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2- and 8-alkynyladenosines: conformational studies and docking to human adenosine A_3 receptor can explain their different biological behavior

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Abstract

Adenosine (Ado) derivatives substituted at the C2 position with an alkynyl chain are endowed with high affinity for A_1, A_{2A} and A_3 human adenosine receptors, while being less active at the low affinity A_{2B} subtype. On the other hand, the introduction of an alkynyl chain at the C8 position of adenosine is detrimental for the affinity and potency at A_1, A_{2A}, and A_{2B} receptors, while is more tolerated by the A_3 receptor. The evaluation of the stimulation of [^{35}S]GTPγS binding revealed that 2-alkynyladenosines behave as adenosine receptors agonists while, on the contrary, 8-alkynyladenosines behave as antagonists.

With this work we demonstrated, by means of an NMR-based and a computational conformational analysis, that 8-alkynyladenosines, differently from 2-alkynyladenosines, cannot adopt the sugar-base *anti* conformation required for adenosine receptor activation.

Furthermore, using the recently reported X-ray crystal structure of bovine rhodopsin as template, we built a 3D model of the seven transmembrane domains of the human adenosine A_3 receptor with the homology modeling. After identification of the binding site we carried out docking experiments, demonstrating that the two class of molecules have different binding modes that explain their different degree of affinity and the shift of their activity from agonism to antagonism.

Keywords: GPCR; Adenosine receptors; Homology modeling; Docking; Conformational analysis; NMR

1. Introduction

Most of adenosine (Ado) actions are mediated by four extracellular receptors termed A_1, A_{2A}, A_{2B}, and A_3 [1,2]. All these subtypes belong to the super-family of seven transmembrane domains G-protein coupled receptors (7TMs GPCR) and are classified by the GPCR database [3] in the family of rhodopsin like receptors (Class A of GPCR).

Among human adenosine receptors, the A_3 subtype was the last one to be identified, and unlike what happened for A_1, A_{2A} and A_{2B}, it was discovered by means of molecular biology techniques followed by pharmacological experiments. In 1991, in fact, Meyerhof et al. [4] identified a novel putative GPCR, expressed during rat spermiogenesis, from a rat testis cDNA library. Even though they did not determine the ligand, they demonstrated a sequence homology greater than 40% with canine A_1 and A_{2A}. One year later the A_3 subtype was cloned from rat striatum, expressed and functionally characterized by Zhou et al. [5]. This novel subtype resulted to correspond to the receptor previously isolated by Meyerhof. Successively, the A_3 receptor was cloned from various human tissues [6,7].

The biological functions of the A_3 subtype are still not very well understood. This is mainly due to the lack of truly selective ligands for in vivo studies and to the poor structural characterization of the receptor itself. In fact, few mutagenesis studies on this subtype have been carried out [8–10].

There are reports that agonists acting via the adenosine A_3 receptor have cardioprotective effects [11,12] although it was proven that the A_3 receptor serves a different cardioprotective function as compared to the adenosine A_1 receptor [13]. Chronic administration of adenosine A_3 receptor agonists has also been shown to have protective actions in the brain, in contrast to acute high-dose administration, which can cause toxicity [14,15].

1.1. 2- and 8-alkynyladenosines as adenosine receptor ligands

In a search for selective adenosine receptor ligands we found that adenosine derivatives substituted at the C2 position with an hexynyl (HE), a phenylethynyl (PE) or a phenylhydroxypropynyl (PHP) chain are endowed with high affinity for A_1, A_{2A} and A_3 human adenosine receptors, while being less active at the low affinity A_{2B} subtype

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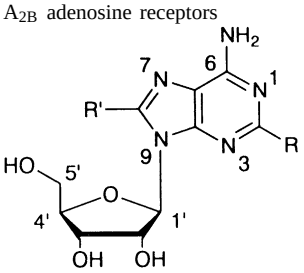
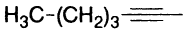
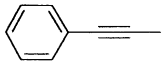
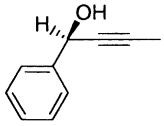
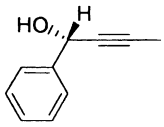
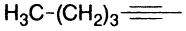
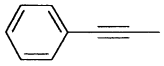
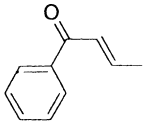
Table 1
Affinities of 2- and 8-alkynyladenosine derivatives in radioligand binding assays at human A_1, A_{2A} and A_3, and in adenylyl cyclase assays at human A_{2B} adenosine receptors



Compound	R	R′	K_i or EC_{50} (nM)			
			K_i A_1[a]	K_i A_{2A}[b]	$EC_{50}A_{2B}$[c]	K_i A_3[d]
1 (2-HEAdo)		H	18	5.7	100,000	4.7
2 (2-PEAdo)		H	391	363	>100,000	16
3 (2-(*R*)-PHPAdo)		H	0.44	29	6,200	5.0
4 (2-(*S*)-PHPAdo)		H	0.67	1.8	920	1.4
5 (8-HEAdo)	H		>100,000	>100,000	>100,000	650
6 (8-PEAdo)	H		>100,000	>100,000	>100,000	790
7[e] (8-PKPAdo)	H		>100,000	46,600	>100,000	9,830

[a] Displacement of specific [^{3}H]CCPA binding in CHO cells, stably transfected with human recombinant A_1 adenosine receptor, expressed as K_i (nM).
[b] Displacement of specific [^{3}H]NECA binding in CHO cells, stably transfected with human recombinant A_{2A} adenosine receptor, expressed as K_i (nM).
[c] Measurement of receptor-stimulated adenylyl cyclase activity in CHO cells, stably transfected with human recombinant A_{2B} adenosine receptor, expressed as EC_{50} (nM).
[d] Displacement of specific [^{3}H]NECA binding in CHO cells, stably transfected with human recombinant A_3 adenosine receptor, expressed as K_i (nM).
[e] The reaction of phenylhydroxypropyne with 8-bromoadenosine did not give the 8-phenylhydroxypropynyl derivative but the phenylketopropenyl analogue **7**, due to a rearrangement on the side chain [17].

(Table 1) [16]. In particular, the compounds that bear the PHP chain at C2 (compounds **3** and **4**, 2-(*R*)-PHPAdo and 2-(*S*)-PHPAdo, respectively), are the most active molecules of the series, while the compound that bears the PE chain at C2 (compound **2**, 2-PEAdo), is the most selective molecule for the A_3 subtype. Furthermore, the evaluation of the stimulation of [^{35}S]GTPγS binding confirmed that the three compounds behave as adenosine receptors agonists.

In a recent work, we investigated the affinity and potency at the four adenosine receptor subtypes of adenosine analogues bearing the above described alkynyl chains but on the C8 position [17]. The results of binding and cyclase studies showed that the introduction of substituents at the C8 position of Ado is detrimental for the affinity and potency at A_1, A_{2A}, and A_{2B} receptors, while is more tolerated by the A_3 receptor (Table 1).

The data obtained from the evaluation of the stimulation or inhibition of [^{35}S]GTPγS binding by these compounds are even more remarkable (Fig. 1). In fact, all three adenosine derivatives do not stimulate basal [^{35}S]GTPγS binding, but inhibit to various extent NECA-stimulated binding. This is the first report of adenosine derivatives with an intact ribose moiety that behave as antagonists of adenosine receptor [17].

With this study, in order to understand the reasons of the very different behavior of such molecules that bear the same substituent but in a different position of the purine ring, we tried to get an insight into the molecular structures of the human A_3 receptor and into the conformation of our ligands.

Since a very important feature which influences the binding of nucleosides to adenosine receptors is the orientation of the sugar moiety with respect to the nucleobase, we carried out a conformational analysis about the glycosidic bond

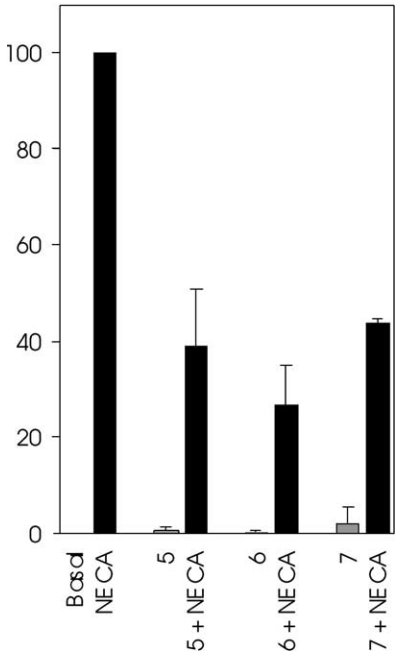


Fig. 1. Percentage of stimulation of [^{35}S]GTPγS binding (gray bars) and percentage of inhibition of NECA-stimulated [^{35}S]GTPγS binding (black bars) by compounds **5**–**7** in membranes of CHO cells which express the human A_3 receptor.

of these C2- and C8-substituted nucleosides using NMR and computational techniques.

Furthermore, we built a model of the seven transmembrane helices of the human A_3 receptor with the homology modeling technique, and after identification of the binding site, by means of an accurate analysis of the available mutagenesis data, we carried out docking experiments, determining the binding mode necessary for the activation of the receptor.

2. Methodologies

2.1. NMR-based conformational studies

Stolarski, Dudicz and their collaborators demonstrated that in purine nucleosides and nucleotides, the H2′ chemical shift can be used as an indicator of the sugar-base orientation [18,19]. Typical δ-values measured in DMSO, in fact, are 5.2 ppm for the compounds restricted in the *syn* conformation, and 4.2 ppm for those which exist only in the *anti* conformation. This significant difference is due to the electronic attraction that nitrogen in 3 position of the purine ring can exert on H2′ when the nucleoside is *syn*.

Since the *syn*/*anti* equilibrium is very rapid respect to the NMR time scale, the chemical shift for a given compound will have an intermediate value determined by the probability of finding the molecule in one of the two rotamers. The H2′ chemical shift of adenosine in DMSO, for example, is 4.62 ppm.

2.2. Semi-empirical conformational studies

Semi-empirical conformational studies were carried out with HyperChem 6.0 [20] program on a Pentium III machine. All the calculations were made utilizing the semi-empirical method AM1 [21] (with an SCF convergence limit of 0.00001 kcal/mol) and the Polak–Ribiere conjugate gradient.

The initial geometries of the molecules were guessed by means of molecular dynamic (MD) simulations at 298 K with the MM+ forcefield, which is an extension of the Allinger MM2 forcefield [22]. In details, nucleosides were built in *anti* conformation and energy minimized (MM+) until an RMS of 0.1 was reached. The molecules, virtually at 0 K, were slowly (1 ps) heated until they reached a temperature of 298 K. The simulation was carried out at constant temperature for 10 ps, using a timestep of 1 fs. During the simulation, snapshots of the system were taken at regular intervals of 1 ps. The structures in each snapshot were energy minimized (MM+), and the one showing the lowest energy was used as starting point for the semi-empirical conformational studies.

The molecules in the initial geometry were previously minimized until an RMS of 0.00001 was reached. Hence, the torsional glycosidic angle was rotated of 360° by steps of 10°, optimizing the geometry at each single step with 200 cycles of minimization and applying an harmonic restraint to the glycosidic angle of 60 kcal/(mol °). The local minima were further optimized until an RMS of 0.00001 kcal/(mol Å) was reached, in order to allow the sugar moiety to adapt its puckering to the new conformation.

2.3. Human A_3 receptor construction and docking experiments

Homology modeling and docking experiments were carried out utilizing SwissPdb Viewer 3.7 [23,24], and HyperChem 6.0 [20] programs on a Pentium III machine. For molecular mechanics calculations the AMBER 96 [25] forcefield was utilized with a distance dependent dielectric constant scaled by a factor of 4. All the energy minimizations were carried out employing the Polak–Ribiere conjugate gradient until an RMS of 0.001 kcal/(mol Å) was reached. This procedure was preceded, wherever necessary, by a few steps of steepest descent to obtain an acceptable starting geometry.

All molecular dynamics simulations were carried at a constant temperature of 298 K, using a timestep of 1 fs. The system, virtually at 0 K, was always slowly (1 ps) heated until it reached the simulation temperature.

The helical bundle of the human A_3 receptor was constructed by homology modeling using the recently reported X-ray crystal structure of bovine rhodopsin [26] (1F88.pdb, freely available at the RCSB Protein Data Bank [27]) with a 2.8 Å resolution as structural template. The seven transmembrane domains were identified analyzing

Table 2
Alignment of the amino acid sequences of the bovine rhodopsin (OPSD_BOVIN) and the four subtypes of human adenosine receptor calculated using the BLOSUM62 substitution matrix, with a gap open penalty of 12 and a gap extension penalty of 4

TM1			
	OPSD_BOVIN	35	WQFSMLAAYMFLLIMLGFPINFLTLYVTVQ 64
	AA3R_HUMAN	10	LANVTYITMEIFIGLCAIVGNVLVICVVKL 39
	AA1R_HUMAN	7	AFQAAYIGIEVLIALVSVPGNVLVIWAVKV 34
	AA2A_HUMAN	4	MGSSVYITVELAIAVLAILGNVLVCWAVWL 33
	AA2B_HUMAN	5	TQDALYVALELVIAALSVAGNVLVCAAVGT34
TM2			
	OPSD_BOVIN	73	NYILLNLAVADLFMVFGGFTTTLYTSLH 100
	AA3R_HUMAN	48	FYFIVSLALADIAVGVLVMPLAIVVSLG 75
	AA1R_HUMAN	45	FCFIVSLAVADVAVGALVIPLAILINIG 72
	AA2A_HUMAN	42	NYFVVSLAAADIAVGVLAIPFAITISTG 69
	AA2B_HUMAN	43	NYFLVSLAAADVAVGLFAIPFAITISLG 70
TM3			
	OPSD_BOVIN	107	PTGCNLEGFFATLGGEIALWSLVVLAIERYVVV 139
	AA3R_HUMAN	80	FYSCLFMTCLLLIFTHASIMSLLAIAVDRYLRV 112
	AA1R_HUMAN	77	FHTCLMVACPVLILTQSSILALLAIAVDRYLRV 109
	AA2A_HUMAN	74	CHGCLFIACFVLVLTQSSIFSLLAIAIDRYIAI 106
	AA2B_HUMAN	75	FYGCLFLACFVLVLTQSSIFSLLAVAVDRYLAI 107
TM4			
	OPSD_BOVIN	151	NHAIMGVAFTWVMALACAAPPLV 173
	AA3R_HUMAN	125	RRIWLALGLCWLVSFLVGLTPMF 147
	AA1R_HUMAN	122	RRAAVAIAGCWILSFVVGLTPMF 144
	AA2A_HUMAN	119	TRAKGIIAICWVLSFAIGLTPML 141
	AA2B_HUMAN	120	TRARGVIAVLWVLAFGIGLTPFL 142
TM5			
	OPSD_BOVIN	200	NESFVIYMFVVHFIIPLIVIFFCYGQL 226
	AA3R_HUMAN	174	MDYMVYFSFLTWIFIPLVVMCAIYLDI 200
	AA1R_HUMAN	177	MEYMVYFNFFVWVLPPLLLMVLIYLEV 203
	AA2A_HUMAN	174	MNYMVYFNFFACVLVPLLLMLGVYLRI 200
	AA2B_HUMAN	179	MSYMVYFNFFGCVLPPLLIMLVIYIKI 205
TM6			
	OPSD_BOVIN	247	EKEVTRMVIIMVIAFLICWLPYAGVAFYIFT 277
	AA3R_HUMAN	225	EFKTAKSLFLVLFLFALSWLPLSIINCIIYF 255
	AA1R_HUMAN	229	ELKIAKSLALILFLFALSWLPLHILNCITLF 259
	AA2A_HUMAN	228	EVHAAKSLAIIVGLFALCWLPLHIINCFTFF 258
	AA2B_HUMAN	229	EIHAAKSLAMIVGIFALCWLPVHAVNCVTLF 259
TM7			
	OPSD_BOVIN	286	IFMTIPAFFAKTSAVYNPVIYIMMN 310
	AA3R_HUMAN	262	LVLYMGILLSHANSMMNPIVYAYKI 286
	AA1R_HUMAN	268	ILTYIAIFLTHGNSAMNPIVYAFRI 292
	AA2A_HUMAN	268	WLMYLAIVLSHTNSVVNPFIYAYRI 292
	AA2B_HUMAN	270	WAMNMAILLSHANSVVNPIVYAYRN 294

The alignment was performed using the program SIM (alignment tool for protein local to the ExPASy server, http://www.expasy.ch/tools/sim-prot.html).

the three-dimensional structure of the rhodopsin crystal. The alignment of the amino acid sequences of the rhodopsin and the A_3 receptor, reported in Table 2, was calculated using the BLOSUM62 substitution matrix, with a gap open penalty of 12 and a gap extension penalty of 4. The alignment was performed using the program SIM (alignment tool for protein local to the ExPASy server, http://www.expasy.ch/tools/sim-prot.html).

Each helix was capped with an acetyl group at the N-terminus and with a *N*-methyl group at the C-terminus. The raw model was then optimized with 1 ps of MD followed by energy minimization.

According to an extensive study of the site-mutagenesis data available in literature reported in Table 3, the ligands were manually docked into the receptor helical bundle and an harmonic restraint of 7 kcal/(mol Å) was initially applied to each of their atoms. Hence, in order to optimize the docked position, only the nucleoside was submitted to 1 ps of MD followed by energy minimization.

Then all the restraints were removed and the whole receptor–ligand complex was submitted to 10 ps of MD and then to energy minimization. The interaction energies between the receptor and the ligand were calculated by subtracting the energy of the separated ligand and receptor to

Table 3
Mutated amino acids in the TMs of human adenosine receptors and correspondences in the A_3 subtype (in italics) derived by sequence alignment

A_1	A_{2A}	A_{2B}	A_3
TM1			
		Val11 [40]	*Ile16*
Glu16 [31]	Glu13 [36,37]		*Glu19*
TM2			
Ser50 [31]			*Ser53*
Asp55 [31]			*Asp58*
		Leu58 [40]	*Val63*
		Phe59 [40]	*Leu64*
TM3			
Cys85 [32]			*Cys88*
Val87 [33]	Val84 [38]		*Leu90*
Leu88 [33]			*Leu91*
Thr91 [33]	Thr88 [39]		*Thr94*
Gln92 [33]	Gln89 [39]		*His95*
Ser93 [31]	Ser90 [39]		*Ala96*
Ser94 [31]	Ser91 [39]		*Ser97*
TM4			
Cys131 [32]			*Cys134*
Ser135 [31]			*Ser138*
TM5			
	Phe180 [30]		*Phe180*
	Asn181 [30]		*Ser181*
	Phe182 [30]		*Phe182*
TM6			
	His250 [30,38]		*Ser247*
	Asn253 [30]		*Asn250*
Cys255 [32]	Cys254 [30]		*Cys251*
	Phe257 [30]		*Tyr254*
TM7			
	Tyr271 [30]	Asn273 [40]	*Tyr265*
	Ile274 [30]		*Ile268*
Thr277 [34,35]	Ser277 [30,39]		*Ser271*
	His278 [30,36,38]		His272 [10]
	Ser281 [30,39]		*Ser275*

the energy of the receptor–ligand complex. These molecular mechanics energies, calculated with the AMBER 96 forcefield, are not rigorous thermodynamic quantities and do not take into account changes in entropy or solvation effect. Consequently, they cannot be used to predict binding affinities but only to compare relative stabilities of the complexes.

3. Results and discussion

3.1. Conformational analysis studies

The orientation of the ribose moiety respect to the nucleobase, as already asserted in Section 1, is an important feature which governs the binding of nucleosides to adenosine receptors [28]. This orientation is characterized by the glycosidic torsional angle χ, which is described by the bonds between the four atoms C8–N9–C1′–O4′. It is well known that nucleosides exist in two predominant rotamers called *syn* and *anti*, respectively. The typical values of the χ angle which are found in crystal structures are about $-130 \pm 30^\circ$ for the *syn* and about $45 \pm 40^\circ$ for the *anti* conformation. On the basis of a detailed structure–activity relationship (SAR) analysis, it has been demonstrated by Bruns [29] that nucleosides bind to adenosine receptors in the *anti* conformation. This evidence has further been confirmed by all the published models of complexes between the various subtypes of adenosines receptors and their agonists, starting with the first model of human A_{2A} receptor published by Kim et al. in 1995 [30], and deposited in the RCSB Protein Data Bank [27] (1MMH.pdb).

3.1.1. NMR studies

In Table 4 we report the H2′ chemical shift of our 2- and 8-substituted adenosines that, as explained in Section 2, is an indicator of the sugar-base orientation. The δ of about 5.0 ppm that we found for our 8-substituted compounds clearly indicates that in DMSO solution, these molecules exist mainly in their *syn* conformation. On the other hand, the 2-substituted compounds show a chemical shift of about 4.5 ppm. Hence, these molecules show a slight preference for *anti* conformation, behaving very similarly to unsubstituted adenosine.

3.1.2. Computational studies

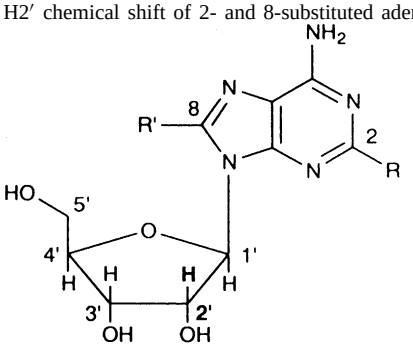
A conformational analysis around the glycosidic bond of these 2- and 8-substituted adenosines was performed utilizing the semi-empirical method AM1, as described in Section 2.

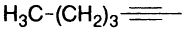
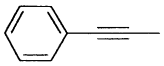
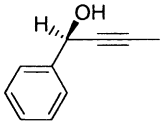
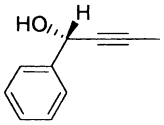
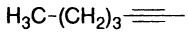
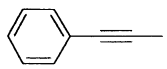
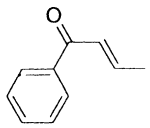
For all the compounds we found three different local minima of energy, one in the *syn* conformation, with $\chi \approx -100^\circ$, and two in the *anti*, with $\chi \approx -10^\circ$ and $\chi \approx 80^\circ$, respectively. As an example, the energetic profiles of 2- and 8-PEAdo are reported in Fig. 2. The energy barriers to cross between *syn* and *anti* conformation are much higher for 8-substituted compounds than for 2-substituted ones. So, 2-alkynyladenosines can easily rotate around the glycosidic bond at room temperature, while 8-substituted adenosines are virtually restricted in the most stable conformation.

Taking into account that the sugar pucker varies considerably with the glycosidic torsion angle, the two most stable conformers were further optimized until they reached an RMS gradient lower than 0.00001 to allow the sugar to assume its most favorable conformation. The difference between the total energies of the two conformations of each molecule are reported in Fig. 3. It can easily be observed that our 2-alkynyl derivatives show a slight preference for the *anti* conformation while our 8-substituted derivatives prefer the *syn* one.

It is worthy to note that the χ angle that we found by this semi-empirical conformational analysis is about -100° for the *syn* conformation of all the examined compounds. This angle makes N3 very close to H2′, explaining very well the H2′ chemical shift of the nucleosides restricted to the *syn* conformation.

Table 4
H2′ chemical shift of 2- and 8-substituted adenosines in DMSO solution



Compound	R	R′	H2′ chemical shift
Adenosine	H	H	4.62
1 (2-HEAdo)		H	4.57
2 (2-PEAdo)		H	4.54
3 (2-(*R*)-PHPAdo)		H	4.51
4 (2-(*S*)-PHPAdo)		H	4.52
5 (8-HEAdo)	H		5.04
6 (8-PEAdo)	H		5.03
7 (8-PKPAdo)	H		4.89

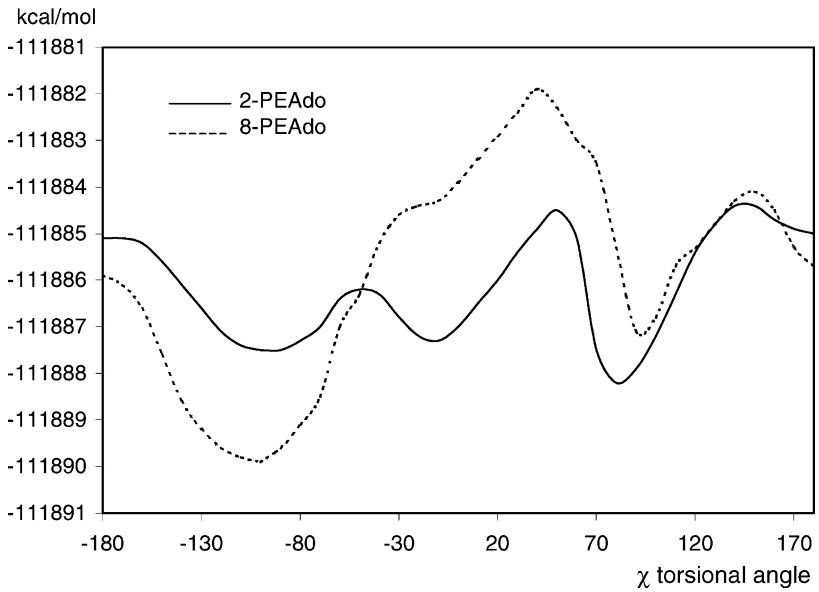


Fig. 2. Total energy (AM1) of 2- and 8-PEAdo in dependence of the C8–N9–C1′–O4′ (χ) torsional angle.

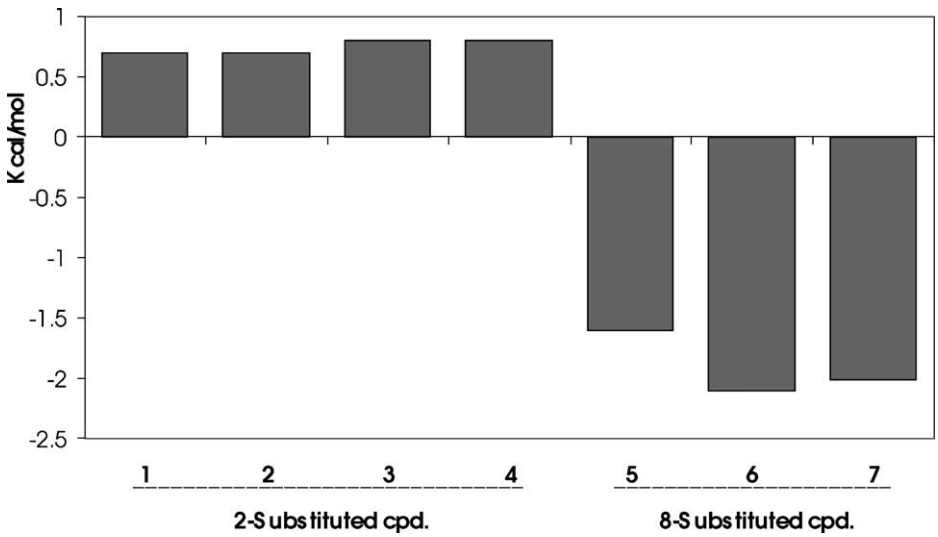


Fig. 3. Differences in total energy (AM1) between the *syn* and the *anti* conformations of 2- and 8-substituted adenosines.

3.2. Homology modeling and docking experiments

Furthermore, in order to understand the reasons of the different behavior of 2- and 8-phenylethynyladenosines, we tried to get an insight into the molecular structure of the human A_3 receptor and its binding site using molecular modeling techniques. For this purpose, we constructed the model of the seven transmembrane helices of the receptor by homology modeling, using the recently reported X-ray crystal structure of bovine rhodopsin with a 2.8 Å resolution as structural template [26].

The binding site was identified using the available site-directed mutagenesis data (see references in Table 3). The major difficulty was the fact that just a few data concerning the A_3 subtype have been published. Thus, in order to have a more complete perspective, after the alignment of the human adenosine receptor sequences, the data of A_1, A_{2A} and A_{2B} subtypes were extended to the A_3 (Table 3).

The agonist 2-PEAdo was manually docked in its *anti* conformation in the cavity among the helices 3, 5, 6 and 7 and the receptor–ligand complex was optimized as described in Section 2. Fig. 4 shows the model obtained for the human A_3 receptor binding site complexed with 2-PEAdo.

The alkynyl chain is accommodated in a hydrophobic region between the helices 3, 4 and 5. The ribose moiety, which is fundamental for the agonist activity, is in strict contact with the helices 3 and 7. In particular, the 5′ hydroxyl group is within hydrogen bond distance respect to Asn274 (TM7). Furthermore, the 3′ hydroxyl group, which has been largely demonstrated to be required for full agonistic activity [40–42], is closely linked to His272 (TM7), corresponding to crucial His278 in the A_{2A} receptor [30,36,38]. The

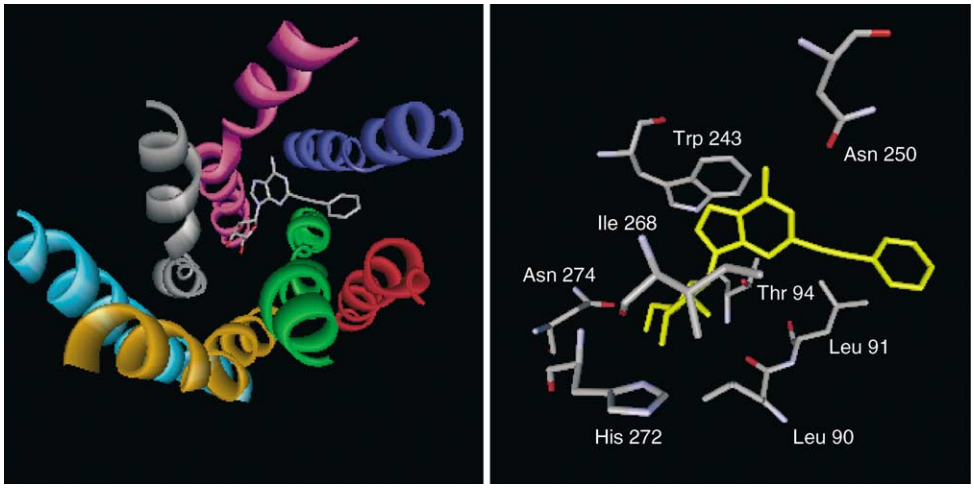


Fig. 4. The agonist 2-PEAdo docked into the seven transmembrane domain of the human A_3 receptor. View from outside the cell (left) and details of the active site (right). Color of helices: cyan (TM1), orange (TM2), green (TM3), red (TM4), blue (TM5), magenta (TM6), and gray (TM7).

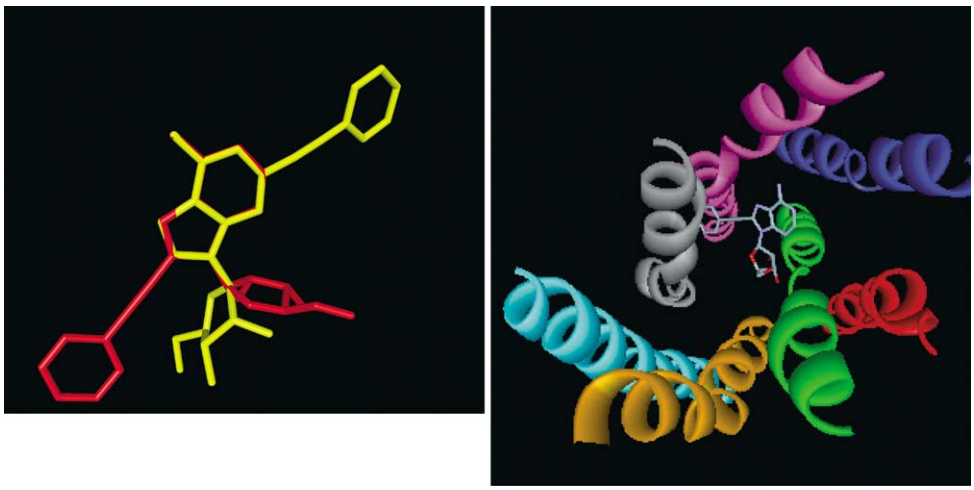


Fig. 5. Superimposition of 2- and 8-PEAdo (left) with exact match of the adenine moieties, and relative docking of 8-PEAdo into the seven transmembrane domains of the human A_3 receptor (right). Color of helices: cyan (TM1), orange (TM2), green (TM3), red (TM4), blue (TM5), magenta (TM6), and gray (TM7).

adenine region is found between the helices 3 and 6, with Asn250 (TM6) hydrogen bonded to the amine in 6 position of the purine ring and Thr94 very close to N3. The calculation of the AMBER potential energies indicates that the receptor–ligand complex is more stable with respect to the separated molecules.

Furthermore, we explored two different binding modes for 8-PEAdo, based on two different superimposition schemes with the agonist 2-PEAdo: the first one explains the reason why our 8-substituted adenosines cannot stimulate the adenosine receptors, while the second one introduces an hypothesis of a possible binding mode, that is in very good accordance with the biological data and could be helpful to project new mutagenesis experiments.

8-PEAdo, in fact, cannot bind to the A_3 receptor in the same way of the 2-PEAdo. If we superimpose the adenine moieties of the two molecules as proposed in Fig. 5, in fact, the alkynyl chain at the C8 position would overlap with helix 6. Furthermore, the ribose moiety of 8-PEAdo, virtually restricted in *syn* conformation as demonstrated by our conformational analysis, would interfere with the amino acids of helix 3. These negative interactions result in a potential energy of the receptor–ligand complex, calculated with the AMBER forcefield, much higher respect to the separated molecules. The binding mode of this molecule, then, should be different.

As a working hypothesis, we propose that the purine moieties of 2- and 8-PEAdo can be superimposed in a way

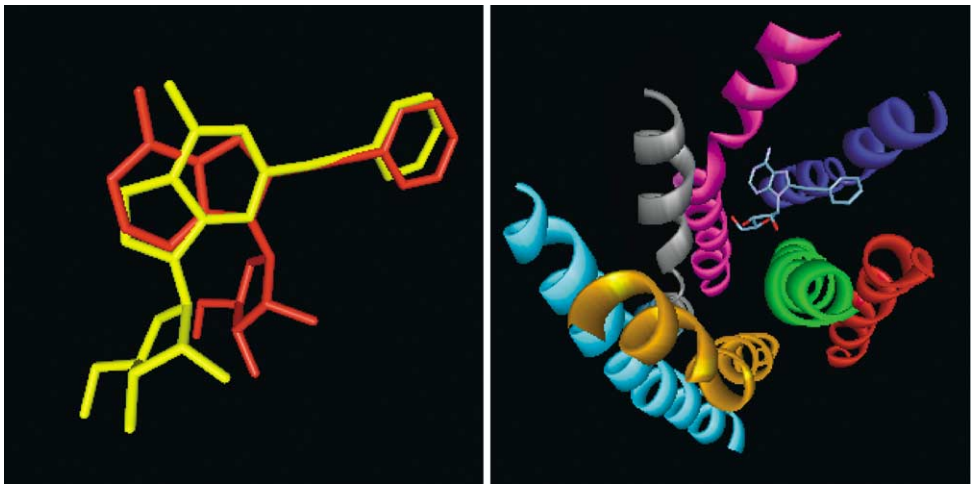


Fig. 6. Superimposition of 2- and 8-PEAdo (left) with the C2 of a molecule matching the C8 of the other one and vice versa, and relative docking of 8-PEAdo into the seven transmembrane domain of the human A_3 receptor (right). Color of helices: cyan (TM1), orange (TM2), green (TM3), red (TM4), blue (TM5), magenta (TM6), and gray (TM7).

that the C2 of a molecule matches the C8 of the other one as shown in Fig. 6. In this way, there is a very good steric and electrostatic correspondence at the level of the purine moieties and the phenylethynyl chains, even though there is no overlap of the ribose moieties.

When 8-PEAdo is docked following this superimposition scheme its adenine region fits in the A_3 receptor cavity exactly as the same region of 2-PEAdo does, the alkynyl chain finds room between the helices 3, 4 and 5, and the sugar is allowed to adopt the *syn* conformation respect to the nucleobase which, as suggested by our conformational analysis data, is the most energetically favorable. With this binding mode, the hydroxyl groups of the ribose, which are supposed to be the responsible for receptor activation, lose the contact with the AA of the helices numbers 3 and 7. As in the case of 2-PEAdo, the AMBER potential energy of the receptor–ligand complex results lower with respect to the energy of the separated molecules.

This is probably the reason why 8-PEAdo can bind to the human A_3 receptor without stimulating it.

The behavior of our 8-substituted adenosines differs markedly between the A_3 receptor and the other adenosine receptor subtypes. In fact, these adenosine derivatives have negligible activity for any receptor other than A_3. The cause of these differences, probably, has to be found in the interactions between the sugar moieties of these nucleosides and the residues in helix 3. According to our docked model described in Fig. 6, helix 3 surrounds the ribose moiety of 8-PEAdo by means of residues Thr87, Leu90, Leu91 and Thr94. The last two residues (Leu91 and Thr94) are conserved among the four adenosine receptor subtypes; on the other hand, Thr87 and Leu90 are peculiar of the A_3 receptor, and in the other three subtypes are substituted with Ala and Val, respectively (Table 2).

4. Conclusions

With this work we give an explanation of the very different behavior of 2-alkynyladenosines and their analogues with the alkynyl chain at the C8 position. In fact, moving the alkynyl chain from the C2 to the C8 position causes, besides a relevant loss of affinity for all the adenosine receptor subtypes, the shift of the activity from agonist to antagonist.

The publication, by Palczewski et al. in 2000 [26], of the X-ray crystal structure of bovine rhodopsin has undoubtedly been a great milestone in the field of 7TMs GPCRs modeling and allowed us to built an accurate model of human adenosine A_3 receptor.

With this model and with an extensive study of the mutagenesis data available in literature we identified the amino acids involved in the recognition of A_3 agonists and determined their binding mode.

With these data we demonstrated, with an NMR-based and a computational conformational analysis, that 8-alkynyladenosines cannot adopt the sugar-base *anti* conformation required for adenosine receptor activation, differently from 2-alkynyladenosines. Furthermore, we showed that the alkynyl chain at the C8 position overlaps sterically with residues in helix 6, preventing binding to the A_3 receptor according to the binding mode proposed in Fig. 5. Hence, we propose an alternative binding mode for these 8-substituted molecules (Fig. 6) that explains their lower affinity and their inability to stimulate this subtype of adenosine receptor. This working hypothesis could be helpful to project new mutagenesis experiments aimed at further understanding the A_3 receptor active site topology.

Acknowledgements

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References

[1] G.L. Stiles, Adenosine receptor subtypes: new insights from cloning and functional studies, in: K.A. Jacobson, M.F. Jarvis (Eds.), Purinergic Approaches in Experimental Therapeutics, Wiley–Liss, New York, 1997, pp. 29–37.
[2] V. Ralevic, G. Burnstock, Receptors for purines and pyrimidines, Pharmacol. Rev. 50 (1998) 413–492.
[3] F. Horn, G. Vriend, F.E. Cohen, Collecting and harvesting biological data: the GPCRDB & NucleaRDB databases, Nucl. Acids Res. 29 (2001) 346–349.
[4] W. Meyerhof, R. Muller-Brechlin, D. Richter, Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis, FEBS Lett. 284 (1991) 155–160.
[5] Q.Y. Zhou, C. Li, M.E. Olah, R.A. Johnson, G.L. Stiles, O. Civelli, Molecular cloning and characterization of an adenosine receptor: the A3 adenosine receptor, Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 7432–7436.
[6] F.G. Sajjadi, G.S. Firestein, cDNA cloning and sequence analysis of the human A3 adenosine receptor, Biochim. Biophys. Acta 1179 (1993) 105–107.
[7] C.A. Salvatore, M.A. Jacobson, H.E. Taylor, J. Linden, R.G. Johnson, Molecular cloning and characterization of the human A3 adenosine receptor, Proc. Natl. Acad. Sci. U.S.A. 90 (1993) 10365–10369.
[8] T.M. Palmer, G.L. Stiles, Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor, Mol. Pharmacol. 57 (2000) 539–545.
[9] A. Chen, Z.G. Gao, D. Barak, B.T. Liang, K.A. Jacobson, Constitutive activation of A(3) adenosine receptors by site-directed mutagenesis, Biochem. Biophys. Res. Commun. 284 (2001) 596–601.
[10] K.A. Jacobson, Z.G. Gao, A. Chen, D. Barak, S.A. Kim, K. Lee, A. Link, P.V. Rompaey, S. van Calenbergh, B.T. Liang, Neoceptor concept based on molecular complementarity in GPCRs: a mutant adenosine A(3) receptor with selectively enhanced affinity for amine-modified nucleosides, J. Med. Chem. 44 (2001) 4125–4136.
[11] B.T. Liang, K.A. Jacobson, Adenosine and ischemic preconditioning, Curr. Pharm. Des. 5 (1999) 1029–1041.
[12] K. Stambaugh, K.A. Jacobson, J.L. Jiang, B.T. Liang, A novel cardioprotective function of adenosine A1 and A3 receptors during prolonged simulated ischemia, Am. J. Physiol. 273 (1997) H501–H505.

[13] B.T. Liang, K.A. Jacobson, A physiological role of the adenosine A3 receptor: sustained cardioprotection, Proc. Natl. Acad. Sci. U.S.A. 95 (1998) 6995–6999.
[14] D.K. Von Lubitz, R.C. Lin, M. Boyd, N. Bischofberger, K.A. Jacobson, Chronic administration of adenosine A3 receptor agonist and cerebral ischemia: neuronal and glial effects, Eur. J. Pharmacol. 367 (1999) 157–163.
[15] K.A. Jacobson, D.K. Von Lubitz, J.W. Daly, B.B. Fredholm, Adenosine receptor ligands: differences with acute versus chronic treatment, Trends Pharmacol. Sci. 17 (1996) 108–113.
[16] R. Volpini, S. Costanzi, V. Lambertucci, S. Vittori, K.N. Klotz, G. Cristalli, N^6-Alkyl-2-alkynyl derivatives of adenosine as potent and selective agonists at human adenosine A_3 receptor and a starting point for searching A_{2B} ligands, J. Med. Chem. 45 (2002) 3271–3279.
[17] R. Volpini, S. Costanzi, C. Lambertucci, S. Vittori, K. Klotz, A. Lorenzen, G. Cristalli, Introduction of alkynyl chains on C8 of adenosine led to very selective antagonists of the A(3) adenosine receptor, Bioorg. Med. Chem. Lett. 11 (2001) 1931–1934.
[18] R. Stolarski, L. Dudycz, D. Shugar, NMR studies in the *syn–anti* dynamic equilibrium in purine nucleosides and nucleotides, Eur. J. Biochem. 108 (1980) 111–121.
[19] L. Dudycz, R. Stolarski, R. Pless, D. Shugar, A ^{1}H NMR study of the *syn–anti* dynamic equilibrium in adenine nucleosides and nucleotides with the aid of some synthetic model analogues with fixed conformations, Z. Naturforsch. [C] 34C (1979) 359–373.
[20] HyperChem, version 6.03, Hypercube Inc., Gainesville, FL, USA, 2000.
[21] M.J.S. Dewar, E.G. Zoebisch, E.F. Healy, J.J.P. Stewart, AM1: a new general purpose quantum mechanical model, J. Am. Chem. Soc. 107 (1985) 3902–3909.
[22] N.L. Allinger, Conformational analysis 130.MM2. A hydrocarbon force field utilizing V1 and V2 torsional terms, J. Am. Chem. Soc. 99 (1977) 8127–8134.
[23] N. Guex, M.C. Peitsch, SWISS-MODEL and the SwissPdbViewer: An environment for comparative protein modeling, Electrophoresis 18 (1997) 2714–2723.
[24] SwissPdb, version 3.7 (beta 2), GlaxoSmithKline R&D, Geneva, Swiss, 2000 (http://www.expasy.ch/spdbv/).
[25] W.D. Cornell, P. Cieplak, C.I. Bayly, I.R. Gould, K.M. Merz Jr., D.M. Ferguson, D.C. Spellmeyer, T. Fox, J.W. Caldwell, P.A. Kollman, A second generation force field for the simulation of proteins nucleic acids, and organic molecules, J. Am. Chem. Soc. 117 (1995) 5179–5197.
[26] K. Palczewski, T. Kumasaka, T. Hori, C.A. Behnke, H. Motoshima, B.A. Fox, I. Le Trong, D.C. Teller, T. Okada, R.E. Stenkamp, M. Yamamoto, M. Miyano, Crystal structure of rhodopsin: a G protein-coupled receptor, Science 289 (2000) 739–745.
[27] H.M. Berman, J. Westbrook, Z. Feng, G. Gilliland, T.N. Bhat, H. Weissig, I.N. Shindyalov, P.E. Bourne, The Protein Data Bank, Nucl. Acids Res. 28 (2000) 235–242.
[28] P.J.M. van Galen, A.P. Ijzerman, W. Soudijin, Xanthine-7-ribosides as adenosine A1 receptor antagonists: further evidence for adenosine's *anti* mode of binding, Nucleosides Nucleotides 9 (1990) 275–291.
[29] R.F. Bruns, Adenosine receptor activation in human fibroblasts: nucleoside agonists and antagonists, Can. J. Physiol. Pharmacol. 58 (1980) 673–691.
[30] J. Kim, J. Wess, A.M. Van Rhee, T. Schoneberg, K.A. Jacobson, Site-directed mutagenesis identifies residues involved in ligand recognition in the human A2a adenosine receptor, J. Biol. Chem. 270 (1995) 13987–13997.
[31] H. Barbhaiya, R. McClain, A. Ijzerman, S.A. Rivkees, Site-directed mutagenesis of the human A1 adenosine receptor: influences of acidic and hydroxy residues in the first four transmembrane domains on ligand binding, Mol. Pharmacol. 50 (1996) 1635–1642.
[32] D.J. Scholl, J.N. Wells, Serine and alanine mutagenesis of the nine native cysteine residues of the human A(1) adenosine receptor, Biochem. Pharmacol. 60 (2000) 1647–1654.
[33] S.A. Rivkees, H. Barbhaiya, A.P. Ijzerman, Identification of the adenine binding site of the human A1 adenosine receptor, J. Biol. Chem. 274 (1999) 3617–3621.
[34] A. Townsend-Nicholson, P.R. Schofield, A threonine residue in the seventh transmembrane domain of the human A1 adenosine receptor mediates specific agonist binding, J. Biol. Chem. 269 (1994) 2373–2376.
[35] A. Dalpiaz, A. Townsend-Nicholson, M.W. Beukers, P.R. Schofield, A.P. Ijzerman, Thermodynamics of full agonist, partial agonist, and antagonist binding to wild-type and mutant adenosine A1 receptors, Biochem. Pharmacol. 56 (1998) 1437–1445.
[36] Z.G. Gao, Q. Jiang, K.A. Jacobson, A.P. Ijzerman, Site-directed mutagenesis studies of human A(2A) adenosine receptors: involvement of glu(13) and his(278) in ligand binding and sodium modulation, Biochem. Pharmacol. 60 (2000) 661–668.
[37] A.P. Ijzerman, J.K. Von Frijtag Drabbe Künzel, J. Kim, Q. Jiang, K.A. Jacobson, Site-directed mutagenesis of the human adenosine A2A receptor. Critical involvement of Glu13 in agonist recognition, Eur. J. Pharmacol. 310 (1996) 269–272.
[38] Q. Jiang, B.X. Lee, M. Glashofer, A.M. Van Rhee, K.A. Jacobson, Mutagenesis reveals structure–activity parallels between human A2A adenosine receptors and biogenic amine G protein-coupled receptors, J. Med. Chem. 40 (1997) 2588–2595.
[39] Q. Jiang, A.M. Van Rhee, J. Kim, S. Yehle, J. Wess, K.A. Jacobson, Hydrophilic side chains in the third and seventh transmembrane helical domains of human A2A adenosine receptors are required for ligand recognition, Mol. Pharmacol. 50 (1996) 512–521.
[40] E.M. van der Wenden, J.K. Von Frijtag Drabbe Künzel, R.A. Mathot, M. Danhof, A.P. Ijzerman, W. Soudijn, Ribose-modified adenosine analogues as potential partial agonists for the adenosine receptor, J. Med. Chem. 38 (1995) 4000–4006.
[41] S. Van Calenbergh, J.K. Von Frijtag Drabbe Künzel, N.M. Blaton, O.M. Peeters, J. Rozenski, A. Van Aerschot, A. De Bruyn, D. De Keukeleire, A.P. Ijzerman, N^6-Cyclopentyl-3′-substituted-xylofuranosyladenosines: a new class of non-xanthine adenosinen A_1 receptor antagonists, J. Med. Chem. 40 (1997) 3765–3772.
[42] S. Vittori, A. Lorenzen, C. Stannek, S. Costanzi, R. Volpini, A.P. Ijzerman, J.K. Von Frijtag Drabbe Künzel, G. Cristalli, *N*-Cycloalkyl derivatives of adenosine and 1-deazaadenosine as agonists and partial agonists of the A(1) adenosine receptor, J. Med. Chem. 43 (2000) 250–260.