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Original article

Synthesis, biological activity and molecular modelling of new trisubstituted 8-azaadenines with high affinity for A_1 adenosine receptors

Irene Giorgi [a,*], Anna Maria Bianucci [a], Giuliana Biagi [a], Oreste Livi [a], Valerio Scartoni [a], Michele Leonardi [a], Daniele Pietra [a], Alessio Coi [a], Ilaria Massarelli [b], Fatena Ahmad Nofal [a], Francesca Lidia Fiamingo [a], Paola Anastasi [a], Giuliano Giannini [a]

[a] *Dipartimento di Scienze Farmaceutiche, Università di Pisa, via Bonanno 6, 56126 Pisa, Italy*
[b] *Dipartimento di Chimica e Chimica Industriale, Università di Pisa, Via Risorgimento 35, 56126 Pisa, Italy*

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Abstract

We describe here the synthesis and biological activity of new 8-azaadenines bearing both a phenyl group on C(2) and a 9-benzyl group substituted in the *ortho* position with a Cl or a F atom or a CF_3 group, to verify the synergistic effect of a combination of these substitution patterns on binding with the A_1 adenosine receptors. In position N^6 aliphatic and cycloaliphatic substituents were chosen which had been shown to bind well with the A_1 receptors. Because of the high lipophilicity of these kinds of molecules, we also introduced a hydroxyalkyl substituent in the same position. The compounds obtained generally showed a very good affinity and selectivity for A_1 receptors. Some of the compounds showed K_i in the nanomolar range, one even in the subnanomolar range (0.6 nM). Molecular docking calculations were performed in order to evaluate the interaction energies between the bovine A_1 receptor model and the selected ligands, and then to correlate these energies with biological activities of the ligands as obtained from the experiments. Molecular docking analysis suggests different binding modes towards A_1 receptors that are plausible for these ligands.

Keywords: 8-Azaadenines; 1,2,3-Triazolo[4,5-*d*]pyrimidines; A_1 adenosine receptor ligands

1. Introduction

Adenosine receptors are member of the P_1 family and are coupled with more subtypes of G proteins which regulate the activity of adenylyl cyclase and other effector systems including calcium or potassium ion channels, phospholipase A or C, and guanylate cyclase [1]. They are classified as A_1, A_{2A}, A_{2B}, A_3 subtypes, and have been characterised using selective ligands. A_1 receptors are widely distributed in the central nervous system and in many peripheral tissues and mediate diverse physiological effects.

The ubiquitous distribution of adenosine receptors in mammalian cell types and the existence of four distinct subtypes together with the variability of physiological responses mean that agonists and antagonists have to be highly selective in their action (with respect to receptor subtype and tissue type) to be of value as therapeutics [2].

The importance of A_1 adenosine receptors is demonstrated by a great number of papers published in these last years about this subject [3–5]. Hundreds of A_1 agonists and antagonists have been assayed to obtain information about the receptor active site and to select more potent and selective compounds

* Corresponding author. Tel.: +39 0502219549.
E-mail address: igiorgi@farm.unipi.it (I. Giorgi).

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with potential therapeutic effects. A_1 antagonists are studied as antihypertensives and potassium sparing diuretics with kidney-protection properties [4], and could be useful in Alzheimer's disease and other CNS disorders [6], and in cardiac therapy and kidney diseases [7].

In the last years we have synthesised a great number of A_1 adenosine receptor ligands, especially 8-azaadenines, varying the substituents in positions 2, 6 and 9. We have shown that an unsubstituted phenyl group, linked in the 2 position, improves very much the activity of 9-benzyl-8-azaadenine, which is inactive with no substituent in the same position; and that the phenyl group is the best one among a series of 2-aryl substituents (2-phenyl-9-benzyl-8-azaadenine $K_i = 40$ nM) [8].

Starting from this very effective nucleus, we have introduced various substituents in position N^6: some N^6-alkyl or N^6-cycloalkyl substituted 2-phenyl-9-benzyladenines demonstrated a very good affinity for A_1 receptors [9,10].

Comparing the affinity of these compounds with that of the corresponding 2-*n*-butyl derivatives we concluded that in position 2 an aromatic group was better than an aliphatic one; instead, in position N^6 the situation is reversed [9]. When the substituent in the N^6 position is a cyclopentyl group, the affinity for the A_1 receptor is very high for both the series, 2-phenyl-9-benzyl and 2-butyl-9-benzyl-8-azaadenines ($K_i = 11$ nM and $K_i = 170$ nM, respectively) showing that the receptor contains at least three lipophilic pockets capable of interacting with lipophilic groups substituted in the 2, N^6 and 9 positions of the 8-azaadenine nucleus [9]. Many other trisubstituted 8-azaadenines were synthesised [11–14] and assayed and some demonstrated a high affinity towards A_1 receptors confirming our hypothesis about the three lipophilic pockets of the A_1 receptors (Fig. 1, **A**) [11–14].

In other papers we have described the synthesis and biological results of 8-azaadenines lacking the substituent in the 2 position but having an *o*-Cl- or *o*-F-benzyl moiety in position 9 (Fig. 1, **B**). Among them, the best compounds as ligands for A_1 receptors were the ones having a cyclohexyl or cyclopentyl ring on the N^6 position [15,16], showing a very good affinity

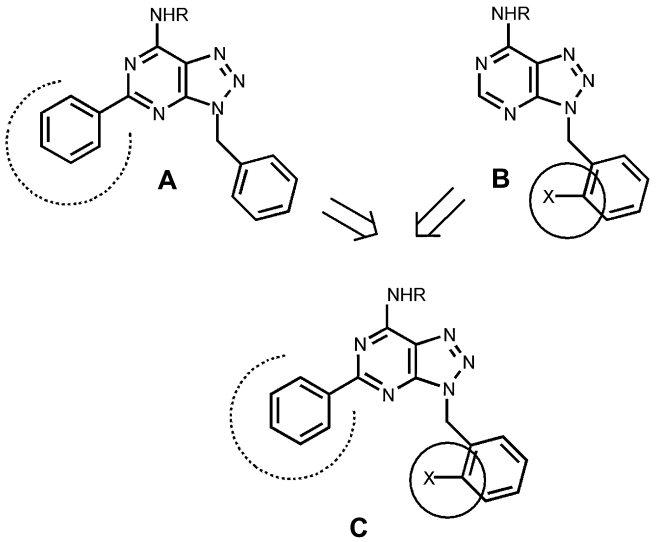


Fig. 1. Design of the new compounds **C**.

($K_i \leq 43$ nM). Comparing these results with those of the analogous lacking the halogen in the *ortho* position of the 9-benzyl substituent, we observed an increase in affinity due to the presence of the halogen. So we hypothesised that the halogen in this position must influence the interaction of the ligand with the receptor by a positive electronic effect on the π–π interaction of the phenyl moiety of the 9-benzyl substituent, or, as in the case of the 9-*ortho*-fluorobenzyl substituent, by a hydrogen bond with the receptor [16].

Considering the results obtained in the past, we describe here the synthesis and biological activity of new 8-azaadenines bearing both a phenyl group on C(2) and a 9-benzyl group substituted in the *ortho* position with a Cl or a F atom or a CF_3 group to verify the synergistic effect of a combination of these substitution patterns on binding with A_1 receptors (Fig. 1, **C**). For the position N^6 aliphatic and cycloaliphatic substituents were chosen which had been shown to bind well with the A_1 receptors. Because of the high lipophilicity of these kinds of molecules, we also introduced hydroxyalkyl substituents in the same position, as in the past we had shown that an hydroxyl group on the N^6 substituent does not lower the affinity of trisubstituted 8-azaadenines [13].

2. Chemistry

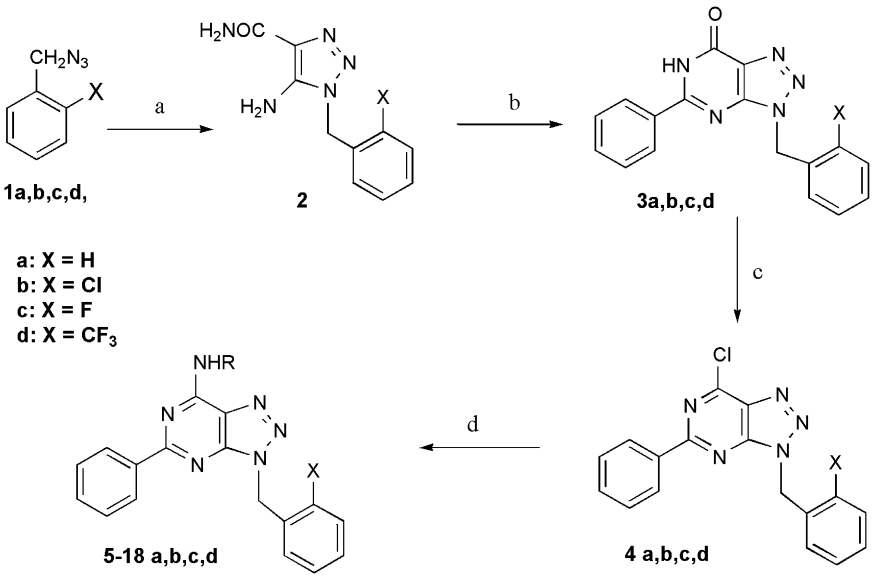
The synthetic route to prepare compounds **9a**, **5–16b** and **5–16c** (Scheme 1) employed a known two-step reaction in the presence of sodium ethoxide [16]: the first step was the 1,3 dipolar addition reaction of the suitable benzylazide and cyanoacetamide to give 1-benzyl or 1-(2-chloro)benzyl or 1-(2 fluoro)benzyl-4-carbamoyl-5-amino-1*H*-1,2,3-triazole (**2a**, **2b**, **2c**) which were not isolated; then, in the same flask, ethyl benzoate was added to obtain the 8-azahypoxanthines **3a** [17], **3b** or **3c** by annulation reaction.

This protocol gave very low yields when 2-trifluorobenzylazide was used, recovering a large amount of 1,2,3-triazole **2d**. Probably the steric hindrance of the *ortho* substituent hinders the electrophile attack of the ethyl benzoate on the amino group of the triazole. So, we put ethyl benzoate in the starting mixture, together with cyanoacetamide and then we added azide dropwise: by this method we obtained the desired product **3d** (25% yield).

The reaction of **3a–d** with phosphorus oxychloride produced the 6-chloro-8-azapurines **4a–d** which, by nucleophilic displacement of the chlorine atom by the suitable amine, gave the desired N^6-substituted-8-azaadenines.

3. Biochemistry

All the new compounds (**9a**, **5–16b**, **c**, **d**) were tested by radioligand binding assays for affinity towards A_1, A_{2A}, and A_3 adenosine receptors. In Table 1 the results of A_1 adenosine receptor binding are reported; in the A_{2A} and A_3 adenosine receptor binding assays, all the compounds showed inhibition values $< 50\%$ at 1 μM, so were considered to have very weak affinity.



R= alkyl, cycloalky, hydroxyalkyl, hydroxycycloalkyl

Scheme 1. Reagents: (a) $CNCH_2CONH_2$, EtONa; (b) $C_6H_5COOC_2H_5$; (c) $POCl_3$; (d) RNH_2.

4. Molecular modelling

The X-ray crystal structure of bovine Rhodopsin with retinal (PDB code: 1U19) was used as a template for complex construction [18]. A homology model for the bovine A_1 receptor was built from this crystal structure, after previous sequence alignment. The construction of a model for the complex of the bovine A_1 receptor subtype with the antagonist DPCPX was accomplished after the binding site had been properly identified. A single-point ab initio calculation with no further geometry optimisation was performed for DPCPX, using the Hartree–Fock method, in order to calculate electrostatic potentials at a grid of points around the ligand. The GAFF force field parameters and partial charges were assigned. Preliminary energy minimizations and molecular dynamics (MD) simulations were then performed in order to optimise the geometry of the model. The lowest energy conformer, collected after MD simulations, was further energy minimised and taken as a reliable 3D theoretical model for the bovine A_1 receptor. It was exploited for the subsequent molecular docking procedure. The complexes, created for each ligand of Table 1, were then constructed starting from DPCPX and screened by molecular docking within the DOCK approach. This approach, in the most recent version of its implementation, allows docking of flexible ligands in the active site of a receptor in order to search for their favourable orientations and conformations. A pre-calculated force field-scoring grid, based on molecular mechanic interaction energies consisting of van der Waals and electrostatic components, is generated to rank each ligand. The energy score is determined both by types and positions of the atoms of the ligand on the energy grids. Subsequently, score optimisation allows the conformation and orientation of the molecule to be adjusted to improve the score. The resulting output file for each screening, based on force field grids, contains the highest scoring compounds ranked in order of their scores. Generalized-Born Surface Area (GBSA), a new scoring function within the most recent version of the program, accounts for the effects of solvation by water. In several systems the DOCK program has been shown to give quite good correlations with respect to the docking scores [19] (see later in Section 7.2).

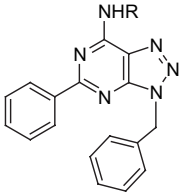
5. Results and discussion

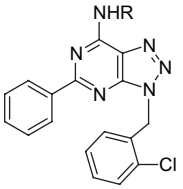
5.1. Structure–activity relationships

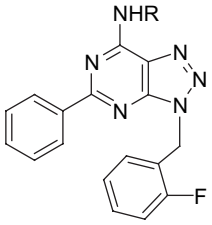
The compounds obtained generally showed very good affinity and selectivity for A_1 receptors. Some of the compounds showed K_i values in the nanomolar range, one even in the subnanomolar range (0.6 nM). We compared the affinities of the new compounds (series **b**, **c**, **d**) with the affinities of similar compounds obtained in the past (series **a**). As A_1 affinity data of series **a** (Table 1) have been performed on bovine membranes, we decided to again use the same membranes to compare the results of the new compounds with those previously described (series **a**, Table 1). To evaluate selectivity, affinities for A_{2A} and A_3 were measured using cloned human receptors. In fact for A_1 subtype there is a good amino acid sequence homology, since standard antagonists as theophilline and DPCPX showed an affinity at bovine A_1 receptors comparable to those reported at the cloned human ones [20,21]. All the compounds were considered to be very weak ligands (% inhibition $< 50\%$ at 1 μM) towards A_{2A} and A_3 receptors and consequently, selective for A_1 subtype.

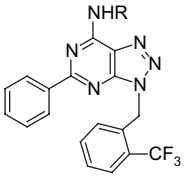
The trend of the A_1 K_i values is interestingly the same for the first three series (series **a**, **b**, **c**) leading to the assumption that these compounds interact with the receptor in a very similar manner. In

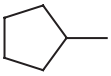
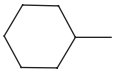
Table 1
Biological results of A_1 adenosine receptor binding









R	Compound	K_i (nM)	Compound	K_i (nM)	Compound	K_i (nM)	Compound	K_i (nM)
$-CH_3$	**5a** [8]	353 ± 30	**5b**	191 ± 15	**5c**	25 ± 2	**5d**	14.9 ± 12
$-C_2H_5$	**6a** [8]	91 ± 8	**6b**	463 ± 18	**6c**	11 ± 1	**6d**	n.d.
$-nC_3H_7$	**7a** [8]	37 ± 4	**7b**	101 ± 9.5	**7c**	3.2 ± 0.2	**7d**	n.d.
$-nC_4H_9$	**8a** [9]	11 ± 1.5	**8b**	79 ± 8	**8c**	4.3 ± 0.3	**8d**	6.5 ± 4
$-nC_6H_{13}$	**9a**	13.2 ± 1.7	**9b**	31 ± 3	**9c**	25 ± 2	**9d**	n.d.
	**10a** [13]	11.3 ± 1	**10b**	11 ± 1	**10c**	15 ± 2	**10d**	7.8 ± 8
	**11a** [13]	5 ± 0.5	**11b**	24 ± 2				
	**12a** [8]	11	**12b**	9 ± 1	**12c**	6.7 ± 0.8	**12d**	0.6 ± 0.2
	**13a** [9]	1.6	**13b**	34 ± 3	**13c**	1.15 ± 0.3	**13d**	6.8 ± 5
$-CH_2CH_2OH$	**14a** [12]	38	**14b**	116 ± 10	**14c**	54 ± 5	**14d**	n.d.
$-CH_2CHOHCH_3$	**15a** [12]	7.5	**15b**	32 ± 3	**15c**	4.8 ± 0.5	**15d**	n.d.
$-CH_2CH_2CH_2OH$	**16a** [12]	21.4	**16b**	33 ± 3	**16c**	3.7 ± 0.2	**16d**	147 ± 20

n.d. = not determined.

order to investigate the structure activity relationships we compared the affinity of compounds **12a–c** and **13a–c** (see Table 1) with the analogous not substituted in position 2 obtained in the past: 9-benzyl-N^6-cyclopentyl-8-azaadenine ($K_i = 127 \pm 8.7$ nM) [15], 9-*o*-chlorobenzyl-N^6-cyclopentyl-8-azaadenine ($K_i = 21 \pm 1.5$ nM) [15], 9-*o*-fluorobenzyl-N^6-cyclopentyl-8-azaadenine ($K_i = 10.5 \pm 0.7$ nM) [16], 9-benzyl-N^6-cyclohexyl-8-azaadenine ($K_i = 121 \pm 5.1$ nM) [15], 9-*o*-chlorobenzyl-N^6-cyclohexyl-8-azaadenine ($K_i = 43 \pm 2.5$ nM) [15], 9-*o*-fluorobenzyl-N^6-cyclohexyl-8-azaadenine ($K_i = 19.5 \pm 1.2$ nM) [16]. 2-Phenyl substituted compounds (see K_i values of the compounds **12a–c** and **13a–c** in Table 1) show better affinity than unsubstituted analogues in the same position. These observations confirm our hypothesis of the presence in the A_1 receptor of three lipophilic pockets in front of positions 2, N^6 and 9 of adenine which can well bind a phenyl, an alkyl and a benzyl group, respectively [11].

Regarding the compounds of the fourth series (**d**), the introduction of a CF_3 group further increased the lipophilicity of the molecules; this fact made the biological assays difficult to perform, because the compounds precipitated in the aqueous environment of the assays, even if in the compound a hydroxyl group is present on the N^6 substituent (compounds **14d–16d**). So we performed the assays after reducing the concentration of the tested compounds and repeated every test till we obtained at least three quantitatively homogeneous results to mediate among them. This technique helped to overcome the problem of low water solubility in some cases. So, probably due to their high affinity, we could measure the K_i values for compounds **5d** ($K_i = 14.9$ nM), **8d** ($K_i = 6.5$ nM), **10d** (7.8 nM), **12d** ($K_i = 0.6$ nM) and **13d** ($K_i = 6.8$ nM). Promising results were obtained.

Regarding the substituent on the 9-benzyl group, we can see that the series 9-(*o*-chlorobenzyl) substituted compounds showed in general less good results, followed by the 9-(unsubstituted) benzyl-8-azaadenines. The 9-(*o*-fluorobenzyl) and the 9-(*o*-trifluoromethylbenzyl) substituted compounds are in general the best ones. Obviously, the order of activity with respect to the *ortho* substituent is not directly related to its steric hindrance or electronegativity. So the high affinity of the compounds of the series **c** and **d** could be partially attributable to a possible hydrogen bond of a fluorine atom, as is confirmed by the molecular modelling study. However, the activity trend cannot be explained in terms of simple molecular descriptors, since the sum of the different contributions to the interaction energy must take into account the possibility of different binding modes, as was suggested by the molecular modelling studies.

5.2. Molecular modelling

The molecular docking procedure was performed in order to evaluate the interaction energies between the bovine A_1

receptor model and the selected ligands, and then to correlate these energies with biological activities of the ligands. The total interaction energy estimated by means of the DOCK program consisted of electrostatic and van der Waals (vdW) contributions.

The values of the correlation coefficient R^2 referred to the total energy, and the two terms with respect to the entire set of ligands and to classes **a**, **b**, **c**, and **d**, are reported in Table 2.

The vdW term seems to give good correlation with the experimental data and the total energy shows the same trend, since the electrostatic term does not give a substantial contribution towards the total energy. The binding of the ligands to the bovine A_1 receptor seems to be driven by favourable vdW interactions and this energetic term could reproduce the experimental data quite well. On the contrary, the electrostatic term does not give a correlation with K_i except for class **d** where the vdW contribution is the highest in terms of mean value along the class (−50.30 kcal/mol). The molecular docking procedure also suggested the binding mode of the ligands. The most straightforward orientation implies that the bicyclic nuclei of the ligands take the same location as the nucleus of the known antagonist DPCPX. Fig. 2 shows the ligand **7c** in the site of the bovine A_1 receptor.

Some major interactions justify its good activity. In particular, hydrogen bonds are observed between the N(3) and the hydroxyl group of Thr91 and between the fluorine of the *ortho*-substituted benzyl group in N(9) and the hydroxyl group of Ser246, thus stabilising the molecule in the binding site.

Furthermore, lipophilic interactions involving the propyl group in N^6 and residues Leu88, Leu250, Val271 are reported; moreover, the phenyl group in C(2) is able to interact with Val189, Trp247, and His251.

The molecular docking procedure gives interesting suggestions about different binding modes of the ligands to the binding site. In a previous work we refer to this above-described orientation as ''adenosine-like'' or *AL* [11]. A second orientation was considered, in which the N(9)-substituent arranges itself in the same binding site as the N^6-substituent. This implies that the phenyl substituent in position 2 of the bicyclic nuclei retains analogous locations in both orientations. This second orientation may be identified as the one obtained from the *AL* orientation after a 180° rotation of the plane containing the bicyclic nucleus around a pseudo-symmetry axis aligned along the straight line containing the C(2) and C(5) atoms. (Fig. 3).

This orientation was referred to as ''reverse'' or *RE* orientation. From the molecular docking, class **d** and series **13a–d** seem to be arranged in *RE* orientation. This fact was expected for **13a–d**, since the cyclohexyl substituent at N^6 is supposed to give a similar hydrophobic contribution as the substituted phenyl at N(9). Furthermore, the CF_3 substituent alone seems to lead to the *RE* orientation, as observed from the results of DOCK within the class **d** (Fig. 4). This could be explained hypothesizing that the CF_3-*ortho*-substituted benzyl group gives better interactions with residues Leu88, Leu250, Val271 than any N^6 substituents characterising the series **5–16**.

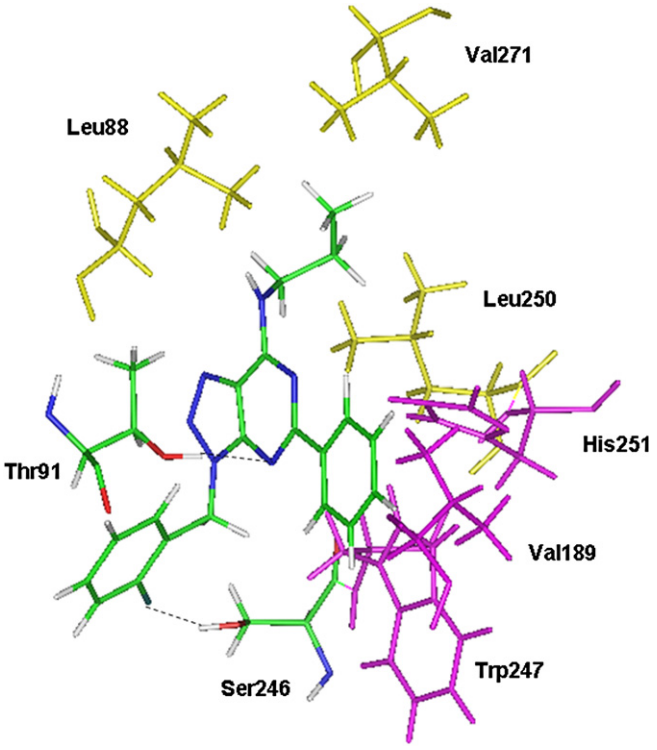


Fig. 2. Stereoview of ligand **7c** and the characteristic residues of its binding site. Thr91 and Ser246 coloured by atom type; residues Leu88, Leu250, Val271 in yellow; and residues Val189, Trp247, and His251 in magenta. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

6. Conclusions

We described herein the synthesis and biological activity of new 8-azaadenines bearing both a phenyl group on C(2) and a 9-benzyl group substituted in the *ortho* position with a Cl or a F atom or a CF_3 group.

The compounds obtained generally showed very good affinity and selectivity for A_1 receptors. The 9-(*o*-fluorobenzyl) (series **c**) and the 9-(*o*-trifluoromethylbenzyl)substituted compounds (series **d**) are more active than the 9-(*o*-chloro or

Table 2
Values of correlation coefficient R^2 between biological activity (K_i) and total interaction energy calculated by DOCK or its single components (electrostatic and van der Waals contributions) for the entire dataset and for each class (**a–d**)

Dataset			Class **a**			Class **b**			Class **c**			Class **d**		
Total	vdW	es	Total	vdW	es	Total	vdW	es	Total	vdW	es	Total	vdW	es
0.68	0.66	0.002	0.82	0.78	0.05	0.79	0.78	0.12	0.64	0.63	0.02	0.64	0.67	0.60

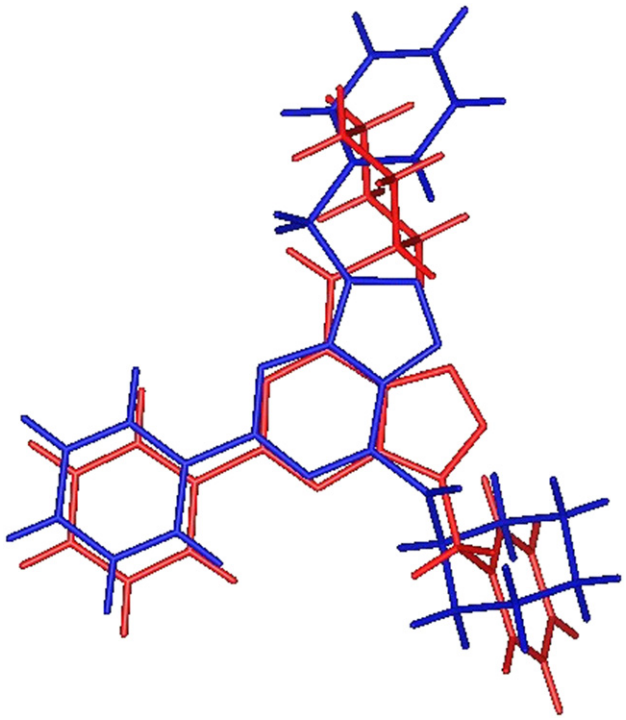


Fig. 3. Starting *AL* orientation of **13c** (red), and its relative *RE* orientation after molecular docking (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

unsubstituted) benzyl ones (series **a** and **b**). The high affinity of the compounds of the series **c** and **d** could be partially attributable to a possible hydrogen bond of a fluorine atom, as is confirmed by the molecular modelling study. The molecular docking analysis suggests different binding modes towards the A_1 receptors that are plausible for these antagonists.

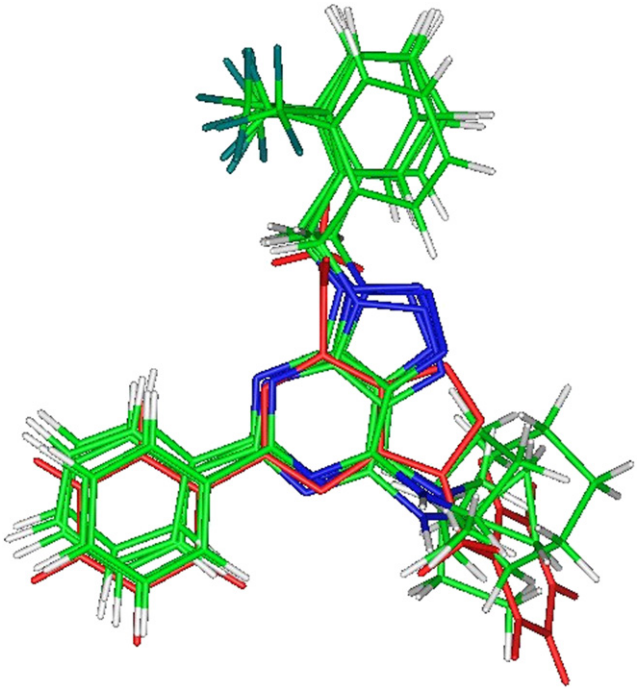


Fig. 4. Starting *AL* orientation of **5a** (red), and the *RE* orientations of class **d** resulting after molecular docking (coloured by atom). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

7. Experimental

7.1. 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 using TMS as an internal standard; the compounds were dissolved in the solvent indicated in Table 3. 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 $\pm$ 0.4% of the theoretical values.

7.1.1. Benzylazide (**1a**), 2-chlorobenzylazide (**1b**) and 2-fluorobenzylazide (**1c**)

These compounds were prepared as described in the literature [22].

7.1.2. 2-Trifluoromethyl-benzylazide (**1d**)

A mixture of 2-(trifluoromethyl)benzyl bromide (0.026 mol), ethanol (18 ml), water (2 ml) and sodium azide (2.80 g, 0.043 mol) was refluxed for 24 h. After cooling, the mixture was filtered and the filtrate was concentrated to 10 ml; then water (5 ml) was added and the mixture was extracted with chloroform (3 × 10 ml). The organic phases were dried and evaporated to obtain a yellow oil; 3.18 g, yield 60%; b.p. 200–201 °C. IR 2102 cm^{-1} (N_3). ^{1}H NMR (DMSO-d_6) 4.625 (s, 2H, CH_2); 7.59–7.80 (m, 4H, arom). Elemental analysis (C, H, N). R_f 0.53 (*n*-hexane–$CHCl_3$ 8:2).

7.1.3. 5-Amino-1-(2-trifluoromethyl-benzyl)-1H-[1,2,3] triazole-4-carboxylic acid amide (**2d**)

To a solution of EtONa (0.05 mol) in 10 ml of absolute EtOH, cyanoacetamide (1.05 g, 0.0125 mol) was added and the mixture was heated at 60 °C for 0.5 h. Then 2-(trifluoromethyl)benzylazide (2.5 g, 0.0125 mol) was added and the mixture was refluxed for 6 h, cooled and concentrated under reduced pressure. To the residue, water (20 ml) was added and the solution acidified with 4 N acetic acid at pH = 5 to obtain a white solid which was crystallised from EtOH–$CHCl_3$ 1:10; yield: 81%; m.p. 198–199 °C. IR 3396, 3307, 3177 (NH); 1640 (C=O) cm^{-1}. ^{1}H NMR 5.60 (s, 2H, CH_2); 6.51 (s, 2H, exch); 7.19 (broad s, 2H, exch); 7.48–7.81 (m, 4H, arom). Elemental analysis (C, H, N).

7.1.4. 2-Phenyl-9-(2-substituted)benzyl-8-azahypoxanthines (**3b**, **c**)

To a stirred solution of EtONa (2.76 g, 0.12 g atom of Na) in 30 ml of absolute EtOH, cyanacetamide (2.51 g, 0.03 mol) was added. The mixture was refluxed for 0.5 h then a solution of the suitable azide (0.03 mol) and ethyl benzoate (0.3 mol) in absolute EtOH (10 ml) was added drop by drop and the

Table 3
Chemical and physical properties of the newly synthesised compounds

Compound	Amine	Yield (%)	R_f	m.p. (°C)	Analysis (C, H, N)
9a	*n*-Hexylamine	58	0.34[b]	175–176[h]	$C_{25}H_{26}N_6$
5b	Methylamine[f]	43	0.55[a]	212–213[h]	$C_{18}H_{15}ClN_6$
6b	Ethylamine[g]	27	0.27[b]	170–172[h]	$C_{19}H_{17}ClN_6$
7b	*n*-Propylamine	41	0.30[b]	203–204[h]	$C_{20}H_{19}ClN_6$
8b	*n*-Butylamine	53	0.30[b]	174–176[h]	$C_{21}H_{21}ClN_6$
9b	*n*-Hexylamine	80	0.36[b]	152–154[h]	$C_{23}H_{25}ClN_6$
10b	Cyclopropylamine	33	0.18[b]	202–203[i]	$C_{20}H_{17}ClN_6$
11b	Cyclobutylamine	25	0.25[b]	208[i]	$C_{21}H_{19}ClN_6$
12b	Cyclopentylamine	31	0.36[b]	156–157[i]	$C_{22}H_{21}ClN_6$
13b	Cyclohexylamine	66	0.39[b]	151–152[i]	$C_{23}H_{23}ClN_6$
14b	Ethanolamine	83	0.25[c]	223–224[i]	$C_{19}H_{17}ClN_6O$
15b	(±)-1-Amino-2-propanol[j]	38	0.14[a]	206–208[i]	$C_{20}H_{19}ClN_6O$
16b	3-Amino-1-propanol[j]	63	0.45[e]	202–203[i]	$C_{20}H_{19}ClN_6O$
5c	Methylamine[f]	78	0.43[a]	223–224[h]	$C_{18}H_{15}FN_6$
6c	Ethylamine[g]	82	0.21[b]	195–197[h]	$C_{19}H_{17}FN_6$
7c	*n*-Propylamine	59	0.31[b]	200–201[h]	$C_{20}H_{19}FN_6$
8c	*n*-Butylamine	66	0.34[b]	148–150[h]	$C_{21}H_{21}FN_6$
9c	*n*-Hexylamine	61	0.38[b]	113–114[h]	$C_{23}H_{25}FN_6$
10c	Cyclopropylamine	15	0.26[b]	180–181[i]	$C_{20}H_{17}FN_6$
12c	Cyclopentylamine	58	0.44[a]	127–128[i]	$C_{22}H_{21}FN_6$
13c	Cyclohexylamine	57	0.43[b]	153–155[i]	$C_{23}H_{23}FN_6$
14c	Ethanolamine	65	0.25[c]	230[i]	$C_{19}H_{17}FN_6O$
15c	(±)-1-Amino-2-propanol[j]	60	0.18[a]	202[i]	$C_{20}H_{19}FN_6O$
16c	3-Amino-1-propanol[j]	39	0.13[a]	195–196[i]	$C_{20}H_{19}FN_6O$
5d	Methylamine[f]	31	0.20[d]	226–227[i]	$C_{19}H_{15}F_3N_6$
6d	Ethylamine[g]	48	0.25[d]	234[i]	$C_{20}H_{17}F_3N_6$
7d	*n*-Propylamine	87	0.29[d]	169–170[i]	$C_{21}H_{19}F_3N_6$
8d	*n*-Butylamine	54	0.34[d]	194[i]	$C_{22}H_{21}F_3N_6$
9d	*n*-Hexylamine	36	0.46[d]	169–170[i]	$C_{24}H_{25}F_3N_6$
10d	Cyclopropylamine	84	0.28[d]	223[i]	$C_{21}H_{17}F_3N_6$
12d	Cyclopentylamine	80	0.47[d]	163–164[i]	$C_{23}H_{21}F_3N_6$
13d	Cyclohexylamine	78	0.49[d]	123[i]	$C_{24}H_{23}F_3N_6$
14d	Ethanolamine	30	0.52[e]	245–246[i]	$C_{20}H_{17}F_3N_6O$
15d	(±)-1-Amino-2-propanol[j]	64	0.60[e]	241[i]	$C_{21}H_{19}F_3N_6O$
16d	3-Amino-1-propanol[j]	65	0.41[e]	213[i]	$C_{21}H_{19}F_3N_6O$

[a] Eluent: chloroform.
[b] Eluent: *n*-hexane–ethyl acetate 9:1.
[c] Eluent: chloroform–methanol 9.8:0.2.
[d] Eluent: hexane–ethyl acetate 8:2.
[e] Eluent: chloroform–methanol 9.9:0.1.
[f] 70% in water.
[g] 40% in water.
[h] Crystallisation solvent: 2-propanol–ethyl ether.
[i] Crystallisation solvent: 2-propanol.
[j] The free base was obtained from the commercial hydrochloride by treating with 10% NaOH and extracting with ethyl acetate.

mixture was refluxed for 6 h, then cooled and concentrated under reduced pressure. To the residue, water (20 ml) was added and the solution acidified with 4 N acetic acid at pH = 5. The solid precipitated was filtered and crystallised from ethanol. **3b**: yield: 50%; m.p. 285 °C. IR 1695 (C=O) cm^{-1}. ^1H NMR (DMSO) 12.96 (s, 1H, exch); 8.15 (m, 2H, arom); 7.55 (m, 4H, arom); 7.36 (m, 3H arom); 5.88 (s, 2H, CH_2). Elemental analysis (C, H, N). **3c**: yield: 50%; m.p. 285 °C. IR 1695 (C=O) cm^{-1}. ^1H NMR (DMSO) 12.85 (s, 1H, exch); 8.16 (m, 2H, arom); 7.59–7.21 (m, 7H, arom); 5.84 (s, 2H, CH_2). Elemental analysis (C, H, N).

*7.1.5. 2-Phenyl-9-(2-trifluoromethyl)benzyl-8-azahypoxanthine (**3d**)*

To a stirred solution of EtONa (1.10 g, 0.048 g atom of Na) in 15 ml of absolute EtOH, cyanacetamide (0.950 g, 0.01 mol) and ethyl benzoate (15 g, 0.1 mol) were added. The mixture was refluxed for 0.5 h then a solution of the suitable azide (0.03 mol) in absolute EtOH (5 ml) was added drop by drop and the mixture refluxed for 6 h, then cooled and concentrated under reduced pressure. To the residue, water (20 ml) was added and the solution acidified with 4 N acetic acid at pH = 5. The solid precipitated was filtered and crystallised from isopropanol to afford 0.705 g. Yield: 22%; m.p. 272 °C. IR 1695 (C=O) cm^{-1}. ^1H NMR (DMSO) 12.89 (s, 1H, exch); 8.13 (m, 2H, arom); 7.85–7.27 (m, 7H, arom); 5.96 (s, 2H, CH_2). Elemental analysis (C, H, N).

*7.1.6. 6-Chloro-2-phenyl-9-(2-substituted)benzyl-8-azapurines (**4a–d**)*

A mixture of 2-phenyl-9-(2-substituted)benzyl-8-azahypoxanthine (**3a–d**) (3.3 mmol), *N*,*N*-diethylaniline (0.8 g, 5.36 mmol) and $POCl_3$ (3.0 ml, 32.55 mmol) was heated at 90 °C for 4 h, then evaporated under reduced pressure. The residue was crystallised from chloroform–ether to give the title compounds (2.18 mmol, 60–70% yield). **4a**: m.p. 184 °C. ^1H NMR ($CDCl_3$) δ: 5.94 (s, 2H, N–CH_2); 7.38 (m, 3H, arom); 7.57 (m, 5H, arom); 8.59 (m, 2H, arom). **4b**: m.p. 165 °C. ^1H NMR (DMSO) δ: 6.10 (s, 2H, N–CH_2); 7.38–7.62 (m, 7H, arom); 8.45 (m, 2H, arom). **4c**: m.p. 163 °C. ^1H NMR ($CDCl_3$) δ: 6.04 (s, 2H, N–CH_2); 7.23–7.64 (m, 7H, arom); 8.46 (m, 2H, arom). **4d**: m.p. 149 °C. ^1H NMR ($CDCl_3$) δ: 6.16 (s, 2H, N–CH_2); 7.15–7.79 (m, 7H); 8.52 (m, 2H).

*7.1.7. 2-Phenyl-N^6-n-hexyl-9-benzyl-8-azaadenines (**9a**)*

In a well-stoppered flask a mixture of **4a** (0.623 mmol), toluene (2 ml), *n*-hexylamine (1 mmol) and *N*,*N*-diethylaniline (0.63 mmol) was heated at 130 °C for 16 h. The mixture was then evaporated, diluted with chloroform, washed with 10% HCl and water. The evaporation of the organic phase gave an oil which was crystallised (see Tables 3 and 4).

*7.1.8. General procedure for preparing 2-phenyl-N^6-alkyl or cycloalkyl-9-(2-substituted-benzyl)-8-azaadenines (**5–16b**, **5–16c**, **5–16d**)*

In a well-stoppered flask a mixture of **4b–d** (0.623 mmol), toluene (2 ml), the suitable amine (1 mmol) and *N*,*N*-diethylaniline (0.09 g, 0.63 mmol) was heated at 130 °C for 16 h. Then the solution was evaporated under reduced pressure, diluted with chloroform and washed with 10% HCl and water. After evaporation of the organic layer the residue was crystallised (see Tables 3 and 4).

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

Compound (solvent)	Aliphatic H	Aromatic H	Benzylic H	Exch. H
9a (DMSO-d_6)	3.66 (m, 2H), 1.73 (m, 2H), 1.60–1.31 (m 6H), 0.94 (t, $J = 7.4$ Hz, 3H)	8.40 (m, 2H), 7.59–7.33 (m, 7H)	5.90 (s, 2H)	9.02 (br t, 1H)
5b (DMSO-d_6)	3.15 (d, $J = 4.6$ Hz, 2H)	8.45 (m, 2H), 7.60–7.31 (m, 7H)	5.91 (s, 2H)	8.93 (br q, 1H)
6b (DMSO-d_6)	3.71 (m, 2H), 1.29 (t, $J = 7.0$ Hz, 3H)	8.43 (m, 2H), 7.60–7.30 (m, 7H)	5.90 (s, 2H)	9.01 (br t, 1H)
7b (DMSO-d_6)	3.64 (m, 2H), 1.73 (m, 2H), 0.97 (t, $J = 7.4$ Hz, 3H)	8.45 (m, 2H), 7.64–7.35 (m, 7H)	5.91 (s, 2H)	9.04 (br t, 1H)
8b (DMSO-d_6)	3.68 (m, 2H), 1.70 (m, 2H), 1.41 (m, 2H), 0.94 (t, $J = 2.8$ Hz, 3H)	8.43 (m, 2H), 7.60–7.31(m, 7H)	5.90 (s, 2H)	9.02 (br t, 1H)
9b (DMSO-d_6)	3.67 (m, 2H), 1.70 (m, 2H), 1.34 (m, 6H), 0.85 (t, $J = 7.4$ Hz, 3H)	8.43 (m, 2H), 7.56–7.32 (m, 7H)	5.90 (s, 2H)	9.03 (br t, 1H)
10b (DMSO-d_6)	3.26 (m, 1H), 0.87 (m, 2H), 0.75 (m, 2H)	8.47 (m, 2H), 7.61–7.35 (m, 7H)	5.91 (s, 1H)	9.13 (br d, 1H)
11b (DMSO-d_6)	4.56 (m, 1H), 3.73 (m, 2H), 3.54 (m, 2H), 1.87 (m, 2H)	8.43 (m, 2H), 7.60–7.35 (m, 7H)	5.91 (s, 2H)	8.98 (br d, 1H)
12b (DMSO-d_6)	4.73 (m, 1H), 2.06 (m, 2H), 1.73 (m, 4H)	8.41 (m, 2H), 7.64–7.28 (m, 7H)	5.90 (s, 2H)	9.03 (br d, 1H)
13b (DMSO-d_6)	4.33 (m, 1H), 1.99–1.20 (m, 10H)	8.40 (m, 2H), 7.52–7.32 (m, 7H)	5.90 (s, 2H)	8.93 (br d, 1H)
14b (DMSO-d_6)	3.71 (m, 4H)	8.42 (m, 2H), 7.56–7.30 (m, 7H)	5.91 (s, 2H)	8.92 (br t, 1H), 4.84 (br t, 1H)
15b (DMSO-d_6)	4.02 (m, 1H), 3.61 (m, 2H), 1.4 (d, $J = 6.2$ Hz, 3H)	8.43 (m, 2H), 7.52–7.32 (m, 7H)	5.91 (s, 2H)	8.90 (br t, 1H), 4.87 (d, 1H)
16b (DMSO-d_6)	3.72 (m, 2H), 3.56 (m, 2H), 1.87 (m, 2H)	8.43 (m, 2H), 7.61–7.35 (m, 7H)	5.91 (s, 2H)	8.97 (br t, 1H), 456 (br t, 1H)
5c (DMSO-d_6)	3.13 (d, $J = 3.8$ Hz, 3H)	8.47 (m, 2H), 7.52–7.17 (m, 7H)	5.87 (s, 2H)	8.92 (br q, 1H)
6c ($CDCl_3$)	3.87 (m, 2H), 1.42 (t, $J = 7.2$ Hz, 3H)	8.54 (m, 2H), 7.50–7.07 (m, 7H)	5.89 (s, 2H)	6.32 (br t, 1H)
7c ($CDCl_3$)	3.80 (m, 2H), 1.84 (m, 2H), 1.08 (t, $J = 7.6$ Hz, 3 H)	8.54 (m, 2 H), 7.51–7.07 (m, 7 H)	5.89 (s, 2H)	6.40 (br t, 1H)
8c ($CDCl_3$)	3.85 (m, 2H), 1.78–1.53 (m, 4H), 1.01 (t, $J = 7.6$ Hz, 3H)	8.55 (m, 2H), 7.49–7.10 (m, 7H)	5.89 (s, 2H)	6.32 (br t, 1H)
9c ($CDCl_3$)	3.80 (m, 2H), 1.74–1.33 (m, 8H), 0.90 (t, $J = 7.0$ Hz, 3H)	8.53 (m, 2H), 7.49–7.10 (m, 7H)	5.88 (s, 2H)	6.37 (br t, 1H)
10c ($CDCl_3$)	3.25 (m, 1H), 1.06 (m, 2H), 0.78 (m, 2H)	8.56 (m, 2H), 7.50–7.05 (m, 7H)	5.89 (s, 2H)	6.48 (br d, 1H)
12c ($CDCl_3$)	4.82 (m, 2H), 2.26 (m, 2 H), 1.78 (m, 6H)	8.54 (m, 2H), 7.51–7.10 (m, 7H)	5.89 (s, 2H)	6.29 (br d, 1H)
13c ($CDCl_3$)	4.43 (m, 1H), 2.24 (m, 2H), 1.84–1.27 (m, 8H)	8.53 (m, 2H), 7.51–7.08 (m, 7H)	5.89 (s, 2H)	6.22 (br d, 1H)
14c (DMSO-d_6)	3.72 (m, 4H)	8.43 (m, 2 H), 7.52–7.32 (m, 7H)	5.91 (s, 2H)	8.91 (br t, 1H), 4.85 (m, 1H)
15c (DMSO-d_6)	4.01 (m, 1H), 3.62 (m, 2H), 1.14 (d, $J = 4.2$ Hz, 3H)	8.47 (m, 2H), 7.52–7.20 (m, 7H)	5.87 (s, 2H)	8.89 m, 1H), 4.0 (m, 1H)
16c (DMSO-d_6)	3.72 (m, 2H), 3.52 (m, 2H), 1.85 (m, 2H)	8.45 (m, 2H), 7.50–7.20 (m, 7H)	5.85 (s, 2H)	8.95 (br t, 1H), 4.54 (m, 1 H)
5d ($CDCl_3$)	3.38 (d, $J = 4.6$ Hz, 3H)	8.54 (m, 2H), 7.78–7.06 (m, 7H)	6.07 (s, 2H)	6.48 (br t, 1H)
6d ($CDCl_3$)	3.89 (m, 2H), 1.44 (t, $J = 7.3$ Hz, 3H)	8.51 (m, 2H), 7.80–7.06 (m, 7H),	6.06 (s, 2H)	6.30 (br t, 1H)
7d ($CDCl_3$)	3.79 (m, 2H), 1.82 (m, 2H), 1.09 (t, $J = 7.4$ Hz, 3H)	8.50 (m, 2H), 7.76–7.07 (m, 7H)	6.04 (s, 2H)	6.32 (m, 1H)
8d (DMSO-d_6)	3.69 (m, 2H), 1.70 (m, 2H), 1.44 (m, 2H), 0.94 (t, $J = 7.4$ Hz, 3H)	8.45 (m, 2H), 7.24–7.60 (m, 7H)	5.99 (s, 1H)	9.02 (br t, 1H)
9d ($CDCl_3$)	3.81 (m, 2H), 1.79 (m, 2H), 1.40–1.25 (m 6H), 0.90 (t, $J = 7.2$ Hz, 3H)	8.48 (m, 2H), 7.76–7.05 (m, 7H)	6.05 (s, 2H)	6.30 (m, 1H)
10d ($CDCl_3$)	3.24 (m, 1H), 1.05 (m, 2H), 0.83 (m, 2H)	8.53 (m, 2H), 7.44–7.10 (m, 7H)	6.07 (s, 2H)	6.41 (br d, 1H)
12d (DMSO-d_6)	4.75 (m, 1H), 2.06 (m, 2 H), 1.89–1.57 (m, 6H)	8.38 (m, 2H), 7.85–7.21 (m, 7H)	5.99 (s, 2H)	9.04 (br d, 1H)
13d (DMSO-d_6)	4.33 (m, 1H), 2.02–1.21 (m, 10 H)	8.37 (m, 2H), 7.85–7.21 (m, 7H)	5.98 (s, 2H)	8.94 (br d, 1H)
14d (DMSO-d_6)	3.73 (m, 4H)	8.40 (m, 2H), 7.85–7.18 (m, 7H)	5.99 (s, 2H)	8.89 (br t, 1H), 4.84 (br t, 1H)
15d (DMSO-d_6)	4.03 (m, 1H), 3.61 (m, 2H), 1.14 (d, $J = 5.8$ Hz, 3H)	8.41 (m, 2H), 7.84–7.22 (m, 7H)	5.98 (s, 2H)	8.89 (br t, 1H), 4.88 (br d, 1H)
16d ($CDCl_3$)	4.06 (m, 2H), 3.73 (m, 2H), 1.98 (m, 2H)	8.42 (m, 2H), 7.77–7.10 (m, 7H)	6.05 (s, 1H)	6.80 (m, 1H)

7.2. Technical details of molecular modelling

All models were built by means of the Sybyl7.0 program (Tripos) on an SGI Octane R12000 workstation [23].

Standard PARM99 [24] force field parameters were assigned to the protein, using the LEAP program in AMBER8.0. The single-point ab initio calculation on DPCPX was performed by using the Gaussian03 program with the 6-31G* basis set [25]. The GAFF force field parameters and partial charges were assigned using the ANTECHAMBER program as implemented in AMBER8.0.

Energy minimization, performed by means of the AMBER8.0 program, was initially run using positional restraints only for the enzyme heavy atoms with a harmonic potential of 1000 kcal/mol Å^2. Subsequently, a second restrained minimization was performed only restraining the backbone atoms with a force constant of 5.0 kcal/mol Å^2. Finally, a further minimization was made to relax all the atoms without constraints. The minimization employed a distance dependent dielectric constant (4r) and the convergence criterion for the energy gradient was 0.01 kcal/mol Å. After the minimization, MD simulations were initiated using the pair wise

Generalised-Born (GB) continuum solvent model introduced by Hawkins and co-workers and implemented in the SANDER module of AMBER8.0 [26,27]. Simulations employed a 1 fs time step for 20 000 steps corresponding to a total of 20.00 ps of GB-MD. The final desired temperature of 298 K was obtained by requesting a heating cycle from 0 to 298 K over the course of the first 5000 MD steps and Langevin dynamics were used to simulate solvent frictional effects with a collision frequency $\gamma = 1.0\ ps^{-1}$. The SHAKE algorithm was applied to constrain bonds involving hydrogen atoms [28].

Dielectric constants of 1 (interior) and 80 (exterior) were employed in all GB-MD simulations. The MD simulation of the bovine A_1 receptor in complex with DPCPX, consisted of 5 ps of heating, 15 ps for equilibration, and 20 ps of MD simulations for data collection. No cutoff (cutoff = 999 Å) was employed during the GB-MD simulations.

All the complexes, each containing one member of the entire class of ligands, were subjected to molecular docking by means of the DOCK approach [29] using the DOCK5.2 version of the program. The GBSA exploited in the calculations is a new scoring function, within the DOCK5.2 release, which has been developed with the aim of accounting for the effects of solvation by water. It works by implicitly using the GB approximation. In spite of the many approximations used, it has been shown that the DOCK program in many cases offers quite good correlations not only with respect to the orientations of molecules inside the receptor cavity but also with respect to the docking scores.

7.3. Biochemical assays

7.3.1. Radioligand binding assays

Membranes of bovine cerebral cortex, that contain adenosine A_1 receptors, were prepared as previously described [30]. Membranes (40 μg of protein) were incubated at 25 °C for 60 min with 0.4 nM of [^{3}H]DPCPX (K_d 0.4 nM), and with increasing concentrations of the compounds in duplicate, in a final volume of 0.4 ml of Tris–HCl buffer. Nonspecific binding was measured in the presence of CPA 10 μM. Binding reactions were terminated by dilution with ice-cold 50 mM Tris–HCl buffer. Samples were then filtered through Whatman GF/C glass-fibre filters using a Brandell cell harvester or a Millipore manifold. Filters were washed three times with 2–3 ml of the same buffer. Bound radioactivity was measured in a liquid scintillation counter (1600 TR Packard) after the addition of 4 ml of scintillation liquid (Emulsifier-Safe, Packard).

The experimental conditions for adenosine A_{2A} and A_3 receptor binding essays were slightly different. Membranes of CHO cells expressing recombinant human A_{2A} or A_3 receptors were prepared as previously described [31]. Membranes (40 μg of protein) were incubated with [^{3}H]ZM241385 4 nM or [^{3}H]NECA 6 nM in the A_{2A} or A_3 experiment, respectively, and compounds at a concentration of 1 μM in duplicate, in a final volume of 0.4 ml of Tris–HCl buffer for 120 min at 25 °C. Nonspecific binding was measured in the presence of 100 μM NECA in the case of A_{2A} binding assay and 100 μM R-PIA in the case of A_3 binding assay. Samples were handled as mentioned before.

7.3.2. Statistical analysis

Binding parameters were estimated by GraphPAD Prism software (GraphPAD, San Diego, CA, USA).

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