Molecular modelling study of 2-phenylethynyladenosine (PEAdo) derivatives as highly selective A_3 adenosine receptor ligands

Diego Dal Ben · Catia Lambertucci · Sara Taffi · Sauro Vittori ·
Rosaria Volpini · Gloria Cristalli

Abstract A series of 2-phenylethynyladenosine (PEAdo) derivatives substituted in the N^6- and $4'$-position was synthesised and the new derivatives were tested at the four human adenosine receptors stably transfected into Chinese hamster ovary (CHO) cells, using radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assay (A_{2B}). Binding studies showed that the presence of a phenyl ethynyl group in the 2 position of adenosine favoured the interaction with A_3 receptors, resulting in compounds endowed with high affinity and selectivity for the A_3 subtype. Additional substitution of the N^6- and $4'$-position increases both A_3 affinity and selectivity. The results showed that the new compounds have a good affinity for the A_3 receptor and in particular, the N^6-methoxy-2-phenylethynyl-5'-N-methylcarboxamidoadenosine, with a K_i at A_3 of 1.9 nM and a selectivity A_1/A_3 and A_{2A}/A_3 of 4,800- and 8,600-fold, respectively. Therefore, it is one of the most potent and selective agonists at the human A_3 adenosine receptor subtype reported so far. Furthermore, functional assays of inhibition of 10 μM forskolin-stimulated cAMP production via the adenosine A_3 receptor revealed that the new trisubstituted adenosine derivatives behave as full agonist of this receptor subtype. Docking analysis of these compounds was performed at a homology model of the human A_3 receptor based on the bovine rhodopsin crystal structure as template, and the results are in accordance with the biological data.

Key words adenosine · adenosine agonists · adenosine receptors · agonists · G-protein-coupled receptors · homology modelling · signal transduction

Introduction

Adenosine (Ado) is an endogenous modulator of a wide range of biological functions in the nervous, cardiovascular, renal and immune systems through interaction with four receptor subtypes, termed A_1, A_{2A}, A_{2B} and A_3, which belong to the family of G-protein-coupled receptors [1]. All four Ado receptor subtypes are coupled to the enzyme adenylyl cyclase in either an inhibitory (A_1 and A_3 subtypes) or stimulatory manner (A_{2A} and A_{2B} subtypes), and their activation leads to a decrease or increase of cAMP levels, respectively. In addition, all four subtypes may positively couple to phospholipase C via different G protein subunits. In particular, the A_3 Ado receptor subtype exerts its action through the stimulation of phospholipases C and D [2]. Although the physiological role of the A_3 receptor subtype is not clearly clarified, due to its recent characterisation and the lack of really selective ligands for in vivo studies, it has recently attracted significant interest as novel drug target. In fact, A_3 agonists may have potential as cerebroprotective and cardioprotective agents and may also be potential drugs for the treatment of asthma, as anti-inflammatory and immunosuppressive agents and in cancer therapy as cytostatics and chemoprotective compounds [3]. In many papers, it has been reported that Ado derivatives bearing in 2 position (ar)alkenyl chains showed good affinity and high degree of selectivity for the human A_3 Ado receptor

D. Dal Ben · C. Lambertucci · S. Taffi · S. Vittori ·
R. Volpini · G. Cristalli (✉)
Dipartimento di Scienze Chimiche, Università di Camerino,
Via S. Agostino, 1, 62032 Camerino, Italy
e-mail: gloria.cristalli@unicam.it

subtype [4 – 6]. Particularly, it has been found that the presence of a phenylethynyl group in the 2 position, combined with small substituents, such as a methyl, in N^6-position of Ado, favours interaction with A_3 receptors, leading to compounds with high affinity and selectivity for the same subtype [7]. In addition, the substitution of the 4′-hydroxymethyl group of the sugar moiety with a methyl- or ethylcarboxamido substituent enhanced A_3 affinity [5, 8]. Starting from these observations and with the aim of finding more potent and selective A_3 Ado receptor agonists, 2-phenylethynyl Ado derivatives with a methoxy group in the N^6-position were synthesised and tested at the four human Ado receptors stably transfected into Chinese hamster ovary (CHO) cells using radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assay (A_{2B}). The ability of the new compounds examined to inhibit 10 μM forskolin-stimulated cAMP production via the Ado A_3 receptor was also studied. As genetic information and molecular modelling of this receptor subtype can facilitate the understanding and the rationalising of the drug–receptor interaction, a molecular docking analysis of these compounds was performed utilising a homology model of human A_3 receptor based on the bovine rhodopsin crystal structure as template. The synthesis of these new compounds will be reported elsewhere.

Materials and methods

Biological evaluation

Binding studies

Dissociation constants of unlabelled compounds (K_i values) were determined in competition experiments in 96-well microplates, as described recently [9]. For A_{2A} and A_3 Ado receptors, the nonselective agonist [^{3}H]NECA (30 nM and 10 nM, respectively) was utilised as radioligand. The A_1-selective agonist 2-chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA; 1 nM) was utilised for the characterisation of A_1 receptor binding. Nonspecific binding was determined in the presence of 100 μM R-PIA and 1 mM theophylline, respectively. For details, see Klotz et al. [9]. All binding data were calculated by nonlinear curve fitting with the programme SCTFIT [10].

Adenylyl cyclase activity

Functional activity of the 2-phenylethynyladenosine (PEAdo) derivatives was determined in adenylyl cyclase experiments. Stimulation of adenylyl cyclase via A_{2B} Ado receptors and inhibition of forskolin-stimulated adenylyl cyclase via A_3 receptors were measured as described earlier [9, 11].

Molecular modelling

Computational methodologies

All molecular modelling studies were carried out on a 2 CPU (PIV 2.0–3.0 GHZ) Linux PC. Homology modelling was done using Molecular Operating Environment (MOE, version 2004.03) suite [12]. Docking studies were done with Schrodinger Macromodel (ver. 8.0) [13, 14] and Schrodinger Maestro interface.

Homology model of the human A_3 receptor

A homology model of the human A_3 receptor was built by using the X-ray crystal structure of the resting state bovine rhodopsin (pdb code: 1L9H [15]; available at the RCSB Protein Data Bank) with a 2.6-Å resolution as structural template.

In order to model the human A_3 receptor, the amino acid sequences of transmembrane (TM) helices of the human A_3 receptor and bovine rhodopsin were aligned. In this phase, some guanine nucleotide-binding protein-coupled receptor (GPCR) highly conserved amino acid residues could work as guide, including the DRY motif (D3.49, R3.50, Y3.51, or D107, R108, Y109 respectively) and three Pro residues (P4.60, P6.50, P7.50 or P145, P189, P245, respectively). Boundaries that were identified from the X-ray crystal structure of bovine rhodopsin were applied for the corresponding sequences of the TM helices of the A_3 receptor. Loop domains of the human A_3 receptor were constructed by the loop search method implemented in MOE. Special care had to be given to the second extracellular (E2) loop, which folds back over TM helices. This loop limits the size of the active site, and their amino acids could be involved in direct interactions with the ligands. The presence of a conserved disulfide link between cysteines in TM domain 3 (TM3) and E2 might be the driving force to this particular fold of the E2 loop, so this domain was modelled using a rhodopsin-like constrained geometry around the E2TM3 disulfide bridge. Because of the limited sequence similarity between the human A_3 receptors and the template in the C-terminal domains, only a short segment of this region was modelled. In particular, the model ends with the Ser306 residue corresponding to the Asp330 residue of the bovine rhodopsin template. Once the heavy atoms were modelled, all hydrogen atoms were added, and the protein coordinates were then minimised with MOE using the AMBER94 [16] force field. The minimisations were carried out by 1,000 steps of steepest descent followed by conjugate gradient minimisation until the RMS gradient of the potential energy was less than 0.05 kJ mol^{-1} Å^{-1}.

Molecular docking of the human A_3 receptor agonists

All agonist structures were manually docked into the hypothetical TM binding site. This receptor region has been established with the aid of published mutational data [17–20]. The searching of favourable binding conformations was conducted by a Monte Carlo Conformational Search protocol implemented in Schrodinger Macromodel. The input structure consisted of the ligand and a shell of receptor residues within the specified distance (6 Å) from the ligand. A second external shell of all the residues within a distance of 8 Å from the first shell was kept fixed. During the Monte Carlo conformational searching, the input structure was modified by random changes in user-specified torsion angles (for all input structure atoms) and molecular position (for the ligand). In this procedure, the ligand was left free to be continuously reoriented within the hypothetical binding site, and both ligand and internal shell residues conformations could be explored and reciprocally relaxed. The method consisted of 10,000 conformational search steps with MMFF94s force field [21–27]. Only unique structures within a 50 kJ mol^{-1} energy window above the found global minimum were saved. The resulting docked complexes were subjected to MMFF94s energy minimisation with Schrodinger Macromodel until the RMS of the conjugate gradient was less than 0.05 kJ mol^{-1} Å^{-1}.

Results and discussion

Binding studies

The new compounds were evaluated at the human recombinant Ado receptors stably transfected into CHO cells utilising radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assay (A_{2B}). Receptor-binding affinity was determined using [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) as radioligand for A_1 receptors whereas [^{3}H]NECA (5′-N-ethylcarboxamidoadenosine) was used for the A_{2A} and A_3 subtypes. The relative potencies of these compounds for the A_{2B} subtype were measured by evaluating the receptor-stimulated adenylyl cyclase activity expressed as EC$_{50}$. None of the tested compounds revealed activity at A_{2B} receptors at concentration up to 100 μM. The results are shown in Table 1.

PEAdo (*1*), 5′-N-methylcarboxamidoadenosine (PEMECA) (*2*), and 5′-N-ethylcarboxamidoadenosine (PENECA) (*3*) have been reported as reference compounds. PEAdo (*1*) displayed a K_i at A_3 of 16 nM and a selectivity A_1/A_3 and A_{2A}/A_3 of 24- and 23-fold while replacement of the hydroxymethyl group in 4′-position of the sugar moiety with a methyl or ethylcarboxamido substituent increased A_3 affinity. In fact, the corresponding PEMECA (*2*) and PENECA (*3*) derivatives showed increased A_3 affinity and selectivity compared with PEAdo with an unmodified ribose (Table 1) [4–6, 28]. The introduction of the methoxy group in N^6-position of *1* led to an enhancement of A_3 affinity of about four fold and a decrease of both A_1 and A_{2A} affinity of three and 12 fold, respectively, leading again to a considerable improvement of A_3 selectivity. The N^6-methoxy-2-phenylethynylAdo (*4*) showed a K_i A_3 = 3.8 nM and a selectivity A_1/A_3 and A_{2A}/A_3 of 320 and 1,100 fold, respectively [29]. Further substitution of the 4′ position of the sugar moiety of *4* enhances the affinity and/or selectivity of the corresponding compounds *5* and *6*.

Thus, N^6-methoxy-2-phenylethynyl-5′-N-ethylcarboxamidoadenosine (*6*) showed the same affinity as the 4′-hydroxymethylderivative *4* at A_3 receptors (*6*; K_i A_3 = 3.5 nM vs. *4*; K_i A_3 = 3.8 nM) combined with improved A_3 selectivity (*6*; selectivity A_1/A_3 = 540 and A_{2A}/A_3 = 1,900 vs. *4*; selectivity A_1/A_3 = 320 and A_{2A}/A_3 = 1,100) whereas an increase of both affinity and selectivity was found for
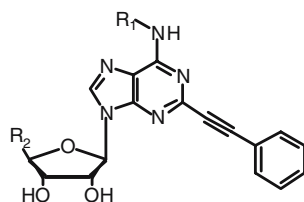
Table 1 Biological data of new synthesised compounds (K_i nM).

Compound	R_1	R_2	$K_i A_1$	$K_i A_{2A}$	$K_i A_3$	A_1/A_3	A_{2A}/A_3
1 PEAdo [4–6]	H	CH_2OH	391 (284–556)	363 (285–462)	16 (13–19)	24	23
2 PEMECA	H	CONHMe	3,920 (2,390–6,430)	1,760 (1,360–2,280)	7.3 (4.2–13)	540	240
3 PENECA	H	CONHEt	560 (480–650)	620 (300–1,300)	6.2 (5.1–7.5)	90	100
4	OCH_3	CH_2OH	1,210 (893–1,630)	4,290 (3,170–5,810)	3.8 (2.6–5.5)	320	1,100
5	OCH_3	CONHMe	9,140 (6,680–12,500)	16,300 (12,100–21,900)	1.9 (1.6–2.2)	4,800	8,600
6	OCH_3	CONHEt	1,880 (1,330–2,660)	6,660 (3,730–11,900)	3.5 (2.9–4.2)	540	1,900

PEAdo 2-phenylethynyladenosine, *PEMECA* 5′-N-methylcarboxamidoadenosine, *PENECA* 5′-N-ethylcarboxamidoadenosine

Table 2 Inhibition of adenylyl cyclase (AC) activity by new compounds.

Compound	% AC activity[a]
4	81 ± 2.3
5	38 ± 3.2
6	35 ± 4.0
Cl-IB-MECA	40 ± 2.2

Shown is the percentage of activity remaining after agonist-mediated inhibition of forskolin- stimulated cyclase activity (100%) ± standard error of mean (SEM) (n = 3)

Cl-IB-MECA 2-chloro-N^6-(3-iodobenzyl)-5′methylcarboxamidoadenosine

[a] After 10 μM forskolin stimulation

compound *5*. In fact, the N^6-methoxy-2phenylethynyl-5′-N-methylcarboxamidoadenosine (*5*), showing K_i A_3 = 1.9 nM and a selectivity A_1/A_3 and A_{2A}/A_3 of 4,800 and 8,600 fold, respectively, resulting in one of the most potent and selective agonist at the human A_3 Ado receptor subtype reported so far. The ability of the new compounds examined to inhibit 10 μM forskolin-stimulated cAMP production via the Ado A_3 receptor was also studied (Table 2).

In particular, the functional assay based on the evaluation of cyclic AMP levels in hA_3 CHO cells shows that the N^6-methoxy-PEAdo derivative *4* is a partial agonists compared with the full agonist 2-chloro-N^6-(3-iodobenzyl)-5′methylcarboxamidoadenosine (Cl-IB-MECA) as a reference [30]. On the other hand, the two N^6-methoxy-PEMECA (*5*) and N^6-methoxy-PENECA (*6*) derivatives show an adenylyl cyclase inhibition comparable to Cl-IB-MECA documenting that they are full A_3 Ado agonists.

Molecular modelling

A molecular docking analysis of these compounds was performed at a homology model of human A_3 receptor built using bovine rhodopsin crystal structure as template, with the aim of obtaining a possible rationalisation of the different binding affinities of the molecules for the human A_3 receptor. The compounds have the same core structure, with different substituents in N^6- and 4′-position. In particular, the different group bound to the 4′ carbon of the ribose moiety seems to influence both affinity and selectivity of the compounds.

In Figure 1, the complexes of the hA_3 receptor model with three agonists are shown, PEAdo (*1*), N^6-methoxy-2-phenylethynylAdo (*4*) and the N^6-methoxy-2-phenyle-thynyl-5′-N-methylcarboxamidoAdo (*5*). These compounds share a common structure apart from the functionalisation of the ribose group and the nitrogen in 6-position. The docking conformations present the adenine scaffold plane almost orthogonally with respect to the receptor axis, with
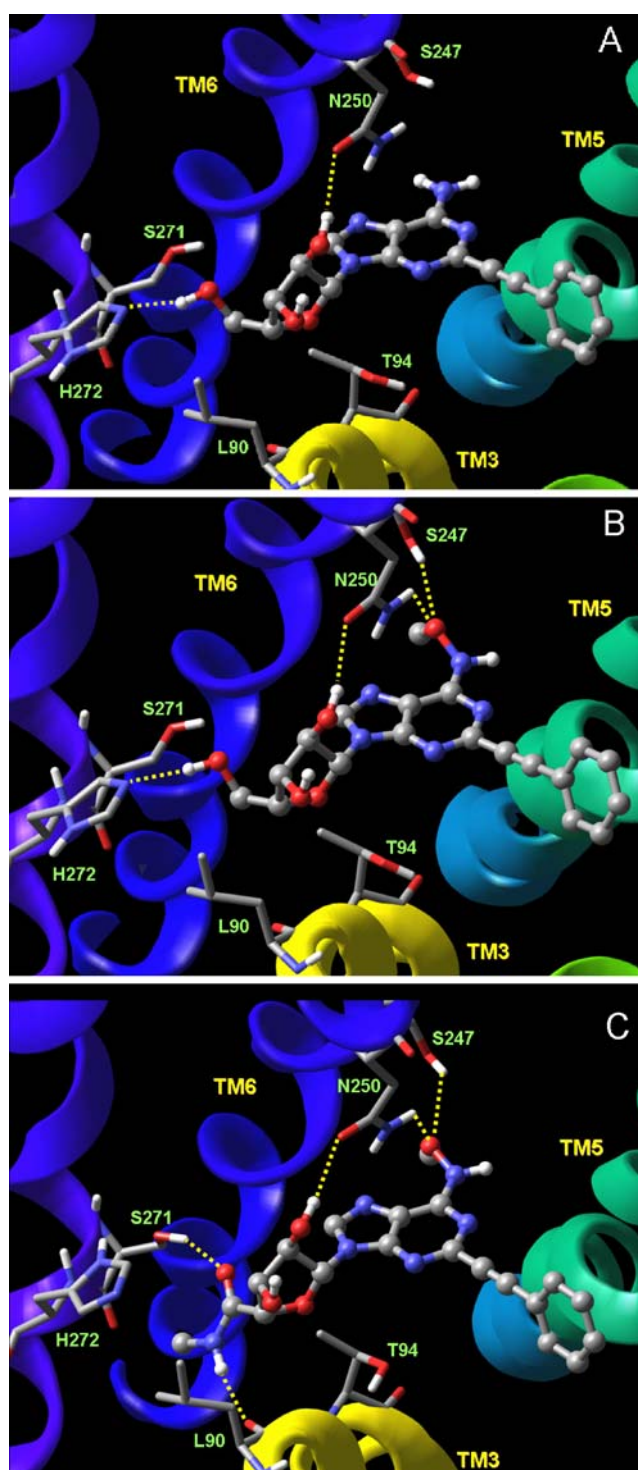
Figure 1 The complex of the hA_3AR with three agonists, (A) (PEAdo) *1*; (B) N^6-methoxy-PEAdo *4*; (C) N^6-methoxy- (PEMECA) *5* (c). The protein is in ribbons representation, and only the protein residues are coloured according to atom type. H-bond interactions (as indicated by the programme) are coloured in *yellow*

the 2-phenylethynyl group inserted in a space between TM3 and TM6. These conformations are in accordance with the already published docking conformation of PEAdo [31]. The presence of a methoxy group in 6-position seems to allow the ligand to interact with the receptor at two points, S247 and N250; this could explain a better affinity of N^6-methoxyAdo derivatives compared with the compounds with an unmodified N^6-position. In addition, the 5′-N-methylcarboxamido group of the 5 derivative leads the molecule to interact with the receptor in two points, L90 and S271, compared with the corresponding hydroxymethyl group in molecule 4, which can have only one H-bond interaction with H272.

Conclusions

A series of PEAdo derivatives substituted in the N^6- and 4′-position was tested at the four human Ado receptors stably transfected into CHO cells using radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assay (A_{2B}). The new compounds were also examined in their ability to inhibit 10 µM forskolin-stimulated cAMP production via the Ado A_3 receptor. Binding studies showed that the presence of a methoxy group in N^6 of Ado derivatives favoured the interaction with A_3 receptors, obtaining compounds endowed with high affinity and selectivity for the A_3 subtype. Additional substitution with an alkylcarboxamido group in 4′-position increases both A_3 affinity and selectivity. In particular, the N^6-methoxy-2-phenylethynyl-5′-N-methylcarboxamidoadenosine (5), showed a K_i at A_3 of 1.9 nM, a selectivity A_1/A_3 and A_{2A}/A_3 of 4,800 and 8,600 fold, respectively, and also an ability comparable to Cl-IB-MECA in inhibiting cAMP production, resulting in one of the most potent and selective agonist at the human A_3 Ado receptor subtype reported so far. Docking analysis of these compounds was performed at a homology model of human A_3 receptor built on the basis of the bovine rhodopsin crystal structure as template, and the results are in accordance with the biological data.

References

1. Cristalli G, Volpini R (2003) Adenosine receptors: chemistry and pharmacology. (eds.) Curr Top Med Chem 3:355–469
2. Ali H, Choi OH, Fraundorfer PF, Yamada K, Gonzaga HM, Beaven MA (1996) Sustained activation of phospholipase D via adenosine A_3 receptors is associated with enhancement of antigen- and Ca^{2+}-ionophore-induced secretion in a rat mast cell line. J Pharmacol Exp Ther 276:837–845
3. Muller CE (2003) Medicinal chemistry of adenosine A_3 receptor ligands. Curr Top Med Chem 3:445–462
4. Cristalli G, Eleuteri A, Vittori S, Volpini R, Lohse MJ, Klotz KN (1992) 2-Alkynyl derivatives of adenosine and adenosine-5′-N-ethyluronamide as selective agonists at A_2 adenosine receptors. J Med Chem 35:2363–2368
5. Volpini R, Costanzi S, Lambertucci C, Vittori S, Cristalli G (2002) Purine nucleosides bearing 1-alkynyl chains as adenosine receptor agonists. Curr Pharm Des 8:2285–2298
6. Cristalli G, Camaioni E, Costanzi S, Vittori S, Volpini R, Klotz KN (1998) Characterization of potent ligands at human recombinant adenosine receptors. Drug Des Res 45:176–181
7. Volpini R, Costanzi S, Lambertucci C, Taffi S, Vittori S, Klotz KN, Cristalli G (2002) N^6-alkyl-2-alkynyl derivatives of adenosine as potent and selective agonists at the human adenosine A_3 receptor and a starting point for searching A_{2B} ligands. J Med Chem 45:3271–3279
8. Cristalli G, Lambertucci C, Taffi S, Vittori S, Volpini R (2003) Medicinal chemistry of adenosine A_{2A} receptor agonists. Curr Top Med Chem 3:387–401
9. Klotz KN, Hessling J, Hegler J, Owman C, Kull B, Fredholm BB, Lohse MJ (1998) Comparative pharmacology of human adenosine receptor subtypes — characterization of stably transfected receptors in CHO cells. Naunyn-Schmiedeberg's Arch Pharmacol 357:1–9
10. De Lean A, Hancock AA, Lefkowitz RJ (1982) Validation and statistical analysis of a computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. Mol Pharmacol 21:5–16
11. Klotz KN, Cristalli G, Grifantini M, Vittori S, Lohse MJ (1985) Photoaffinity labeling of A_1-adenosine receptors. J Biol Chem 260:14659–14664
12. Molecular Operating Environment, (MOE 2004.03); C.C.G., Inc., 1255 University St., Suite 1600, Montreal, Quebec, Canada, H3B 3X3
13. Macromodel, 8.0; Schrodinger, Inc., 1500 SW First Ave. Suite 1180, Portland OR 97201
14. Mohamadi F, Richards NGJ, Guida WC, Liskamp R, Lipton M, Caufield C, Chang G, Hendrickson T, Still WC (1990) Macro-Model—an integrated software system for modeling organic and bioorganic molecules using molecular mechanics. J Comput Chem 11:440–467
15. Okada T, Fujiyoshi Y, Silow M, Navarro J, Landau EM, Shichida Y (2002) Functional role of internal water molecules in rhodopsin revealed by X-ray crystallography. Proc Natl Acad Sci USA 99:5982–5987
16. Cornell WD, Cieplak P, Bayly CI, Gould IR, Merz KM, Ferguson DM, Spellmeyer DC, Fox T, Caldwell JW, Kollman PA (1995) A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. J Am Chem Soc 117:5179–5197
17. Gao ZG, Kim SK, Biadatti T, Chen W, Lee K, Barak D, Kim SG, Johnson CR, Jacobson KA (2002) Structural determinants of A_3 adenosine receptor activation: nucleoside ligands at the agonist/antagonist boundary. J Med Chem 45:4471–4484
18. Gao ZG, Chen A, Barak D, Kim SK, Muller CE, Jacobson KA (2002) Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A_3 adenosine receptor. J Biol Chem 277:19056–19063
19. Moro S, Spalluto G, Jacobson KA (2005) Techniques: Recent developments in computer-aided engineering of GPCR ligands using the human adenosine A_3 receptor as an example. Trends Pharmacol Sci 26:44–51
20. Jacobson KA, Gao ZG, Chen A, Barak D, Kim SA, Lee K, Link A, Rompaey PV, van Calenbergh S, Liang BT (2001) 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 44:412–536

21. Halgren TA (1996) Merck Molecular Force Field. I. Basis, form, scope, parameterization, and performance of MMFF94. J Comput Chem 17:490–519

22. Halgren TA (1996) Merck Molecular Force Field. II. MMFF94 van der Waals and electrostatic parameters for intermolecular interactions. J Comput Chem 17:520–552

23. Halgren TA (1996) Merck Molecular Force Field. III. Molecular geometries and vibrational frequencies for MMFF94. J Comput Chem 17:553–586

24. Halgren TA (1996) Merck Molecular Force Field. IV. Conformational energies and geometries for MMFF94. J Comput Chem 17:587–615

25. Halgren TA, Nachbar R (1996) Merck Molecular Force Field. V. Extension of MMFF94 using experimental data, additional computational data, and empirical rules. J Comput Chem 17:616–641

26. Halgren TA (1999) MMFF VI. MMFF94s option for energy minimization studies. J Comput Chem 20:720–729

27. Halgren TA (1999) MMFF VII. Characterization of MMFF94, MMFF94s, and other widely available force fields for conformational energies and for intermolecular-interaction energies and geometries. J Comput Chem 20:730–748

28. Vittori S, Volpini R, Lambertucci C, Taffi S, Klotz KN, Cristalli G (2005) 2-substituted 5′-N-methylcarboxamidoadenosine (MECA) derivatives as A$_3$ adenosine receptor ligands. Nucleosides Nucleotides Nucleic Acids 24:935–938

29. Volpini R, Lambertucci C, Taffi S, Vittori S, Klotz KN, Cristalli G (2005) In A$_3$ Adenosine Receptors: Synthesis and Biological Evaluation of New Potent and Selective Ligands, Chemistry of Nucleic Acid Components, XIIIth Symposium, Spindleruv Mlyn, Czech Republic, September 3–9, 2005, pp 297–300

30. Brambilla R, Cattabeni F, Ceruti S, Barbieri D, Franceschi C, Kim YC, Jacobson KA, Klotz KN, Lohse MJ, Abbracchio MP (2000) Activation of the A3 adenosine receptor affects cell cycle progression and cell growth. Naunyn-Schmiedeberg's Arch Pharmacol 361:225–234

31. Costanzi S, Lambertucci C, Vittori S, Volpini R, Cristalli G (2003) 2- and 8-alkynyladenosines: conformational studies and docking to human adenosine A$_3$ receptor can explain their different biological behavior. J Mol Graph Model 21:253–262