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Synthesis and structure–activity relationships of 2-hydrazinyladenosine derivatives as A_{2A} adenosine receptor ligands

Ali El-Tayeb [a,b,*,†], Sabrina Gollos [c]

[a] Pharmaceutical Institute, Pharmaceutical Chemistry I, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany
[b] Department of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, University of Al-Azhar, Assiut Branch, 71524 Assiut, Egypt
[c] Isotope Laboratory, PharmaCenter Bonn, Pharmaceutical Institute, University of Bonn, An der Immenburg 4, D-53121 Bonn, Germany

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ABSTRACT

A series of 2-hydrazinyladenosine derivatives was synthesized and investigated in radioligand binding studies for their affinity at the adenosine receptor subtypes with the goal to obtain potent and A_{2A}AR selective agonists and to explore the structure–activity relationships of this class of compounds at A_{2A}AR. Modifications included introduction of a second sugar moiety at position 2 of adenosine to form new bis-sugar nucleosides and/or modifications of the 2-position linker in different ways. The performed modifications were found to produce compounds with relatively high A_{2A}AR affinity and very high selectivity toward A_{2A}AR. The most potent bis-sugar nucleoside was obtained with the D-galactose derivative **16** which exhibited a K_i value of 329 nM at A_{2A}AR with marked selectivity against the other AR subtypes. In another set of compounds, compound **3** was modified via replacement of its cyclic structure with mono- and disubstituted phenyl moieties and the resulting hydrazones **10**–**14** were found to have low nanomolar affinity for A_{2A}AR. In addition to **3**, compounds **10**, **11** and **13** have been identified as the most potent compounds in the present series with K_i values of 16.1, 24.4, and 12.0 nM, respectively, at rat A_{2A}AR. Species differences were tested and found to exist in different rates. Functional properties of the most potent compounds **10**, **11**, **13** and **16** were assessed showing that the compounds acted as agonists at A_{2A}AR.

1. Introduction

Nucleoside adenosine (**1**, Fig. 1) is a naturally occurring extracellular purine nucleoside, which modulates multiple physiological and pathophysiological processes through its action on cell-surface G protein-coupled adenosine receptors.[1–3] Four adenosine receptor (AR) subtypes have been cloned and characterized, A_1, A_{2A}, A_{2B} and A_3. The A_1 and A_3 receptor subtypes are G_i-coupled receptors, activation of these receptors leads to inhibition of adenylate cyclase, while activation of A_{2A} and A_{2B} receptor subtypes stimulate the adenylate cyclase through G_s protein. In addition, coupling to other second messenger systems has been described.[1–3] A_1AR are highly expressed in the brain (cortex, cerebellum, and hippocampus), dorsal horn of spinal cord, eye, adrenal gland and atria. A_{2A}AR are highly expressed in spleen, thymus, leukocytes, blood platelets, striatopallidal GABAergic neurons, and olfactory bulb.[1,2] In the brain, A_{2A}AR are expressed in high density in the striatum, while in the periphery A_{2A}AR are highly expressed in the intestinal mucosa, enteric neurons, hepatocytes and a variety of immune cells. In the intestine, A_{2A}AR are expressed in the jejunum, ileum, and cecum.[1,2] A_{2B}AR are highly expressed in cecum, colon, bladder and in intermediate or low levels in other regions, for example, lung, blood vessels, mast cells and ovary. A_3AR are highly expressed in testis, mast cells, lung, and spleen.[1,2] The crystal structure of the human A_{2A} receptors has been solved via number of successive studies in the last few years. The structure of A_{2A}AR

Abbreviations: AR, adenosine receptor(s); binodenoson, 2-((cyclohexylmethylene)hydrazino)adenosine; BSA, bovine serum albumin; CADO, 2-chloroadenosine; [^{3}H]CCPA, [^{3}H]2-chloro-N^6-cyclopentyladenosine; CHO, Chinese hamster ovary; [^{3}H]CGS21680, [^{3}H](2-(4-(2-carboxyethyl)phenyl)ethylamino)-5′-*N*-ethylcarboxamido-adenosine; CPA, N^6-cyclopentyladenosine; DMSO, dimethylsulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; ESI, electrospray ionization; h, human; HEK, human embryonic kidney; NECA, 5′-(*N*-ethylcarboxamido)adenosine; *R*-PIA, (*R*)-N^6-phenylisopropyladenosine; PSB, Pharmaceutical Science Bonn; [^{3}H]PSB-603, [^{3}H]8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine; PSB-0777, 4-(2-(6-amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-ylthio)ethyl)benzene sulfonic acid; r, rat; SAR, structure–activity relationships; TLC, thin layer chromatography; TRIS, tris(hydroxymethyl)aminomethane; vs., versus.

* Corresponding author. Present address: Pharmazeutisches Institut, Pharmazeutische Chemie I, An der Immenburg 4, D-53121 Bonn, Germany. Tel.: +49 228 73 2564; fax: +49 228 73 2567.

E-mail addresses: a.el-tayeb@uni-bonn.de, ali72eg@yahoo.com (A. El-Tayeb).

† Permanent address: Department of Pharmaceutical Medicinal Chemistry, Faculty of Pharmacy, Al-Azhar University, Assiut Branch, 71524 Assiut, Egypt. Tel.: +2 088 2312191; fax: +2 088 2181191.

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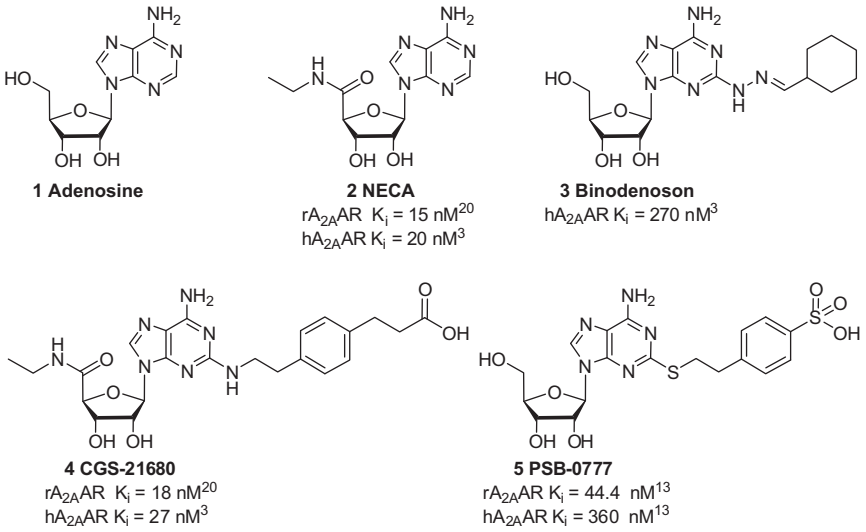


Figure 1. Structures of selected potent and selective A_{2A}AR agonists.

have been solved in complex with the conformationally selective A_{2A}AR agonist, that capable of receptor stabilization in a specific active-state configuration, 2-(3-(1-(pyridin-2-yl)piperidin-4-yl) ureido)ethyl-6-*N*-(2,2-diphenylethyl)-5′-*N*-ethylcarboxamidoadenosine-2-carboxamide (UK-432097)[4] and with the A_{2A}AR antagonist 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5] triazin-5-ylamino]ethyl)phenol (ZM241385),[5] in 2.7, and 2.6 Å resolution, respectively. In addition, X-ray structures of the A_{2A}AR bound to its endogenous agonist adenosine (**1**, Fig. 1, 3 Å resolution), and the synthetic analogue 5′-(*N*-ethylcarboxamido)adenosine (NECA, **2**, Fig. 1, 2.6 Å resolution) have recently been obtained showing that the ribose residue of agonists occupies a distinct hydrophilic binding pocket from that occupied by antagonists. The agonists induce motion of the helices 3, 5 and 7 and the antagonists prevent conformational change of the receptor.[6]

Due to the wide distribution of the AR subtypes, selective AR agonists are recommended for the treatment of a variety of pathological conditions. After more than three decades of research, selective agonists and antagonists for almost all four adenosine receptor subtypes are available.[7] A considerable number of synthetic selective AR agonists have been discovered and clinically evaluated. For example, the A_1AR agonist GW493838 was investigated for the treatment of peripheral neuropathic pain in patients with postherpetic neuralgia or peripheral nerve injury.[3,7] The A_3AR agonist CF101 was designed for the treatment of patients suffering from rheumatoid arthritis.[3,7] Regadenoson, an A_{2A}AR agonist, has been approved in 2008 by the food and drug administration (FDA) for stress testing in conjunction with myocardial perfusion imaging because of its vasodilatory effects via its activation of A_{2A}AR.[3,7]

Adenosine A_{2A} receptors mediate the potent immunosuppressive and hypotensive effects of adenosine.[3,8] One of the main therapeutic potentials of A_{2A}AR agonists is due to their anti-inflammatory and immunosuppressive effects. On the other hand, A_{2A}AR agonists are potent vasodilators and are applied as diagnostics for pharmacologic stress testing in the heart. Further potential therapeutic applications of A_{2A}AR agonists are the treatment of psychosis and Huntingtoń s disease.[9–11] Activation of the A_{2A}AR on a variety of inflammatory cell types leads to anti-inflammatory effects. However, the systemic use of A_{2A}AR agonists as anti-inflammatory drugs is limited by their potent hypotensive activity due to activation of A_{2A}AR expressed in heart and blood vessels.[1,8] Recently, we have developed phosphorylated adenosine-derived A_{2A}AR agonists, which would be preferably dephosphorylated in inflamed tissues with high expression of *ecto*-5′-nucleotidase.[12a,b] This is thought to allow a separation of anti-inflammatory and hypotensive effects since the concentration of the active drug, the A_{2A}AR agonist, would only be achieved locally at the sites of inflammation with high *ecto*-5′-nucleotidase activity.[12a,b] In a further study and to avoid the vasodilatory side effects we have developed local highly polar, perorally non-absorbable A_{2A}-selective agonists as a local therapy, for example, of inflammatory bowel disease, avoiding the hypotensive effects of centrally acting anti-inflammatory A_{2A}AR agonists.[13]

Most of the potent and selective A_{2A}AR agonists are adenosine derivatives modified in the 2-position of the adenine moiety by introducing bulky substituents and/or in the 5′-position of the riboside of the adenosine scaffold as shown in Figure 1. N^6,2-Disubstitution and substitution in position 8 may also lead to potent A_{2A}AR agonists. Introduction of *N*-ethylcarboxamide moiety at the 5′-position (e.g., NECA, **2**, Fig. 1) or a tetrazole ring at the 5′-position of the ribose ring[9a] represents the most structural modifications at the ribose moiety of the adenosine scaffold. At position 2 of the purine moiety, variations include the introduction of large cyclic substituents (aromatic or cycloalkyl) or a simple long lipophilic alkyl chain, and in most cases a three-membered linker between the large cyclic substituents and the purine moiety of the adenosine scaffold often containing an electron-rich function (e.g., O, NH, triple bond) attached to the purine ring, for example, Binodenoson (**3**), CGS-21680 (**4**) and PSB-0777 (**5**) as shown in Figure 1. However, substitution at 2-position of purine moiety represents the essential substitution site and the shared common structural features in all known potent and selective A_{2A}AR agonists.[3,7,9b–18]

The recently published crystal structures of A_{2A}AR showed the importance of the extracellular loops, for example, EL3 of A_{2A}AR in the binding with the substituents in the 2-position of the purine moiety.[4–6,19] Therefore, in the present study, our goal was to explore the structure–activity relationships (SAR) of A_{2A}AR agonists modified at 2-position of the purine moiety of the adenosine scaffold via synthesis of some 2-substituted adenosine derivatives and evaluation of their affinity at A_{2A}AR as well as at the other adenosine receptor subtypes.

As starting structure for modifications, we have selected 2-((cyclohexylmethylene)hydrazino)adenosine (Binodenoson, CorVue™, **3**, Fig. 1). Binodenoson (**3**) has been identified as a potent and selective A_{2A}AR agonist and has been clinically evaluated to

be used as a stress agent in conjunction with myocardial perfusion imaging.[21,22] To achieve our goals, we planned to resynthesize and evaluate nucleoside **3** at A_{2A}AR, and then to replace the cyclohexyl moiety of **3** with different mono- or disubstituted aromatic moieties. As common feature in the most known potent A_{2A}AR agonists (Fig. 1) a three atom linker between the purine moiety and the cyclic structure was observed and seems to be required for A_{2A}AR activity. In this study our goal was to explore the SAR of this linker via its extension by one more atom, to remove the hydrazone double bond and to introduce hydrogen bond acceptors in the linker via introduction of urea and thiourea moieties within the linker. Removal of the hydrogen bond donor at the nitrogen atom of the 2-hydrazinoadenosine via introduction of methyl moiety was also designed. In another set of compounds, our goal was to introduce monosaccharide pentose and hexose moieties (aldose or ketose) as a second sugar moiety at the 2-position of adenosine scaffold to replace the cyclohexyl moiety of **3** to form bis-sugar nucleosides. Formation of such bis-sugar nucleosides connected to the adenine moiety via different lengths and possessing multiple hydroxyl groups distributed with different stereogenic centers will give more information about the receptor ligand interaction. For example, the binding mode, the possibility of hydrogen bond formation with A_{2A}AR and the sterogenic requirements which might be involved in the agonist receptor interaction. In addition to SAR exploration, formation of the bis-sugar nucleosides with multiple hydroxyl groups will markedly increase the hydrophilicity of the produced compounds and accordingly, the resulting highly polar and less absorbable A_{2A}AR agonists can work locally as anti-inflammatory, for example, for the treatment of inflammatory bowel disease, avoiding the hypotensive effects of centrally acting anti-inflammatory A_{2A}AR agonists.[13] Finally, the effect of the new modifications on the A_{2A}AR as well as on the other AR subtypes were evaluated and reported in the present study.

2. Results and discussion

2.1. Syntheses

2-Chloroadenosine (**7**) was synthesized starting from guanosine (**6**) in four steps according to published procedures[23–26] with few modifications, later it was bought from commercial sources. At first, protection of the 2′-, 3′- and 5′-hydroxyl groups of guanosine (**6**) was achieved via acetylation using acetic anhydride in acetonitrile under mild conditions to give 2′,3′,5′-acetylguanosine (**6a**).[23,24] The 6-chloro-2′,3′,5′-acetylguanosine derivative (**6b**) was obtained via nucleophilic displacement reaction of 2′,3′,5′-acetylguanosine with phosphorus oxychloride at 100 °C to give the product in a good yield.[25] 2,6-Dichloro-2′,3′,5′-acetyl adenosine nucleoside (**6c**) was obtained via diazotization/chloro-dediazoniation of 6-chloro-2′,3′,5′-acetylguanosine according to published procedures[25,26] which was used as such for the next reaction steps after preliminary purification and without further structure elucidation. An N^6-amino function was introduced and 2′-, 3′- and 5′-acetyl groups were removed in one reaction step using saturated ammonia in ethanol solution to afford the 2-chloroadenosine nucleoside **7** in low yields (20%) after reaction time of 3 days as shown in Scheme 1. For detailed reaction procedures, NMR characterization and LCMS data of the intermediate compounds see Supplementary data.

2-Chloroadenosine (**7**) was treated with hydrazine[21,27] or methylhydrazine to give the corresponding 2-hydrazinoadenosine (**8**) and 2-*N*-methylhydrazinoadenosine (**9**), respectively. Refluxing of **8** or **9** with aromatic aldehydes, aldose monosaccharides (D-glucose, D-galactose, D-mannose and D-ribose) or ketose monosaccharide (D-fructose) in methanol for several hours afforded the corresponding hydrazones **3**, **10**–**24** in high yields (Scheme 1). The nucleoside hydrazones **3**, **10**–**11**, **13**–**14** were precipitated from methanol after cooling while compound **12** was further purified using silica gel column chromatography. The bis-sugar nucleosides **15**–**24** were purified by high performance liquid chromatography (HPLC) on reverse phase C18 material in order to remove the traces of the unreacted sugars.

N-Phenylhydrazinecarboxamide and *N*-phenylhydrazinecarbothioamide moieties were introduced into the 2-position of adenosine via reaction of **9** with phenylisocyanate or phenylisothiocyanate in methanol under mild condition to give the desired products **25** and **26**, respectively, in high yields as shown in Scheme 2. Compounds **25** and **26** were precipitated from the reaction mixture, washed with methanol and crystalized by addition of ethyl acetate and excess *n*-hexane without further need for chromatographic purifications.

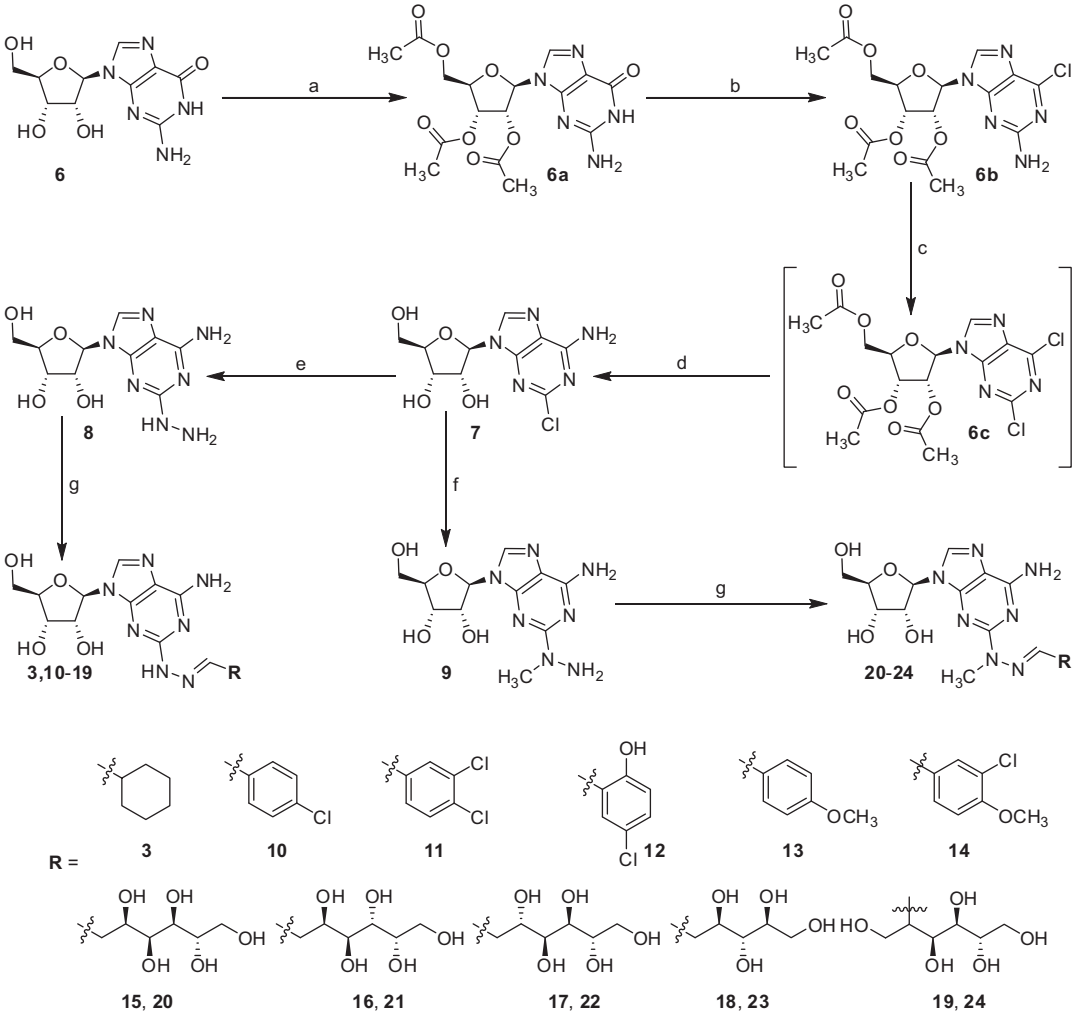
The structures of the synthesized nucleosides were confirmed by ^{1}H- and ^{13}C NMR spectroscopy, in addition to HPLC analysis coupled to electrospray ionization mass spectrometry (LC/ESI-MS) performed in both, positive and negative mode. The purity of the products was generally $\geqslant$95%.

3. Biological activity

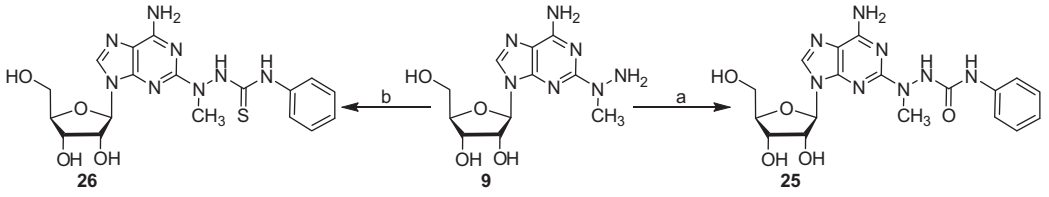
The synthesized 2-hydrazinyladenosine derivatives were in vitro investigated in radioligand binding studies at the AR subtypes. The binding studies were performed at rat brain cortical membranes for A_1AR using [^{3}H]CCPA as a radioligand, at rat brain striatal A_{2A}AR using the agonist radioligand [^{3}H]CGS21680 and at membrane preparations of human recombinant A_3AR expressed in Chinese hamster ovary (CHO) cells using [^{3}H]NECA as a radioligand. Two nucleosides were additionally tested at human recombinant A_1 and A_{2A} receptors using [^{3}H]CCPA and [^{3}H]CGS21680 as radioligands, respectively, in order to investigate the potential species differences. Bis-sugar nucleosides **15**–**24** as well as nucleoside **3** were additionally investigated at human recombinant A_{2B}AR using the antagonist radioligand [^{3}H]PSB-603 because the agonist radioligand for A_{2B}AR does not exist. Functional properties of the most potent compounds **10**, **11**, **13** and **16** were assessed by adenylate cyclase assays measuring cAMP accumulation in human CHO cells stably expressing the human A_{2A}AR. The test results of all synthesized nucleosides at the four adenosine receptor subtypes are depicted in Table 1.

3.1. Adenosine receptor affinity

In general, the determined K_i values in the present series of compounds confirm the previous observations and the reported SAR at A_{2A}AR in which the 2-substituted adenosine derivatives possess a higher affinity toward the A_{2A}AR than the other AR subtypes. This affinity is strongly depending on the nature of the substituent, which should be in accordance with the general SAR requirements at A_{2A}AR, for example, presence of cyclic structure or long chain separated from the adenine moiety of the position-2 of the adenosine scaffold via a linker consists of mostly three atoms. Introduction of a second sugar moiety linked to position 2 of adenosine via a hydrazone linker to form a bis-sugar nucleoside structure with different sugar lengths and distinguished stereogenic centers (nucleosides **15**–**24**) generally led to compounds with a relatively higher affinity toward A_{2A}AR than the other AR subtypes as shown in Table 1 (see also *Supplementary* Fig. S1). However, the affinities still lower than that of the parent compound **3** (Fig. 1), probably due to a lack of the cyclic structure observed at the most potent A_{2A}AR agonists. The rank order of potency of bis-sugar nucleosides **15**–**19** at A_{2A}AR was **16** (D-galactose; 329 nM) > **15**



Scheme 1. Synthesis of nucleosides (**7**–**24**), Reagents and conditions: (a) acetic anhydride, 4-dimethylaminopyridine, ethyldimethylamine, acetonitrile, 1 h, rt; (b) $POCl_3$, tetraethylammonium chloride, *N*,*N*-dimethylaniline, 15–30 min, 100 °C; (c) benzyltriethylammonium nitrite, acetylchloride, dichloromethane, 1 h, 0 °C; (d) saturated ammonia in ethanol solution, 3 days, rt; (e) hydrazine hydrate, rt, 10 h; (f) methylhydrazine, rt, 7 h; (g) **R**-CHO or **R**-C=O for compounds **19** and **24**, methanol, reflux, 3–12 h.

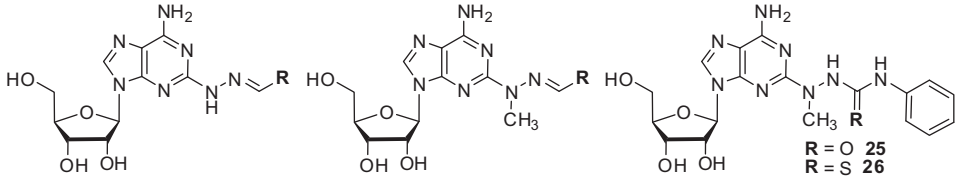


Scheme 2. Synthesis of nucleosides (**25**–**26**), Reagents and conditions: (a) phenylisocyanate, methanol, 2 h, rt; (b) phenylisothiocyanate, methanol, 2 h, rt.

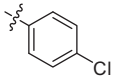
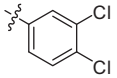
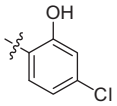
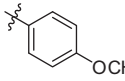
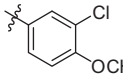
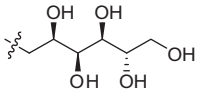
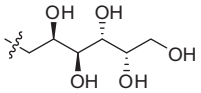
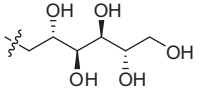
(D-glucose; 42% inhibition) > **19** (D-fructose; 34% inhibition) > **17** (D-mannose; 33% inhibition) ≈ **18** (D-ribose; 33% inhibition). The most potent compound in the present bis-sugar nucleoside series at A_{2A}AR was obtained with D-galactose modification, compound **16**, which exhibited a K_i value of 329 nM (Fig. 2B). Compound **16** was only 13-fold less potent than **3** but **16** possessed higher selectivity toward the other AR subtypes in comparison with **3**. The stereogenic distribution of the hydroxyl groups of the second sugar moiety seems to be important for activity and selectivity; the favored stereogenic distribution at A_{2A}AR was obtained with D-galactose sugar. Removal of the hydrogen bond donor at the nitrogen atom of the 2-hydrazinoadenosine via introduction of a methyl moiety at the nitrogen atom of the hydrazine structure led to relatively lower affinity bis-sugar nucleosides, compare **15**–**19** versus (vs.) **20**–**24**, this was also observed in compounds **25** and **26**. The methylated analogue of the most potent nucleoside **16**, compound **21**, exhibited only 25% inhibition at A_{2A}AR (Table 1). Further modifications and experimental evaluations of **16** including functional assays and binding studies using human receptors are aimed in order to have more potent A_{2A}AR agonists while keeping their A_{2A}AR selectivity.

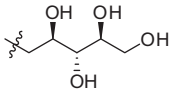
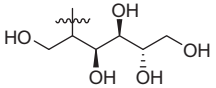
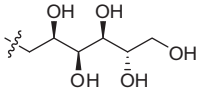
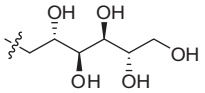
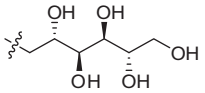
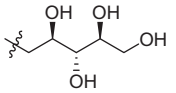
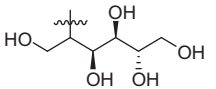
The synthesized bis-sugar nucleosides were screened at A_{2B}AR using the antagonist radioligand [^{3}H]PSB-603 and the compounds showed almost low or no affinity at this low affinity AR subtype. The promising substituent was observed in the case of compounds **19** (D-fructose; 17% inhibition) ≈ **21** (D-galactose; 17% inhibition)

Table 1
Adenosine receptor affinities of the synthesized nucleosides **3** and **10–26**



3, **10-19** **20-24** **25-26**

Nucleoside	R	A_1AR rat brain cortical membranes vs. [^{3}H]CCPA[a]	A_{2A}AR rat brain striatal membranes vs. [^{3}H]CGS21680[a]	A_{2B}AR human recombinant vs. [^{3}H]PSB-603[b,c,d]	A_3AR human recombinant vs. [^{3}H]NECA[a]
		K_i ± SEM (nM) or (% inhibition ± SEM at 1 μM), (n = 3)			
2 (NECA)	-	5.1 [3] 13.6[f] [28]	15 [20] 20[g] [3]	1890 [29]	6.2 [28]
4 (CGS21680)	-	1800 [3] 289[f] [3]	18 [20] 27[g] [3]	>10,000 [29]	114[h] [30]
5 (PSB-0777)	-	⩾10,000 [13] 54[f] [13]	44.4 [13] 360[g] [13]	>10,000 [13]	>1000 [13]
3 (Binodenoson)		70.8 ± 3.6 169 ± 13[f] *48,000*[f] [3]	25.5 ± 3.5 81 ± 34[g] *270*[g] [3]	>10,000 (17 ± 3) *430,000* [3]	52.6 ± 5.1 *903* [3]
10		108 ± 31	16.1 ± 1.9	n.d.[e]	71.9 ± 4.7
11		263 ± 53	24.4 ± 7.2	n.d.	88.8 ± 8.6
12		58.0 ± 9.2	91.7 ± 22.9	n.d.	131 ± 25
13		65.7 ± 11.6 723 ± 280[f]	12.0 ± 4.1 687 ± 127[g]	n.d.	112 ± 47
14		32.1 ± 2.1	53.7 ± 15.1	n.d.	51.1 ± 17.5
15		>1000 (11 ± 8)	>1000 (42 ± 1)	>10,000 (10 ± 1)	>1000 (3 ± 2)
16		>10,000[d] (31 ± 2)	329 ± 104	>10,000 (6 ± 7)	>10,000[d] (38 ± 4)
17		>1000 (34 ± 1)	>1000 (33 ± 1)	>10,000 (12 ± 5)	>1000 (16 ± 2)

18		>1000 (24 ± 2)	>1000 (33 ± 5)	>10,000 (14 ± 3)	>1000 (23 ± 11)
19		>1000 (25 ± 2)	>1000 (34 ± 3)	>10,000 (17 ± 5)	>1000 (15 ± 5)
20		>1000 (2 ± 4)	>1000 (31 ± 3)	>10,000 (7 ± 5)	>1000 (19 ± 6)
21		>1000 (12 ± 2)	>1000 (25 ± 1)	>10,000 (17 ± 1)	>1000 (13 ± 4)
22		>1000 (15 ± 4)	>1000 (2 ± 1)	>10,000 (5 ± 10)	>1000 (11 ± 3)
23		>1000 (9 ± 3)	>1000 (7 ± 1)	>10,000 (9 ± 8)	>1000 (1 ± 3)
24		>1000 (17 ± 4)	>1000 (25 ± 2)	>10,000 (6 ± 4)	>1000 (5 ± 2)
25	O	>1000 (3 ± 8)	>1000 (11 ± 7)	n.d.	>1000 (23 ± 5)
26	S	>1000 (1 ± 11)	>1000 (16 ± 6)	n.d.	>1000 (36 ± 7)

[a] Agonist radioligand.
[b] Antagonist radioligand.
[c] Affinities of the agonists will be underestimated since an antagonist radioligand was used for the determination.
[d] At 10 μM.
[e] Not determined.
[f] Using human recombinant A_1AR vs. [^{3}H]CCPA.
[g] Using human recombinant A_{2A}AR vs. [^{3}H]CGS21680.
[h] [^{125}I]I-AB-MECA was used as a radioligand.

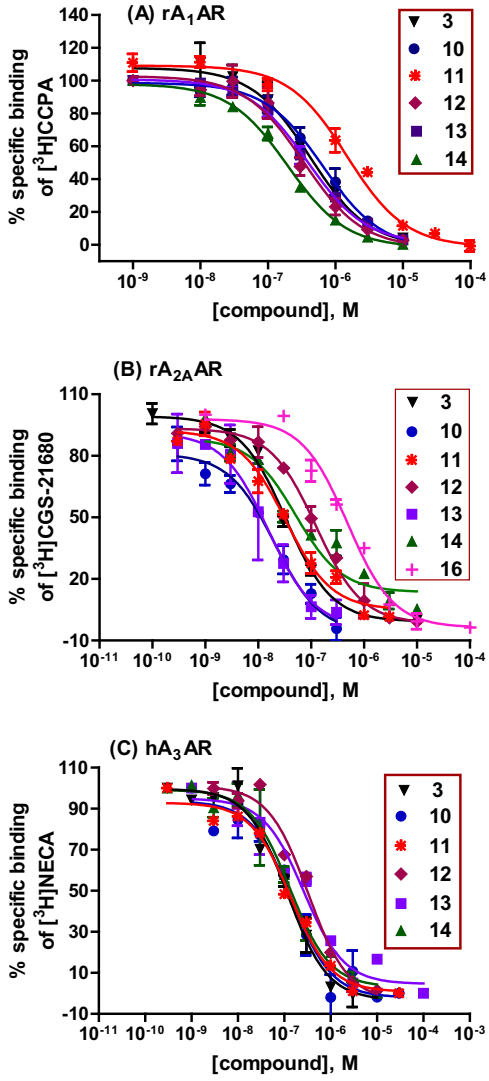


Figure 2. Results of competition curves of potent adenine nucleoside derivatives **3**, **10**–**14**, **16** as ligands at A_1AR versus [^{3}H]CCPA at rat brain cortical membranes (A), A_{2A}AR versus [^{3}H]CGS21680 at rat brain striatal membranes (B) and A_3AR versus [^{3}H]NECA at human recombinant A_3AR (C). Data points represent means of three independent experiments performed in triplicate.

but still weak A_{2B}AR ligands (Table 1). The bis-sugar nucleosides **19** and **21** exhibited 17% inhibition at 10 μM at the low affinity A_{2B}AR, the estimated K_i value of both compounds is expected to be in micromolar range and probably comparable with that of adenosine itself at A_{2B}AR (15 μM).[3] These results turn compounds **19** and **21** into promising starting compounds for the discovery of potent A_{2B}AR agonists, especially if we know that the present data were obtained from assays using the antagonist radioligand [^{3}H]PSB-603 and accordingly the affinities of the A_{2B}AR agonists will be underestimated, that is, compounds **19** and **21** should show higher affinities if the agonist radioligand was used which does not exist so far. Therefore, functional assays were aimed after further optimization of this kind of compounds based on **19** and **21**, for example, via combined modification with N^6-substitution and/or introduction of *N*-ethylcarboxamide moiety at 5′-position of the first ribose–sugar moiety which may lead to the discovery of potent A_{2B}AR agonists. No significant difference in affinity was observed between aldose and ketose bis-sugar nucleosides at the four AR subtypes.

Further modifications based on **3** were obtained via extension of the linker between the adenine moiety and the cyclic structure by one more atom and removal of hydrazone double bond via introduction of urea and thiourea moieties within the linker together with introduction of methyl residue into the nitrogen atom of 2-hydazinoadenosine in compounds **25** and **26**. The results showed a complete loss of activity at A_1AR and A_{2A}AR compared to the parent compound **3**, probably due to the shift of the hydrogen bond donor between the two adjacent nitrogen atoms of the hydrazine residue and/or introduction of an unfavorable hydrogen bond acceptor within the linker. These results proved the importance of keeping the linker length within three atoms in this class of compounds. However, compounds **25** and **26** showed somehow activity at A_3AR (Table 1).

In the present study, the hydrazone nucleoside **3** was resynthesized and evaluated in radioligand binding studies at the four AR subtypes and the obtained K_i value of this compound showed in our hand higher affinity of **3** at A_1, A_{2A} and A_3AR subtypes exhibiting 70.8, 25.5 and 52.6 nM, respectively (Fig. 2). This is not consistent with the reported data in which compound **3** showed very low affinity at A_1AR (48 μM)[3] and relatively low affinity at A_3AR (0.903 μM).[3] Compound **3** showed low affinity (17% inhibition) at A_{2B}AR as shown in Table 1 and in Supplementary Figure 1. Analogues of hydrazone **3** (compounds **10**–**14**) were synthesized and evaluated at AR subtypes and the results showed that, as in case of **3**, all tested 2-hydrazinyloadenosine derivatives **10**–**14** possessed very high affinity at all three AR subtypes. The quantified K_i values were generally high at A_{2A}AR except in case of compounds **12** and **14**, which showed higher affinity for A_1AR with K_i values of 58 and 32.1 nM, respectively. At A_1AR, the rank order of potency was **14** (32.1 nM) > **12** (58.0 nM) ⩾ **13** (65.7 nM) ⩾ **3** (70.8 nM) > **10** (108 nM) > **11** (263 nM) as shown in Table 1 and Figure 2A.

At A_{2A}AR, replacement of cyclohexyl-moiety of **3** (K_i = 25.5 nM) by 4-chloro-2-hydroxyphenyl-moiety resulted in a 3.6-fold less potent compound (compound **12**, K_i = 91.7 nM). The difference in affinity was reduced to about twofold by the replacement of cyclohexyl-moiety with 3-chloro-4-methoxyphenyl-moiety (compound **14**, K_i = 53.7 nM). A comparable affinity was achieved by the replacement with 3,4-dichlorophenyl-moiety in compound **11** with a K_i value of 24.4 nM. The monochloro-substituted phenyl moiety derivative **10** showed a higher affinity than that of **3** with a K_i value of 16.1 nM. The most potent compound at A_{2A}AR in the present series was obtained with compound **13** in which the cyclohexyl-moiety of **3** was replaced by 4-methoxyphenyl-moiety. Compound **13** showed about twofold more potency at A_{2A}AR exhibiting a K_i value of 12.0 nM.

The obtained results from the present short series of compounds **10**–**14**, showed that the monosubstituted phenyl moiety generally possessed higher affinities than that of disubstituted moieties. The presence of a chloro-residue in the phenyl moiety seems to increase the activity at A_{2A}AR. The rank order of potency at A_{2A}AR was **13** (12.0 nM) > **10** (16.1 nM) > **11** (24.4 nM) ⩾ **3** (25.5 nM) > **14** (53.7 nM) > **12** (91.7 nM) as shown in Table 1 and Figure 2B. At A_3AR, the results showed that compound **14** was the most potent compound with a K_i value of 51 nM, the rank order of potency at this receptor was **14** (51.1 nM) > **10** (71.9 nM) > **11** (88.8 nM) > **13** (112 nM) > **12** (131 nM) >> **3** (903 nM) see Table 1 and Figure 2C.

The potential species differences were evaluated using selected potent compounds of this series at A_{2A}AR. Therefore, in addition to the parent compound **3**, compound **13** was tested at human recombinant A_1 and A_{2A} receptors using [^{3}H]CCPA and [^{3}H]CGS21680 as radioligands, respectively. The results showed that species differences existed in the case of compound **13** at both A_1 and A_{2A}AR. A sharp decrease in affinity was observed at both receptor subtypes. At A_{2A}AR, compound **13** showed about 57-fold

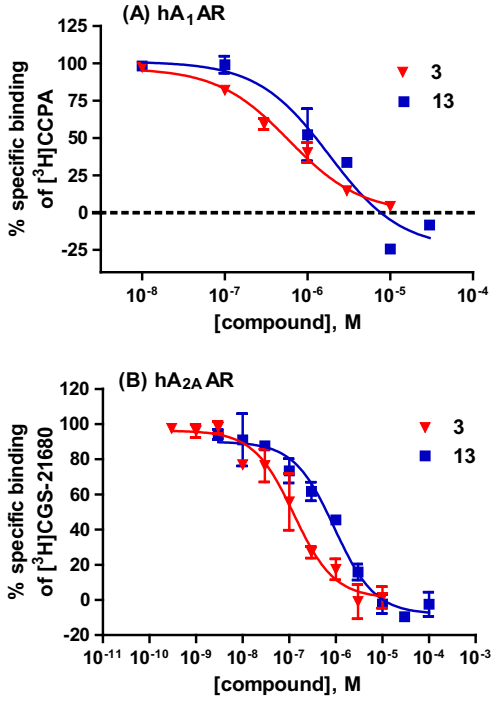


Figure 3. Results of competition curves of potent adenine nucleoside derivatives **3** and **13** as ligands at human recombinant A_1AR versus [^{3}H]CCPA (A) and at human recombinant A_{2A}AR versus [^{3}H]CGS21680 (B). Data points represent means of three independent experiments performed in triplicate.

less potency and exhibited a K_i value of 687 nM at human A_{2A}AR compared with 12.0 nM at rat A_{2A}AR. The same was observed at A_1AR, compound **13** showed affinities of 723 nM at human and 65.7 nM at rat A_1AR (almost 11-fold less potent) as shown in Figure 3. These species differences were observed with much less extent in case of compound **3** since the compound showed about 2.5-fold less potency in human than that in rat receptors with K_i values of 81 nM and 25.5 nM, respectively. Our results of compound **3** at hA_{2A}AR were somehow in the same range of the literature values (270 nM at hA_{2A}AR)[3] and also in agreement with the literature data obtained from similar reported A_{2A}AR agonists (e.g., **5**, Table 1).[13] At hA_1AR, compound **3** showed in our hand very high affinity in comparison to the literature values (48 μM),[3] compound **3** exhibited a K_i value of 169 nM as shown in Table 1 and Figure 3 (compare also **3** vs. **4** at hA_1AR, Table 1).

Functional properties of the most potent compounds **10**, **11**, **13** and **16** were assessed by adenylate cyclase assays measuring cAMP

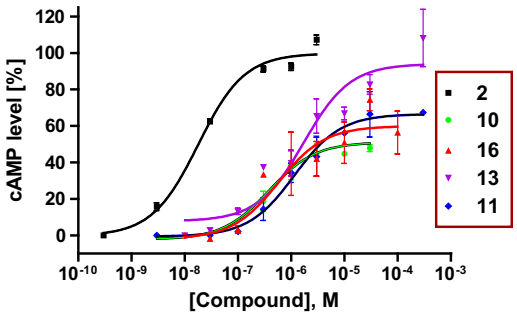


Figure 4. Concentration–response curves of **2**, **10**, **11**, **13** and **16** in cAMP accumulation assays using CHO cells expressing the human A_{2A}AR ($n = 3$); EC_{50}: 0.018 ± 1.22 μM (**2**), 0.493 ± 0.21 μM (**10**), 1.66 ± 0.59 μM (**11**), 1.36 ± 0.89 μM (**13**), 1.44 ± 1.04 μM (**16**). All data represent means ± SEM of three separate experiments performed in triplicates.

accumulation in human CHO cells stably expressing the human A_{2A}AR.[13] For comparison, the full agonist NECA (**2**, Fig. 1) was investigated under the same condition. Concentration–response curves of the test compounds were obtained showing that **10**, **11**, **13** and **16** acted as agonists at A_{2A}AR. For NECA (**2**) an EC_{50} value of 0.018 μM was determined, while compounds **10**, **11**, **13** and **16** showed EC_{50} values of 0.493, 1.66, 1.36 and 1.44 μM, respectively, (Fig. 4). The species differences between human and rat may explain the lower potencies of the test compounds in cAMP assay than that obtained from the binding assays. The efficacy of **13** was not significantly different from that of NECA (**2**), indicating that **13** is full agonists at A_{2A}AR while compounds **10**, **11** and **16** are partial agonists at A_{2A}AR.

4. Conclusions

A series of 2-hydrazinyladenosine derivatives based on modification of the well-known A_{2A}AR-potent agonist **3** was designed, synthesized and evaluated for their affinities at different AR subtypes with the aim of exploring the SAR of this class of compounds as A_{2A}AR agonists. Modifications included formation of bis-sugar nucleosides (compounds **15**–**24**), modification of the linker between the cyclic structure and the adenine moiety via introduction of methyl group in the nitrogen atom of the hydrazine structure (e.g., compounds **20**–**26**) and/or modification and extension of the linker length (compounds **25**–**26**). The synthesized compounds were pharmacologically evaluated and found to produce compounds with relatively higher A_{2A}AR affinity but still lower than that of nucleoside **3**. The most potent bis-sugar nucleoside at A_{2A}AR was **16** which exhibited a K_i value of 329 nM with marked selectivity against the other AR subtypes. Compound **16** represents a lead compound for synthesizing more potent, highly polar and selective A_{2A}AR agonists to be used, for example, as a local anti-inflammatory agent. In another set of compounds, hydrazones **3**, **10**–**14** were synthesized and were found to have low nanomolar affinity at the A_{2A}AR as well as at the other AR subtypes. Compound **13** has been identified as the most potent compound in the present series at rA_{2A}AR with a K_i value of 12.0 nM. However, compounds **3**, **10**–**14** are characterized by the lack of selectivity against the other AR subtypes. Species differences were tested and found to exist between human and rat A_1 and A_{2A}AR in different rates. At A_{2A}AR, compound **3** showed 2.5-fold less potency in human than that in rat receptors while compound **13** showed 57-fold less potency in human than in rat A_{2A}AR. Functional properties of the most potent compounds **10**, **11**, **13** and **16** were assessed by adenylate cyclase assays measuring cAMP accumulation in human CHO cells stably expressing the human A_{2A}AR and the results showed that compound **13** (EC_{50} = 1.36 ± 0.89 μM) is full agonists at A_{2A}AR while compounds **10**, **11** and **16** (EC_{50} = 0.493 ± 0.21, 1.36 ± 0.89 and 1.44 ± 1.04 μM, respectively) are partial agonists at A_{2A}AR. Further studies will be aimed at improving the A_{2A}AR affinity and selectivity based on modifications of bis-sugar nucleoside **16**, this will include, for example, combined modifications at 2- and 5′-positions in the adenosine scaffold.

5. Experimental section

All commercially available reagents were obtained from various producers (Acros, Aldrich, Fluka, Merck, and Sigma) and used without further purification. Solvents were used without additional purification or drying, unless otherwise noted. The reactions were monitored by thin layer chromatography (TLC) using aluminum sheets with silica gel 60 F_{254} (Merck). Column chromatography was carried out with silica gel 0.060–0.200 mm, pore diameter

ca. 6 nm. Mass spectra were recorded on an API 2000 (Applied Biosystems, Darmstadt, Germany) mass spectrometer (turbo ion spray ion source) coupled with an HPLC system (Agilent 1100) using a Phenomenex Luna 3μ C18 column. ^{1}H- and ^{13}C NMR spectra were performed on a Bruker Avance 500 MHz spectrometer. DMSO-d_6 or D_2O were used as solvents as indicated below. Shifts are given in ppm relative to the remaining protons of the deuterated solvents used as internal standard. Purity of the prepared nucleosides was checked by TLC on silica gel 60 F_{254} (Merck) aluminum plates, using dichloromethane/methanol (9:1 or 3:1) or *n*-butanol/CH_3COOH/H_2O = 2:1:1 as the mobile phase. Purity of the final nucleosides was confirmed by HPLC by dissolving 1 mg/ml in H_2O/MeOH = 1:1, containing 2 mM ammonium acetate. A sample of 10 μL was injected into an HPLC instrument (Agilent 1100) using a Phenomenex Luna 3 μ C18 column. Elution was performed with a gradient of water/methanol (containing 2 mM ammonium acetate) from 90:10 to 0:100 for 30 min at a flow rate of 250 μL/min, starting the gradient after 10 min. UV absorption was detected from 220 to 400 nm using a diode array detector. The purity of the products was generally ⩾95%.

Compounds **3**,[21] **8**,[21,27] **10**,[21] and **13**,[21] have previously been synthesized and we provide detailed spectral data in the present study while the other compounds are new.

5.1. General procedure for preparation of 2-(methyl) hydrazinoadenosine derivatives: Synthesis of compounds 8 and 9

Solution of **7** (1.5 g, 5 mmol) in 5 mL of hydrazine hydrate for synthesis of **8** or methylhydrazine for synthesis of **9** was allowed to stir over night till disappearance of **7** determined by TLC (CH_2Cl_2:MeOH = 3:1). 2-Propanol (50 mL) was added to the reaction mixture and the formed gum was taken in water (100 mL) and stirred for additional 5 h. The precipitated product was filtered and washed with water and dried to give the pure product in about 80% overall yield after concentration of the mother liquor and collection of the remaining product.

5.2. General procedure for preparation of 2-(*N*′-ar(alk) ylidenehydrazino)adenosines 3, 10–24

A mixture of **8** or **9** (0.5 mmol) and 1.1 equiv of aromatic aldehyde, sugar aldehyde or ketone in 30 mL methanol were refluxed for 3–15 h till the disappearance of the starting material and the completion of the reaction was determined by TLC using CH_2Cl_2:MeOH = 3:1 for compounds **3**, **10**–**14** and using *n*-butanol:CH_3COOH:H_2O = 2:1:1 for compounds **15**–**24**. In case of aromatic aldehydes, the products were crystallized out on cooling and filtered off, washed with methanol, and dried to give the pure products. Products of sugar aldehyde or ketone were precipitated after concentration under vacuum and the crude product was crystallized by adding ethyl acetate (1 mL) and excess *n*-hexane. The products were further purified by reverse phase HPLC chromatography to afford the pure product in about 70% overall yield.

5.3. General procedure for preparation of the adenosine derivatives *N*-phenylhydrazinecarboxamide (25) and carbothioamide (26)

To a solution of **9** (1 mmol) in methanol (20 mL), 1.1 equiv of phenylisocyanate was added for preparation of compound **25**, or phenylisothiocyanate for preparation of compound **26**. The reaction mixture was stirred at room temperature for 2 h and the precipitated compound was filtered off, washed with methanol and dried to give the pure products in 70% yield.

5.4. Preparative HPLC purification of 15–24

Crude nucleosides **15**–**24** were dissolved in 5 mL of deionized water:methanol (1:1) and injected into an RP-HPLC column (Knauer 20 mm ID, Eurospher-100 C18). The column was eluted with a solvent gradient of 0–100% of water/methanol for 30 min at a flow rate of 5 mL/min. The UV absorption was detected at 254 nm. Fractions were collected and appropriate fractions pooled and evaporated under vacuum.

5.4.1. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-(1-methylhydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (9)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.17 (s, 3H), 3.50–3.64 (m, 2H), 3.87–3.89 (q, 1H, J = 3.99 Hz), 4.12–4.14 (m, 1H), 4.59–4.62 (q, 1H, J = 5.56 Hz), 4.66 (s, 2H), 4.93–4.95 (t, 1H, J = 5.83 Hz), 5.08–5.09 (d, 1H, J = 5.04 Hz), 5.29–5.30 (d, 1H, J = 6.30 Hz), 5.74–5.76 (d, 1H, J = 5.99 Hz), 6.84 (s, 2H), 7.93 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 39.40, 61.86, 70.75, 73.10, 85.32, 87.26, 113.36, 137.04, 151.60, 155.80, 160.97. LC/ESI-MS: negative mode 310.1 ([M−H]$^-$), positive mode 312.3 ([M+H]$^+$).

5.4.2. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-((*E*)-2-(cyclohexylmethylene) hydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (3)

^{1}H NMR (500 MHz, DMSO-d_6) δ 1.15–1.26 (m, 5H), 1.60–1.62 (m, 1H), 1.68–1.73 (m, 4H), 2.17–2.21 (m, 1H), 3.52–3.64 (m, 2H), 3.89–3.91 (q, 1H, J = 3.36 Hz), 4.12 (m, 1H), 4.52–4.53 (m, 1H), 5.03–5.09 (m, 2H), 5.36 (m, 1H), 5.74–5.75 (d, 1H, J = 6.30 Hz), 7.13 (s, 2H), 7.23–7.24 (d, 1H, J = 5.99 Hz), 8.01 (s, 1H), 10.16 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 24.95, 25.24, 25.64, 30.41, 61.82, 70.84, 73.54, 85.82, 87.08, 114.49, 137.43, 138.09, 148.39, 151.41, 155.41. LC/ESI-MS: negative mode 390.4 ([M−H]$^-$), positive mode 392.4 ([M+H]$^+$).

5.4.3. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-((*E*)-2-(4-chlorobenzylidene) hydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl) tetrahydrofuran-3,4-diol (10)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.56–3.71 (m, 2H), 3.94–3.96 (q, 1H, J = 3.04 Hz), 4.18–4.21 (q, 1H, J = 3.15 Hz), 4.65–4.68 (q, 1H, J = 5.25 Hz), 5.08–5.09 (d, 1H, J = 4.41 Hz), 5.20–5.22 (q, 1H, J = 3.36 Hz), 5.38–5.39 (d, 1H, J = 6.30 Hz), 5.77–5.79 (d, 1H, J = 6.30 Hz), 7.02 (s, 2H), 7.41–7.43 (d, 2H, J = 8.51 Hz), 7.73–7.75 (d, 2H, J = 8.51 Hz), 8.01 (s, 1H), 8.04 (s, 1H), 10.68 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 62.06, 71.15, 73.23, 86.14, 87.90, 115.61, 128.03, 128.72, 132.77, 134.72, 138.07, 138.52, 151.02, 156.31, 156.37. LC/ESI-MS: negative mode 418.0 ([M−H]$^-$), positive mode 420.1 ([M+H]$^+$).

5.4.4. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-((*E*)-2-(3,4-dichlorobenzylidene)hydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (11)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.58–3.73 (m, 2H), 3.97–3.99 (q, 1H, J = 2.73 Hz), 4.17–4.19 (m, 1H), 4.69–4.73 (q, 1H, J = 5.25 Hz), 5.08–5.09 (d, 1H, J = 4.09 Hz), 5.36–5.37 (d, 1H, J = 6.30 Hz), 5.38–5.40 (m, 1H), 5.76–5.77 (d, 1H, J = 6.93 Hz), 7.07 (s, 2H), 7.60–7.62 (m, 2H), 8.01 (d, 2H, J = 2.20 Hz), 8.04 (s, 1H), 10.84 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 62.20, 71.32, 73.09, 86.39, 88.34, 115.94, 126.73, 127.62, 130.37, 130.79, 131.64, 136.63, 137.17, 138.52, 150.76, 156.13, 156.40. LC/ESI-MS: negative mode 451.94 ([M−H]$^-$), positive mode 454.07 ([M+H]$^+$).

5.4.5. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-((*E*)-2-(4-chloro-2-hydroxybenzylidene)hydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (12)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.54–3.67 (m, 2H), 3.91–3.93 (q, 1H, J = 3.67 Hz), 4.12–4.15 (q, 1H, J = 3.67 Hz), 4.61–4.64 (q, 1H,

J = 5.56 Hz), 5.08–5.10 (m, 2H), 5.34–5.36 (d, 1H, *J* = 6.30 Hz), 5.79–5.80 (d, 1H, *J* = 6.30 Hz), 6.88–6.90 (d, 1H, *J* = 8.82 Hz), 7.05 (s, 2H), 7.17–7.19 (m, 1H), 7.59–7.60 (d, 1H, *J* = 2.83 Hz), 8.07 (s, 1H), 8.20 (s, 1H), 10.97 (s, 1H), 11.59 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 61.85, 70.77, 73.35, 85.74, 87.27, 115.40, 117.97, 121.65, 122.77, 127.61, 128.95, 137.80, 138.69, 151.17, 155.44, 155.89, 156.36. LC/ESI-MS: negative mode 434.0 ([M−H]$^-$), positive mode 436.3 ([M+H]$^+$).

5.4.6. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-((*E*)-2-(4-methoxybenzylidene)hydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (13)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.56–3.71 (m, 2H), 3.77 (s, 3H), 3.93–3.95 (q, 1H, *J* = 2.52 Hz), 4.19 (m, 1H), 4.64–4.68 (q, 1H, *J* = 5.25 Hz), 5.06–5.07 (d, 1H, *J* = 4.41 Hz), 5.20–5.22 (q, 1H, *J* = 3.36 Hz), 5.38–5.40 (d, 1H, *J* = 6.30 Hz), 5.77–5.78 (d, 1H, *J* = 6.30 Hz), 6.93–6.95 (m, 4H), 7.65–7.66 (m, 2H), 7.98 (s, 1H), 8.0 (s, 1H), 10.41 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 55.31, 62.08, 71.16, 73.26, 86.15, 87.86, 114.20, 115.37, 127.93, 128.40, 137.79, 140.0, 151.12, 156.32, 156.57, 159.76. LC/ESI-MS: negative mode 413.9 ([M−H]$^-$), positive mode 416.0 ([M+H]$^+$).

5.4.7. (2*R*,3*R*,4*S*,5*R*)-2-(6-Amino-2-((*E*)-2-(3-chloro-4-methoxybenzylidene)hydrazinyl)-9*H*-purin-9-yl)-5-(hydroxymethyl)tetrahydrofuran-3,4-diol (14)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.58–3.73 (m, 2H), 3.87 (s, 3H), 3.97–3.99 (q, 1H, *J* = 2.73 Hz), 4.16–4.18 (m, 1H), 4.69–4.72 (q, 1H, *J* = 5.56 Hz), 5.07 (d, 1H, *J* = 4.091 Hz), 5.35–5.39 (m, 2H), 5.76–5.77 (d, 1H, *J* = 6.62 Hz), 7.0 (s, 2H), 7.13–7.15 (d, 1H, *J* = 8.51 Hz), 7.53–7.55 (m, 1H), 7.97 (s, 1H), 7.98 (s, 1H), 8.02–8.03 (d, 1H, *J* = 1.89 Hz), 10.57 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 56.33, 62.21, 71.33, 73.08, 86.39, 88.26, 112.77, 115.69, 121.78, 127.11, 127.21, 129.47, 138.22, 138.52, 150.89, 154.71, 156.37, 156.41. LC/ESI-MS: negative mode 448.0 ([M−H]$^-$), positive mode 450.0 ([M+H]$^+$).

5.4.8. (2*S*,3*S*,4*S*,5*R*,*E*)-6-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)hydrazono)hexane-1,2,3,4,5-pentaol (15)

^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 3.04–3.08 (m, 2H), 3.13–3.16 (m, 1H), 3.65–3.39 (m, 1H), 3.41–3.44 (m, 1H), 3.53–3.54 (m, 1H), 3.59–3.69 (m, 2H), 3.90–3.91 (m, 2H), 4.08–4.09 (t, 1H, *J* = 4.88 Hz), 4.39–4.41 (t, 1H, *J* = 5.51 Hz), 5.72–5.73 (d, 1H, *J* = 5.99 Hz), 8.22 (s, 1H). LC/ESI-MS: negative mode 458.44 ([M−H]$^-$), positive mode 460.31 ([M+H]$^+$).

5.4.9. (2*S*,3*R*,4*S*,5*R*,*E*)-6-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)hydrazono)hexane-1,2,3,4,5-pentaol (16)

^{1}H NMR (500 MHz, DMSO-d_6) 3.37–3.42 (m, 3H), 3.49–3.54 (m, 3H), 3.60–3.72 (m, 2H), 3.95 (q, 1H, *J* = 1.68 Hz), 4.07–4.13 (m, 4H), 4.38–4.40 (m, 2H), 4.62–4.63 (q, 1H, *J* = 5.14 Hz), 5.08–5.09 (d, 2H, *J* = 4.09 Hz), 5.32 (d, 1H, *J* = 6.30 Hz), 5.72–5.73 (d, 1H, *J* = 6.93 Hz), 7.0 (s, 2H), 7.43–7.44 (d, 1H, *J* = 4.41 Hz), 7.95 (s, 1H), 10.31 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 62.28, 63.28, 69.40, 70.05, 70.18, 71.32, 71.88, 73.0, 86.30, 88.06, 115.33, 137.97, 145.11, 150.82, 156.35, 156.58. LC/ESI-MS: negative mode 458.38 ([M−H]$^-$), positive mode 460.22 ([M+H]$^+$).

5.4.10. (2*S*,3*S*,4*S*,5*S*,*E*)-6-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)hydrazono)hexane-1,2,3,4,5-pentaol (17)

^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) 3.21–3.25 (m, 1H), 4.08 (m, 1H), 3.29–3.33 (m, 1H), 3.46–3.60 (m, 3H), 3.88–3.95 (m, 1H), 4.08–4.11 (m, 2H), 4.15–4.20 (m, 1H), 4.38–4.41 (m, 1H), 5.72–5.74 (m, 1H), 7.54–7.57 (d, 1H, *J* = 5.67 Hz), 8.28 (s, 1H). LC/ESI-MS: negative mode 458.36 ([M−H]$^-$), positive mode 460.22 ([M+H]$^+$).

5.4.11. (2*S*,3*R*,4*R*,*E*)-5-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)hydrazono)pentane-1,2,3,4-tetraol (18)

^{1}H NMR (500 MHz, DMSO-d_6) 3.38–3.67 (m, 7H), 3.96 (d, 1H, *J* = 1.57 Hz), 4.08 (m, 1H), 4.25–4.28 (m, 2H), 4.58 (d, 1H, *J* = 3.46 Hz), 4.63–4.64 (q, 1H, *J* = 5.25 Hz), 4.72–4.73 (d, 1H, *J* = 4.72 Hz), 5.08–5.09 (d, 1H, *J* = 3.78 Hz), 5.31–5.33 (d, 1H, *J* = 6.30 Hz), 5.71–5.73 (d, 1H, *J* = 7.25 Hz), 5.89 (s, 1H), 6.99 (s, 2H), 7.43–7.45 (d, 1H, *J* = 5.04 Hz), 7.94 (s, 1H), 10.32 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 62.27, 63.31, 71.36, 71.53, 72.38, 72.94, 74.72, 86.33, 88.19, 115.44, 138.05, 143.85, 150.73, 156.42, 156.70. LC/ESI-MS: negative mode 428.23 ([M−H]$^-$), positive mode 430.15 ([M+H]$^+$).

5.4.12. (2*S*,3*R*,4*S*,*E*)-5-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)hydrazono)hexane-1,2,3,4,6-pentaol (19)

^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 3.39–3.42 (m, 2H), 3.51–3.52 (m, 2H), 3.59–3.61 (m, 2H), 3.90–3.91 (m, 1H), 3.98–3.99 (m, 1H), 4.08–4.09 (m, 2H), 4.40 (m, 1H), 4.59 (m, 1H), 5.70–5.72 (d, 1H, *J* = 6.93 Hz), 7.98 (s, 1H). LC/ESI-MS: negative mode 458.39 ([M−H]$^-$), positive mode 460.22 ([M+H]$^+$).

5.4.13. (2*S*,3*S*,4*S*,5*R*,*E*)-6-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methylhydrazono)hexane-1,2,3,4,5-pentaol (20)

^{1}H NMR (500 MHz, DMSO-d_6) δ 3.03–3.10 (m, 2H), 3.19–3.21 (t, 1H, *J* = 8.82 Hz), 3.27 (s, 3H), 3.35 (m, 2H), 3.41–3.47 (m, 2H), 3.51–3.56 (m, 2H), 3.61–3.68 (m, 2H), 3.89–3.91 (q, 1H, *J* = 3.99 Hz), 4.01–4.04 (m, 1H), 4.12 (m, 1H), 4.38 (m, 1H), 4.49–4.50 (m, 1H), 4.89 (m, 1H), 4.95 (m, 1H), 5.17 (m, 1H), 5.40 (m, 1H), 5.75–5.76 (d, 1H, *J* = 5.76 Hz), 7.0 (m, 2H) 8.21 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 38.65, 61.46, 70.41, 72.05, 73.77, 76.85, 78.41, 85.57, 87.21, 92.33, 97.01, 112.64, 139.10, 151.50, 155.06, 159.46, 160.84. LC/ESI-MS: negative mode 472.37 ([M−H]$^-$), positive mode 474.48 ([M+H]$^+$).

5.4.14. (2*R*,3*S*,4*R*,5*S*,*E*)-6-(2-(6-Amino-9-((2*S*,3*S*,4*R*,5*S*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methylhydrazono)hexane-1,2,3,4,5-pentaol (21)

^{1}H NMR (500 MHz, DMSO-d_6) 3.38–3.44 (m, 2H), 3.47 (s, 3H), 3.50–3.57 (m, 3H), 3.64–3.67 (m, 1H), 3.73–3.75 (m, 1H), 3.15–3.19 (m, 1H), 3.94–3.96 (q, 1H, *J* = 2.73 Hz), 4.11–4.13 (m, 2H), 4.18–4.19 (m, 1H), 4.37–4.40 (m, 2H), 4.51 (t, 1H, *J* = 5.20 Hz), 4.57–4.62 (m, 1H), 4.67–4.70 (m, 1H), 5.10–5.11 (d, 1H, *J* = 4.09 Hz), 5.19–5.21 (d, 1H, *J* = 5.99 Hz), 5.35–5.36 (d, 1H, *J* = 6.30 Hz), 5.75–5.76 (d, 1H, *J* = 6.93 Hz), 7.10–7.11 (s, 2H), 8.01 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 31.34, 62.20, 63.29, 69.38, 70.12, 70.65, 71.30, 72.33, 72.96, 86.12, 88.19, 115.37, 138.79, 141.50, 150.94, 155.97, 156.59. LC/ESI-MS: negative mode 472.31 ([M−H]$^-$), positive mode 474.41 ([M+H]$^+$).

5.4.15. (2*S*,3*S*,4*S*,5*S*,*E*)-6-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methylhydrazono)hexane-1,2,3,4,5-pentaol (22)

^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) 3.45 (s, 3H), 3.48–3.54 (m, 3H), 3.59–3.61 (m, 2H), 3.67–3.68 (m, 2H), 3.94–3.96 (q, 1H, *J* = 2.73 Hz), 4.10–4.11 (m, 1H), 4.22–4.26 (m, 1H), 4.62–4.65 (m, 1H), 5.75–5.76 (d, 1H, *J* = 6.62 Hz), 7.12–7.13 (d, 1H, *J* = 5.35 Hz), 8.01 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 31.25, 62.22, 64.02, 69.76, 70.70, 71.35, 71.41, 71.65, 72.79, 86.18, 88.24, 115.39, 138.92, 141.65, 150.91, 155.95, 156.85. LC/ESI-MS: negative mode 472.33 ([M−H]$^-$), positive mode 474.42 ([M+H]$^+$).

5.4.16. (2*R*,3*S*,4*S*,*E*)-5-(2-(6-Amino-9-((2*S*,3*S*,4*R*,5*S*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methylhydrazono)pentane-1,2,3,4-tetraol (23)

^{1}H NMR (500 MHz, DMSO-d_6) 3.40–3.43 (m, 2H), 3.46 (s, 3H), 3.50–3.55 (m, 3H), 3.58–3.61 (m, 1H), 3.66–3.69 (m, 1H), 3.86–3.88 (m, 1H), 3.97 (q, 1H, J = 2.41 Hz), 4.10–4.12 (m, 1H), 4.38–4.40 (t, 1H, J = 5.04 Hz), 4.67–4.69 (m, 2H), 5.10–5.15 (m, 1H), 5.32–5.44 (m, 2H), 5.74–5.75 (d, 1H, J = 6.93 Hz), 5.85 (s, 1H), 7.07–7.08 (d, 1H, J = 5.35 Hz), 7.13 (s, 2H), 8.0 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 31.29, 62.22, 63.29, 71.38, 71.84, 72.52, 72.94, 74.76, 86.22, 88.46, 115.51, 138.98, 140.35, 150.86, 155.93, 156.80. LC/ESI-MS: negative mode 442.40 ([M−H]$^-$), positive mode 444.22 ([M+H]$^+$).

5.4.17. (2*S*,3*R*,4*S*,*E*)-5-(2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methylhydrazono)hexane-1,2,3,4,6-pentaol (24)

^{1}H NMR (500 MHz, DMSO-d_6 + D_2O) δ 3.14 (s, 3H), 3.23–3.24 (m, 2H), 3.37–3.93 (m, 1H), 3.47–3.48 (m, 1H), 3.49 (m, 1H), 3.51–3.52 (m, 1H), 3.58–3.63 (m, 2H), 3.79–3.81 (t, 1H, J = 5.99 Hz), 3.87–3.89 (q, 1H, J = 3.99 Hz), 4.10–4.12 (m, 1H), 4.57–4.59 (t, 1H, J = 5.51 Hz), 5.73 (d, 1H, J = 5.99 Hz), 7.92 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 39.41, 61.86, 63.13, 64.56, 68.01, 69.33, 70.75, 73.09, 75.90, 82.08, 87.26, 102.13, 113.36, 137.04, 151.60, 155.80, 160.97. LC/ESI-MS: negative mode 472.32 ([M−H]$^-$), positive mode 474.33 ([M+H]$^+$).

5.4.18. 2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methyl-*N*-phenylhydrazinecarboxamide (25)

^{1}H NMR (500 MHz, DMSO-d_6) 3.28 (s, 3H), 3.47–3.62 (m, 2H), 3.85–3.87 (q, 1H, J = 3.99 Hz), 4.11–4.14 (q, 1H, J = 3.99 Hz), 4.57–4.61 (q, 1H, J = 5.56 Hz), 4.87–4.89 (t, 1H, J = 5.67 Hz), 5.07–5.08 (d, 1H, J = 4.72 Hz), 5.28–5.29 (d, 1H, J = 5.99 Hz), 5.76–5.77 (d, 1H, J = 5.99 Hz), 6.90–6.92 (m, 3H), 7.19–7.22 (m, 2H), 7.47–7.49 (d, 2H, J = 7.88 Hz), 8.01 (s, 1H), 8.18 (s, 1H), 8.70 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 39.78, 61.88, 70.71, 73.21, 85.30, 87.08, 114.48, 118.74, 121.72, 128.59, 137.61, 140.09, 151.43, 155.72, 156.01, 160.45. LC/ESI-MS: negative mode 429.18 ([M−H]$^-$), positive mode 431.35 ([M+H]$^+$).

5.4.19. 2-(6-Amino-9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9*H*-purin-2-yl)-2-methyl-*N*-phenylhydrazinecarbothioamide (26)

^{1}H NMR (500 MHz, DMSO-d_6) 3.24 (s, 3H), 3.51–3.65 (m, 2H), 3.87–3.89 (q, 1H, J = 3.99 Hz), 4.17–4.18 (q, 1H, J = 3.88 Hz), 4.62–4.63 (q, 1H, J = 5.25 Hz), 4.81 (m, 1H), 5.07–5.08 (d, 1H, J = 4.72 Hz), 5.25–5.26 (d, 1H, J = 5.35 Hz), 5.78–5.80 (d, 1H, J = 5.67 Hz), 7.05 (s, 2H), 7.08–7.11 (m, 1H), 7.26–7.28 (t, 2H, J = 7.40 Hz), 7.54–7.55 (d, 2H, J = 7.56 Hz), 8.06 (s, 1H), 9.61 (s, 1H), 9.68 (s, 1H). ^{13}C NMR (125 MHz, DMSO-d_6) δ 39.12, 61.90, 70.64, 73.23, 85.31, 87.19, 115.01, 124.70, 125.56, 127.89, 138.03, 139.55, 151.23, 156.10, 159.84, 179.85. LC/ESI-MS: negative mode 445.13 ([M−H]$^-$), positive mode 447.26 ([M+H]$^+$).

5.5. Adenosine receptor binding assays

5.5.1. Chemicals

Tri(hydroxymethyl)aminomethan (Tris-buffer) was obtained from Acros Organics (Leverkusen, Germany); DMSO was from Fluka (Switzerland), HCl was from Merck, HAT supplement from Gibco. Radioligands were commercially available or custom-labelled from appropriate precursors as described.[12,13] [^{3}H]CCPA ([^{3}H]2-chloro-*N*^{6}-cyclopentyladenosine (42.6 Ci/mmol),[28] [^{3}H]CGS21680 ((2-(4-(2-carboxyethyl)phenyl)ethylamino)-5′-*N*-ethylcarboxamido-adenosine, 41 Ci/mmol),[20] and [^{3}H]PSB-603 ([^{3}H]8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine, 73 Ci/mmol)[29] and [^{3}H]NECA ([^{3}H]5′-(*N*-ethylcarboxamido)adenosine, 15.3 Ci/mmol),[31] were used as A_1, A_{2A}, A_{2B} and A_3 radioligands, respectively. All other chemical reagents, cell culture materials and adenosine receptor ligands were obtained from Sigma.

5.5.2. Receptor-radioligand binding studies

Rat brain cortical membrane preparations were used as a source for A_1 and rat brain striatal membrane preparations as a source for A_{2A} receptors as previously described.[32,33] Membrane preparations of CHO cells expressing the human A_1, A_{2B} or A_3 receptors were used for hA_1, hA_{2B} and hA_3 assays.[12,13,31,34,35] Membrane preparation of human embryonic kidney (HEK) cells expressing human A_{2A} receptors obtained from PerkinElmer (Product No.: RBHA2A-M400UA), was used for human A_{2A} assays. Stock solutions of the compounds were prepared in dimethyl sulfoxide (DMSO); the final concentration of DMSO was 2.5%. The radioligand concentrations and incubation times (incubation at rt) were as follows: [^{3}H]CCPA: 42.6 Ci/mmol, 1 nM (rat and human A_1), incubation for 90 min; [^{3}H]CGS21680: 41 Ci/mmol 5.0 nM (rat and human A_{2A}), incubation for 60 min; [^{3}H]NECA: 15.3 Ci/mmol 10.0 nM (human A_3), incubation for 180 min; [^{3}H]PSB-603 73 Ci/mmol 0.3 nM (human A_{2B}), incubation for 75 min. About 50−125 μg of protein/vial were used in the assays. Membranes were preincubated for 10–15 min with 0.12 IU/mL of adenosine deaminase in order to remove endogenous adenosine. Binding assays were performed essentially as previously described.[12,13,32,33]

Binding assays at A_1, A_{2A} and A_3 receptors were carried out using PE-vials in a total volume of 400 μL assay buffer (50 mM TRIS–HCl, pH 7.4) containing 100 μL of membrane protein suspension and 100 μL of radioligand solution in the presence of 10 μL of various concentrations of test compound. Nonspecific binding was determined in the presence of 10 μM 2-chloroadenosine (CADO) in A_1AR assays, 50 μM NECA in A_{2A}AR assays, or 100 μM (*R*)-*N*^{6}-phenyl-isopropyladenosine (*R*-PIA) in A_3AR assays. Incubation was terminated by rapid filtration using a Brandel 48-channel cell harvester (Brandel, Gaithersburg, MD) through Whatman GF/B glass fiber filters. Filters were rinsed three times with 2 mL each of ice-cold TRIS–HCl buffer (50 mM, pH 7.4) and incubated for 6–9 h with 2.5 μL of scintillation cocktail (Ready Safe™, Coulter) per well before radioactivity was counted in a liquid scintillation counter (Tricarb 2900TR, Canberra Packard). A_{2B}AR binding assays were carried out in a total volume of 1000 μL containing 25 μL of test compound dissolved in 775 μL TRIS–HCl buffer (50 mM, pH 7.4), 100 μL radioligand solution, and 100 μL of membrane suspension. Nonspecific binding was determined in the presence of 10 μM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). The assay was incubated for 75 min at rt. The A_{2B}AR assay was filtered through GF/B glass fiber filters using a 48-channel cell harvester, and filters were washed four times with ice-cold TRIS–HCl buffer (50 mM, pH 7.4) containing 0.1% bovine serum albumin (BSA). Then filters were transferred to vials and incubated for 9 h with 2.5 mL of scintillation cocktail (Beckman Coulter). Radioactivity was counted in a liquid scintillation counter (Tricarb 2900TR, Canberra Packard) with a counting efficiency of 53%. Curves were determined using 6−7 different concentrations of test compounds spanning 3 orders of magnitude. At least three independent experiments were performed, each in duplicate (human receptors) or triplicate (rat receptors). Data were analyzed with GraphPad Prism, Version 5.0 (GraphPAD, San Diego, CA, USA). For the calculation of K_i values by nonlinear regression analysis, the Cheng-Prusoff equation and K_D values of 0.2 nM (rat A_1AR) and 0.61 nM (human A_1AR) for [^{3}H]CCPA, 15.9 nM (rat A_{2A}AR) and 26.76 nM (human A_{2A}AR) for [^{3}H]CGS21680, 0.41 nM (human A_{2B}AR) for [^{3}H]PSB-603 and 6.2 nM (human A_3AR) for [^{3}H]NECA were used.[12,13,20,29–34]

5.6. Functional assays

5.6.1. Culture of CHO cells

CHO cells expressing the human A_{2A} receptor were stably grown adherent and maintained in a DMEM F-12, supplemented with 10% FCS, 100 IU/mL penicillin G, 100 μg/ml streptomycin, 1 mM glutamine and 200 μg/ml G 418, containing at 37 °C in 5% CO_2. Before recultivation, the cells were washed twice with PBS, trypsinized, and resuspended in new medium and counted.

5.6.2. Measurement of cAMP accumulation

The cells were cultured on 24-well (150,000–200,000) overnight in the culture medium at 37 °C, 5% CO_2. After removal of the culture medium, cells were washed with HBSS buffer (containing 20 mM HEPES; pH 7.3) and then incubated with HBSS buffer containing adenosine 1 IU/mL deaminase for 120 min at 37 °C, 5% CO_2. The cells were then preincubated with phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidone (Ro 20–1724), 40 μM, for 15 min. Compounds were added in a volume of 0.1 mL HBSS buffer and incubations were continued for 15 min. at 37 °C. The reaction was stopped by removal of the reaction buffer followed by the addition of a hot lysis buffer (500 μL; 90 °C; 4 mM Na_2EDTA; 0.1 ‰ Triton X100). The multi-well plates were incubated at room temperature for 5 min and freezed at −20 °C. The plates were thawed on ice and the cells were homogenized. cAMP levels were quantified by incubation of 50 μL each well with cAMP binding protein prepared from calf adrenal glands (75 μg/well) and [^{3}H]cAMP (final concentration 3 nM). The plates were incubated at 4 °C for 60 min and the samples were harvested by filtration through Whatman GF/B filters using a harvester (Brandel 48-channel cell harvester). Each filter was rinsed three times with 1 mL, 50 mM TRIS–HCl, pH 7.4, the filters were punched out into scintillation vials and counted in a liquid scintillation counter with 2.5 ml Ultima Gold® scintillation cocktail. The samples were counted after 6 h for 1 min. The amount of cAMP was determined using standard cAMP curves of three independent experiments each in triplicates.[13,36,37]

Contributions of authors

Ali El-Tayeb planned the experiments, synthesized all compounds, analyzed the structure–activity relationships, and wrote the manuscript. Sabrina Gollos did the pharmacological testing of the compounds.

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Supplementary data

These data include synthetic procedures, synthetic scheme, ^{1}H and ^{13}C NMR spectral data and mass spectra of nucleosides **6a**, **6b**, **7** and **8**, the results of the initial screening of some tested compounds at A_1, A_{2A}, A_{2B} and A_3 receptors as well as examples of LC/ESI-MS purity determination of selected nucleosides (compounds **11**, **18**, **23**, **24**, **25** and **26**). Supplementary material associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.11.021.

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