New A2A adenosine receptor antagonists: a structure-based upside-down interaction in the receptor cavity

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Adenosine receptor antagonists are generally based on heterocyclic core structures presenting substituents of various volumes and chemical-physical profiles. Adenine and purine-based adenosine receptor antagonists have been reported in literature. In this work we combined various substituents in the 2, 6, and 8-positions of 9-ethylpurine to depict a structure-affinity relationship analysis at the human adenosine receptors. Compounds were rationally designed through molecular modeling analysis and then synthesized and evaluated at radioligand binding studies at human adenosine receptors. The new compounds showed affinity for the human adenosine receptors, with some derivatives endowed with low nanomolar Ki data, in particular at the A2AAR subtype. The purine core proves to be a versatile core structure for the development of novel adenosine receptor antagonists with nanomolar affinity for these membrane proteins.

1. Introduction

Extracellular adenosine (Ado) plays a number of physiological roles through the stimulation of G Protein-Coupled Receptors called adenosine receptors (ARs) and known as four subtypes: A1, A2A, A2B, and A3 ARs [1,2]. Each subtype has unique pharmacological profile due to endogenous ligand affinity, intracellular effector coupling, and synthetic ligands specificity. In fact, A1 and A2A ARs are endowed with nanomolar affinity for Ado, while A2B and A3 ARs need micromolar concentrations (i.e. due to tissue damage) of the endogenous ligand to be activated. Furthermore, while the activation of A2A and A2B ARs takes an increase of intracellular levels of cAMP due to stimulation of adenylyl cyclase (AC) activity, the stimulation of A1 and A3 ARs takes to reduction of intracellular cAMP and stimulation of phospholipase C (PLC) activity [1,2]. Each AR subtype presents different levels of expression in various tissues. Through the modulation of the activity of the different ARs, extracellular Ado regulates vascular smooth muscle tone and blood flow [3,4], heart rate and contractility [5], inflammation [6–8], sleep [9,10], and cognitive mechanisms [11]. Within the CNS, Ado modulates also neuronal plasticity and development, neuronal excitability and arousal, release of neurotransmitters, and neuroinflammation [12–16]. All together, these factors make the ARs potential (if not promising) therapeutic targets for a number of conditions including cardiovascular, inflammatory, and neurodegenerative diseases [17–21]. Besides Ado itself, therapeutically used as injectable antiarrhythmic agent, natural or synthetic AR ligands are available on the market as agonists (i.e. the coronary vasodilator Regadenoson [22]) and antagonists (the CNS stimulator caffeine [23], the bronchodilator theophylline [24], the anti-Parkinson tool istradefylline [25]). The prototypical AR antagonists are xanthines and methylxanthines, whose modifications led to derivatives endowed with potency and AR subtype selectivity [26]. Among these compounds are the above cited caffeine, theophylline, and istradefylline. A number of classes of non-xanthine derivatives have also been developed as potent and selective AR antagonists [27–30].

In the last years we developed 9-alkyladenine derivatives substituted in the 2, N6, and 8-positions. These compounds presented different degrees of affinity and selectivity for the various AR subtypes [31–37]. More in detail, the 8-bromo-9-ethyladenine showed good affinity and moderate selectivity for the A2AAR (I; Ki A2A = 52 nM, Fig. 1); substitution of the bromine atom with a 2-furyl ring enhanced both the A2A affinity and selectivity, especially versus the A3 receptor.
subtype (II; \( K_i A_{2A} = 3.7 \text{nM} \)). On the other hand, even the presence of a phenylethylamino or a phenylethyloxy chain in the 2-position of 9-ethyladenine favoured the interaction with the \( A_{2A} \)-AR subtype leading to compound III and IV, which are endowed with a similar selectivity profile (see details within the Biological activity section). The combination of a phenylethylamino or a phenylethyloxy group in the 2-position and a bromine atom in the 8-position led to compounds endowed with enhanced \( A_{2A} \)-AR affinity (V; \( K_i = 19 \text{nM} \) and VI; \( K_i = 1.7 \text{nM} \), Fig. 1) [33,38].

Since the replacement of the 8-substituent of the 8-bromo-9-ethyladenine with a 2-furyl ring led to an improvement of the \( A_{2A} \)-AR affinity [39], we hence synthesized (and here we present) 9-ethyladenine derivatives bearing a 2-furyl group in the 8-position and a phenylethylamino or a phenylethyloxy group in the 2-position (designed molecules of general formula A, Fig. 1). On the other hand, the displacement of the phenylethylamino or the phenylethyloxy chain of V and VI from the 2- to the 6-position led to a significative decrease of the \( A_{2A} \)-AR affinity (compounds VII; \( K_i = 280 \text{nM} \) and VIII; \( K_i = 19,300 \text{nM} \), Fig. 2) [31].

For compounds VII and VIII, modeling studies suggested a particular binding mode in which the 6-substituents mimicked the role of the analogue 2-substituents of V and VI, while the 8- and 9-substituents were located almost in the same position (Fig. 2). This resulted in a sort of upside-down binding mode respect to the “canonical” binding orientation observed i.e. for the reference \( A_{2A} \) receptor ligand ZM241385. In this binding mode, however, the 6-substituted derivatives lacked the H-bond donor function corresponding to the free amine function of the 2-substituted derivatives [40]. On these bases, we designed and developed 9-ethyladenines bearing a phenylethylamino or a phenylethyloxy chain in the 6-position, a bromine atom in the 8-position, and an amine function in the 2-position (designed molecules of general formula B, Fig. 2). The presence of the amino group in the 2-position is aimed at restoring the H-bond donor function suggested by molecular modeling studies previously reported [40] and according to the “pharmacophore” hypothesis showed in Fig. 2. The new compound bearing the phenylethylamino chain in the 6-position and an 8-bromo substituent was further modified by replacing the bromine atom with a 2-furyl ring.

Finally, we also designed and synthesized 9-ethyladenines bearing various groups in the 8-position and the phenylethylamino substituent in both the 2- and 6-positions to test the effect of the combined presence of the two arylalkyl groups on the AR affinity (designed molecules of general formula C, Fig. 3). All the compounds were tested by radioligand binding studies at the human recombinant \( A_1, A_{2A} \) and \( A_3 \) ARs, and the results were analysed with molecular docking studies.

### 2. Results and discussion

#### 2.1. Chemistry

The synthesis of the 9-ethyl-8-(2-furyl)-2-phenylethylamine-adenine (1) and its 2-alkoxy analogue 2 was carried out by Suzuki coupling reaction conditions on the 8-bromo-9-ethyl-2-substituted adenine

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![Fig. 1. Known and designed compounds of general formula A.](image1)

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![Fig. 2. Design of \( N^8,8 \)-disubstituted 2-amino-9-ethyladenines. A. Previously reported \( N^8,8 \)-disubstituted 9-ethyladenines [31]. B. Comparison of compounds in panel A (compound VII as example) with 8,8-disubstituted 9-ethyladenines (compound V as example) and definition of respective pharmacophore features, where Ha, Hd, and Hy are H-bond acceptor, H-bond donor and Hydrophobic pharmacophoric features, respectively. C. Superimposition of compounds VII and V with matching of pharmacophore features; compound V presents and additional H-bond donor group indicated by the arrow. D. Design of \( N^8 \)-phenylalkyl substituted 2-amino-9-ethyl adenine derivatives; the 2-amino group is inserted to restore the H-bond donor feature suggested in panel; example of designed compound; E. The same example compound, rotated view. F. Designed \( N^8,8 \)-disubstituted 2-amino-9-ethyladenines to be synthesized and tested in this work.](image2)
The two compounds were in turn treated with 2-(tributylstannyl)furan and bis triphenylphosphine palladium dichloride in tetrahydrofuran as solvent at 60°C for 5h in anhydrous conditions (Scheme 2). Compound 3 was reacted with ethyl iodide in dry dimethylformamide (DMF), using potassium carbonate as base, under anhydrous conditions at room temperature (r.t.) as reported before [31].

After chromatography of the reaction mixture, 4 and 4a were obtained as pure products with 66% and 21% yield, respectively. These compounds were confirmed previously [31] and the structures were confirmed by UV spectra and NMR chemical shifts. In this work, for a deeper characterization, the alkylation site of the two isomers was assigned by 1H-NOE difference spectroscopy in DMSO of the N6-methyl analogues 5 and 6, obtained by reacting 4 and 4a with methanol (Scheme 2). The choice to use the N6-methyl derivatives 5 and 6 for the alkylation site assignment was due to the fact that in the case of the N7-isomer the NOE effect easily takes place when in the N8-position there is a small group (i.e. a methyl group) that makes the H-N(6) less exchangeable. In fact, irradiation of the CH2 protons of 5 (at 3.95 ppm) yielded a NOE at the H-C(8) and H3-C(CH2) protons while there was no effect on the H-N(6) proton in the resulting spectra. On the contrary, irradiation of CH2 protons of 6 (at 4.26 ppm) yielded a NOE at the H-C(8), H2-C(CH2) and also at the H-N(6) protons in the resulting spectra. These data allowed an unambiguous identification of 5 as the 9-isomer and 6 as the 7-isomer (Fig. 4).

Hence, 4 was treated with phenylethylamine or phenylethyl alcohol in order to substitute the 6-chlorine atom and to obtain compounds 7 and 8, respectively (Scheme 2). The reactions were performed using the reagents as solvents at r.t. In the case of reaction with phenylethyl alcohol, sodium hydroxide was added to catalyse the reaction.

The 6-substituted 2-aminooctahydrobenzol[e]purine derivatives 7 and 8 were obtained with a yield of 78% and 57%, respectively. Reaction of these compounds with phenylethylamine, at r.t., furnished the desired 8-bromo derivatives 9 and 10 with 80 and 35% yield, respectively (Scheme 2).

To obtain the 8-furyl derivative 13, a different synthetic pathway was set up, which allows to synthesise 2-aminooctahydrobenzol[e]purine derivatives substituted in the 6-position with various chains. In fact, compound 4 was brominated to obtain the polyfunctionalized intermediate 11 (Scheme 3). The 8-bromo-6-chlora adenine derivative 11 was then reacted with 2-(tributylstannyl)furan in the presence of bis(triphenylphosphine)palladium (II) dichloride in DMF using Stille coupling reaction conditions.

The selective substitution of the 8-bromine atom was obtained using 2-(tributylstannyl)furan in a 1:1 equivalent ratio with the purine derivative 11; the 8-furyl analogue 12 was obtained with a 54% yield. Replacement of the 6-chlorine atom of 12 with phenylethylamine, at r.t., furnished the desired tetrasubstituted adenine derivative 13 in 90% yield. This approach could be very useful to obtain in good yield further 6-substituted derivatives of compound 12. Compounds 17–20 were obtained by 3 synthetic steps starting from 2,6-dichloro-9-ethylnitrobenzene (14, Scheme 4) obtained with the earlier reported procedure [38]. 14 was treated with 2-phenylethylamine at 120°C for 16 h to get the desired 9-ethyl-2-phenylethylamine-6-phenylethyladenine (15), with 90% yield, which was then treated with bromine and sodium acetate in acetic acid and left to react at r.t. for 12 h.

The 8-bromoderivative 16 was obtained with 85% yield as pure compound. The substitution of the 8-bromine atom with a 2-furyl group was obtained as previously described for compounds 1 and 2 (Scheme 1), to get the 9-ethyl-8-(2-furyl)-2-phenylethylamine-6-phenylethyladenine (17) with 29% yield. Finally, reaction of 16 with methanol or ethanol, used as solvent, or phenethyl alcohol (using tetrahydrofuran as solvent) in the presence of sodium hydroxide, furnished the 8-alkyloxy purine derivatives 18–20 (Scheme 4). These compounds were obtained by reaction at 70°C or reflux for 17–72 h, with yield ranging from 37 to 80%.

2.2. Biological activity and molecular modeling studies

The novel adenine derivatives (1, 2, 7–10, 13, 15–20) were tested by evaluating their affinity at the human A1, A2A and A3 ARs stably expressed in Chinese hamster ovary (CHO) cells. Previously published derivatives V–VIII were reported as reference compounds. A set of derivatives was also selected and tested at the hA2BAR subtype by measuring their inhibitory effects on NECA-stimulated cAMP levels in hA2BAR CHO cells. The results are presented in Table 1. Molecular docking analyses were performed to simulate the binding mode of the synthesized compounds at the hA2BARAR. As molecular target, we chose the high-resolution crystal structure of the hA2BAR in complex with the antagonist ZM241385 (http://www.rcsb.org; pdb code: 5NM4; 1.7Å resolution [41]). Gold software [42] was employed to perform docking analyses to depict the potential binding mode of the compounds at the hA2BARAR binding cavity. For a subset of compounds, the binding modes at a homology model of the hA2BAR [43] were also simulated with the same tools and protocols.

As already reported in the introduction part (Fig. 1), the presence of a bromine atom in the 8-position of 9-ethyl-2-phenylethylamine or 9-ethyl-2-phenylethyladrenochrome enhanced both the A2BAR affinity and selectivity (III; Ki A2BAR = 150 nM, select. A1/A2A = 2, A3/A2A = 21 vs V; Ki A2AAR = 19 nM, select. A1/A2A = 8, A3/A2A = 163; and IV; Ki A2AAR = 120 nM, select. A1/A2A = 1, A3/A2A = 60 vs VI; Ki A2AAR = 1.7 nM, select. A1/A2A = 13, A3/A2A = 640) [33]. Replacement of the 8-bromine atom of V and VI with a 2-furyl ring led to

![Diagram](https://example.com/diagram.png)

**Scheme 1.** Reagents and conditions: nBut3Sn-(2-furyl), (Pb3P)2PdCl2, THF, 60°C, 5 h.

![Diagram](https://example.com/diagram.png)

**Fig. 3.** Designed compounds of general formula C.
compounds with generally enhanced affinity at ARs, but decreased selectivity for the A2AAR subtype. In particular, the 9-ethyl-8-furyl-2-phenylethylaminoadenine (1: Ki A2AAR = 3.8 nM, select. A1/A2A = 2, A1/A3 = 5) displayed a five-fold increased affinity respect to its 8-bromo analogue, while the 9-ethyl-8-furyl-2-phenylethoxyadenine (2: Ki A2AAR = 2.2 nM, select. A1/A2A = 3, A1/A3 = 7) maintained the same A2AAR affinity of VI.

The results of biological evaluation of compounds 7–10 (Table 1) showed that the combination of a phenylalkylamino or phenylalkyloxy group in the 6-position with a 2-amine function generally takes to compounds endowed with micromolar or high nanomolar affinity for the human ARs, with a low selectivity in some cases for the A2AAR subtype.

As expected, the introduction of an 8-bromo substituent improves the affinity at all the AR subtypes with respect to the corresponding 8-unsubstituted derivatives. Among these molecules, compound 9 showed the best A2AAR affinity and selectivity (9, Table 1; Ki A2AAR = 266 nM;
$A_1/A_{2A} = 7; A_3/A_{2A} = 4$). Based on the observation that compound 1 and 2, bearing a 2-furyl group in the 8-position, showed an improved $A_{2AAR}$ affinity with respect to the corresponding 8-bromo substituted analogues V and VI, we analogously modified compound 9 to obtain the derivative 13. This compound showed a remarkable improvement of affinity for the ARs with respect to the corresponding 8-bromo substituted analogue 9 and resulted the compound endowed with the highest $A_{2AAR}$ affinity (13, Table 2; $K_i A_{2AAR} = 12\text{nM}$) among the newly 6-substituted 2-amino synthesized compounds. Among these derivatives, compounds 9 and 13 showed activity as $A_{2BAR}$ inhibitors, even if only at micromolar level.

Results of docking studies performed at the human $A_{2AAR}$ crystal structure showed that the 2-amino derivatives 7–10, 13 may interact with the receptor by adopting two kinds of conformations (Fig. 5). The first one (binding mode 1, Fig. 5A) presents the purine core oriented similarly to the co-crystallized $A_{2AAR}$ antagonist ZM241385 and similarly also to other adenine-based $A_{2AAR}$ antagonists previously developed and reported. In detail, the bicyclic core is inserted between

\[
\begin{align*}
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\end{align*}
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\end{align*}
\]
residues of transmembrane (TM) domains 3, 6, and 7, making polar and hydrophobic interactions with the receptor residues.

The 8- and 9-substituents point toward the depth of the cavity while the N6-substituent is oriented toward the extracellular environment. The alternative docking conformation (binding mode 2, Fig. 5B), presents the compound inserted in the cavity with an upside-down orientation with respect to the binding mode 1 arrangement. The 8- and 9-substituents are again pointing toward the depth of the cavity, while the 2-amino group is externally oriented. The comparison (Fig. 5A and B) and superimposition (Fig. 5C) of the two arrangements demonstrate a good agreement with the rationale presented in Fig. 2 (considering i.e. compounds V and 9, or 1 and 13). In fact, the phenylalkylamine group in the 6-position of compound 13 (binding mode 2, Fig. 5B) is located in almost the same position of the analogue group in the 2-position of 1 (Fig. 5D); analogously, the exocyclic amine groups of the same two compounds are similarly located, making analogue polar interaction with the receptor residue Asn253.

We further developed this series by introducing the phenylalkylamino group in both the 2- and 6-position, with additional 8-modifications. Results (Table 1) show that these compounds (15–20) are generally endowed with nanomolar affinity for the ARs, but with some selectivity for the A3AR subtype. Compound 17 (Fig. 6), presenting a 2-furyl group in the 8-position, is endowed with a significantly improved A2AAR affinity (17, Table 1; K_i A2AAR = 104 nM) with respect to the 8-unsubstituted derivative 15 (15, Table 2; K_i A2AAR = 593 nM); on the other hand, compound 17 lacks selectivity for the A2AAR and it preferentially binds the A3AR subtype (17, Table 1; K_i A3AR = 45 nM).

Compound 15 showed a low nanomolar A2AAR affinity (15, Table 1; K_i A2AAR = 13 nM) with 35- and 47-fold selectivity vs the A1 and A3A AR subtypes, resulting the most A3AR selective ligand of the whole group. An improved affinity for this AR subtype was not totally unexpected, as

<table>
<thead>
<tr>
<th>CPD</th>
<th>hA2AAR (K_i nM)</th>
<th>hA2AR (IC50 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>19</td>
<td>411 (398–424)</td>
</tr>
<tr>
<td>VI</td>
<td>1,7</td>
<td>108 (50–166)</td>
</tr>
<tr>
<td>1</td>
<td>3.8</td>
<td>171 (135–207)</td>
</tr>
<tr>
<td>7</td>
<td>4.635</td>
<td>&gt; 50,000</td>
</tr>
<tr>
<td>8</td>
<td>5.044</td>
<td>&gt; 50,000</td>
</tr>
<tr>
<td>9</td>
<td>266</td>
<td>5,753 (4,776–6,730)</td>
</tr>
<tr>
<td>10</td>
<td>1,080</td>
<td>&gt; 50,000</td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td>962 (696–1,228)</td>
</tr>
</tbody>
</table>

Fig. 5. Docking studies at the A2AAR, with key receptor residues indicated. A and B. Docking conformations of compound 13. Binding mode 1 (A) and 2 (B) are represented. C. Superimposition of the two binding modes of compound 13 suggested by docking studies. D. Docking conformation of compound 1.
Docking studies at the A3AR were performed with several A3AR ligands reported in literature, featuring arylalkyl groups in a position corresponding to the N^6-substituent of the here reported compound series (i.e., the reference A3AR agonist IBMEC/A or 2-Cl-IB-MECA, or other Ado/NECA derivatives [21,44]). Docking studies at a homology model of A3AR showed that this molecule may adopt both the above described binding modes (Fig. 7A and B). Considering the binding mode 1 arrangement, the N^6-arylalkyl substituent is located in proximity of a hydrophobic valine residue (Val169) that is substituted by a negatively charged glutamate in the other AR subtypes (i.e., Glu169 in the A2AAR [45]). This feature takes on an improved interaction of the A3AR with the N^6-arylalkyl substituent considering the binding mode 1 arrangement, but also with the 2-arylalkyl substituent considering the binding 2 docking conformations. Docking results at the A3AR show that favourable docking scores are assigned to both binding modes and hence both binding modes could easily take place within this receptor cavity. The ability of compounds presenting some symmetry to adopt various binding modes within the A3AR cavity was already observed and reported [46]. Introduction of substituents in the 8-position of 15 led to a significant decrease of A3AR affinity and selectivity, while the A2AAR affinity resulted generally slightly improved. This may due to different interaction of the compounds with the depth of the A2A and A3 ARs binding cavity, but also with the effect of the 8-substituent to make the compound able (or not) to adopt various binding modes within the cavities, with consequent higher or lower interaction with the receptor. Among the derivatives of the second set, compounds 15, 17, and 19 were tested as A3AR inhibitors but they did not show any activity at this AR subtype.

Selected derivatives (V, VI, 1, 7–10, 13) were tested in CHO cells, stably expressing hA2AAR, in order to assess their ability to inhibit or stimulate the receptor through the evaluation of cAMP production. The results showed that the compounds were not able to stimulate cAMP production (data not shown) and inhibited the NECA-induced increase of cAMP accumulation (see Table 2), behaving as antagonists of the A2AAR. For three of them, 7, 8, 10, endowed with micromolar A2AAR affinity, it was not possible to obtain an IC50 value below 50 μM concentration. For the other compounds, the obtained IC50 values present an analogue trend of activity compared to the affinity data at the same receptor.

Furthermore, in order to evaluate the efficacy of some new compounds in native tissues, 1 and 13 were tested at rat aorta rings to evaluate their ability to counteract relaxation induced by the selective A2AAR agonist CGS 21680 [47]. The A2AAR antagonist ZM 241385 was also tested for comparison. CGS 21680 showed an EC50 = 280 nM whereas ZM 241385 exhibited an IC50 = 64 nM. Results confirmed the antagonistic behaviour of the new compounds 1 and 13, which counteracted the relaxation induced by CGS 21680 with an IC50 of 136 nM and 589 nM, respectively (Table 3). These data reveal that 1 and 13 have analogue effect at rat and human A2AARs demonstrating their usefulness as tools to study pathologies involving the adenosine A2A receptor.

### Table 3

<table>
<thead>
<tr>
<th>CPD</th>
<th>EC50 (nM)</th>
<th>IC50 (nM)</th>
<th>K_{i} (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGS 21680</td>
<td>280 (228–332)</td>
<td>23 (18–27)</td>
<td></td>
</tr>
<tr>
<td>ZM 241385</td>
<td>64 (50–77)</td>
<td>1.2 (0.9–1.4)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>136 (93–179)</td>
<td>3.8 (3.4–4.2)</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>589 (487–690)</td>
<td>12 (7.3–16)</td>
<td></td>
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| **Note**: EC50 is the concentration of agonists that gives half-maximal response. IC50 is the concentration of antagonists required to produce 50% inhibition of the CGS 21680 relaxation effect. Data are means of 2–4 separated experiments. Values are given as geometric means with 95% confidence intervals in parentheses.

### 3. Conclusion

Based on the observation that previously reported 9-ethyladenine derivatives bearing arylalkyl groups in the 2-position and an 8-bromo substituent are endowed with nanomolar affinity for the human A3AR, in this work we further modified these compounds by substituting the 8-bromo atom with a 2-furyl ring, very often associated in literature with high A3AR affinity. The obtained compounds showed low nanomolar A3AR K_{i} data, confirming the hypothesis. From the further observation that 9-ethyladenine derivatives bearing arylalkyl groups in the 2-position or in the N^6-position and an 8-bromo substituent present nanomolar affinity as AR ligands, and following a pharmacophore-based rationale, we developed also a set of 9-ethylpurine derivatives bearing a phenylethylamino- or phenylethoxy-group in the 6-position and a 2-amine function, to test the effect of these substituents on the affinity at human ARs. Compounds showed low nanomolar affinity in particular for the A2AAR, with the presence of a further 8-substituent modulating the affinity at all the ARs. Docking studies suggested that the substituents inserted in the purine core could make the compounds adopt a sort of upside-down arrangement within the receptor cavity, as compared to the “canonical” binding mode of the reference A2AAR ligand ZM241385. Following these results and observations, we developed a second group of compounds presenting arylalkyl groups at both 2- and 6-positions, to test the effect on AR affinity and subtype selectivity. The second group proved to be slightly A3AR selective, and again the 8-substituent gave a significant modulation of the AR affinity, with different impact at the various AR subtypes. Docking studies suggested more than one potential binding mode for these compounds, similarly to the first group. These data again suggest the purine core as...
a versatile scaffold to develop AR ligands with nanomolar affinity and various degrees of selectivity for the different AR subtypes.

4. Materials and methods

4.1. Chemistry

Melting points were determined with a Büchi apparatus and are uncorrected. 1H NMR spectra were obtained with Varian Gemini 200 or VX 300 MHz spectrometers (1H NMR), (13C NMR); δ values are in ppm, J values are in Hz. All exchangeable protons were confirmed by the addition of D2O. Mass spectra were recorded on an HP 1100-MSD series instrument. All measurements were performed using electrospray ionization (ESI-MS) on a single quadrupole analyzer. Thin-layer chromatography (TLC) was carried out on pre-coated TLC plates with silica gel 60 F254 (Fluka). For column chromatography, silica gel 60 (Merck) was used. For preparative TLC, silica gel 20 × 20 cm plates (UV 254 indicator) from Analtech were used. Elemental analyses were determined on Fisons Instruments Model EA 1108 CHNS-O model indicator and are within 0.4% of theoretical values. Purity of the compounds was ≥98% according to elemental analysis data.

4.1.1. General procedure for the synthesis of 8-(furan-2-yl) purine derivatives 1, 2, 12, and 17

To the suitable 8-bromo derivatives V, VI, [33] 11, or 16 (70 mg, 0.19 mmol), in anhydrous THF (10 mL), 2-(tributylstannyl)furan (in the appropriate amount) and (PPh3)2PdCl2 (8 mg, 0.011 mmol) were added and the reaction was left under stirring, in nitrogen atmosphere, at 60 °C, unless otherwise noted, in the oil bath, for the time necessary to reach the reaction completion. The solvent was removed under vacuum and the crude worked-up as described below.

4.1.1.1. 9-Ethyl-8-(furan-2-yl)-N6-phenethyl-9H-purine-2,6-diamine (1). The title compound was obtained by reaction of V with 5 equivalents of 2-(tributylstannyl)furan, in 5 h the reaction was complete. The crude residue was purified by preparative thin layer chromatography (TLC) eluting with AcOEt-cC6H12-CH3OH (60:40 to AcOEt 100%). Compound 1 was obtained pure, with 61% yield, after crystallization from CHCl3–MeCN (99:5). Compound 1 was obtained pure, with 39% yield, after crystallization from CH3CN. M.p. = 173–175 °C; 1H NMR (DMSO-d6) δ = 1.31 (t, J = 7.2 Hz, 3H, CH3); 4.34 (m, 2H, N-CH2); 6.76 (m, 1H, H-Furyl); 7.01 (s, 2H, NH2); 7.26 (d, J = 3.6 Hz, 1H, H-Furyl); 8.00 (s, 1H, H-Furyl). ESI-MS: positive mode m/z 264.0 [M + H]+, 549.0 [2M + Na]1.

4.1.1.2. 9-Ethyl-8-(furan-2-yl)-N6,6-di(phenethyl)-9H-purine-2,6-diamine (17). The title compound was obtained by reaction of 16 with 5 equivalents of 2-(tributylstannyl)furan in an oil bath under reflux for 2.5 h till the reaction was complete. The crude residue was purified by flash column chromatography, dry silica slurry, eluting with nC6H14–AcOEt (90:10 to 85:15). Compound 17 was obtained as a white solid in 29% yield after crystallization from AcOEt – Et2O to get. M.p. = 171–173 °C; 1H NMR (DMSO-d6) δ = 1.32 (m, 3H, NCH2CH3), 2.88 (t, J = 6.8 Hz, 2H, CH2Ph), 2.93 (t, J = 8.0 Hz, 2H, CH2Ph), 3.56 (m, 2H, NCH2CH3), 3.67 (m, 2H, NCH2CH3), 4.29 (m, 2H, NCH2CH3), 6.72 (m, 1H, H-Furyl), 7.03 (m, 1H, H-Furyl), 7.19–7.36 (m, 12H, H-Ph, N6H, and N8H), 7.92 (m, 1H, H-Furyl). ESI-MS: positive mode m/z 453.0 [M + H]+, 474.9 [M + Na]1. Elemental analysis calculated for C22H13N6O: C, 71.66; H, 6.24; N, 18.57; found: C, 71.86; H, 6.46; N, 18.34.

4.1.1.3. 6-Chloro-9-ethyl-9H-purin-6-amine (6a) and 7-ethyl-N6-methyl-7H-purine-2,6-diamine (6b). To a solution of 2-amino-6-chloropurine (3, 210 mg, 1.24 mmol) in 6.2 mL of dry DMF K2CO3 (215 mg, 1.56 mmol) and 1 equivalent of iodoethane (99 μL, 1.24 mmol) were added. The mixture was left under stirring and under nitrogen atmosphere at r.t. After 5 h the reaction was complete. After evaporation of the solvent, a dry silica slurry was prepared and flash chromatography, eluting with nC6H14–AcOEt (60:40 to AcOEt 100%), was used to separate the reaction products. Compounds 4 and 4a were obtained after crystallization from MeCN as pure product with the yield of 66 and 21%, respectively. 4: m.p. = 154–156 °C; 1H NMR (DMSO-d6, 400 MHz) δ = 1.35 (t, J = 7.2 Hz, 3H, CH3); 4.05 (m, 2H, N-CH2); 6.88 (bs, 2H, NH2); 8.13 (s, 1H, H-8). 4a: m.p. = 178 °C (dec.); 1H NMR (DMSO-d6, 400 MHz) δ = 1.38 (t, J = 7.2 Hz, 3H, CH3); 4.30 (m, 2H, N-CH2); 6.62 (br s, 2H, NH2); 8.37 (s, 1H, H-8).

4.1.2. General procedure for the synthesis of 9-ethyl-N6-methyl-9H-purine-2,6-diamine (5) and 7-ethyl-N6-methyl-7H-purine-2,6-diamine (6)

To 2 mL of methylamine, compound 4 or 4a (100 mg, 0.51 mmol) were in turn added in a steel vial at ~80 °C. The vial, well closed, was then brought to r.t. and the reaction was left for 16 h until completion. After evaporation of methylamine at r.t. and coevaporation with MeOH, compound 5 was obtained after crystallization from MeOH in 96% yield; the crude mixture of the reaction of 4a was chromatographed on silica gel flash column, eluting with CHCl3–MeOH (93:7–90:10) to get compound 6 in 64% yield.

5: m.p. = 212–214 °C; 1H NMR (DMSO-d6, 400 MHz) δ = 1.30 (t, J = 7.2 Hz, 3H, CH3); 2.86 (bs, 3H, N-CH3); 3.95 (m, 2H, N-CH3); 5.81 (bs, 2H, NH2); 7.11 (bs, 1H, NH), 7.67 (s, 1H, H-8). Elemental analysis calculated for C8H12N6O: C, 49.99; H, 6.29; N, 43.72; found: C, 50.32; H, 6.32; N, 43.65.

6: m.p. = 223 °C; 1H NMR (DMSO-d6, 400 MHz) δ = 1.27 (t, J = 7.2 Hz, 3H, CH3); 2.88 (d, J = 4.8 Hz, 3H, N-CH3); 4.26 (q, J = 6.0 Hz, 2H, N-CH2); 5.55 (bs, 2H, NH2); 6.45 (m, 1H, NH), 7.84 (s, 1H, H-8).

4.1.2.1. Synthesis of 9-ethyl-N6-phenethyl-9H-purine-2,6-diamine (12). The title compound was obtained by reaction with 6 with 5 equivalents of 2-(tributylstannyl)furan in a 1:1 equivalent ratio. The reaction was complete within 3 h. The crude residue was purified by normal column chromatography using a dry silica slurry and eluting with CHCl3–MeCN (99:5). Compound 12 was obtained pure, with 54% yield, after crystallization from CHCl3–cC6H12. M.p. = 220–222 °C; 1H NMR = (DMSO-d6) δ = 1.31 (t, J = 7.2 Hz, 3H, CH3); 4.34 (m, 2H, N-CH2); 6.76 (m, 1H, H-Furyl); 7.01 (s, 2H, NH2); 7.26 (d, J = 3.6 Hz, 1H, H-Furyl); 8.00 (s, 1H, H-Furyl). ESI-MS: positive mode m/z 264.0 [M + H]+, 549.0 [2M + Na]1.

4.1.2.2. Synthesis of 9-ethyl-N6-phenethyl-9H-purine-2,6-diamine (7). Phenethylamine (7 mL) was added to compound 4 (200 mg, 1.01 mmol). The reaction was left under stirring at r.t. for 3.5 h. The phenethylamine was removed under vacuum at high temperature. After preparation of a dry silica slurry, the crude mixture was chromatographed on a flash column, eluting with AcOEt – cC6H12 – MeOH (60:37:3). Compound 7 was obtained after crystallization from
MeCN in 80% yield. M.p. = 106–108 °C; 1H NMR (DMSO-d6, 400 MHz) δ = 1.31 (t, J = 7.2 Hz, 2H, CH3); 2.87 (t, J = 7.6 Hz, 2H, CH2-Ph); 3.59 (br. s, 2H, NH-CH2); 3.96 (q, J = 7.2, 2H, N-CH2); 5.84 (s, 2H, NH2); 7.17 (m, 1H, H-Ph); 7.28 (m, 5H, H-Ph and H-8); 7.69 (s, 1H, NH). Elemental analysis calcld for C31H31N14: C, 69.23; H, 5.40; N, 19.47. Found: C, 69.25; H, 5.39; N, 19.46.

4.1.2.2. Synthesis of 9-ethyl-6-phenetoxy-9H-purin-2-amine (4). Phenethyl alcohol (5 mL) was added to compound 3 (200 mg; 0.92 mmol) and the reaction mixture was left for 8 h, after which it was filtered. The volatile compounds were removed under vacuum, and the dry crude was dissolved in acetonitrile (MeCN) and used for the flash chromatography eluted with suitable solvents.

4.1.2.3. Synthesis of 9-ethyl-8-bromo-6-chloro-9H-purin-2-amine (5). To a mixture of 2-chloro-9H-purin-2-amine (50 mg; 0.13 mmol), sodium acetate (23mg; 0.25 mmol) and sodium hydrogen carbonate (200 mg; 5 mmol), 18.3 mL of acetonitrile (MeCN) was added. The mixture was stirred at room temperature for 3 h. After completion, the reaction solution was evaporated, and the dry residue was dissolved in acetonitrile (MeCN) and used for the flash chromatography eluted with suitable solvents.

4.1.2.4. Synthesis of 9-ethyl-8-(furan-2-yl)-N6-phenethyl-9H-purine-2,6-diamine (6). Phenethylamine (2 mL) was added to a mixture of 9-ethyl-9H-purin-2-amine (16) (50 mg; 0.13 mmol), sodium acetate (23 mg; 0.25 mmol) and sodium hydrogen carbonate (200 mg; 5 mmol), and the reaction mixture was left for 8 h. After completion, the mixture was evaporated, and the dry residue was dissolved in acetonitrile (MeCN) and used for the flash chromatography eluted with suitable solvents.

4.1.2.5. Synthesis of 9-ethyl-6-chloro-9H-purin-2-amine (7). To a mixture of 9-ethyl-9H-purin-2-amine (16) (50 mg; 0.13 mmol), sodium acetate (23 mg; 0.25 mmol) and sodium hydrogen carbonate (200 mg; 5 mmol), 18.3 mL of acetonitrile (MeCN) was added. The mixture was stirred at room temperature for 3 h. After completion, the reaction solution was evaporated, and the dry residue was dissolved in acetonitrile (MeCN) and used for the flash chromatography eluted with suitable solvents.

4.1.2.6. Synthesis of 8-bromo-9-ethyl-N2,N6-diphenethyl-9H-purine-2,6-diamine (11). A mixture of 9-ethyl-9H-purin-2-amine (16) (50 mg; 0.13 mmol) and sodium acetate (23 mg; 0.25 mmol) was added to acetonitrile (MeCN) and sodium hydrogen carbonate (200 mg; 5 mmol), and the reaction mixture was left for 8 h. After completion, the mixture was evaporated, and the dry residue was dissolved in acetonitrile (MeCN) and used for the flash chromatography eluted with suitable solvents.

4.1.3. General procedure for the preparation of the 8-bromoderivatives 9–11

To a solution (1 mmol) of the suitable purine derivative 7, 8, or 4 in dry DMF (4 mL) the NBS (202 mg; 1.1 mmol) was added and the reaction mixture was left under stirring and under nitrogen atmosphere at r.t. for the time necessary to the reaction completion. The volatiles were evaporated and a dry silica slurry was prepared with the crude residue and used for the flash chromatography eluted with the suitable solvent.

4.1.3.1. 8-Bromo-9-ethyl-N6-phenethyl-9H-purine-2,6-diamine (9). Compound 9 was prepared from compound 7. The reaction took 10 min for the completion. The crude residue was chromatographed eluting with CHCl3 - MeCN (98:2) to afford compound 9 in 75% yield. M.p. = 182°C (dec); 1H NMR (DMSO-d6, 400 MHz) δ = 1.27 (t, J = 7.4 Hz, 3H, CH3); 2.89 (t, J = 7.6 Hz, 2H, CH2-Ph); 3.61 (bs, 2H, NH2); 7.18 (m, 1H, H-Ph); 7.28 (m, 4H, H-Ph); 7.45 (bs, 1H, NH). ESI-MS: positive mode m/z 349.1 [M + H]+, 371.1 [M + Na]+. Elemental analysis calcld for C18H14N6O: C, 65.11; H, 5.79; N, 24.12. Found: C, 65.13; H, 5.78; N, 24.13.

4.1.3.2. 8-Bromo-6-chloro-9-ethyl-9H-purin-2-amine (10). Compound 10 was prepared from compound 8. The reaction took 70 min for the completion. The crude residue was chromatographed eluting with CHCl3 - MeOH (98:2) to afford compound 10 in 90% yield. 1H NMR (DMSO-d6, 400 MHz) δ = 1.24 (t, J = 7.0 Hz, 3H, CH3); 2.85 (t, J = 7.8 Hz, 2H, CH2-Ph); 3.57 (br. s, 2H, NH-CH2); 3.96 (q, J = 7.0 Hz, 2H, N-CH2); 6.01 (s, 2H, NH2); 7.18 (m, 6H, H-Ph and H-8); 7.44 (s, 1H, NH). ESI-MS: positive mode m/z 361.0 [M + H]+, 383.0 [M + Na]+. Elemental analysis calcld for C19H16N6O: C, 67.03; H, 5.93; N, 24.04. Found: C, 67.05; H, 5.93; N, 24.03.

4.1.3.3. 8-Bromo-6-chloro-9-ethyl-9H-purin-2-amino (11). Compound 11 was prepared from compound 4. The reaction was left for 8 h, then stopped due to the formation of side products. The DMF was removed under vacuum and the crude mixture was partitioned between H2O and AcOEt. The organic layers were collected, dried over Na2SO4, and concentrated to dryness under vacuum. The crude residue was chromatographed eluting with nC6H14 - AcOEt (75:25). The collected product was crystallized from CH2Cl2 and compound 11 was obtained in 75% yield. M.p. = 182°C (dec); 1H NMR (DMSO-d6, 400 MHz) δ = 1.27 (t, J = 7.4 Hz, 3H, CH3); 4.04 (q, J = 7.2 Hz, 2H, N-CH2); 7.06 (s, 2H, NH2). ESI-MS: positive mode m/z 276.0 [M + H]+, 297.9 [M + Na]+, 315.9 [M + K]+.
4.1.4.1. 9-Ethyl-8-methoxy-N²,N⁶-diphenethyl-9H-purine-2,6-diamine (18).

The title compound was obtained by reaction of 16 with methanol for 17 h. Purification by column chromatography eluted with nCH₂₄-NaOAc (90:10 to 70:30) gave 18 as vitreous solid with 80% yield. ¹H NMR (DMSO-d₆) δ = 1.21 (t, J = 7.2 Hz, 3H, NCH₂CH₃), 2.85 (m, 4H, 2 × CH₂Ph), 3.44 (q, J = 6.4 Hz, 2H, NCH₂CH₃), 3.62 (br s, 2H, NCH₂CH₃), 3.81 (q, J = 6.8 Hz, 2H, NCH₂CH₃), 4.00 (s, 3H, OCH₃), 6.29 (br s, 1H, N=H), 6.87 (br s, 1H, N=H), 7.17–7.26 ppm (m, 10H, H-Ph). ESI-MS: positive mode m/z 417.0 [M+H]+, 438.9 [M+Na]+. Elemental analysis calcd for C₂₅H₃₀N₆O: C, 69.74; H, 7.02; N, 19.31.

4.1.4.2. 8-Ethoxy-9-ethyl-N²,N⁶-diphenethyl-9H-purine-2,6-diamine (19).
The title compound was obtained by reaction of 16 with ethanol for 19 h. Purification by column chromatography eluted with nCH₂₄-NaOAc (90:10 to 80:20) gave 19 as vitreous solid with 67% yield. ¹H NMR (DMSO-d₆) δ = 1.22 (t, J = 6.8 Hz, 3H, NCH₂CH₃), 1.35 (t, J = 6.8 Hz, 3H, OCH₃CH₂), 2.85 (m, 4H, 2 × CH₂Ph), 3.44 (q, J = 6.0 Hz, 2H, NCH₂CH₃), 3.62 (br s, 2H, NCH₂CH₃), 3.80 (q, J = 7.2 Hz, 2H, OCH₂CH₃), 4.41 (q, J = 7.2 Hz, 2H, OCH₂CH₃), 6.27 (br s, 1H, N=H), 6.82 (br s, 1H, N=H), 7.15–7.26 ppm (m, 10H, H-Ph). ESI-MS: positive mode m/z 431.1 [M+H]+. Elemental analysis calcd for C₂₅H₃₀N₆O: C, 69.74; H, 7.02; N, 19.52; found: C, 69.95; H, 6.92; N, 19.99.

4.1.4.3. Synthesis of 9-ethyl-8-phenoxy-N²,N⁶-diphenethyl-9H-purine-2,6-diamine (20). To a solution of 16 (70 mg; 0.15 mmol) in dry tetrahydrofuran (2 mL) sodium hydroxide (30 mg; 0.75 mmol) and 2-phenylethanol (120 µL; 1.00 mmol) were added. The reaction was poured at reflux for 72 h. The solvent was removed under vacuum and the crude residue purified by silica gel column, dry silica slurry, poured at reflux for 72 h. The solvent was distilled under vacuum to yield 20 as vitreous solid with 67% yield. ¹H NMR (DMSO-d₆) δ = 1.12 (t, J = 6.8 Hz, 3H, NCH₂CH₃), 2.84 (m, 4H, 2 × CH₂Ph), 3.08 (t, J = 6.4 Hz, 2H, OCH₂CH₂Ph), 3.43 (q, J = 6.8 Hz, 2H, NCH₂CH₃), 3.61 (br s, 2H, NCH₂CH₃), 3.74 (q, J = 8.0 Hz, 2H, NCH₂CH₃), 4.56 (q, J = 7.2 Hz, 2H, OCH₂CH₃), 6.28 (br s, 1H, N=H), 6.83 (br s, 1H, N=H), 7.16–7.30 ppm (m, 15H, H-Ph). ESI-MS: positive mode m/z 507.1 [M+H]+, 529.0 [M+Na]+. Elemental analysis calcd for C₂₅H₂₄N₆O: C, 73.49; H, 6.76; N, 16.59; found: C, 73.68; H, 6.97; N, 16.35.

4.2. Biological evaluation

4.2.1. Cell culture

CHO cells stable transfected with hARs were grown in Dulbecco’s modified Eagle’s medium (DMEM) with nutrient mixture F12 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml amphotericin B, 0.1 mg/ml gentamicine and 1 mM sodium pyruvate. They were cultured at 37°C in a humidified atmosphere of 5% CO₂/95% air [38,48].

4.2.2. Membrane preparation

Crude membranes for radioligand binding experiments were prepared by collecting cells (CHO stably transfected with hA₁, hA₂A and hA₃ ARs) in ice-cold hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized on ice (Ultra-Turrax, 2 × 20 s at full speed) and the homogenate was spun for 10 min (4°C) at 3200 rpm. The supernatant was centrifuged for 50 min at 37000 rpm at 4°C. The membrane pellet was resuspended in the specific binding buffer (hA₁AR: 50 mM Tris/HCl buffer, pH 7.4; hA₂AAR: 50 mM Tris/HCl, 50 mM MgCl₂, pH 7.4; hA₃AR: 50 mM Tris/HCl, 10 mM MgCl₂, 1 mM EDTA, pH 8.25), frozen in liquid nitrogen at a protein concentration of 2–4 mg/mL and stored at −80°C.

4.2.3. Radioligand binding

Dissociation constants of unlabelled compounds (Kᵥ₅) were determined in radioligand competition experiments, where a fixed concentration of radioligand (1 nM [³H]CPPA, Kᵥ₅ = 1.1 nM; 10 nM [³H]NECA, Kᵥ₅ = 20 nM; [³H]HEMADO, Kᵥ₅ = 1.5 nM) was incubated in a 96-well plate with 10 µg of specific receptor (hA₁, hA₂A, and hA₃ ARs, respectively) cell membrane preparations and increasing concentrations of the compound understudy. Non-specific binding was determined in the presence of 1 mM theophylline for hA₁AR and 100 µM (R)-N²-phenyl-propyladenosine (R-PIA) for both hA₂A and hA₃AR. All binding data were calculated by non-linear curve fitting with Prism 5.0 programme (GraphPAD Software, San Diego, CA, USA). Each concentration was tested three-five times in triplicate and the values are given as the mean ± standard errors (S.E.). The activity of the compounds at the hA₂AAR was evaluated as inhibition of NECA-stimulated adenyl cyclase activity.

4.2.4. GloSensor cAMP Assay

Cells, stably expressing the hA₂A or hA₂B AR and transiently the biosensor, were harvested in CO₂-independent medium and were counted in a Neubauer chamber. The desired number of cells was incubated in equilibration medium containing a 3% v/v GloSensor cAMP reagent stock solution, 10% FBS, and 87% CO₂ independent medium. After 2 h of incubation at rt, the cells were dispensed in the wells of a 384-well plate and, when a steady-state basal signal was obtained, the NECA reference agonist or the understudy compounds, at different concentrations, were added. Initially, the ability of the selected compounds to stimulate the cAMP production was evaluated, but any results were obtained. Subsequently, their antagonist profile was evaluated by assessing their ability to counteract NECA-induced increase of cAMP accumulation. The cells were incubated in the reaction medium (10 min at rt) with different understudy molecule concentrations and then treated with NECA. After 10 min, various luminescence measurements were performed at different incubation times [38,48].

4.2.5. Functional antagonism in isolated rat aorta

All animal testing was carried out according to European Communities Council Directive of 24 November 1986 (86/609/EEC).

Male Wistar rats (275–300 g) were killed by cervical dislocation and the aorta was isolated, freed from adhering connective tissue, and set up rapidly under 1 g resting tension in 10 mL organ baths containing Krebs solution of the following composition (mM): NaCl, 118.4; KCl, 4.7; CaCl₂, 1.9; MgSO₄, 1.2; NaH₂PO₄, 1.2; NaHCO₃, 25; glucose, 11.7. The preparation was kept at 37°C and aerated with 5% CO₂, 95% O₂ at pH 7.4 [49]. After that a 2-hs equilibirum period a sub-maximal contractions of aorta rings were obtained by PGE₂a (3 µM).

Concentration-response curves were constructed by cumulative addition of the A₂A selective agonist CGS 21680 which gives relaxation of the tissue [47]. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a minimal level and remained steady. Relaxations were recorded by means of an isometric transducer connected to the MacLab system PowerLab/800. After 30 min the dose-response curve was repeated in the presence of compounds 1, 13 (used at 1000 nM) or the reference antagonist ZM 241385 (used at 300 nM). In addition, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

The IC₅₀ values were obtained according to the following equation:

\[ Kᵥ₅ = IC₅₀/[1 + (A/EC₅₀)²] \]

where DR is the ratio of EC₅₀ values of agonist after and before treatment with antagonist concentration [B].
4.2.6. Statistical analysis

Responses were expressed as percentage of the maximal relative luminescence units (RLU). Concentration–response curves were fitted by a nonlinear regression with the Prism 5.0 program (GraphPAD Software, San Diego, CA, USA). The antagonist profile of the two compounds was expressed as IC50. The IC50 value is the concentration of antagonists that produces 50% inhibition of the agonist effect. Each concentration was tested three-five times in triplicate and the values are given as geometric means with 95% confidence intervals in parentheses [50].

4.3. Molecular modeling

A high-resolution crystal structures of the hA2AAR in complex with ZM241385 was retrieved from the Protein Data Bank (http://www.rcsb.org; pdb code: 5NNM; 1.7 Å resolution [41]). The structure was modified by rebuilding the missing IL3 segment and by mutating some residues to restore the wild type sequence. The Homology Modeling tool of MOE [51] was used for this task. Hydrogen atoms were added and energetically minimized. A homology model of the human A1AR was built following a procedure previously reported [43]. The Homology Modeling tool of MOE was employed even for this task. All compound structures were docked into the binding site of the hA2A and hA3AR structures using the genetic algorithm docking tool of CDK Gold [42] with default efficiency settings and by selecting GoldScore as scoring function [42].

Declaration of Competing Interest

The authors declare no conflict of interest.

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