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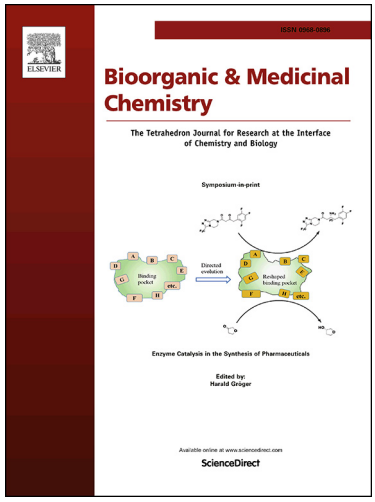

2-Aryladenine derivatives as a potent scaffold for A_1, A_3 and dual A_1/A_3 adenosine receptor antagonists: synthesis and structure-activity relationships

Filipe Areias, Carla Correia, Ashly Rocha, José Brea, Marián Castro, Maria I. Loza, M. Fernanda Proença, M. Alice Carvalho

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Graphical Abstract

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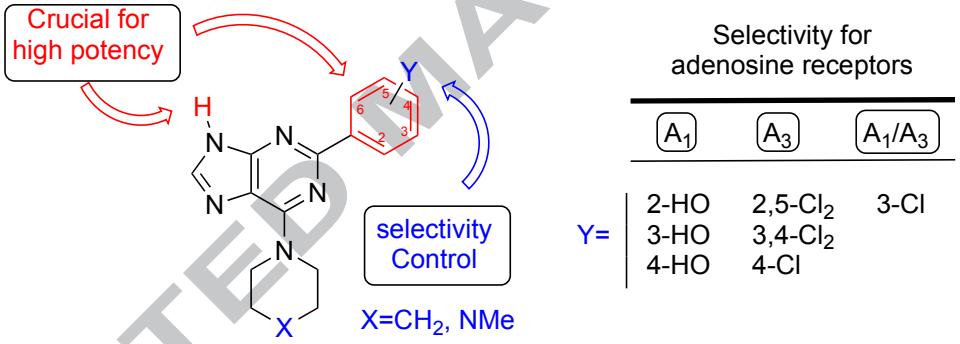

2-Aryladenine derivatives as a potent scaffold for A_1, A_3 and dual A_1/A_3 adenosine receptor antagonists: synthesis and structure-activity relationships

Filipe Areias[a,b,c,#], Carla Correia[a,#], Ashly Rocha[a], José Brea[b], Marián Castro[b], Maria I. Loza[b], M. Fernanda Proença[a,*], M. Alice Carvalho[a,*]

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Abstract

From a collection containing more than 1500 academic compounds, *in silico* screening identified a hit for the human A_1 adenosine receptor containing a new purine scaffold. To study the structure activity relationships of this new chemical series for adenosine receptors, a library of 24 purines was synthesized and tested in radioligand binding assays at human A_1, A_{2A}, A_{2B} and A_3 adenosine receptor subtypes. Fourteen molecules showed potent antagonism at A_1, A_3 or dual A_1/A_3 adenosine receptors. This purine scaffold is an important source for novel biochemical tools and/or therapeutic drugs.

1. Introduction

Adenosine is an endogenous purinergic nucleoside, occurring in all body cells and modulates many physiological and pathological conditions related to cardiovascular, immune, metabolic and neurological functions [1,2]. Cellular signalling by adenosine occurs through four adenosine receptor subtypes (A_1, A_{2A}, A_{2B}, and A_3), belonging to the G protein-coupled receptor (GPCR) superfamily [3]. The A_1 and A_3

receptors principally couple to $G_{i/o}$ proteins, inducing an inhibitory effect on adenylyl cyclase activity, while the A_{2A} and A_{2B} receptors couple to G_s proteins, stimulating cyclic AMP production [4-5]. Therefore, it is of therapeutic interest to develop new drugs active on adenosine receptors, as evidenced by the continuous patent claims on new compounds modulating adenosine receptors or new uses for selective ligands [6-8]. Intensive efforts in medicinal chemistry in the adenosine receptor field yielded selective agonists and antagonists for the four receptor subtypes as well as allosteric modulators [3,9], some of them advancing to clinical trials or to FDA approval for diagnostic or therapeutic uses [2,3,5,8-9]. For example, A_1 ligands are under development for cardiovascular diseases, pain indications, glaucoma and PET imaging agents [3,5,8-10]. Also, A_{2B} antagonists and dual A_{2B}/A_3 antagonists are being investigated for their use in asthma, diabetes, and cancer. In addition, A_{2A} agonists are in clinical trials for cardiac imaging diagnostic and wound healing and some have already been approved for cardiac perfusion imaging [3,5,8-9], while A_{2A} antagonists are under development for the treatment of cancer [11]. Finally, A_3 agonists have been linked to inflammatory diseases, such as rheumatoid arthritis and psoriasis, liver cancer, hepatitis, and liver regeneration, and showed efficacy in clinical trials for dry eye syndrome [3,5,8-9,12].

Typically, selective adenosine ligands were developed as heterocyclic-based scaffolds, comprising one, two or three fused rings having nitrogen and sometimes sulphur as heteroatoms [5, 13-14]. In particular, adenosine receptor ligands based on purine scaffold were reported as adenine derivatives and aza-and deazapurines. Furthermore, the reported adenine derivatives have a hydrogen atom, an alkyl, an aryl, a sugar or a sugar-like unit on N_9, an amino group, primary or secundary amine on C_6 and an aryl, an alkyl or alkynyl, a proton or a chlorine atom on C_2. As far as we know adenine derivatives having a proton or a methyl group on N_9, combined with a secondary amine on C_6 and different aryl units on C_2 were never reported as adenosine receptor ligands.

The current work is part of an ongoing interdisciplinary project integrating organic and medicinal chemistry with *in silico* and *in vitro* pharmacology. In a previous study, we showed that *in silico* target profiling was able to identify the targets at which a chemical library of biologically-orphan molecules should be tested, leading to the identification of novel antagonists for all members of the adenosine receptor family [15]. Subsequently *in silico* target profiling of the library of 1584 compounds led to the

identification of compound **3a** as a new hit for the A_1 receptor subtype containing a purine scaffold (Fig. 1).

In this work we introduce the new family of compounds based on the general structure of the purine that was present in the original hit (**3a**). This purine series showed a rich variety of affinity profiles for adenosine receptors, some with marked selectivity for the A_1, A_3 and dual A_1/A_3 receptor subtypes.

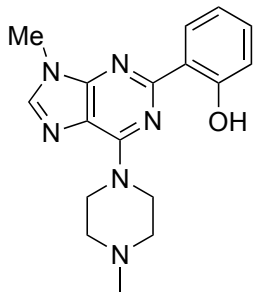


Figure 1. Purine hit (**3a**) for A_1 receptor subtype identified by *in silico* target profiling.

2. Results and discussion

2.1. *In silico* target profiling

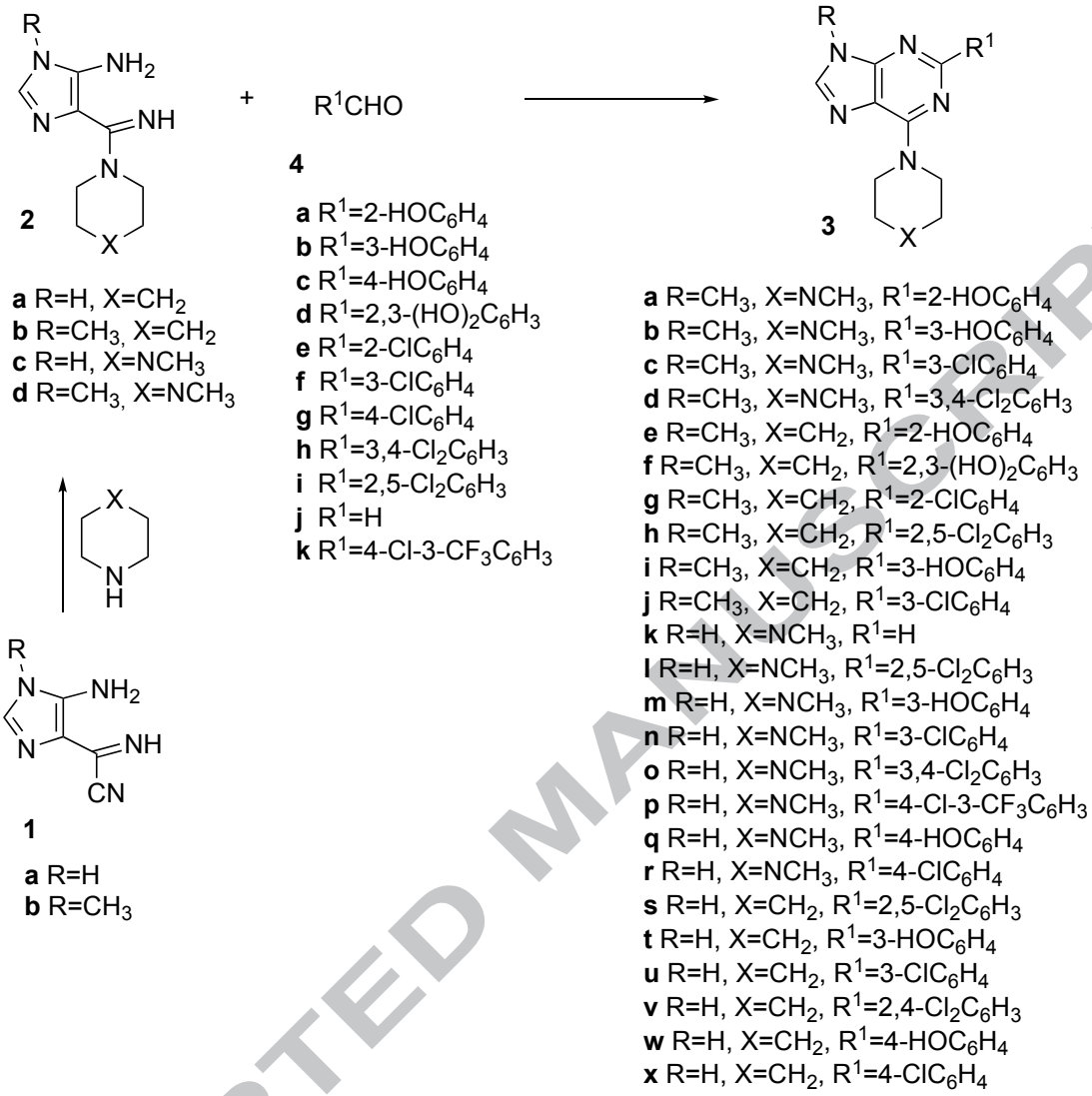
A collection of 1584 biologically-orphan molecules was profiled against ligand-based models available for 4643 proteins [15]. This *in silico* target profiling identified one molecule (**3a**) containing a novel purine scaffold for which affinity for adenosine receptor subtypes was predicted. The compound was then synthesized and tested *in vitro* on all human adenosine receptors (A_1, A_{2A}, A_{2B} and A_3) and presented submicromolar affinity for the A_1 adenosine receptor (**Table 1**). These results set the basis for initiating a medicinal chemistry program around a novel purine series.

The new purine series **3a-x** was built by combining different groups at N_9, C_6 and C_2 of the purine nucleus. A hydrogen or a methyl group were incorporated at N_9, a piperidinyl or a 4-methylpiperazinyl group were introduced in C_6 and different substituent groups were incorporated in the aryl substituent present in C_2 of the purine ring.

2.2. Chemistry

The synthetic approach followed to prepare the target molecules **3a-x** is described in Scheme 1. The synthesis of starting materials **1a-b**, was described in previous works [16-17] and compound **2a** was also described [18]. Compounds **3c**, **d**, **g** and **j** were previously reported [19] however their synthesis is described here for the first time. The new derivatives **2b-d** were obtained following an experimental protocol similar to that used for the synthesis of **2a**. The imidazoles **1**, in acetonitrile at room temperature, were reacted with an excess of the corresponding secondary amine. The pure products precipitated from solution and were isolated, in very good to excellent yield, by simple filtration.

In order to obtain compounds **3a-x**, imidazoles **2a-d** were reacted with different aldehydes (**Scheme 1**) using ethanol as solvent and triethylamine as base. The reactions started at room temperature until the reagents evolved to a yellow solution. The temperature was then raised to 40 ^{o}C and the reactions continued at that temperature to complete consumption. These reactions led to black-greyish solids that required purification. This was achieved by filtration of a dichloromethane solution of the solids through a silica gel column.



Scheme 1 Synthesis of 2-aryl-adenine derivatives **3a-x**

When the same methodology was applied to the reaction of compounds **2b** and **2d** with phenolic aldehydes, a black solution resulted shortly after mixing the reagents that evolved to a black oil. In order to obtain the phenolic derivatives the reactions were performed in acidic medium until complete consumption of reagents (evidenced by TLC) and then continued in basic medium, at 40 °C, according to the previously described procedure [20]. The pure products were obtained following the purification approach described above for the other derivatives.

2.3. Pharmacology

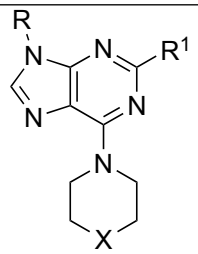
All synthesized compounds **(3a-x)** were screened by radioligand binding experiments at all human adenosine receptor subtypes (A_1, A_{2A}, A_{2B} and A_3) expressed

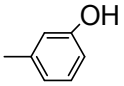
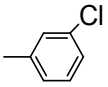
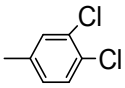
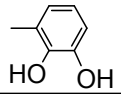
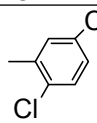
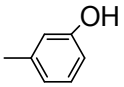
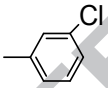
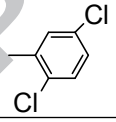
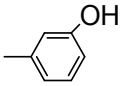
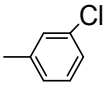
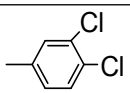
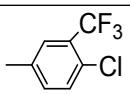
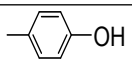
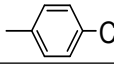
in mammalian cell lines. The percentage of inhibition of radioligand binding ($\%_{inhib}$) was obtained for all the tested compounds at a concentration of 10 μM. Those compounds showing a $\%_{inhib}$ higher than 80 % were assayed at different concentrations for the calculation of their affinity (measured as pK_i) at the studied receptor (**Table 1**).

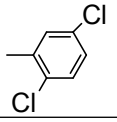
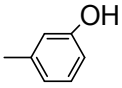
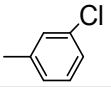
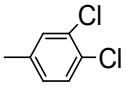
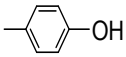
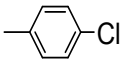
Binding tests confirmed the virtual study results and the 9-methyl-6-(4-methypiperazinyl)purine **3a** presented a pK_i value of 6.2 ± 0.3 for A_1 receptor with a good selectivity for this receptor (see **Table 1**). When the affinity of **3a** for adenosine receptor subtypes was compared with that of the other 9-methyl-6-(4-methypiperazinyl)purines (**3b**, **3c** and **3d**), we observed that **3a** was the most potent compound of this group. We observed also that **3c** and **3d** had higher affinity for A_1 receptor than **3b**, while **3b** had greater affinity for A_3 receptor.

Some 9-methyl-6-piperidinylpurines (**3e-j**) were also synthesized and tested. The purines **3e** and **3i** had higher affinity for A_{2A} and A_1 receptors, respectively, with inhibition percentages of 74.8 ± 4.6 % and 78.5 ± 1.3 % at 10 μM, respectively. The other purine derivatives of this group (**3f**, **3g**, **3h** and **3j**) presented weak affinities for all adenosine receptor subtypes. When the results of 9-methyl-6-piperidinylpurines **3e**, **3i** and **3j** were compared to the respective 6-(4-methypiperazinyl) derivatives (**3a**, **3b** and **3c**), we observed only small to moderate changes in the affinity of these compounds for adenosine receptor subtypes. These results indicated that piperidine versus *N*-methypiperazine subunit in C_6 of the purine nucleus, having a methyl group in N_9, had only limited importance for the affinity at adenosine receptors. Indeed, all purines belonging to these two groups had weak to moderate affinities for the adenosine receptors except **3a**.

Table 1
Binding affinities of the new compounds **3** at all human adenosine receptors ($\%_{inhib}$ at 10 μM or **pK_i**, mean ± SEM).

				**Human adenosine receptors**			
	R	X	**R^1**	**hA_1**	**hA_{2A}**	**hA_{2B}**	**hA_3**

3a	Me	NMe		**6.2 ± 0.3**	59.6 ± 1.0 %	36.0 ± 3.2 %	15.3 ± 1.1 %
3b	Me	NMe		27.1 ± 3.0 %	4.2 ± 0.3 %	47.8 ± 2.4 %	63.8 ± 1.4 %
3c	Me	NMe		45.7 ± 0.9 %	21.0 ± 1.8 %	19.2 ± 1.1 %	38.3 ± 3.9 %
3d	Me	NMe		65.2 ± 1.9 %	25.0 ± 2.1 %	28.3 ± 1.7 %	35.3 ± 3.0 %
3e	Me	CH_2		50.5 ± 3.0 %	74.8 ± 4.6 %	30.2 ± 2.5 %	20.0 ± 2.1 %
3f	Me	CH_2		17.2 ± 0.4 %	24.1 ± 1.9 %	26.0 ± 1.5 %	33.7 ± 2.0 %
3g	Me	CH_2		35.2 ± 2.9 %	36.4 ± 3.5 %	32.7 ± 0.7 %	2.1 ± 0.5 %
3h	Me	CH_2		13.2 ± 1.0 %	2.4 ± 0.5 %	31.0 ± 2.7 %	5.4 ± 0.6 %
3i	Me	CH_2		78.5 ± 1.3 %	54.8 ± 2.0 %	29.3 ± 0.5 %	49.1 ± 5.0 %
3j	Me	CH_2		22.4 ± 0.5 %	17.0 ± 0.3 %	24.2 ± 1.9 %	6.1 ± 0.7 %
3k	H	NMe	H	9.2 ± 0.7 %	19.1 ± 2.0 %	14.0 ± 0.9 %	26.9 ± 4.6 %
3l	H	NMe		**5.6 ± 0.3**	9.1 ± 0.2 %	13.0 ± 0.47 %	**6.6 ± 0.2**
3m	H	NMe		**6.8 ± 0.1**	1.0 ± 1.0 %	27.0 ± 2.0 %	**6.3 ± 0.5**
3n	H	NMe		**7.1 ± 0.2**	52.7 ± 3.0 %	29.9 ± 2.6 %	**7.0 ± 0.1**
3o	H	NMe		**6.2 ± 0.3**	12.0 ± 0.6 %	24.1 ± 2.3 %	**7.4 ± 0.2**
3p	H	NMe		52.4 ± 2.1 %	32.0 ± 3.3 %	25.1 ± 2.7 %	**6.1 ± 0.4**
3q	H	NMe		**6.4 ± 0.2**	37.0 ± 3.0 %	53.0 ± 2.0 %	48 ± 1 %
3r	H	NMe		**6.4 ± 0.3**	15.9 ± 1.7 %	6.2 ± 0.5 %	**7.4 ± 0.1**

3s	H	CH_2		1.4 ± 0.1 %	21.0 ± 0.5 %	12.1 ± 1.0 %	**6.7 ± 0.4**
3t	H	CH_2		**8.0 ± 0.1**	**6.5 ± 0.4**	**6.8 ± 0.1**	**7.2 ± 0.2**
3u	H	CH_2		**7.3 ± 0.1**	43.5 ± 1.3 %	**6.0 ± 0.4**	**7.4 ± 0.3**
3v	H	CH_2		24.1 ± 4.8 %	7.0 ± 0.6 %	0.7 ± 0.1 %	**7.1 ± 0.1**
3w	H	CH_2		**8.0 ± 0.2**	**7.0 ± 0.5**	**7.1 ± 0.2**	**7.4 ± 0.3**
3x	H	CH_2		**6.9 ± 0.4**	**5.5 ± 0.3**	1.4 ± 0.7 %	**8.1 ± 0.1**

In order to evaluate the importance of the group present in N_9 of the purine nucleus (hydrogen atom versus methyl group) in the affinity of these compounds for adenosine receptors the new derivatives 9-H-6-(4-methypiperazinyl)purine (**3l-r**) were synthesized, tested and the results are presented in Table 1. The first observation that should be highlighted is that by comparing the affinities of 9-*H*-6-(4-methypiperazinyl)purines (**3m**, **3n** and **3o**) with the respective 9-methyl-6-(4-methypiperazinyl) derivatives (**3b**, **3c** and **3d**) for adenosine receptors, we observed a great increase in the affinity values for A_1 and A_3 receptors. These results showed the importance of the group present in N_9 (hydrogen atom over methyl group) for activity. Furthermore, the purine **3q** (pK_i = 6.4 ± 0.2) was selective for A_1 receptors. The compounds **3m** and **3n** had similar affinity for both A_1 (pK_i = 6.8 ± 0.1; pK_i = 7.1 ± 0.2) and A_3 (pK_i = 6.3 ± 0.5; pK_i = 7.0 ± 0.1) receptors, respectively. On the other side, the purines **3o** and **3r** were the most potent compounds for A_3 receptors in this chemical series (pK_i = 7.4 ± 0.2 and 7.4 ± 0.1, respectively), being **3o** slightly more selective than **3r**. In addition, compounds **3l** (pK_i = 6.6 ± 0.2) and **3p** (pK_i = 6.1 ± 0.4) were also selective for A_3 receptors but they were less potent than **3o** and **3r**.

The series 9-*H*-6-piperidinylpurines (**3s-x**) was synthesized to assess the importance of the group present in C_6 of the purine nucleus in the affinity of these compounds for adenosine receptors. The results, presented in Table 1, showed that the

presence of a piridinyl group in C_6 of the purine nucleus instead of a 4-methylpiperidinyl group led to compounds with higher potency (**3m** *vs* **3t**, **3n** *vs* **3u**, **3r** *vs* **3x**) or to compounds with similar potency but having higher selectivity (**3l** *vs* **3s**, **3o** *vs* **3v**). Compounds **3t** and **3w**, with the same affinity for A_1 receptors (pK_i = 8.0), were the most potent and **3t** was more selective for this receptor. Figure **2(a)** shows a representative competition binding curve for compound **3t** at A_1 receptors. Compound **3x** was the most potent and, at the same time, the more selective compound for A_3 receptors (pK_i = 8.1 ± 0.1). The purine **3u** had almost the same affinity for A_1 (pK_i = 7.3 ± 0.1) and A_3 (pK_i = 7.4 ± 0.3) receptors. Compounds **3s** and **3v** were selective for A_3 receptors with pK_i values of 6.7 ± 0.4 and 7.1 ± 0.1, respectively. Additionally, we observed a high increase in the affinities of 9-*H*-6-piperidinylpurines (**3s**, **3t** and **3u**) over their respective 9-methyl-6-piperidinylpurines (**3h**, **3i** and **3j**) for A_1 and A_3 adenosine receptors, confirming the importance of the hydrogen atom over methyl group in N_9 position of purine as observed previously in this work.

In order to complete our structure-activity relationship (SAR) study, compound **3k** was also synthesized and tested. The results (**Table 1**) showed that compound **3k** had little affinity for all adenosine receptor subtypes, showing that the aromatic ring present in position C_2 of the purine ring is crucial for the affinity of these purines for adenosine receptors. For purines with submicromolar affinity for adenosine receptors, the aromatic substituents (R^1) present in C_2 of the purine ring are also important to obtain potent and selective ligands. When R^1 = 2-HOC_6H_4, 3-HOC_6H_4, 4-HOC_6H_4, selective compounds for A_1 receptors were obtained (**3a**, **3m**, **3q**, **3t** and **3w**). Selective ligands for A_3 receptors were achieved when R^3 = 2,5-$Cl_2C_6H_3$, 3,4-$Cl_2C_6H_3$, 4-ClC_6H_4 (**3l**, **3s**, **3o**, **3v**, **3r**, **3x**). Dual ligands for A_1/A_3 receptors were generated when R^3 = 3-ClC_6H_4 (purines **3n** and **3u**). This dual affinity at A_1/A_3 receptors can be of therapeutic interest because A_1 and A_3 receptors principally couple to $G_{i/o}$ proteins and induce a complementary inhibition on adenylyl cyclase activity [4-6]. Therefore, the compounds **3n** and **3u** are dual ligands of A_1/A_3 receptors and they can antagonize simultaneously the inhibitor effect on adenylyl cyclase activity by A_1 and A_3 receptors.

From the most potent and selective compounds for A_1 and A_3 receptors, two representative compounds were selected and tested in cyclic AMP assays to study their functional activity. The functional behaviour of compounds **3t** and **3x** at A_1 and A_3 receptors, respectively, was examined by measuring their effect on the modulation of intracellular cAMP levels by the non-selective adenosine receptor agonist NECA.

Figure 2(b) shows the results of a representative experiment for the antagonist potency of compound **3x** at A_3 receptors. The results confirmed that the compounds **3t** and **3x** were antagonists at A_1 and A_3 receptors, respectively. The pK_B values express the antagonist potency of these compounds (**Table 2**), which was in good agreement with their affinity values (pK_i) at the corresponding receptor. We extrapolated that all compounds of our series are antagonists of adenosine receptors based on their structural similarity.

Table 2
Antagonistic potency (pK_B) of selected compounds at human hA_1 and hA_3 adenosine receptors in cAMP assays.

Compound	**Human adenosine receptors**	
	hA_1	**hA_3**
3t	**7.9 ± 0.2**	ND
3x	ND	**8.2 ± 0.1**

ND – Not determined.

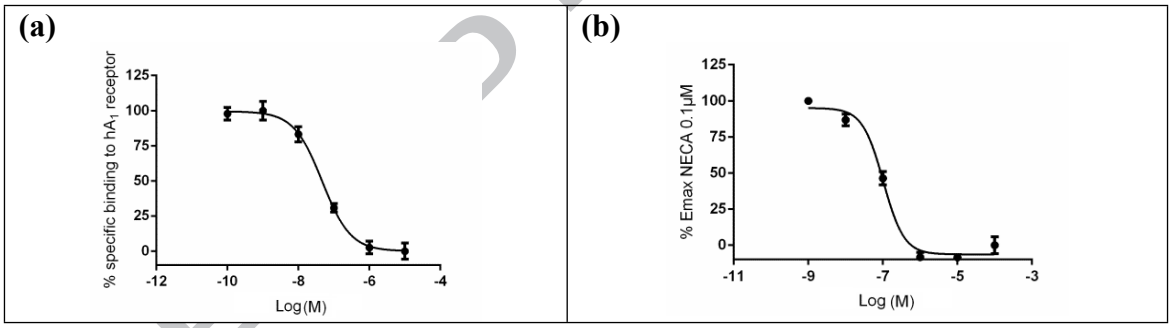


Figure 2. **(a)** Competition binding curve for compound **3t** at A_1 receptors. **(b)** Concentration-response curve of **3x** in the presence of **0.1 µM** NECA at human A_3 receptors expressed in CHO cells. Points represent the mean ± standard deviation (vertical bars) of duplicate measurements.

3. Conclusion

In summary, several purine-based compounds having different substituents in C_2, C_6 and N_9 of the purine nucleus were designed, synthesized and pharmacologically tested for adenosine receptors. From the synthesized compounds, five showed high potency and selectivity for A_1 receptors and seven showed high potency and selectivity for A_3 receptors. Two of the compounds showed high potency for both receptors A_1/A_3

and represent compounds with dual affinity for those receptors. From SAR analysis, we observed that the substituent groups present in C_2, C_6 and N_9 of the purine ring affect the potency and selectivity of the compounds for adenosine receptors. In order to generate highly potent and selective ligands for A_1, A_3 and dual A_1/A_3 receptors a hydrogen atom must be present in N_9 and an aryl group in C_2. The group in C_6 (piperidinyl *vs* 4-methylpirazinyl) combined with a specific aryl group in C_2 seems to govern the selectivity however a complex relationship between this substituents is observed. Functional studies indicated that these structurally related ligands have antagonist activity at A_1 and A_3 receptors. In conclusion, a number of purine-based compounds are described in this study, several of them showed selective antagonism for A_1, A_3 and dual A_1/A_3 receptors at submicromolar concentration.

4. Experimental protocols

4.1. Chemistry

The 5-amino-4-(cyanoformimidoyl)imidazoles **1** used in this work were synthesized according to previously described procedures [16-18] and compound **2a** was synthesized according to procedure described in [18]. Solvents and other chemicals commercially available were used as shipped. The melting points were determined with a Gallenkamp melting point apparatus and are uncorrected. The reactions were monitored by thin layer chromatography (TLC) using Silica Gel 60 F_{254} (Merck) with detection by UV light. The NMR spectra were recorded on a Varian Unity Plus (^{1}H: 300 MHz, ^{13}C: 75 MHz) or on a Bruker Avance III NMR spectrometer (^{1}H: 400 MHz, ^{13}C: 100 MHz) including the ^{1}H and ^{13}C correlation spectra (HMQC and HMBC), for solutions in [D_6]-DMSO [residual [D_6]-DMSO (δ_H = 2.49 ppm) or [D_6]-DMSO (δ_C = 39.5 ppm) as internal standard] at 298 K. Chemical shifts (δ) were reported in parts per million (ppm) and the coupling constants, *J,* were reported in hertz (Hz). The purities of all tested compounds were higher than 95 % by elemental analysis, which were reported to be within 0.4 % of calculated values. IR spectra were recorded with a FT-IR Bomem MB 104 using nujol mulls and NaCl cells. Elemental analyses were performed with a LECO CHNS-932 instrument.

*4.1. General procedure for the synthesis of **2b-d***

The secondary amine (5.0 molar equivalent) was added to a suspension of 5-amino-4-cyanoformimidoylimidazole **1a-b** in acetonitrile. The mixture was stirred at room temperature until all the starting material was consumed (evidence by TLC. The off-white solid that precipitated from the reaction mixture was filtered and washed with acetonitrile and diethyl ether.

4.1.1. 4-(Imino(piperidin-1-yl)methyl)-1-methyl-1*H*-imidazol-5-amine (**2b**)

Off-white solid; Yield: 0.60 g, 72 %; m.p. 133 – 135 ^{o}C (Found: C, 57.80; H, 8.30; N, 33.90. $C_{10}H_{17}N_5$ requires C, 57.97; H, 8.21; N, 33.82); IR (Nujol mull) ν_{max}: 3332, 3109, 1578, 1522 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 7.24 (s, 1H, H-2), 4.80 – 6.80 (br s, 3H, NH), 3.41 (m, 7H), 1.57 (s, 6H); ^{13}C (75 MHz, DMSO-d6) δ: 160.33, 141.67, 132.32, 110.25, 48.41, 30.00, 25.56, 23.92.

4.1.2. 4-(Imino(4-methylpiperazin-1-yl)methyl)-1*H*-imidazol-5-amine (**2c**)

Off-white solid; Yield: 0.65 g, 94 %; m.p. = 182 – 184 ^{o}C (Found: C, 55.34; H, 6.48; N, 38.42. $C_{10}H_{14}N_6$ requires C, 55.05; H, 6.42; N, 38.53); IR (Nujol mull) ν_{max}: 3328, 3212, 1498 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 10.00 – 7.20 (br s, 1H, NH), 6.99 (s, 1H, H-2), 4.81 (br s, 2H, NH), 3.68 (m, 4H), 2.37 (t, *J* = 4.8 Hz, 4H), 2.18 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 159.57, 147.63, 135.19, 107.10, 54.52, 46.56, 45.76.

4.1.3. 4-(Imino(4-methylpiperazin-1-yl)methyl)-1-methyl-1*H*-imidazol-5-amine (**2d**)

Off-white solid; Yield: 0.16 g, 74 %; m.p. = 131 – 133 ^{o}C (Found: C, 50.06; H, 8.23; N, 34.78. $C_{10}H_{18}N_6.H_2O$ requires C, 50.00; H, 8.33; N, 35.00); IR (Nujol mull) ν_{max}: 3375, 3257, 3148, 1596, 1557, 1533 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 7.12 (s, 1H, H-2), 6.80 – 4.80 (br s, 3H, NH), 3.38 (s, 3H), 3.28 (t, *J* = 4.8 Hz, 4H), 2.34 (t, *J* = 4.8 Hz, 4H), 2.17 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 163.18, 139.85, 130.68, 113.80, 54.56, 46.51, 45.77, 29.71.

*4.2. General procedure for the synthesis of **3a-x***

Method A: The aldehyde (1.1 molar equivalent) and trifluoroacetic acid (1.3 molar equivalent) were added to a suspension of **2**, in ethanol, under efficient magnetic

stirring, at room temperature. A yellow solution resulted and was maintained under the previous reaction conditions until TLC showed absence of starting material and the presence of a new spot. The solution was concentrated to dryness in the rotary evaporator. Ethanol (1 mL) and triethylamine (10 molar equivalent) were added to the solid residue and the reaction was maintained at 40 ^{o}C until TLC showed only one spot for the product. The dark solution was concentrated and water was added. A dark solid precipitated, was filtered, washed with water followed by diethyl ether and then dissolved in DCM. The dark solution obtained was filtered through a column of silica gel (1 cm height). The solution was concentrated, diethyl ether was added and an off white solid precipitated. The solid was filtered and washed with diethyl ether and identified as **3**.

Method B: The aldehyde (1.1 molar equivalent) and triethylamine (10 molar equivalent) were added to a suspension of **2**, in ethanol, under efficient magnetic stirring, at room temperature. A yellow solution resulted and the reaction was mantained at 40 ^{o}C until TLC showed the absence of starting material. The solution was concentrated to dryness in the rotary evaporator. The solid residue was dissolved in DCM and filtered through a column of silica gel (1 cm height). The solution was concentrated, diethyl ether was added and an off white solid precipitated. The solid was filtered and washed with diethyl ether and identified as **3**.

4.2.1. 2-(9-Methyl-6-(4-methylpiperazin-1-yl)-9*H*-purin-2-yl)phenol **3a**

Method A: Off-white solid; Yield: 0.08 g, 67 %; m.p. = 193 – 195 ^{o}C (Found: C, 63.33; H, 5.85; N, 25.73. $C_{17}H_{20}N_6O$ requires C, 62.96; H, 6.17; N, 25.92); IR (Nujol mull) ν_{max}: 3013, 1599, 1581, 1513 cm^{-1}; ^{1}H (300 MHz, DMSO-d$_6$) δ: 13.56 (s, 1H, OH), 8.36 (dd, *J* = 7.6 Hz, *J* = 1.2 Hz, 1H), 8.16 (s, 1H), 7.31 (dt, *J* = 7.6 Hz, *J* = 1.2 Hz, 1H), 6.90 (t, *J* = 7.6 Hz, 1H), 6.89 (d, *J* = 7.6 Hz, 1H), 4.22 (br s, 4H), 3.76 (s, 3H), 2.43 – 2.50 (m, 4H), 2.22 (s, 3H); ^{13}C (75 MHz, DMSO-d$_6$) δ: 157.41, 152.18, 149.68, 141.11, 131.82, 119.31, 117.66, 117.12, 54.41, 45.66, 44.75, 29.56.

4.2.2. 3-(9-Methyl-6-(4-methylpiperazin-1-yl)-9*H*-purin-2-yl)phenol **3b**

Method A: Off-white solid; Yield: 0.10 g, 55 %; m.p. = 234 – 235 ^{o}C (Found: C, 62.61; H, 6.33; N, 25.73. $C_{17}H_{20}N_6O$ requires C, 62.96; H, 6.17; N, 25.93); IR (Nujol mull) ν_{max}: 3108, 3084, 1599, 1569 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) 9.51 (s, 1H), 8.14 (s, 1H), 7.80 – 7.85 (m, 2H), 7.24 (t, *J* = 8.4 Hz, 1H), 6.81 (dt, *J* = 8.1 Hz, *J* = 1.8 Hz, 1H), 4.28 (br s, 4H), 3.78 (s, 3H), 2.45 – 2.48 (m, 4H), 2.22 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 157.33, 156.85, 152.84, 151.98, 140.92, 139.74, 129.07, 118.53, 118.05, 116.66, 114.48, 54.60, 45.79, 44.43, 29.38.

4.2.3. 2-(3-Chlorophenyl)-9-methyl-6-(4-methylpiperazin-1-yl)-9*H*-purine **3c**

Method B: Off-white solid; Yield: 0.12 g, 72 %; m.p. = 132 – 134 ^{o}C (Found: C, 59.25; H, 5.66; N, 24.28. $C_{17}H_{19}N_6Cl$ requires C, 59.56; H, 5.55; N, 24.50); IR (Nujol mull) ν_{max}: 1670, 1592, 1570 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 8.20-8.40 (m, 2H), 8.17 (s, 1H), 7.48 – 7.51 (m, 2H), 4.28 (br s, 4H), 3.79 (s, 3H), 2.45 (t, *J* = 5.1 Hz, 4H), 2.22 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 155.26, 152.90, 151.81, 141.27, 140.49, 133.14, 130.22, 129.38, 127.02, 126.19, 118.33, 54.58, 45.76, 44.49, 29.45.

4.2.4. 2-(3,4-Dichlorophenyl)-9-methyl-6-(4-methylpiperazin-1-yl)-9*H*-purine **3d**

Method B: Off-white solid; Yield: 0.12 g, 60 %; m.p. = 136 – 138 ^{o}C (Found: C, 54.04; H, 4.67; N, 22.16. $C_{17}H_{18}N_6Cl_2$ requires C, 54.11; H, 4.47; N, 22.28); IR (Nujol mull) ν_{max}: 1727, 1591, 1560 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 8.47 (d, *J* = 1.8 Hz, 1H), 8.31 (dd, *J* = 8.5 Hz, *J* = 2.4 Hz, 1H), 8.17 (s, 1H), 7.71 (d, *J* = 8.4 Hz, 1H), 4.27 (br s, 4H), 3.78 (s, 3H), 2.44 (m, 4H), 2.22 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 154.41, 152.86, 151.72, 141.38, 138.96, 132.21, 131.13, 131.59, 128.95, 127.60, 118.37, 54.60, 45.79, 44.54, 29.49 .

4.2.5. 2-(9-Methyl-6-(piperidin-1-yl)-9*H*-purin-2-yl)phenol **3e**

Method A: Off-white solid; Yield: 0.13 g, 70 %; m.p. = 184 – 186 ^{o}C (Found: C, 65.63; H, 6.17; N, 22.62. $C_{17}H_{19}N_5O.0.1H_2O$ requires C, 65.64; H, 6.18; N, 22.52); IR (Nujol mull) ν_{max}: 3013, 1560, 1576 cm^{-1}; ^{1}H (300 MHz, DMSO-d6 δ: 9.83 (br s, 1H), 8.50 (s, 1H), 8.17 (d, *J* = 8.7 Hz, 2H), 7.79 (d, *J* = 8.4 Hz, 2H), 7.42 (d, *J* = 8.4 Hz, 2H), 6.82 (d, *J* = 8.7 Hz, 2H), 4.30 (br s, 4H), 3.77 (s, 4H), 2.40 (s, 3H); ^{13}C (75 MHz, DMSO-d6)

δ: 159.35, 157.47, 152.03, 149.72, 140.85, 131.77, 128.85, 119.40, 118.49, 117.54, 117.12, 46.08, 29.55, 25.57, 24.06.

4.2.6. 3-(9-Methyl-6-(piperidin-1-yl)-9*H*-purin-2-yl)benzene-1,2-diol **3f**

Method A: Off-white solid; Yield: 0.12 g, 41 %; m.p. = 222 – 224 ^{o}C (Found: C, 62.90; H, 5.55; N, 21.78. $C_{17}H_{19}N_5O_2$ requires C, 62.77; H, 5.85; N, 21.54); IR (Nujol mull) ν_{max}: 3462, 1611, 1580 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 13.95 (s, 1H), 8.80 (br s, 1H), 8.17 (s, 1H), 7.83 (dd, *J* = 8.0 Hz, *J* = 1.5 Hz, 1H), 6.84 (dd, *J* = 7.8 Hz, *J* = 1.5 Hz, 1H), 6.70 (t, *J* = 7.8 Hz, 1H), 4.22 (s, 4H), 3.78 (s, 3H), 1.66 (s, 6H); ^{13}C (75 MHz, DMSO-d6) δ: 157.95, 151.97, 149.86, 148.34, 146.07, 140.89, 119.56, 118.96, 117.82, 117.42, 117.20, 46.11, 29.55, 25.59, 24.07.

4.2.7. 2-(2-Chlorophenyl)-9-methyl-6-(piperidin-1-yl)-9*H*-purine **3g**

Method B: Off-white solid; Yield: 0.14 g, 40 %; m.p. = 145 – 147 ^{o}C (Found: C, 62.47; H, 5.63; N, 21.65. $C_{17}H_{18}N_5Cl$ requires C, 62.29; H, 5.50; N, 21.37); IR (Nujol mull) ν_{max}: 1582 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 8.16 (s, 1H), 7.71 – 7.74 (m, 1H), 7.49 – 7.53 (m, 1H), 7.39 – 7.44 (m, 2H), 4.21 (br s, 4H), 3.74 (s, 3H), 1.66 (d, *J* = 4.2 Hz, 2H), 1.57 (d, *J* = 3.9 Hz, 4H); ^{13}C (75 MHz, DMSO-d6) δ: 157.71, 152.62, 151.47, 140.78, 138.92, 131.62, 131.48, 130.02, 129.75, 126.81, 117.55, 45.59, 29.50, 25.71, 24.27.

4.2.8. 2-(2,5-Dichlorophenyl)-9-methyl-6-(piperidin-1-yl)-9*H*-purine **3h**

Method B: Off-white solid; Yield: 0.15 g, 45 %; m.p. = 116 – 118 ^{o}C (Found: C, 56.15; H, 4.70; N, 18.93. $C_{17}H_{17}N_5Cl_2$ requires C, 56.35; H, 4.69; N, 19.34); IR (Nujol mull) ν_{max}: 3052, 1692, 1592 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 8.18 (s, 1H), 7.77 (d, *J* = 2.4 Hz, 1H), 7.57 (d, *J* = 8.7 Hz, 1H), 7.51 (dd, *J* = 8.7 Hz, *J* = 2.4 Hz, 1H), 4.22 (br s, 4H), 3.75 (s, 3H), 1.67 (d, *J* = 4.8 Hz, 2H), 1.59 (d, *J* = 4.2 Hz, 4H); ^{13}C (75 MHz, DMSO-d6) δ: 156.31, 152.60, 151.36, 140.98, 140.28, 131.89, 131.37, 130.86, 130.43, 129.56, 117.70, 45.71, 29.53, 25.68, 24.22.

4.2.9. 3-(9-Methyl-6-(piperidin-1-yl)-9*H*-purin-2-yl)phenol **3i**

Method A: Off-white solid; Yield: 0.15 g, 67 %; m.p. = 210 – 212 ^{o}C (Found: C, 64.78; H, 5.93; N, 21.97. $C_{17}H_{19}N_5O.0.3H_2O$ requires C, 64.88; H, 6.23; N, 22.26); IR (Nujol mull) ν_{max}: 3165, 1569 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 13.45 (s, 1H), 8.56 (s, 1H), 8.82 (br s, 1H), 7.79 (dd, *J* = 8.2 Hz, *J* = 1.8 Hz, 1H), 7.67 (d, *J* = 8.1 Hz, 2H), 7.46 (d, *J* = 8.1 Hz, 2H), 6.83 (dd, *J* = 8.0 Hz, *J* = 1.5 Hz, 1H), 6.69 (t, *J* = 7.8 Hz, 1H), 4.30 (br s, 4H), 3.81 (s, 4H), 2.42 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 157.24, 156.85, 152.79, 151.89, 140.56, 139.90, 129.05, 118.53, 117.93, 116.54, 114.44, 45.42, 29.36, 25.66, 24.33.

4.2.10. 2-(3-Chlorophenyl)-9-methyl-6-(piperidin-1-yl)-9*H*-purine **3j**

Method B: Off-white solid; Yield: 0.10 g, 35 %; m.p. = 139 – 141 ^{o}C (Found: C, 61.03; H, 5.40; N, 20.66. $C_{17}H_{18}N_5Cl.0.4H_2O$ requires C, 60.95; H, 5.62; N, 20.91); IR (Nujol mull) ν_{max}: 1699, 1595, 1585, 1569 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 8.14 (s, 1H), 8.30 – 8.35 (m, 2H), 7.40 – 7.52 (m, 2H), 4.27 (br s, 4H), 3.79 (s, 3H), 1.68 (t, *J* = 4.0 Hz, 2H), 1.57 (d, *J* = 4.4 Hz, 4H); ^{13}C (75 MHz, DMSO-d6) δ: 155.26, 152.83, 151.73, 140.90, 140.65, 133.10, 130.19, 129.28, 126.99, 126.14, 118.21, 45.59, 29.42, 25.71, 24.27.

4.2.11. 6-(4-Methylpiperazin-1-yl)-9*H*-purine **3k**

Method B: Off-white solid; Yield: 0.03 g, 72 %; m.p. = 274 – 276 ^{o}C (Found: C, 55.34; H, 6.48; N, 38.42. $C_{10}H_{14}N_6$ requires C, 55.05; H, 6.42; N, 38.53); IR (Nujol mull) ν_{max}: 3067, 1582, 1517 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 12.99 8br s, 1H), 8.19 (s, 1H), 8.10 (s, 1H), 4.20 (br s, 4H), 3.38 (s, 3H), 2.40 – 2.50 (m, 4H), 2.20 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 153.12, 151.73, 151.44, 138.14, 118.73, 54.59, 45.77, 44.45.

4.2.12. 2-(2,5-Dichlorophenyl)-6-(4-methylpiperazin-1-yl)-9*H*-purine **3l**

Method B: Off-white solid; Yield: 0.090 g, 53 %; m.p. = 271 – 273 ^{o}C (Found: C, 52.77; H, 4.35; N, 22.95. $C_{16}H_{16}N_6Cl_2$ requires C, 52.91; H, 4.44; N, 23.14); IR (Nujol mull) ν_{max}: 3257, 3148, 1596, 1557 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 8.08 (s, 1H),

7.74 (d, *J* = 2.7 Hz, 1H), 7.46 (dd, *J* = 8.7 Hz, *J* = 2.7 Hz, 1H), 4.80 (br s, 4H), 2.41 (m, 4H), 2.19 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 155.34, 154.11, 152.54, 141.61, 140.77, 131.85, 131.33, 130.97, 130.39, 129.28, 118.31, 54.69, 45.83, 44.58.

4.2.13. 3-(6-(4-Methylpiperazin-1-yl)-9*H*-purin-2-yl)phenol **3m**

Method B: Off-white solid; Yield: 0.23 g, 68 %; m.p. = 266 – 268 °C (Found: C, 57.93; H, 5.10; N, 24.26. $C_{16}H_{18}N_6O$ requires C, 58.01; H, 5.07; N, 24.09.); IR (Nujol mull) ν_{max}: 3125, 1596, 1568, 1516 cm^{-1}; ^{1}H (400 MHz, DMSO-d6) δ: 13.03 (br s, 1H), 9.44 (br s, 1H), 8.11 (s, 1H), 7.77-7.78 (m, 2H), 7.23 (t, *J* = 8.0 Hz, 1H), 6.81 (dd, *J* = 2.4 Hz, *J* = 8.0 Hz, 1H), 4.28 (br s, 4H), 2.45 (s, 4H), 2.22 (s, 3H); ^{13}C (100 MHz, DMSO-d6) δ: 157.28, 156.93, 152.86, 152.54, 139.97, 138.57, 129.13, 118.45, 117.91, 116.54, 114.43, 54.65, 45.83, 44.46.

4.2.14. 2-(3-Chlorophenyl)-6-(4-methylpiperazin-1-yl)-9*H*-purine **3n**

Method B: Off-white solid; Yield: 0.11 g, 43 %; m.p. = 239 – 241 °C (Found: C, 58.26; H, 5,11; N, 25.38. $C_{16}H_{17}N_6Cl$ requires C, 58.45; H, 5.18; N, 25.57); IR (Nujol mull) ν_{max}: 3110, 1588, 1573 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 13.13 (br s, 1H), 8.25 – 8.31 (m, 2H), 8.16 (s, 1H), 7.49 – 7.51 (m, 2H), 4.29 (br s, 4H), 4.20 – 4.50 (m, 4H), 2.23 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 155.34, 152.89, 152.40, 140.70, 138.92, 133.12, 130.21, 129.33, 126.98, 126.04, 118.14, 55.60, 45.77, 44.42.

4.2.15. 2-(3,4-Dichlorophenyl)-6-(4-methylpiperazin-1-yl)-9*H*-purine **3o**

Method B: Off-white solid; Yield: 0.13 g, 48 %; m.p. = 257 – 259 °C (Found: C, 52.88; H, 4.64; N, 22.98. $C_{16}H_{16}N_6Cl_2$ requires C, 52.89; H, 4.41; N, 23.14); IR (Nujol mull) ν_{max}: 3093, 1696, 1584, 1559 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 13.13 (br s, 1H), 8.43 (d, *J* = 1.8 Hz, 1H), 8.27 (dd, *J* = 8.7 Hz, *J* = 2.1 Hz, 1H), 8.16 (s, 1H), 7.69 (d, *J* = 8.7 Hz, 1H), 4.28 (br s, 4H), 2.40 – 2.50 (m, 4H), 2.22 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 154.49, 152.85, 152.34, 139.18, 139.08, 132.07, 131.10, 130.59, 128.90, 127.47, 118.18, 54.60, 45.78, 44.47.

4.2.16. 2-(4-Chloro-3-(trifluoromethyl)phenyl)-6-(4-methylpiperazin-1-yl)-9*H*-purine **3p**

Method B: Off-white solid; Yield: 0.12 g, 48 %; m.p. = 232 – 234 °C (Found: C, 51.38; H, 4.00; N, 21.33. $C_{17}H_{16}N_6ClF_3$ requires C, 51.45; H, 4.03; N, 21.19); IR (Nujol mull) ν_{max}: 3093, 1600, 1586, 1575 cm^{-1}; ^1H (300 MHz, DMSO-d6) δ: 13.13 (br s, 1H), 8.72 (d, *J* = 2.1 Hz, 1H), 8.60 (dd, *J* = 8.1 Hz, *J* = 1.8 Hz, 1H), 8.18 (s, 1H), 7.83 (d, *J* = 8.4 Hz, 1H), 4.30 (br s, 4H), 2.43 – 2.50 (m, 4H), 2.23 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 154.32, 152.87, 152.33, 139.14, 137.95, 132.63, 131.84, 131.58 (d, *J* = 1Hz), 126.55 (q, *J* = 31Hz), 126.04 (q, *J* = 5 Hz), 122.89 (q, *J* = 272 Hz), 118.25, 54.57, 45.74, 44.54.

4.2.17. 4-(6-(4-Methylpiperazin-1-yl)-9*H*-purin-2-yl)phenol **3q**

Method B: Dark orange solid; Yield: 0. 23 g, 76 %; m.p. = 253 – 255 °C (Found: C, 58.12; H, 4.99; N, 24.23. $C_{16}H_{18}N_6O$ requires C, 58.01; H, 5.07; N, 24.09.); IR (Nujol mull) ν_{max}: 3188, 1660, 1572 cm^{-1}; ^1H (400 MHz, DMSO-d6) δ: 12.96 (br s, 1H), 9.77 (br s, 1H), 8.17 (d, *J* = 8.8 Hz, 2H), 8.05 (s, 1H), 6.82 (d, *J* = 8.8 Hz, 2H), 4.27 (br s, 4H), 2.50-2.49 (m, 4H), 2.22 (s, 3H); ^{13}C (100 MHz, DMSO-d6) δ: 158.96, 157.19, 152.88, 152.68, 138.08, 129.56, 129.14, 117.40, 114.95, 54.67, 45.83, 44.40.

4.2.18. 2-(4-Chlorophenyl)-6-(4-methylpiperazin-1-yl)-9*H*-purine **3r**

Method B: Off-white solid; Yield: 0.12 g, 42 %; m.p. = 133 – 134 °C (Found: C, 58.35; H, 5.33; N, 25.87. $C_{16}H_{17}N_6Cl$ requires C, 58.44; H, 5.22; N, 25.56); IR (Nujol mull) ν_{max}: 3191, 3087, 1583 cm^{-1}; ^1H (300 MHz, DMSO-d6) δ: 13.08 (s, 1H), 8.34 (d, *J* = 8.4 Hz, 2H), 8.14 (s, 1H), 7.50 (d, *J* = 8.4 Hz, 2H), 4.29 (br s, 4H), 2.45 (m, 4H), 2.22 (s, 3H); ^{13}C (75 MHz, DMSO-d6) δ: 155.80, 152.90, 152.49, 138.82, 137.41, 134.30, 129.23, 128.31, 117.99, 54.63, 45.80, 44.46.

4.2.19. 2-(2,5-Dichlorophenyl)-6-(piperidin-1-yl)-9*H*-purine **3s**

Method B: Off-white solid; Yield: 0.05 g, 45 %; m.p. = 288 – 290 °C (Found: C, 54.98; H, 4.44; N, 20.35. $C_{16}H_{15}N_5Cl_2$ requires C, 55.17; H, 4.31; N, 20.11); IR (Nujol mull) ν_{max}: 3049, 1594, 1573, 1557 cm^{-1}; ^1H (300 MHz, DMSO-d6) δ: 13.11 (br s, 1H), 8.16

(s, 1H), 7.74 (d, *J* = 2.4 Hz, 1H), 7.55 (d, *J* = 8.4 Hz, 1H), 7.49 (dd, *J* = 8.4 Hz, *J* = 2.8 Hz, 1H), 4.22 (br s, 4H), 1.67 (d, *J* = 4.8 Hz, 2H), 1.59 (d, *J* = 4.0 Hz, 4H); ^{13}C (75 MHz, DMSO-d6) δ: 156.33, 152.61, 151.80, 140.44, 138.60, 131.87, 131.35, 130.89, 130.35, 129.46, 117.52, 45.55, 25.68, 24.25.

4.2.20. 3-(6-(Piperidin-1-yl)-9*H*-purin-2-yl)phenol **3t**

Method B: Light yellow solid; Yield: 0.17 g, 63 %; m.p. = 291 – 293 °C (Found: C, 64.93; H, 5.74; N, 23.93. $C_{16}H_{17}N_5O$ requires C, 65.07; H, 5.80; N, 23.71); IR (Nujol mull) ν_{max}: 3165, 3109, 1569, 1510 cm^{-1}; ^{1}H (400 MHz, DMSO-d6) δ: 12.97(br s, 1H), 9.43 (br s, 1H), 8.08 (s, 1H), 7.76-7.78 (m, 2H), 7.23 (t, *J* = 8.0 Hz, 1H), 6.80 (m, 1H), 4.28 (br s, 4H), 1.70 – 1.62 (m, 6H); ^{13}C (100 MHz, DMSO-d6) δ: 157.25, 156.94, 152.80, 152.44, 140.13, 138.17, 129.08, 118.40, 117.77, 116.43, 114.42, 45.48, 25.70, 24.37.

4.2.21. 2-(3-Chlorophenyl)-6-(piperidin-1-yl)-9*H*-purine **3u**

Method B: Off-white solid; Yield: 0.05 g, 20 %; m.p. = 245 – 247 °C (Found: C, 60.97; H, 5.10; N, 21.85. $C_{16}H_{16}N_5Cl.0.1H_2O$ requires C, 60.89; H, 5.14; N, 22.20); IR (Nujol mull) ν_{max}: 3055, 1600, 1581, 1567 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 13.05 (br s, 1H), 8.26 – 8.30 (m, 2H), 8.13 (s, 1H), 7.48 – 7.51(m, 2H), 4.28 (br s, 4H), 1.60 – 1.70 (m, 6H); ^{13}C (75 MHz, DMSO-d6) δ: 155.36, 152.83, 152.35, 140.87, 138.61, 133.11, 130.24, 129.18, 126.97, 126.02, 118.02, 45.62, 25.68, 24.32.

4.2.22. 2-(3,4-Dichlorophenyl)-6-(piperidin-1-yl)-9*H*-purine **3v**

Method B: Off-white solid; Yield: 0.11 g, 33 %; m.p. = 142 – 143 °C (Found: C, 55.45; H, 4.45; N, 20.49. $C_{16}H_{15}N_5Cl_2$ requires C, 55.18; H, 4.35; N, 20.11); IR (Nujol mull) ν_{max}: 3202, 3111, 1599, 1576, 1556 cm^{-1}; ^{1}H (300 MHz, DMSO-d6) δ: 13.08 (br s, 1H), 8.42 (d, *J* = 2.1 Hz, 1H), 8.27 (dd, *J* = 8.4 Hz, *J* = 2.1 Hz, 1H), 8.14 (s, 1H), 7.70 (d, *J* = 8.4 Hz, 1H), 4.27 (br s, 4H), 1.63 (m, 6H); ^{13}C (75 MHz, DMSO-d6) δ: 154.54, 152.81, 152.18, 139.33, 138.67, 132.01, 131.09, 130.61, 128.88, 127.45, 118.11, 45.61, 25.68, 24.29.

4.2.23. 4-(6-(Piperidin-1-yl)-9*H*-purin-2-yl)phenol **3w**

Method B: Light yellow solid; Yield: 0. 19 g, 72 %; m.p. > 300 °C (Found: C, 58.34; H, 6.65; N, 20.88. $C_{16}H_{17}N_5O$. 1.90H_2O requires C, 58.31; H, 6.36; N, 21.25); IR (Nujol mull) ν_{max}: 3208, 3125, 1608, 1569, 1518 cm^{-1}; ^1H (400 MHz, DMSO-d6) δ: 12.86 (br s, 1H), 9.69 (br s, 1H), 8.17 (d, *J* = 8.8 Hz, 2H), 8.02 (s, 1H), 6.81 (d, *J* = 8.8 Hz, 2H), 4.25 (br s, 4H), 1.69 – 1.60 (m, 6H); ^{13}C (100 MHz, DMSO-d6) δ: 158.87, 157.21, 152.83, 152.60, 140.13, 137.71, 129.10, 117.28, 114.93, 45.49, 25.71, 24.40.

4.2.24. 2-(4-Chlorophenyl)-6-(piperidin-1-yl)-9*H*-purine **3x**

Method B: Off-white solid; Yield: 0.06 g, 18 %; m.p. = 141 – 143 °C (Found: C, 61.06; H, 5.17; N, 22.46. $C_{16}H_{16}N_5Cl$ requires C, 61.23; H, 5.15; N, 22.32); IR (Nujol mull) ν_{max}: 3250, 1622, 1581 cm^{-1}; ^1H (300 MHz, DMSO-d6) δ: 8.32 (d, *J* = 6.9 Hz, 2H), 8.18 (s, 1H), 7.52 (d, *J* = 6.9 Hz, 2H), 4.26 (br s, 4H), 1.63 (m, 6H); ^{13}C (75 MHz, DMSO-d6) δ: 155.69, 152.69, 152.01, 138.71, 137.13, 134.46, 129.29, 128.38, 117.28, 45.71, 25.71, 24.28.

4.2. Radioligand binding assays

The percentage of inhibition of specific radioligand binding at the different receptors by the compounds was assayed at the compound concentration of 10 μM at all adenosine receptors following the conditions stated below. Competition binding curves at all receptors were carried out by assaying 6 different concentrations (range from 10 nM to 100 μM) for all the compounds showing an inhibition percentage above 80%. The -log of the inhibition constant (pK_i) of each compound was calculated by the Cheng-Prusoff equation, $K_i = IC_{50}/(1 + [L]/K_d)$, where IC_{50} is the concentration of compound that displaces the binding of the radioligand by 50 %, [L] is the free radioligand concentration and K_d is the dissociation constant of each radioligand. IC_{50} values were obtained by non-linear regression fitting the data, using Prism 2.1 software (GraphPad, San Diego, CA).

4.2.1. Human A_1 receptors

Adenosine A_1 receptor competition binding experiments were carried out in membranes from CHO-A_1 cells (Euroscreen, Gosselies, Belgium). On the day of assay, membranes were defrosted and re-suspended in incubation buffer 20 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, 2 UI/ml adenosine deaminase (pH = 7.4). Each reaction well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in duplicate, contained 15 μg of protein, 2 nM [^{3}H]DPCPX and test compound. Non-specific binding was determined in the presence of 10 μM (R)-PIA. The reaction mixture was incubated at 25 °C for 60 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

4.2.2. Human A_{2A} receptors

Adenosine A_{2A} receptor competition binding experiments were carried out in membranes from HeLa-A_{2A} cells. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$ and 2 UI/mL adenosine deaminase (pH = 7.4). Each reaction well of a GF/C multiscreen plate (Millipore, Madrid, Spain), prepared in duplicate, contained 10 μg of protein, 3 nM [^{3}H]ZM241385 and test compound. Nonspecific binding was determined in the presence of 50 μM NECA. The reaction mixture was incubated at 25 °C for 30 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

4.2.3. Human A_{2B} receptors

Adenosine A_{2B} receptor competition binding experiments were carried out in membranes from HEK-293-A_{2B} cells (Euroscreen, Gosselies, Belgium) prepared following the provider's protocol. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris-HCl, 1 mM EDTA, 10 mM $MgCl_2$, 0.1 mM benzamidine, 10 μg/mL bacitracine and 2 UI/mL adenosine deaminase (pH = 6.5). Each reaction well prepared in duplicate, contained 18 μg of protein, 35 nM

[^{3}H]DPCPX and test compound. Non-specific binding was determined in the presence of 400 μM NECA. The reaction mixture was incubated at 25 °C for 30 min, after which samples were filtered through a multiscreen GF/C microplate and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

4.2.4. Human A_3 receptors

Adenosine A_3 receptor competition binding experiments were carried out in membranes from HeLa-A_3 cells. On the day of assay, membranes were defrosted and re-suspended in incubation buffer 50 mM Tris-HCl, 1 mM EDTA, 5 mM $MgCl_2$ and 2 UI/mL adenosine deaminase (pH = 7.4). Each reaction well of a GF/B multiscreen plate (Millipore, Madrid, Spain), prepared in triplicate, contained 90 μg of protein, 30 nM [^{3}H]NECA and test compound. Non-specific binding was determined in the presence of 100 μM (R)-PIA. The reaction mixture was incubated at 25 °C for 180 min, after which samples were filtered and measured in a microplate beta scintillation counter (Microbeta Trilux, Perkin Elmer, Madrid, Spain).

4.3 Functional studies

4.3.1. Human A_1 receptors

The antagonist behaviour of test compounds at A_1 receptors was evaluated in CHO-A_1 cells stably expressing the receptors, by measuring NECA-mediated inhibition of forkolin-stimulated cAMP accumulation, in the absence or presence of test compounds. Briefly, cells grown in 96 well plates with growth medium containing dialyzed fetal bovine serum, were washed twice with F-12 nutrient mixture medium containing 25 mM HEPES pH 7.4 and 20 μM of the phosphodiesterase inhibitor rolipram (incubation buffer). Then the test compounds were preincubated for 15 min in assay medium and after this time, different concentrations (0.1 nM - 1 mM) of the agonist NECA and 3 μM of forskolin were added to each well. The incubation was continued for 15 min and reaction was stopped with a lysis buffer included in a cAMP

enzyme immunoassay kit (GE Healthcare). Cell lysates were transferred to a plate coated with anti-rabbit IgG and rabbit-anticAMP in the presence of cAMP conjugated with peroxidase for 60 min. After this time wells were washed four times with the wash buffer provided in the kit and TMB was added to each well and incubated for 60 min. Peroxidase reaction was stopped with 1 M sulphuric acid and the cAMP quantity was calculate from the optical density at 450 nm read on a Tecan M100 reader.

4.3.2. Human A_3 receptors

The antagonist behaviour of test compounds at A_3 receptors was evaluated in CHO- A_3 cells stably expressing the receptors, by measuring NECA-mediated inhibition of forskolin-stimulated cAMP accumulation, in the absence or presence of test compounds. Briefly, cells grown in 96 well plates with growth medium containing dialyzed fetal bovine serum, were washed twice with DMEM F-12 nutrient mixture medium containing 25 mM HEPES pH 7.4 and 30 μM of the phosphodiesterase inhibitor rolipram (incubation buffer). Then the test compounds were preincubated for 15 min in assay medium and after this time, different concentrations (0.1 nM - 1 mM) of the agonist NECA and 10 μM of forskolin were added to each well. The incubation was continued for 15 min and cAMP was quantified by an enzyme immunoassay (Perkin Elmer).

NECA concentration-response curves in cAMP assays were fitted to the following equations with Prism 2.1 (Graph Pad, San Diego, CA) and Kaleidagraph software (Synergy Software, Reading, PA), respectively:

$$E = E_{max} \times [A]^s/(EC^s_{50} + [A]^s)$$

where E_{max}, [A], and s represent the maximum response, agonist concentration, and curve slope, respectively. EC_{50} is the concentration of agonist that produces 50 % of the maximal response. The antagonist potency was expressed as pK_B (-log of the dissociation constant, K_B), calculated from a concentration-response curve of the antagonist over the effect elicited by NECA following the equation:

$$K_B = IC_{50}/(2+(A/A_{50})^n)^{1/n}-1$$

where IC_{50} is concentration of antagonist that inhibits the agonist effect by 50 %, A is the concentration of agonist, A_{50} is the concentration of agonist that exerts 50 % of the maximal effect and n is the Hill-slope of the inhibition curve.

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