CH_2C_6H_5$	4-CF_3	15% ± 2	25% ± 3	10% ± 1	—
71	$(CH_2)_2CH_3$	$CH_2C_6H_5$	4-F	16% ± 2	30% ± 3	25% ± 3	—
72	CH_3	$CH_2C_6H_5$	4-OCH_3	10% ± 1	17% ± 2	26% ± 3	—
73	CH_3	$CH_2C_6H_5$	4-CF_3	25% ± 3	14% ± 2	3% ± 1	—
74	CH_3	$CH_2C_6H_5$	4-F	32% ± 3	2600 ± 240	11% ± 1	—
75	H	$CH_2C_6H_5$	4-OCH_3	10% ± 1	5% ± 1	3% ± 0.3	—
76	H	$CH_2C_6H_5$	4-CF_3	32% ± 4	4% ± 0.3	30% ± 5	—
77	H	$CH_2C_6H_5$	4-F	27% ± 3	13% ± 1	6% ± 1	—
78	*p*-$C_6H_4CH_3$	$CH_2C_6H_5$	4-OCH_3	100 ± 9	13% ± 1	93 ± 8	1.1
79	*p*-$C_6H_4CH_3$	$CH_2C_6H_5$	4-CF_3	80 ± 7	0%	24 ± 3	3.3
80	*p*-$C_6H_4CH_3$	$CH_2C_6H_5$	F	678 ± 57	29% ± 3	28 ± 3	24.2
81	*p*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4Cl$	4-CF_3	0%	0%	33 ± 4	c.s.
82	*p*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4Cl$	4-F	12% ± 1	29% ± 3	35 ± 3	c.s.
83	*p*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4F$	4-CF_3	4100 ± 320	0%	28 ± 4	146
84	*p*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4F$	4-F	430 ± 39	8050 ± 740	6 ± 01	71.7
85	*p*-$C_6H_4CH_3$	*p*-$CH_2C_6H_4CH_3$	4-CF_3	5000 ± 510	0%	284 ± 29	17.6
86	*p*-$C_6H_4CH_3$	*p*-$CH_2C_6H_4CH_3$	4F	700 ± 63	7030 ± 670	22 ± 2	31.8
87	*m*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4Cl$	4-CF_3	4270 ± 380	0%	437 ± 40	9.8
88	*m*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4Cl$	4-F	1480 ± 150	25% ± 2	355 ± 35	4.2
89	*m*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4F$	4-CF_3	4750 ± 380	0%	248 ± 25	19.2

Table 1 (*continued*)

Compd	R	R^1	R^2	K_i[a] (nM) or *I* (%)			A_1/A_3[e]
				hA_1[b]	hA_{2A}[c]	hA_3[d]	
90	*m*-$C_6H_4CH_3$	*o*-$CH_2C_6H_4F$	4-F	1600 ± 160	20% ± 2	45 ± 5	35.9
Theophilline[f]				6200 ± 530	21,000 ± 1800[g]	86,000 ± 1800	
DPCPX[f]				3.2 ± 0.2	337 ± 28[g]	1300 ± 125	

[a] The K_i values are the mean ± SEM of four separate assays, each performed in triplicate.
[b] Inhibition of specific [^{3}H]DPCPX binding in human A_1 recombinant adenosine receptor or inhibition percentage (*I* %) at 10 μM concentration.
[c] Inhibition of specific [^{3}H]NECA binding in human A_{2A} recombinant adenosine receptor or inhibition percentage (*I* %) at 10 μM concentration.
[d] Inhibition of specific [^{125}I]AB-MECA binding in human A_3 recombinant adenosine receptor or inhibition percentage (*I* %) at 10 μM concentration.
[e] A_1/A_3 selectivity ratio values, c.s.: completely selective.
[f] Ref. 43.
[g] Value obtained using bovine striatal membranes.



5-17 **18-34**

	R	R^1
5[25], **18**[25]	C_6H_5	$CH_2C_6H_5$
6[44], **19**[44]	C_6H_5	$(CH_2)_6CH_3$
7[26], **20**[26]	C_6H_5	$(CH_2)_2C_6H_5$
8, 21	C_6H_5	o-$CH_2C_6H_4$-Cl
9, 22	C_6H_5	o-$CH_2C_6H_4F$
10, 23	C_6H_5	p-$CH_2C_6H_4CH_3$
11[29], **24**	$CH_2C_6H_5$	$CH_2C_6H_5$
12[45], **25**[45]	$(CH_2)_3CH_3$	$CH_2C_6H_5$
13[29], **26**[29]	$(CH_2)_2CH_3$	$CH_2C_6H_5$
14[29], **27**[46]	CH_3	$CH_2C_6H_5$
15[29], **28**[45]	H	$CH_2C_6H_5$
16, 29[46]	p-$C_6H_4CH_3$	$CH_2C_6H_5$
30	p-$C_6H_4CH_3$	o-$CH_2C_6H_4Cl$
31	p-$C_6H_4CH_3$	o-$CH_2C_6H_4F$
17, 32	p-$C_6H_4CH_3$	p-$CH_2C_6H_4CH_3$
33	m-$C_6H_4CH_3$	o-$CH_2C_6H_4Cl$
34	m-$C_6H_4CH_3$	o-$CH_2C_6H_4F$

Figure 2. Chemical structures of compounds **5**–**34**. See Refs. 25,26,29, 44–46.

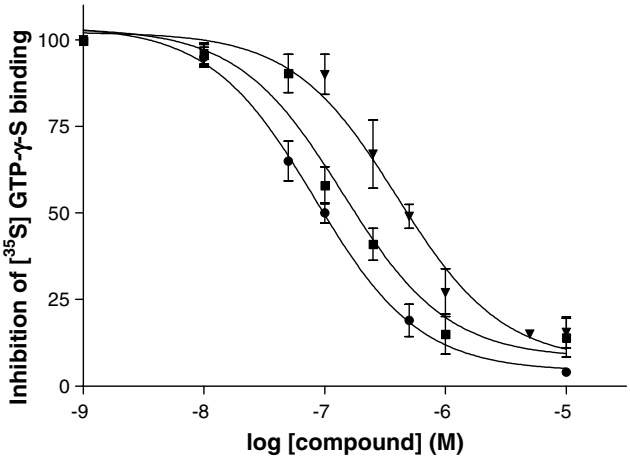


Figure 3. Concentration–response curves for inhibition of binding of [^{35}S]GTPγS by new compounds in the presence of a single concentration of the agonist NECA. Membranes of CHO cells expressing human A_3 adenosine receptors were incubated in the presence of 10 μM NECA and increasing concentrations of the new compounds **81** (■), **82** (▼) and **84** (●) as described in Experimental procedures. Each data point represents the mean ± SEM of at least three independent experiments.

Table 2. Ability of new ligands to inhibit [^{35}S]GTP-γ-binding in membranes of cells expressing the cloned human A_3 adenosine receptor compared with receptor binding affinities

Compound	A_3 receptor binding affinity K_i (nM)[a]	[^{35}S]GTP-γ-S binding IC_{50} (nM)[b]
81	33 ± 3	141 ± 15
82	35 ± 4	398 ± 40
83	6 ± 1	83 ± 10
84	28 ± 3	148 ± 16

[a] K_i values determined in radioligand binding assays in membranes from transfected CHO cells (±SEM).
[b] IC_{50} for inhibition by antagonists in the presence of 10 μM NECA in membranes of CHO cells expressing the human A_3 adenosine receptors (±SEM).

bonds involving at least one hydrogen atom. A cut-off of 12 Å for the non-bonded interactions was used. The receptors were then minimised with harmonic position constraints for all heavy atoms. The constraining force constants applied were 5000, 1000, 100 and 10 kcal/mol/Å^2. Subsequently, a cycle of minimisation was done to relax all the atoms without constraints. The maximum number of minimisation steps was set to 10,000 and the convergence criterion for energy gradient was set to 0.01 kcal/mol/Å^2. All molecular dynamics (MD) simulations were performed with AMBER. The MD simulation involved: (1) a 20 ps equilibration step, (2) a 50 ps molecular dynamics simulation for the actual data collection. The time step of the simulations was 1.5 fs with a cut-off of 12 Å for the non-bonded interactions. In the data collection stage, the snapshot was recorded in the trajectory file every 200 fs. The 10 best complexes for both the receptors were considered and further minimised through decreasing constraints, followed by an additional cycle of minimisation with no constraints (relaxation). After the models for human A_1 and A_3 receptors were obtained, ligand **82** was then docked into the binding site by using the DOCK program[37] exploiting the flexible docking option. The docking step was followed by a further sequence of energy minimisation, using the same gradient threshold as in the previous one, thus leading to the optimised three-di-

```
CLUSTAL W (1.82) multiple sequence alignment
AA1R_HUMAN      -------------------------MP---PSISAFQAAYIGIEVLIALVSVPGNVLVIW 32
AA3R_HUMAN      -------------------------MPNNSTALSLANVTYITMEIFIGLCAIVGNVLVIC 35
OPSD_BOVIN      MNGTEGPNFYVPFSNKTGVVRSPFEAPQYYLAEPWQFSMLAAYMFLLIMLGFPINFLTLY 60
                                        *    : .          .:: : ..  *.*.:

AA1R_HUMAN      AVKVNQALRDATFCFIVSLAVADVAVGALVIPLAILINIGPQTYFHT--CLMVACPVLIL 90
AA3R_HUMAN      VVKLNPSLQTTTFYFIVSLALADIAVGVLVMPLAIVVSLGITIHFYS--CLFMTCLLLIF 93
OPSD_BOVIN      VTVQHKKLRTPLNYILLNLAVADLFMVFGGFTTTLYTSLHGYFVFGPTGCNLEGFFATLG 120
                ..  :  *: .   :::.**:**: :    :. ::  .:     * .  * :      :

AA1R_HUMAN      TQSSILALLAIAVDRYLRVKIPLRYKMVVTPRRAAVAIAGCWILSFVVGLTPMFGWNNLS 150
AA3R_HUMAN      THASIMSLLAIAVDRYLRVKLTVRYKRVTTHRRIWLALGLCWLVSFLVGLTPMFGWN--M 151
OPSD_BOVIN      GEIALWSLVVLAIERYVVVCKPMSNFRFGEN-HAIMGVAFTWVMALACAAPPLVGWSRYI 179
                 . :: :*:.:*::**: *  .:    .    :  :.:.  *::::  . .*:.**.

AA1R_HUMAN      AVERAWAANGSMGEPVIKCEFEKVISMEYMVYFNFFVWVLPPLLLMVLIYLEVFYLIRKQ 210
AA3R_HUMAN      KLTSEYHRN----VTFLSCQFVSVMRMDYMVYFSFLTWIFIPLVVMCAIYLDIFYIIRNK 207
OPSD_BOVIN      PEGMQCSCG------IDYYTPHEETNNESFVIYMFVVHFIIPLIVIFFCYGQLVFTVKEA 233
                        .      .      .    : :* : *.. .: **:::   * ::.: :::

AA1R_HUMAN      LNKKVSASSGDPQKYYGKELKIAKSLALILFLFALSWLPLHILNCITLFCPSCHKPSILT 270
AA3R_HUMAN      LSLNLSNSK-ETGAFYGREFKTAKSLFLVLFLFALSWLPLSIINCIIYFNG--EVPQLVL 264
OPSD_BOVIN      AAQQQ-----ESATTQKAEKEVTRMVIIMVIAFLICWLPYAGVAFYIFTHQGSDFGPIFM 288
                   :      :.      * : :: : :::: * :.***   :          .   :.

AA1R_HUMAN      YIAIFLTHGNSAMNPIVYAFRIQKFRVTFLKIWNDHFRCQPAPPIDEDLPEERPDD---- 326
AA3R_HUMAN      YMGILLSHANSMMNPIVYAYKIKKFKETYLLILKACVVCHPSDSLDTSIEKNSE------ 318
OPSD_BOVIN      TIPAFFAKTSAVYNPVIYIMMNKQFRNCMVTTLCCGKNPLGDDEASTTVSKTETSQVAPA 348
                 :  :::: .:  **::*    ::*:   :               .  : :
```

"*" means that the residues or nucleotides in that column are identical in all sequences in the alignment.
":" means that conserved substitutions are observed.
"." means that semi-conserved substitutions are observed.

Figure 4. Multiple alignment of human A_1, human A_3 receptors and bovine rhodopsin.

mensional model for both A_1 and A_3 human receptors. The computational analysis was carried out on compounds **41**, **43**, **82**, **84**, **86** and **90**, selected as significant molecules. The selection was made to achieve a good sampling of both selective and non-selective molecules, thus, ensuring the discovery of selectivity requirements (see Technical Details for the description of the minimisation and docking protocols). The critical amino acids surrounding the full antagonist **82** (Leu91, His95, Phe182, Ile186, Val235, Leu236, Asn250, Asn274 and Asn278) are shown in Figure 5. They appear to be the most significant moieties of the binding site for the molecules analysed, according to details highlighted below. The purine ring is located in a hydrophobic pocket defined by Leu91 and His95. The carbamoyl moiety is oriented towards Asn274 and Asn278, both located on TM7. Interestingly, these two asparagine residues are conserved among a large number of G protein-coupled receptors. The interactions with this site orient the carbamoyl phenyl ring in the middle of TM6 and TM7. The substituent at position 2 of the adenine nucleus, mostly a phenyl or a *p*-methyl-phenyl group, is placed in a hydrophobic pocket, delimited by apolar amino acids, such as Ile186, Leu190 (TM5) and Leu236 (TM6).

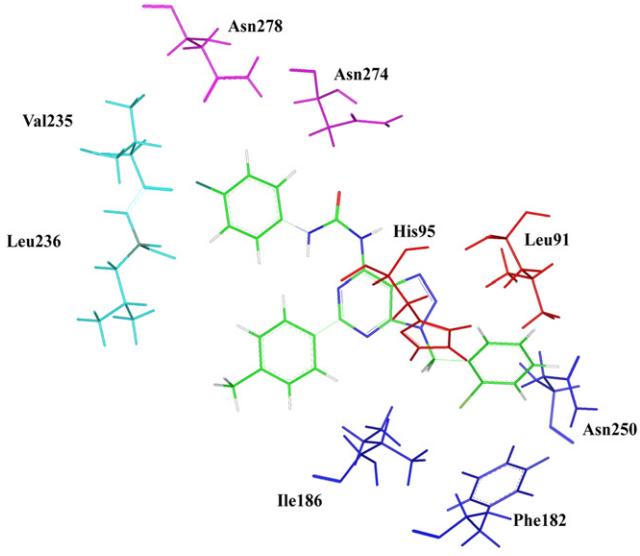


Figure 5. Binding site for **82** in human A_3 receptor.

Additional aromatic–aromatic stabilisation of the 9-benzyl group, interacting with Phe182, may be responsible for the increase in binding affinity. Site-directed

mutagenesis showed the inability of the N250A mutant of A_3 to bind a radio-labelled antagonist, leading to a proposed direct interaction of this residue with ligands.[38] This could suggests a better interaction for ligands carrying an H-bond acceptor in the *o*-position of the substituent at position 9.

The *p*-position of the phenyl ring belonging to the N^6-substituent (aromatic moiety attached to the carbamoyl substituent), turns out to face a mostly hydrophobic area, due to the presence of Val235 (TM6) and Asn278 (TM7).

We exploited the DOCK program with the aim of identifying the most favourable binding conformations and orientations for the selected ligands on both models of A_1 and A_3 receptors. Moreover, the DOCK program enables a rough evaluation of interaction energies. These values do not represent rigorous thermodynamic quantities, since changes in entropy and solvation effects are only approximately taken into account. Nevertheless, they can be reasonably used in comparisons with relative interaction energies obtained from binding assay experiments, in order to validate three-dimensional (3D) theoretical models, that subsequently may be exploited with predictive purposes. Taking into account the above considerations, we then compared the DOCK scores with biological data. An energetically favourable model correlating well with the experimental results was selected for both receptor subtypes.

3. Results and discussion

3.1. Structure–activity relationships

Modification on the 8-azaadenine nucleus made in the series of compounds synthesised in this paper is at positions 2, 6 and 9. On the basis of the biological results (Table 1) of affinity on A_1, A_{2A} and A_3 some interesting considerations about structure–activity relationships are possible.

3.1.1. The 6-position. The series of compounds **35–53** were synthesised to confirm the hypothesis that carbamoylation of the 6-amino group of 2-phenyl-6-amino-9-benzyl-8-azaadenines would confer A_3 ligand activity on the molecules. Various substituents (Cl, CF_3, F, OCH_3 and I) were introduced to the *o*-, *m*- and *p*- positions of the phenylcarbamoyl function to ascertain the influence of the type and position of the substituent on affinity towards the adenosine receptors. The biological data were encouraging as they revealed activity in the nanomolar range. For example, compounds **38**, **39**, **40**, **41**, **42**, **43** and **46** showed K_i values ranging from 18 nM (compound **43**) to 54 nM (compound **40**) (see Table 1). These compounds showed very low affinity towards A_{2A} receptors, but retained good affinity for A_1 receptor subtypes with K_i values between 91 and 1600 nM. It is evident from Table 1 that the chlorophenyl groups do not contribute significantly to the affinity for the adenosine receptors, nor does the *p*-iodophenyl moiety. The potent electron-withdrawing groups CF_3 and the F groups produce nearly the same response, independent of their position in the phenyl ring. The A_1/A_3receptor selectivity for these compounds ranged from 3 to 7. Replacement of the *p*-CF_3 or *p*-F with a OCH_3 substituent did not markedly change the activity, but the OCH_3 group in the *o*- and *m*-positions proved detrimental to A_3 and A_1 receptor affinity. The fact that the F atom can be an electron-donating group, whereas the CF_3 cannot, suggests that electron-withdrawing abilities of both substituents is an important consideration in their interactions with macromolecular species. Thus, the electron-withdrawing properties of the *p*-OCH_3 may contribute to affinity for the A_3 receptors. Compounds **40**, **43** and **46** having *p*-CF_3, *p*-F and *p*-OCH_3, respectively, as substituents on the phenylcarbamoyl group were selected as lead compounds for the next phase of the study. Maintaining the N^6 substituent unchanged, which would assure A_3 affinity, we sought to vary the 9- and 2-positions on these molecules to lower affinity towards the A_1 subtype and enhance selectivity.

3.1.2. The 9-position. Replacement of the 9-benzyl group with alkyl or aralkyl substituents (compounds **48–53**) resulted in a dramatic fall in affinity for the three adenosine subtypes. These results reveal the 9-benzyl group as an important structural criterion for binding to A_1 and A_3 receptors in these types of adenosine ligands. A substituted benzyl group, however, those with *o*-Cl, *o*-F or *p*-CH_3, confers very little affinity for A_{2A} receptors, low affinity for A_1 receptors, and an affinity for A_3 receptors in the submicromolar range (compounds **56**, **59** and **62**). Based on the conformational considerations for the fit of isoquinoline and quinazoline derivatives at the A_3 receptors,[23] one may deduce that the benzyl substituent is oriented away from the pyrimidine portion of the azapurine ring and can be in the plane or rotated at 90° to the plane of the azapurine ring.

3.1.3. The 2-position. At position 2, replacement of the phenyl with a variety of substituents such as H, benzyl, or alkyl groups (butyl, propyl, methyl) caused nearly complete loss of activity. Biological assays revealed a low percentage of displacement at 10 μM (<32%) towards A_1, A_{2A} and A_3 adenosine receptors. However, insertion of a methyl group on the *m*- or *p*-position of the phenyl ring resulted in compounds with good affinity for A_3 receptors and showed a very good A_1/A_3 and A_{2A}/A_3 selectivity (compounds **80–84**, **86** and **90**). In fact, compounds **81** and **82**, which resulted in complete selectivity showing no affinity on A_1 and A_{2A} receptors and good affinity for A_3 (K_i 32.5 and 35 nM, respectively), bear a *p*-CH_3 group at the phenyl moiety on C(2), an *o*-chlorobenzyl at N(9) and a *p*-CF_3 or *p*-F on the phenyl moiety at the C(6)-position, respectively. Compound **84**, the most potent of the A_3-receptor blockers, also displays a *p*-methylphenyl group at the C(2), an *o*-fluorobenzyl at N(9) and a *p*-F at phenylcarbamoyl moiety. Also compounds **86** and **90** can be considered very interesting as A_3 selective ligands having KiA_1 700 nM and 1600 nM and KiA_3 22.4 and 44.6 nM, respectively. All these compounds have low affinity for A_{2A} receptors showing percentage inhibition at 10 μM concentrations between 59 and 0.

3.2. Molecular modelling

From molecular modelling studies the following findings were obtained.

3.2.1. Analysis of tautomerism. For each one of the six antagonists of both human A_1 and A_3 receptors, subjected to the docking procedure by means of the DOCK program, three runs were performed, corresponding to one of the three tautomeric forms, here referred as to ureic, iminol-1 and iminol-2 (Fig. 6, b–d). The DOCK results suggest the ureic form as the preferred one for compounds **41**, **84** and **86** in A_1 and compounds **43**, **86** and **82** in A_3. The iminol-1 tautomeric form appears to be preferred for compounds **90** and **82** in A_1. Finally, the iminol-2 tautomeric form was suggested to be the preferred one for compound **43** in A_1 and compounds **84**, **41** and **90** in A_3. In most of the cases, DOCK suggested the ureic form as the best for the interaction in both receptors. On the other hand, the iminol-2 form turns out to be the best for the interactions of **43** and **84**, the most active antagonist of A_1 and A_3, respectively. Finally, the iminol-1 tautomeric form seems to show the lowest population in interaction with the binding site.

As an example, Figure 7a shows the different orientation of **86** in the A_1 receptor. The ureic form seems to take a more relaxed (quite planar) arrangement in the binding site; on the other hand the iminol-2 tautomeric form is more distorted along the carbamoyl moiety, leading to a rotation of the aromatic ring. This fact can justify lower interaction energy predicted by DOCK for the ureic form. Figures 7b and c show a stereo-view for two different orientations of **86** in the A_3 receptor. Also in this case, the ureic form seems to keep a quite planar conformation into the binding site, whereas the iminol-2 tautomeric form results to be slightly distorted. It may account for the less favourable interaction energy predicted by DOCK with regard to the ureic form, even though the scores for the two inhibitors differ very slightly, as in the case of the A_1 receptor.

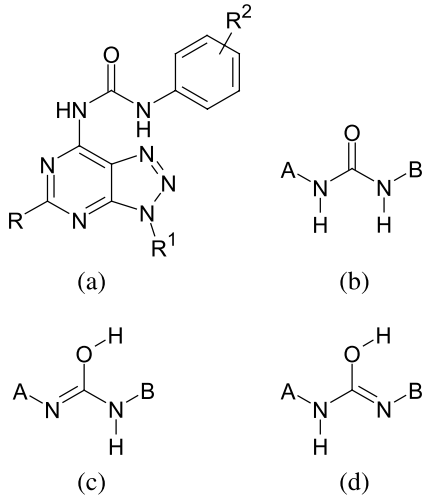


Figure 6. (a) Main template shared by all the analysed antagonists of A_1 and A_3 adenosine receptors; (b) ureic form; (c) iminolic-1 form; (d) iminolic-2 form.

3.2.2. Quality fitting and prediction reliability. With regard to the predictive power of the theoretical models developed here, it has to be pointed out that the criterion followed in assessing their validity consisted of splitting the receptor antagonists into two classes on the basis of their affinity value. For example, in the case of the human A_1 receptor, the trend of calculated interaction energies was found to be in good accordance with data coming from binding experiments for all the high affinity antagonists (K_i up to 90 nM). For the other compounds showing lower affinity (higher than 400 nM) also the calculated interaction energies were observed to have quite unfavourable values. An analogous observation can be made with regard to the A_3 antagonists. So both models appear capable of discriminating between high and low affinity molecules.

4. Conclusions

After the first findings regarding compounds **35–46**, which showed in some cases remarkable A_3 affinity, our efforts were addressed to improving the A_1/A_3 selectivity. This goal was reached maintaining the N^6 substituent unchanged to ensure A_3 affinity and varying the structure of groups at N(9) and C(2) positions. In several cases, especially for compounds **80–86**, **89** and **90**, we managed to lower affinity towards the A_1 subtype and to enhance A_3/A_1 selectivity, obtaining the first examples of A_3 potent and selective antagonists having an adenine-like nucleus. Further, by molecular modelling methods we could obtain some information regarding the preferred tautomeric structure of the ureic group in interaction with binding sites, and good correlation between selectivity ratio values and differences in calculated interaction energies with A_3 and A_1 subtypes.

The molecular modelling work, carried out by the DOCK program, supplied two receptor models, which appear to be extremely useful for understanding the binding modes, and suggesting the most probable tautomeric forms of antagonists for human A_1 and A_3 receptors. In particular, the ureic form is thought to be the most relevant for the interaction in both the receptors, whereas the iminol-2 form seems to be the best for the binding of **43** and **84** in A_1 and A_3 receptors, respectively. The good correlation between the experimental and calculated values for the A_1 receptor and the good estimation of the A_1/A_3 selectivity, can identify DOCK as a suitable tool for the design of new selective antagonists towards human A_3 receptor.

5. Experimental

5.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra in Nujol mulls were recorded on a Mattson Genesis series FTIR spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer in δ units from TMS as an

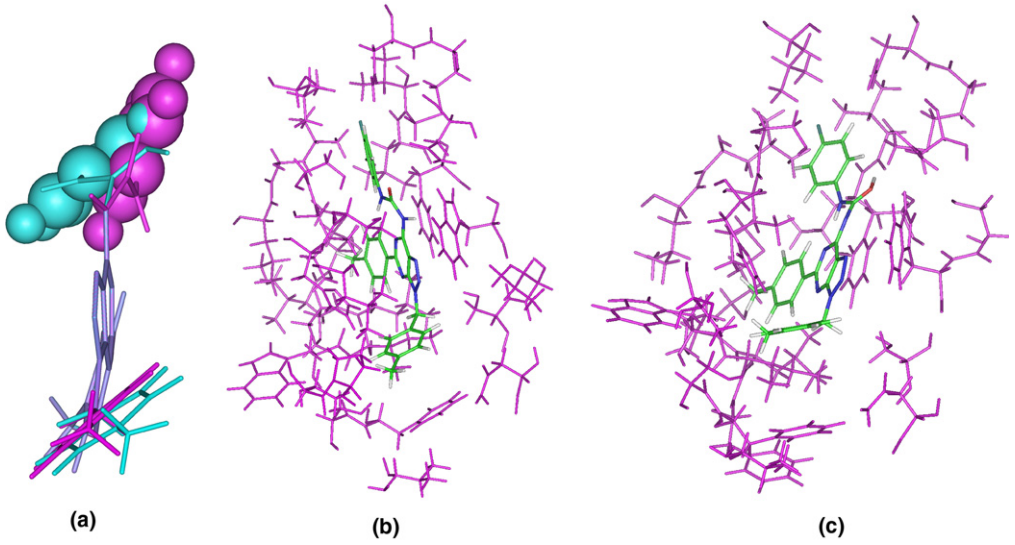


Figure 7. (a) Different conformations for ureic and iminol-2 forms of compound **86** in human A_1 receptor, respectively coloured in magenta and light blue. (b) Best conformer of the ureic form of compound **86** in human A_3 receptor. (c) Best conformer of the iminol-2 form of compound **86** in human A_3 receptor.

internal standard; the compounds were dissolved in the solvent indicated in Table 4. TLC was performed on precoated silica gel F_{254} plates (Merck). Microanalyses (C H N) were carried out on a Carlo Erba elemental analyser (Model 1106) and were within ±0.4% of the theoretical values.

5.2. General procedure to prepare 2,9-disubstituted-8-azahypoxanthines 5–17

To a stirred solution of sodium ethoxide obtained from sodium (0.46 g, 20 mmol) and absolute ethanol (20 mL), cyanoacetamide (0.84 g, 0.01 mol) and suitable azide (0.01 mol) were added and the mixture was refluxed for 1 h. Then the appropriate ethyl ester (0.05 mol) was added and heating was continued for 8 h. Evaporation of the solvent at reduced pressure, dilution with water and acidification with 10% acetic acid gave a solid which was filtered and crystallised or flash-chromatographed (see Tables 3 and 4).

5.3. General procedure to prepare 6-aminosubstituted-2,9-disubstituted-8-azaadenines 18–29, 32

A mixture of a suitable 2,9-disubstituted-8-azahypoxanthine (2 mmol), *N*,*N*-diethylaniline (2 mmol) and $POCl_3$ (1.5 mL, 16.3 mmol) was heated at 90 °C for 6 h. After cooling, the residue was diluted with diethyl ether and then filtered. The solid obtained (6-chloro-2,9-disubstituted-8-azaadenine) was not characterised but reacted as soon as possible with absolute ethanol saturated with NH_3 (15 mL) in a well-stoppered steel flask. After 10 h at 110 °C the mixture was concentrated at reduced pressure, and the solid precipitated filtered, washed with water and crystallised or flash-chromatographed (see Tables 3 and 4).

Table 3. Chemical and physical properties of compounds **8**–**10**, **16**, **17**, **20**–**24** and **30**–**33**

Compound	Yield (%)	Crystall. Solvent	Mp (°C)	Formula	Analysis
8	26.3	MeOH/Isopropanol	283–285	$C_{17}H_{12}ClN_5O$	C, H, N
9	29	MeOH/Isopropanol	280	$C_{17}H_{12}FN_5O$	C, H, N
10	50	MeOH/Isopropanol	262–264	$C_{18}H_{15}N_5O$	C, H, N
16	40	MeOH	270	$C_{18}H_{15}N_5O$	C, H, N
17	55	[a]	240–242	$C_{19}H_{17}N_5O$	C, H, N
20	64	[b]	165	$C_{18}H_{16}N_6$	C, H, N
21	46	CH_3CN	198	$C_{17}H_{13}ClN_6$	C, H, N
22	50	Isopropanol	180–182	$C_{17}H_{13}FN_6$	C, H, N
23	71	Isopropanol	193–195	$C_{18}H_{16}N_6$	C, H, N
24	47.5	Isopropanol	202	$C_{18}H_{16}N_6$	C, H, N
30	26	THF/*n*-hexane	223–225	$C_{18}H_{15}ClN_6$	C, H, N
31	20	Isopropanol	205	$C_{18}H_{15}FN_6$	C, H, N
32	50	Isopropanol	212	$C_{19}H_{18}N_6$	C, H, N
33	37	THF/*n*-hexane	215–216	$C_{18}H_{15}ClN_6$	C, H, N
34	35	[c]	195	$C_{18}H_{15}FN_6$	C, H, N

[a] Purification by flash-chromatography (eluent $CHCl_3$/MeOH 100:4).
[b] Purification by flash-chromatography (eluent $CHCl_3$/MeOH 100:2).
[c] Purification by flash-chromatography (eluent $CHCl_3$).

Table 4. ^{1}H NMR of the new synthesised compounds

	Aliphatic H	Aromatic H	Benzylic H	Exch. H
8[a]		8.15 (m, 2H), 7.56 (m, 4H), 7.36 (m, 3H)	5.88 (s, 2H)	12.86 (s, 1H)
9[a]		8.17 (m, 2H), 7.60–7.17 (m, 7H)	5.83 (s, 2H)	12.85 (s, 1H)
10[a]	2.27 (s, 3H)	8.17 (m, 2H), 7.60 (m, 3H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.17 (d, *J* = 8.0 Hz, 2H)	5.75 (s, 2H)	12.84 (s, 1H)
16[b]	2.48 (s, 3H)	8.10 (d, *J* = 8.0 Hz, 2H), 7.53–7.28 (m, 7H)	5.80 (s, 2H)	11.19 (s, 1H)
17[a]	2.38 (s, 3H), 2.26 (s, 3H)	8.13 (d, *J* = 8.4 Hz, 2H), 7.33 (d, *J* = 8.4 Hz, 2H), 7.28 (d, *J* = 8.4 Hz, 2H), 7.15 (d, *J* = 8.4 Hz, 2H)	5.69 (s, 2H)	12.82 (s, 1H)
20[a]	4.84 (t, *J* = 7.2 Hz, 2H)	8.35 (m, 2H), 7.50 (m, 3H), 7.15 (m, 5H)	3.32 (t, *J* = 7.0 Hz, 2H)	8.38 (br s, 1H) 8.07 (br s, 1H)
21[b]		8.46 (m, 2H), 7.47 (m, 4H), 7.25 (m, 3H)	5.98 (s, 2H)	6.17 (br s, 2H)
22[b]		8.47 (m, 2H), 7.50–7.07 (m, 7H),	5.89 (s, 2H)	6.22 (s, 2H)
23[b]	2.33 (s, 3H)	8.48 (m, 2H), 7.50 (m, 3H), 7.43 (d, *J* = 8.0 Hz, 2H), 7.16 (d, *J* = 8.0 Hz, 2H)	5.78 (s, 2H)	5.89 (s, 2H)
24[b]		7.47–7.31 (m, 10H)	5.73 (s, 2H), 4.17 (s, 2H)	6.06 (s, 2H)
30[b]	2.43 (s, 3H)	8.35 (d, *J* = 8.4 Hz, 2H), 7.46–7.20 (m, 6H)	5.96 (s, 2H)	6.14 (s, 2H)
31[a]	2.37 (s, 3H)	8.29 (d, *J* = 8.2 Hz, 2H), 7.45–7.20 (m, 6H)	5.84 (s, 2H)	8.45 (br s, 1H) 8.13 (br s, 1H)
32[a]	2.37 (s, 3H), 2.24 (s, 3H)	8.31 (d, *J* = 8.0 Hz, 2H), 7.30 (d, *J* = 8.0 Hz, 2H), 7.29 (d, *J* = 7.8 Hz, 2H) 7.15 (d, *J* = 7.8 Hz, 2H)	5.74 (s, 2H)	8.43 (br s, 1H) 8.10 (br s, 1H)
33[a]	2.40 (s, 3H)	7.96 (m, 2H), 7.56–7.36 (m, 6H)	5.89 (s, 2H)	3.45 (s, 2H)
34[a]	2.40 (s, 3H)	8.20 (m, 2H), 7.46–7.17 (m, 6H)	5.85 (s, 2H)	8.48 (br s, 1H) 8.20 (br s, 1H)
35[a]		7.14–7.60 (m, 11H), 8.14 (d, 1H), 8.39 (m, 2H + 1H exch.)	5.95 (s, 2H)	10.99 (1H)
36[a]		7.18–7.82 (m, 12H), 8.41 (m, 2H + 1H exch.)	5.95 (s, 2H)	11.34 (1H)
37[b]		7.25–7.65 (m, 12H), 8.40 (m, 2H + 1H exch.)	5.90 (s, 2H)	11.87 (1H)
38[b]		7.37–7.88 (m, 12H), 8.33 (m, 2H)	5.91 (s, 2H)	8.52 (1H) 11.30 (1H)
39[b]		7.36–8.05 (m, 12H), 8.43 (m, 2H)	5.91 (s, 2H)	8.48 (1H) 12.14 (1H)
40[b]		7.34–7.83 (m, 12H) , 8.42 (m, 2H)	5.91 (s, 2H)	8.48 (1H) 12.08 (1H)
41[b]		7.15–7.60 (m, 12H), 8.45–8.50 (m, 2H + 1H exch.)	5.90 (s, 2H)	11.54 (1H)
42[b]		6.90 (m, 1H), 7.35–7.64 (m, 11H), 8.42 (m, 2H + 1H exch.)	5.90 (s, 2H)	11.93 (1H)
43[b]		7.07–7.67 (m, 12H), 8.42 (m, 2H + 1H exch.)	5.90 (s, 2H)	11.80 (1H)
44[b]	3.85 (s, 3H)	6.92–7.59 (m, 11H), 8.25 (m, 1H), 8.55 (m, 2H)	5.89 (s, 2H)	8.38 (s, 1H) 11.44 (s, 1H)
45[b]	3.86 (s, 3H)	6.75 (m, 1H), 7.21–7.62 (m, 11H), 8.44 (m, 2H + 1H exch.)	5.90 (s, 2H)	11.81 (s, 1H)
46[b]	3.85 (s, 3H)	6.95–7.62 (m, 12H), 8.42 (m, 2H + 1H exch.)	5.91 (s, 2H)	12.08 (s, 1H)
47[b]		7.37–7.74 (m, 12H), 8.37 (m, 2H + 1H exch.)	5.90 (s, 2H)	11.87 (s, 1H)
48[b]	4.73 (t, *J* = 7.0 Hz, 3H), 3.85 (s, 3H), 2.10 (m, 2H), 1.41–1.27 (m, 8H) 0.89 (t, *J* = 6.4 Hz, 3H)	8.41 (m, 2H), 7.60 (m, 5H), 6.96 (d, 2H)		11.69 (s, 1H) 8.27 (s, 1H)
49[a]	4.71 (t, *J* = 7.0 Hz, 2H), 2.02 (m, 2H), 1.28 (m, 8H),0.82 (t, *J* = 6.4 Hz, 3H)	8.41 (m, 2H), 7.81–7.61 (m, 7H + 1H exch.)		11.46 (s, 1H)
50[b]	4.74 (t, *J* = 7.0 Hz, 2H), 2.12 (m, 2H), 1.41–1.27 (m, 8H), 0.89 (t, *J* = 6.4 Hz, 3H)	8.43–8.35 (m, 2H), 7.69–7.57 (m, 5H), 7.13 (d, 2H)		11.83 (s, 1H) 8.34 (s, 1H)
51[a]	3.74 (s 3H), 4.96 (t, *J* = 6.6 Hz, 2H)	8.31 (m, 2H), 7.62 (m, 2H), 7.51 (d, *J* = 9.0 Hz, 2H), 720–7.11 (m, 5H), 6.98 (d, *J* = 9.0 Hz, 2H)	3.32 (t, *J* = 6.6 Hz, 2H)	11.23 (s, 1H) 11.12 (s, 1H)
52[b]	5.00 (t, *J* = 7.2 Hz, 2H)	8.35 (m, 2H), 7.82 (d, *J* = 9.0 Hz, 2H), 7.69–7.60 (m, 4H), 7.29–7.20 (m, 6H)	3.44 (t, *J* = 7.2 Hz, 2H)	12.08 (s, 1H) 8.56 (s, 1H)
53[a]	4.97 (t, *J* = 6.6 Hz, 2H)	8.34 (m, 2H), 7.63 (m, 5H), 7.30–7.13 (m, 7H)	3.37 (t, *J* = 6.6 Hz, 2H)	11.27 (s, 1H) 11.10 (br s, 1H)

Table 4 (*continued*)

	Aliphatic H	Aromatic H	Benzylic H	Exch. H
54[a]	3.75 (s, 3H)	8.35 (m, 2H), 7.61–7.38 (m, 9H), 6.98 (d, J = 8.8 Hz, 2H)	6.01 (s, 2H)	11.20 (s, 2H)
55[a]		8.39 (m, 2H), 7.81–7.38 (m, 11H)	6.03 (s, 2H)	11.38 (s, 2H)
56[a]		8.38 (m, 2H), 7.67–7.22 (m, 11H)	6.03 (s, 2H)	11.24 (s, 2H)
57[a]	3.75 (s, 3H)	8.39 (m, 2H), 7.63–6.20 (m, 9H), 6.98 (d, J = 9.2 Hz, 2H)	5.98 (s, 2H)	11.20 (s, 2H)
58[b]		8.40 (m, 2H), 7.84–7.15 (m, 11H)	5.98 (s, 2H)	12.08 (s, 1H) 8.55 (s, 1H)
59[a]		8.40 (m, 2H), 7.66–7.19 (m, 11H)	5.99 (s, 2H)	11.26 (s, 1H) 11.24 (s, 1H)
60[b]	3.85 (s, 3H) 2.34 (s, 3H)	8.41 (m, 2H + 1H exch.), 7.61–6.93 (m, 11H)	5.86 (s, 2H)	11.65 (s, 1H)
61[b]	2.34 (s, 3H)	8.40 (m, 2H), 7.82 (d, J = 8.4 Hz, 2H), 7.61 (m, 5H), 7.44 (d, J = 8.2 Hz, 2H), 7.18 (d, J = 8.2 Hz, 2H)	5.86 (s, 2H) 2.34 (s, 3H)	12.09 (s, 1H) 8.63 (s, 1H)
62[a]	2.26 (s, 3H)	8.41 (m, 2H), 7.62 (m, 5H), 7.36–7.16 (m, 6H)	5.89 (s, 2H)	11.26 (s, 2H)
63[b]	3.83 (s, 3H)	7.50–7.27 (m, 10H), 7.19 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 8.8 Hz, 2H)	5.82 (s, 2H) 4.36 (s, 2H)	11.14 (s, 1H) 8.28 (s, 1H)
64[b]		7.55–7.27 (m, 14H)	5.84 (s, 2H) 4.41 (s, 2H)	11.56 (s, 1H) 8.35 (s, 1H)
65[b]		7.49–7.14 (m, 14H)	5.83 (s, 2H) 4.38 (s, 2H)	11.26 (s, 1H) 8.20 (s, 1H)
66[a]	3.74 (s, 3H), 3.00 (t, J = 6.8 Hz, 2H), 1.84 (m, 2H), 1.38 (m, 2H), 0.91 (t, J = 7.2 Hz, 3H)	7.50 (d, J = 9.0 Hz, 2H), 7.35 (m, 5H), 6.96 (d, J = 9.0 Hz, 2H)	5.84 (s, 2H)	11.73 (s, 1H) 11.17 (s, 1H)
67[a]	3.01 (t, J = 6.8 Hz, 2H), 1.83 (m, 2H), 1.41 (m, 2H), 0,91 (t, J = 7.2 Hz, 3H)	7.77 (m, 4H), 7.34 (m, 5H)	5.84 (s, 2H)	11.85 (s, 1H) 11.26 (s, 1H)
68[a]	3.00 (t, J = 6.8 Hz, 2H), 1.81 (m, 2H), 1.36 (m, 2H), 0.89 (t, J = 7.2 Hz, 3H)	7.59 (m, 2H), 7.34–7.18 (m, 7H)	5.84 (s, 2H)	11.85 (s, 1H) 11.15 (s, 1H)
69[a]	3.74 (s, 3H), 2.97 (t, J = 7.2 Hz, 2H), 1.87 (m, 2H), 0.97 (t, J = 7.2 Hz, 3H)	7.50 (d, J = 9.0 Hz, 2H), 7.34 (m, 5H), 6.95 (d, J = 9.0 Hz, 2H),	5.85 (s, 2H)	11.72 (s, 1H) 11.15 (s, 1H)
70[b]	3.07 (t, J = 7.2 Hz, 2H), 2.00 (m, 2H), 1.11 (t, J = 7.2 Hz, 3H)	7.75–7.31 (m, 9H)	5.84 (s, 2H)	12.14 (s, 1H) 8.40 (s, 1H)
71[a]	2.99 (t, J = 7.2 Hz, 2H), 1.88 (m, 2H), 0.97 (t, J = 7.2 Hz, 3H)	7.61 (m, 2H), 7.37–7.19 (m, 7H)	5.85 (s, 2H)	11.85 (s, 1H) 11.26 (s, 1H)
72[a]	3.74 (s, 3H), 3.73 (s, 3H)	7.52 (d, J = 8.0 Hz, 2H), 7.34 (m, 5H), 6.94 (d, J = 8.0 Hz, 2H)	5.83 (s, 2H)	11.62 (s, 2H)
73[b]	2.86 (s, 3H)		5.81 (s, 2H)	12.12 (s, 1H) 8.35 (s, 1H)
74[a]	3.34 (s, 3H)	8.74 (m, 2H), 8.22 (m, 2H), 7.34 (m, 5H)	5.92 (s, 2H)	8.35 (s, 2H)
75[a]	3.74 (s, 3H)	8.84 (s, 1H), 7.53 (d, J = 8.8 Hz, 2H), 7.34 (m, 5H) 6.94 (d, J = 8.8 Hz, 2H)	5.88 (s, 2H)	11.44 (s, 1H) 11.19 (s, 1H)
76[a]		8.88 (s, 1H), 7.85 (d, J = 8.8 Hz, 2H), 7.72 (d, J = 8.8 Hz, 2H), 7.35 (m, 5H)	5.90 (s, 2H)	11.86 (s, 1H) 11.42 (s, 1H)
77[a]		8.86 (s, 1H), 7.67 (m, 2H), 7.36–7.22 (m, 7H)	5.90 (s, 2H)	11.61 (s, 1H) 11.30 (s, 1H)
78[b]	3.85 (s, 3H) 2.50 (s, 3H)	8.30 (d, J = 8.2 Hz, 2H + 1H exch.), 7.61–7.35 (m, 9H), 6.96 (d, J = 9.0 Hz, 2H)	5.88 (s, 2H)	11.65 (s, 1H)
79[b]	2.52 (s, 3H)	8.30 (d, J = 8.2 Hz, 2H + 1H exch.), 7.81 (d, J = 8.8 Hz, 2H), 7.67 (d, J = 8.8 Hz, 2H), 7.56–7.35 (m, 7H)	5.89 (s, 2H)	12.10 (s, 1H)
80[b]	2.51 (s, 3H)	8.29 (d, J = 8.2 Hz, 2H + 1H exch.), 7.64–7.07 (m, 11H)	5.89 (s, 1H)	11.82 (s, 1H)
81[b]	2.51 (s, 3H)	8.28 (d, J = 8.8 Hz, 2H), 7.82 (d, J = 8.4 Hz, 2H), 7.68 (d, J = 8.4 Hz, 2H) 7.42–7.30 (m, 6H)	6.04 (s, 2H)	12.11 (s, 1H) 8.54 (s, 1H)

(*continued on next page*)

Table 4 (*continued*)

	Aliphatic H	Aromatic H	Benzylic H	Exch. H
82[a]	2.41 (s, 3H)	8.27 (d, J = 8.2 Hz, 2H), 7.67–7.22 (m, 10H)	6.01 (s, 2H)	11.28 (s, 1H) 11.25 (s, 1H)
83[a]	2.51 (s, 3H)	8.29 (d, J = 8.4 Hz, 2H), 7.82 (d, J = 8.6 Hz, 2H), 7.67 (d, J = 8.6 Hz, 2H), 7.57–7.15 (m, 6H)	5.97 (s, 2H)	12.11 (s, 1H) 8.57 (s, 1H)
84[a]	2.40 (s, 3H)	8.28 (d, J = 8.4 Hz, 2H), 7.78–7.06 (m, 10H)	5.90 (s, 2H)	11.30 (s, 2H)
85[a]	2.43 (s, 3H) 2.26 (s, 3H)	8.32 (d, J = 8.0 Hz, 2H), 7.80 (m, 4H), 7.44 (d, J = 8.0 Hz, 2H), 7.33 (d, J = 7.8 Hz, 2H) 7.17 (d, J = 7.8 Hz, 2H)	5.88 (s, 2H)	11.47 (s, 1H) 11.32 (s, 1H)
86[a]	2.42 (s, 3H) 2.26 (s, 3H)	8.30 (d, J = 8.0 Hz, 2H), 7.63 (m, 2H), 7.43 (d, J = 8.0 Hz, 2H) 7.31–7.16 (m, 6H)	5.88 (s, 2H)	11.31 (s, 1H) 11.22 (s, 1H)
87[a]	2.42 (s, 3H)	8.23 (s, 2H), 7.95–7.38 (m, 10H)	5.98 (s, 2H)	12.68 (s, 2H)
88[a]	2.42 (s, 3H)	8.17 (m, 2H), 7.64–7.27 (m, 10H)	6.02 (s, 2H)	11.45 (s, 1H) 11.25 (s, 1H)
89[b]	2.54 (s, 3H)	8.21 (m, 2H), 7.84 (d, J = 8.6 Hz, 2H) 7.66 (d, J = 8.6 Hz, 2H) 7.51–7.16 (m, 6H)	5.98 (s, 2H)	12.15 (s, 1H) 8.54 (s, 1H)
90[b]	2.52 (s, 3H)	8.20 (m, 2H), 7.66–7.07 (m, 10H)	5.98 (s, 2H)	11.90 (s, 1H) 8.48 (s, 1H)

[a] Solvent = DMSO.
[b] Solvent = $CDCl_3$.

5.4. General procedure to prepare 2,9-disubstituted-8-azaadenines 30, 31, 33 and 34

To a stirred solution of sodium ethoxide obtained from sodium (0.46 g, 20 mmol) and absolute ethanol (20 mL), malononitrile (0.66 g, 0.01 mol) was added and the mixture was heated at reflux for 15 min. Then a solution of a suitable azide (0.01 mol) and a suitable nitrile (0.1 mol) in absolute ethanol (2 mL) was added and the mixture was refluxed for 5 h. Evaporation of the solvent at reduced pressure, dilution with water and acidification with 10% acetic acid gave a solid, which was repeatedly washed with hot diethyl ether and then with water. The product obtained was purified by crystallisation or flash chromatography (see Tables 3 and 4).

5.5. General procedure to prepare 2,9-disubstituted-6-[(aryl)amino]carbonyl]amino-8-azapurines 35–90

A solution of **1** (100 mg, 0.34 mmol) and of a suitable phenylisocyanate (0.68 mmol) in acetonitrile (5 mL) was refluxed for 1 h. Before cooling, the hot reaction mixture was filtered to collect the solid precipitated, which resulted in the pure reaction product. Some of these products were not recrystallised (see Tables 4 and 5).

6. Biochemistry

6.1. Materials and methods

N^6-[4-Amino-3-[^{125}I]iodobenzyl) 5′-*N*-methycarbamoyladenosine ([^{125}I]ABMECA), [^{3}H]-8-cyclopenty-1,3-dipropylxanthine ([^{3}H]DCPCX) and [^{3}H]-5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) were obtained from NEN Life Science Products, Inc. (Koln, Germany). Adenosine deaminase (ADA) was obtained from Boeringer–Mannhein (Mannhein, Germany). Cell culture media and fetal calf serum were obtained from Bio-Wittaker (Walkersville, MD, USA). (−)-N^6-(2-phenylisopropyl) adenosine (R-PIA), 5′-*N*-ethylcarbamoyladenosine (NECA) and other agents were purchased from Sigma–Aldrich, srl.

Affinity of new synthesised compounds towards human A_1 adenosine receptors (ARs) was evaluated by competition experiments as described by Baraldi et al.,[39] using [^{3}H]DPCPX (111Ci/mmol) as a radioligand.

CHO cells, stably transfected with human A_1ARs, were grown and maintained in Dulbecco's modified Eagle's medium with F12 nutrient mixture without nucleosides, containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine (2 mM), and geneticin (0.2 mg/mL) at 37 °C in 5% CO_2/95% air. The cells were washed with phosphate-buffered saline solution and were scraped from flasks in ice-cold hypotonic buffer (5 mM Tris–HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenised with Polytron, and the homogenate was centrifuged at 48,000*g* for 30 min. The membrane pellet was resuspended in T_1 buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, 2 U/mL ADA, pH 7.4). Aliquots of membranes (50 μg of proteins) obtained from A_1 CHO cells were incubated at 25 °C for 180 min in 500 μL T_1 buffer, containing 3nM [^{3}H]DPCPX and different concentrations of new synthesised compounds. Nonspecific binding was determined in the presence of 50 μM R-PIA. The dissociation constant (K_d) of [^{3}H]DPCPX in CHO cell membranes was 3 nM.

Affinity towards human A_{2A}ARs was evaluated by competition experiments as described by Klotz et al.,[40] using [^{3}H]NECA as a radioligand. CHO cells, stably transfected with human A_{2A}ARs, were grown adherently and the membrane fraction was prepared as described above. Aliquots of cell membranes (80 μg of proteins) were incubated at 25 °C for 90 min in 500 μL T_2 buffer (50 mM Tris–HCl, 2 mM $MgCl_2$, 2 U/mL ADA,

Table 5. Chemical and physical properties of the compounds **35–90**

Compd	Yield (%)	Crystall. Solvent[a]	Mp (°C)	Formula	Analysis
35	96	Isopropanol	183	$C_{24}H_{18}ClN_7O$	(C,H,N)
36	80	Isopropanol	193	$C_{24}H_{18}ClN_7O$	(C,H,N)
37	90	Isopropanol	224	$C_{24}H_{18}ClN_7O$	(C,H,N)
38	95	Isopropanol	169	$C_{25}H_{18}F_3N_7O$	(C,H,N)
39	92	Isopropanol	177–180	$C_{25}H_{18}F_3N_7O$	(C,H,N)
40	86	Isopropanol	235–237	$C_{25}H_{18}F_3N_7O$	(C,H,N)
41	90	Isopropanol	206	$C_{24}H_{18}FN_7O$	(C,H,N)
42	91	Isopropanol	210	$C_{24}H_{18}FN_7O$	(C,H,N)
43	90	Isopropanol	220	$C_{24}H_{18}FN_7O$	(C,H,N)
44	94	Isopropanol	176	$C_{25}H_{21}N_7O_2$	(C,H,N)
45	92	Isopropanol	190–191	$C_{25}H_{21}N_7O_2$	(C,H,N)
46	85	Isopropanol	187	$C_{25}H_{21}N_7O_2$	(C,H,N)
47	92	Isopropanol	242–245	$C_{24}H_{18}IN_7O$	(C,H,N)
48	75	Isopropanol	182	$C_{22}H_{29}N_7O_2$	(C,H,N)
49	58		200	$C_{25}H_{26}F_3N_7O$	(C,H,N)
50	58		188	$C_{24}H_{26}FN_7O$	(C,H,N)
51	84		202	$C_{26}H_{23}N_7O_2$	(C,H,N)
52	53		212	$C_{26}H_{20}F_3N_7O$	(C,H,N)
53	99		210–211	$C_{25}H_{20}FN_7O$	(C,H,N)
54	55	THF/*n*-hexane	225	$C_{25}H_{20}ClN_7O_2$	(C,H,N)
55	60	THF	268	$C_{25}H_{17}ClF_3N_7O$	(C,H,N)
56	70	THF/*n*-hexane	210	$C_{24}H_{17}ClFN_7O$	(C,H,N)
57	55		220	$C_{25}H_{20}FN_7O_2$	(C,H,N)
58	60	THF	240	$C_{25}H_{17}F_4N_7O$	(C,H,N)
59	92	THF/*n*-hexane	215	$C_{24}H_{17}F_2N_7O$	(C,H,N)
60	40		230	$C_{26}H_{23}N_7O_2$	(C,H,N)
61	56		255	$C_{26}H_{20}F_3N_7O$	(C,H,N)
62	89	THF/*n*-hexane	216	$C_{25}H_{20}FN_7O$	(C,H,N)
63	95	EtOH	174	$C_{26}H_{23}N_7O_2$	(C,H,N)
64	56		183–185	$C_{26}H_{20}F_3N_7O$	(C,H,N)
65	55		183	$C_{25}H_{20}FN_7O$	(C,H,N)
66	96		180	$C_{23}H_{25}N_7O_2$	(C,H,N)
67	90		185	$C_{23}H_{22}F_3N_7O$	(C,H,N)
68	85		180–181	$C_{22}H_{22}FN_7O$	(C,H,N)
69	85		190	$C_{22}H_{23}N_7O_2$	(C,H,N)
70	80		198	$C_{22}H_{20}F_3N_7O$	(C,H,N)
71	80		190–193	$C_{21}H_{20}FN_7O$	(C,H,N)
72	50		260	$C_{20}H_{19}N_7O_2$	(C,H,N)
73	65		191	$C_{20}H_{16}F_3N_7O$	(C,H,N)
74	62	THF	232–233	$C_{19}H_{16}FN_7O$	(C,H,N)
75	82	THF/Isopropanol	218	$C_{19}H_{17}N_7O_2$	(C,H,N)
76	80	Isopropanol	225	$C_{19}H_{14}F_3N_7O$	(C,H,N)
77	72		224	$C_{18}H_{14}FN_7O$	(C,H,N)
78	61	THF/*n*-hexane	235	$C_{26}H_{23}N_7O_2$	(C,H,N)
79	70	THF/*n*-hexane	232	$C_{26}H_{20}F_3N_7O$	(C,H,N)
80	69	THF/*n*-hexane	222	$C_{25}H_{20}FN_7O$	(C,H,N)
81	80	THF/isopropanol	244	$C_{26}H_{19}ClF_4N_7O$	(C,H,N)
82	53	THF/isopropanol	220	$C_{25}H_{19}ClFN_7O$	(C,H,N)
83	50		250	$C_{26}H_{19}F_4N_7O$	(C,H,N)
84	42		246–248	$C_{25}H_{19}F_2N_7O$	(C,H,N)
85	58		253	$C_{27}H_{22}F_3N_7O$	(C,H,N)
86	80	$CHCl_3$/MeOH	250	$C_{26}H_{22}FN_7O$	(C,H,N)
87	70		243–244	$C_{26}H_{19}ClF_3N_7O$	(C,H,N)
88	40		262 dec.	$C_{25}H_{19}ClFN_7O$	(C,H,N)
89	30		222	$C_{26}H_{19}F_4N_7O$	(C,H,N)
90	50	$CHCl_3$	245	$C_{25}H_{19}F_2N_7O$	(C,H,N)

[a] Where crystallisation solvent is not indicated, mp was determined on the pure crystalline product directly precipitated from reaction mixture and not recrystallised.

pH 7.4) in the presence of 30 nM [^{3}H]NECA and different concentrations of new synthesised compounds. Non-specific binding was determined in the presence of 100 μM NECA. The dissociation (K_d) of [^{3}H]NECA in A_{2A} CHO cell membranes was 30 nM.

Affinity towards human A_3ARs was evaluated by competition experiments as described by Colotta et al.,[41] using [^{125}I]ABMECA as a radioligand. CHO cells, stably transfected with human A_3ARs, were grown adherently and the membrane fraction was prepared as described above.

Aliquots of cell membranes (40 μg of proteins) were incubated at 25 °C for 90 min in 100 μL T_3 buffer (50 mM Tris–HCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 U/mL ADA, pH 7.4) in the presence of 1.4 nM [^{125}I]ABMECA and different concentrations of new synthetised compounds. Nonspecific binding was determined in the presence of 50 μM R-PIA. The dissociation (K_d) of [^{125}I]ABMECA in A_3 CHO cell membranes was 1.4 nM.

In all competition experiments, the compounds were dissolved in DMSO and added to the assay mixture (DMSO concentration maximum 2%). The experiments ($n = 3$), carried out in triplicate, were analysed by an iterative curve-fitting procedure (GraphPad, Prism program, San Diego, CA), which provided IC_{50}, and SEM values for compounds tested. IC_{50} values were converted to K_i values using the Cheng and Prusoff equation.[42] Protein levels were evaluated through the method of Lowry, using bovine serum albumin as standard.

The binding of [^{35}S]GTPγS was carried out as described by Jacobson et al.[32] using CHO cells expressing human A_3 receptors. Briefly, membranes were suspended in a buffer containing 50 mM Tris, 3 U/mL adenosine deaminase, 100 mM NaCl, and 10 mM $MgCl_2$, pH 7.4 at a protein concentration of 5–10 μg per tube. The membrane suspension was preincubated with 0.5 μM GDP, 10 μM NECA and an antagonist in a final volume of 450 μL buffer at 30 °C for 20 min and transferred to ice for 20 min. [^{35}S]GTPγS was added to a final concentration of 0.1 nM in a total volume of 500 μL and the mixture was determined in the presence of 10 μM GTPγS. Incubation of the reaction mixture was terminated by filtration over GF/C glass fibres using a Brandel cell harvester and washed with the same buffer.

6.2. Molecular modelling (technical details)

All calculations were carried out on a Silicon Graphics Octane R12000 workstation, equipped with the Accelrys package containing a graphic tool (Insight) [Insight, Accelrys Inc., San Diego CA] and a computation tool (Discover) [Discover, Accelrys Inc., San Diego CA], which is capable of performing molecular mechanics (MM) calculations and molecular dynamics (MD) simulations. Insight was exploited for building the three-dimensional models for human A_1 and A_3 receptors and some antagonists, while energy minimisation was performed through Discover.

The two initial models for complexes involving ligand **82** and both receptors were energy minimised by using the cff91 force field of Discover according to the protocol described below. A sphere of water molecules of 18 Å radius, centred on the ligand, was added to each model of the complexes. As a consequence of explicitly representing the solvent, a value of 1 was used for the dielectric constant in the MM calculations aimed at energy minimisation. The dielectric constant was also set to as distance dependent. First of all 100 steps of iterations based on the steepest descents algorithm were performed until the threshold on the energy derivative with respect to the atomic coordinates was less than 1 kcal/Å. They were followed by at least 20,000 steps of conjugated gradient, with a threshold of 0.01 kcal/Å. The subsequent molecular docking procedure was performed by using the DOCK program (version 4.0.1).[37] The search for optimal orientations and conformations of the ligands in the binding site was carried out by the use of the flexible docking option in the program. All torsion angles of the ligands were so allowed to change and many complex configurations were collected. The corresponding approximate interaction energies were also collected and compared in order to identify optimal orientations and conformations of the ligands. Further, energy minimisations (same protocol as above) were performed on the best configurations of both models for complexes involving A_1 and A_3 receptors, so that the whole structure could relax to the best near energy minima. It made the analysis of interactions, detailed at molecular level, more reliable.

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