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Brief Article

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5′-*C*-Ethyl-tetrazolyl-*N*^{6}-Substituted Adenosine and 2-Chloro-adenosine Derivatives as Highly Potent Dual Acting A_1 Adenosine Receptor Agonists and A_3 Adenosine Receptor Antagonists†

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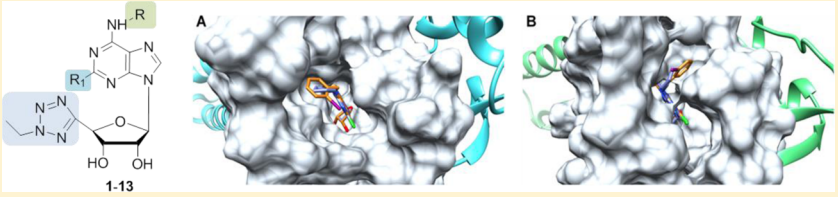
Supporting Information



ABSTRACT: A series of *N*^{6}-substituted-5′-*C*-(2-ethyl-2*H*-tetrazol-5-yl)-adenosine and 2-chloro-adenosine derivatives was synthesized as novel, highly potent dual acting hA_1AR agonists and hA_3AR antagonists, potentially useful in the treatment of glaucoma and other diseases. The best affinity and selectivity profiles were achieved by *N*^{6}-substitution with a 2-fluoro-4-chloro-phenyl- or a methyl- group. Through an in silico receptor-driven approach, the molecular bases of the hA_1- and hA_3AR recognition and activation of this series of 5′-*C*-ethyl-tetrazolyl derivatives were explained.

INTRODUCTION

Adenosine receptors (ARs) belong to the G protein-coupled receptor (GPCR) family, with A_1AR and A_3AR coupled to G_i, and A_{2A} and A_{2B} coupled to G_s.[1] The A_1 adenosine receptor (A_1AR) is the best characterized subtype of the four known ARs and the most conserved AR subtype among species.[1] Selective A_1AR agonists have antiarrhythmic, antinociceptive, and neuro- and cardioprotective effects. Moreover, A_1AR agonists reduce lipolysis in adipose tissue and reduce elevated intraocular pressure (IOP), the most widely recognized risk factor for the onset and progression of glaucoma.[2] The A_3AR is the most recently identified AR subtype and is involved in a variety of physiological and pathophysiological processes.[1] A_3AR agonists are useful in several autoimmune inflammatory conditions such as arthritis, psoriasis, and inflammatory bowel disease.[3] Instead, A_3AR antagonists may be beneficial in the treatment of glaucoma[4] and respiratory tract diseases such as asthma.[5] Recent findings indicated that some compounds might act through two different subtypes of a receptor family, with both pathways leading to beneficial effects. A dual $A_{2B}AR$ and A_3AR antagonist was developed by Novartis as an antiasthmatic agent,[6] while GlaxoSmithKline has investigated a dual $A_{2A}AR$ agonist and A_3AR antagonist as anti-inflammatory agent.[7] More recently, Jacobson et al.[8] reported dual acting human (h) $A_{2A}AR$ agonists and hA_3AR antagonists that might be advantageous for asthma or other inflammatory diseases. To date, no examples of dual acting hA_1AR agonists and hA_3AR antagonists are known. Only a combination of INO-8879, a potent A_1AR agonist in phase I/II of clinical trials for the treatment of glaucoma, with an A_3AR antagonist, was claimed in WO2010127210 A1.[9] Therefore, the availability of dual acting hA_1AR full agonists and hA_3AR antagonists may be of great interest in the treatment of some pathological conditions such as glaucoma. Selectivity for the A_1AR may be achieved by substitution of the *N*^{6}-position of adenosine with a wide range of cycloalkyl-, bicycloalkyl-, and arylalkyl groups.[10] Moreover, modifications at the ribose moiety, serving as a recognition domain of A_1 receptors, contributes to A_1AR affinity, selectivity, and efficacy, leading to full or partial A_1 agonists.[11] Our previous work showed that replacement of the 5′-hydroxy group by a chlorine atom in *N*^{6}-substituted adenosine derivatives increased selectivity for A_1AR.[12] 5′-Chloro-5′-deoxy-*N*^{6}-(±)-(*endo*-norborn-2-yl)-adenosine (5′Cl5′d-(±)-ENBA) displayed high A_1AR affinity and selectivity. It was shown to reduce both mechanical allodynia and thermal hyperalgesia in a mice model of neuropathic pain without affecting motor and cardiovascular functions[13] and to reduce

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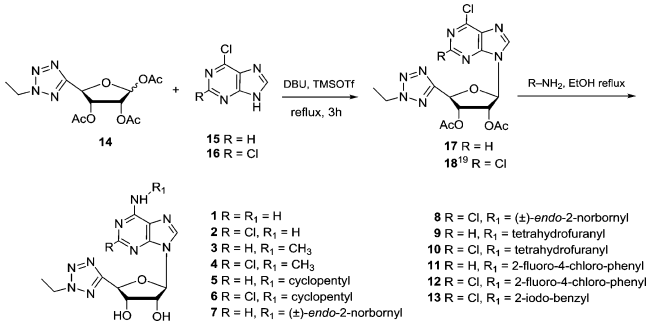
dyskinesia evoked by L-DOPA in a mice model of Parkinson's disease.[14] Structure−activity relationships of A_3AR, extensively reviewed by Jacobson et al.,[15] have pointed out that potent and selective A_3AR agonists were obtained by combining sterically bulky N^6 groups with a 5′-uronamide moiety on the ribose. However, a smaller N^6-methyl group also maintained hA_3AR binding affinity with the beneficial effect of more favorable physicochemical properties that are predictive of greater drug-like properties in vivo.[16] At the ribose moiety, the removal of the ability of a 5′-*N*-alkyluronamide to donate an H-bond (e.g., through a *N*,*N*-dimethylation) or the complete removal of substituents at the 4′-position (e.g., truncated adenosine derivatives) switched the efficacy from hA_3AR agonists to hA_3AR antagonists.[17,18] On the basis of these findings and as a proof of the concept, we designed and synthesized a new series of N^6-substituted-5′-*C*-(2-ethyl-tetrazol-5-yl)-adenosine and 2-chloro-adenosine derivatives (compounds **3**−**13**) as highly potent dual acting hA_1AR agonists and hA_3AR antagonists. 5′-*C*-(2-ethyl-2*H*-tetrazol-5-yl)-adenosine (**1**) and 2-chloro analogue **2** were also synthesized. The latter (**2**) has been reported already as intermediate in the synthesis of hA_{2A}AR agonists,[19] but no biological studies were provided. Compounds **1**−**13** were evaluated for affinity, selectivity, and efficacy at all cloned human adenosine receptor subtypes. Finally, an in silico receptor-driven approach was used to gain insight into the structural basis for AR recognition and activation in this series of 5′-*C*-ethyl-tetrazolyl derivatives.

RESULTS AND DISCUSSION

Chemistry. The 5′-*C*-ethyl-tetrazolyl adenosine derivatives **1**−**13** were synthesized starting from 2-ethyl-5-(1,2,3-tri-*O*-acetyl-D-ribofuranosyl)-2*H*-tetrazole (**14**, mixture of α and β anomers) as described in Scheme 1. Compound **14** was

Scheme 1. Synthesis of Target Compounds 1−13



synthesized following the method described in the literature.[19] Coupling of **14** with 6-chloropurine (**15**) or 2,6-dichloropurine (**16**) was performed by trimethylsilyl trifluoromethanesulfonate (TMSiOTf) mediated N-glycosylation in acetonitrile and in the presence of 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) to obtain the protected compounds **17** and **18**,[19] respectively, in high yields. The synthesis of derivatives **17** and **18** resulted in a stereoselective ribosylation (β/α, 99:1). Nucleophilic displacement of a 6-chlorine atom in **17** and **18** by ammonia or the appropriate amine, followed by deprotection, furnished the nucleoside analogues **1**−**13**. Assignment of the β-anomeric configuration of nucleosides **1**−**13** was performed by proton NOE data. In particular, the β-anomeric configuration was determined by selective irradiation of the H-1′ signal that increased the intensity of the H-4′ signal; this indicates that H-1′ and H-4′ are located on the same face of the ribosyl ring.[11]

Binding Affinity. All the synthesized compounds (**1**−**13**) were tested in radioligand binding assays for affinity at the human recombinant adenosine receptors, stably transfected into Chinese hamster ovary (CHO) cells, utilizing radioligandbinding assays (A_1, A_{2A}, and A_3) or an adenylyl cyclase activity assay (A_{2B}),[20,21] and the results are reported in Tables 1 and 2. As shown in Table 1, all compounds bearing in the N^6 position a substituent such as cyclopentyl (**5**, **6**), *endo*-(±)-norbornyl (**7**, **8**), tetrahydrofuran-2-yl (**9**, **10**), and 2-fluoro-4-chloro-phenyl (**11**, **12**) displayed K_i values ranging from 0.31 to 1.67 nM at hA_1AR, with **6** as the most potent one. However, the replacement of the 5′-hydroxy-methyl group in N^6-substituted adenosine derivatives with the 2-ethyl-2*H*-tetrazol-5-yl one provided also potent hA_3AR ligands (**5**−**12**) with K_i values in the low nanomolar range (K_i = 2.61−16.1 nM). As expected, substitution in N^6 position with a 3-iodo-benzyl- or a methyl- group increased hA_3AR affinity and reduced hA_1AR affinity. **3**, **4**, and **13** displayed hA_3AR affinities in the subnanomolar range (K_i = 0.43−0.59 nM) and hA_1AR affinities in the low nanomolar range (K_i = 2.39−8.59 nM). It is interesting to note that replacing the 5′-hydroxymethyl in adenosine derivatives with the 5′-*C*-(2-ethyl)-2*H*-tetrazol-5-yl group, high affinities at both hA_1 and hA_3AR were maintained, even without N^6 substitution (**1** and **2**, K_i = 0.92 and 0.97 nM at hA_1AR, and 3.86 and 1.80 nM at hA_3AR, respectively). Unexpectedly, adenosine derivatives **1** and **2** showed a binding affinity in the low nanomolar range, also at hA_{2A}AR, resulting in potent but nonselective human adenosine receptor ligands. It is noteworthy that 2-substituted derivatives of **1** and **2** have been previously reported as potent and selective A_{2A}AR agonists.[19] Our results showed that the omission of the 2-amino-alcohol or its substitution with a chlorine in the 5′-*C*-ethyl-tetrazolyl-adenosine derivatives maintained A_{2A}AR affinity and restored A_1AR and A_3AR affinities (Table 1). The introduction of a chlorine atom in the 2-position of the adenine ring resulted in a 2.2-fold increase in hA_1AR affinity of N^6-cyclopentyl adenosine derivatives (e.g., **6** vs **5**), but not of N^6-*endo*-norbornyl (**8** vs **7**), N^6-tetrahydrofuranyl- (**10** vs **9**), and N^6-(2-fluoro-4-chloro-phenyl)-adenosine derivatives (**12** vs **11**). A_1AR affinity does not seem to be deeply influenced by N^6-substitution in the 5′-*C*-ethyl-tetrazolyl-adenosine derivatives because unsubstituted compounds **1** and **2** showed high hA_1AR affinity (K_i = 0.92 and 0.97 nM, respectively) and behaved as hA_1AR full agonists (EC_{50} = 26.3 and 19.8 nM, respectively). However, N^6-substitution with a cyclopentyl (**5**, **6**), *endo*-(±)-norbornyl (**7**, **8**), or 2-fluoro-4-chloro-phenyl (**11**) group provided hA_1AR agonists more potent than **1** and **2**, with **6** as the most potent hA_1AR agonist of the series (K_i = 0.31 nM). Analogously, N^6-substitution with a methyl (**3**, **4**) or a 3-iodo-benzyl (**13**) group decreased hA_1AR and hA_{2A}AR affinities and increased hA_3AR affinity, resulting in **4** as the most potent hA_3AR ligand (K_i = 0.43 nM) of the series. Very interestingly, as shown in the adenylyl cyclase assay (Table 2), all tested compounds behaved as hA_3AR antagonists. **3**, **4** and **13** resulted the most potent, with EC_{50} values of 6.38, 7.69, and 13.3 nM, respectively. **3**, **4**, **11**, and **12** showed the best selectivity and affinity profile at both hA_1AR and hA_3AR, resulting in the first very potent and selective dual acting hA_1AR agonists and hA_3AR antagonists (for selectivities see Supporting Information (SI), Table S1). It is noteworthy that **4** presented the highest selectivity toward A_{2A} (390 and 7800, respectively).

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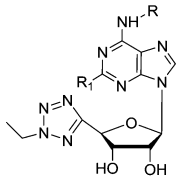
Table 1. Binding Affinity of 5′-*C*-Ethyl-tetrazolyl-adenosine Derivatives



1-13

compd	R	R_1	K_i (nM)[a] A_1[b]	A_{2A}[c]	A_3[d]
1	H	H	0.919 (0.716−1.18)	8.12 (6.01−11.0)	3.86 (3.49−4.28)
2	H	Cl	0.966 (0.770−1.21)	20.7 (16.9−25.3)	1.80 (1.42−2.29)
3	CH_3	H	3.56 (3.02−4.19)	778 (685−883)	0.478 (0.421−0.541)
4	CH_3	Cl	8.59 (8.11−9.10)	3350 (2920−3840)	0.429 (0.361−0.509)
5	cyclopentyl	H	0.682 (0.425−1.09)	93.0 (74.4−116)	7.97 (4.68−13.6)
6	cyclopentyl	Cl	0.311 (0.285−0.340)	62.2 (44.2−87.3)	3.90 (3.42−4.46)
7	(±)-*endo*-2-norbornyl	H	0.453 (0.336−0.610)	64.5 (44.3−94.0)	8.85 (7.87−10.0)
8	(±)-*endo*-2-norbornyl	Cl	0.728 (0.616−0.860)	164.0 (128−211)	16.1 (11.6−22.4)
9	tetrahydrofuranyl	H	1.23 (0.946−1.60)	554 (484−634)	15.4 (12.2−19.5)
10	tetrahydrofuranyl	Cl	1.06 (0.737−1.52)	870 (777−974)	9.62 (6.54−14.1)
11	2-fluoro-4-chloro-phenyl	H	0.432 (0.330−0.565)	77.5 (69.4−86.6)	2.61 (1.84−3.71)
12	2-fluoro-4-chloro-phenyl	Cl	1.67 (1.42−1.97)	223 (206−241)	4.71 (3.49−6.36)
13	2-iodo-benzyl	Cl	2.39 (2.18−2.62)	108 (68.5−171)	0.588 (0.540−0.641)

[a]K_i values are given in nM with 95% confidence intervals in parentheses. [b]Displacement of specific [^{3}H]CCPA binding in CHO cells transfected with the human recombinant A_1 adenosine receptor. [c]Displacement of specific [^{3}H]NECA binding in CHO cells transfected with human recombinant A_{2A} adenosine receptor. [d]Displacement of specific [^{3}H]HEMADO binding in CHO cells transfected with human recombinant A_3 adenosine receptor.

Table 2. EC_{50} Values for Adenylyl Cyclase Activation (A_{2A} and A_{2B}) or Inhibition (A_1 and A_3)[a]

compd	R	R_1	EC_{50} (nM) A_1	A_{2A}	A_{2B}	A_3
1	H	H	26.3 (20.8−33.3)	2.36 (2.00−2.78)	347 (266−454)	93.6 (79.7−110)
2	H	Cl	19.8 (14.3−27.5)	3.84 (3.32−4.44)	542 (340−865)	31.7 (23.2−43.2)
3	CH_3	H	97.2 (84.4−112)	1710 (1150−2530)	1480 (923−2390)	6.38 (5.03−8.07)
4	CH_3	Cl	250 (204−307)	238 (206−274)	3510 (2290−5360) pag (75%)	7.69 (6.34−9.32)
5	cyclopentyl	H	8.61 (6.68−11.1)	9.75 (6.42−14.8)	675 (528−862)	51.5 (29.9−88.6)
6	cyclopentyl	Cl	3.62 (3.31−3.96)	5.31 (3.96−7.13)	1150 (820−1620)	39.5 (33.0−47.3)
7	(±)-*endo*-2-norbornyl	H	4.66 (3.33−6.54)	7.71 (6.76−8.80)	1,010 (675−1510)	83.9 (45.9−153)
8	(±)-*endo*-2-norbornyl	Cl	10.2 (8.97−11.5)	12.8 (9.41−17.4)	>30000	721 (637−816)
9	tetrahydrofuranyl	H	8.74 (7.24−10.5)	25.1 (19.8−31.9)	1340 (716−2510)	61.9 (53.6−71.4)
10	tetrahydrofuranyl	Cl	7.42 (4.38−12.6)	33.2 (28.7−38.4)	3150 (1780−5560)	104 (67.0−162)
11	2-fluoro-4-chloro-phenyl	H	10.2 (9.48−11.1)	8.40 (7.41−9.53)	530 (314−896)	33.9 (21.1−54.5)
12	2-fluoro-4-chloro-phenyl	Cl	64.8 (47.9−87.8)	28.8 (20.8−39.9)	1110 (888−1400)	37.0 (27.1−50.7)
13	2-iodo-benzyl	Cl	64.9 (54.2−77.8)	9.04 (6.37−12.8) pag (75%)	834 (563−1230) pag (76%)	13.3 (6.72−26.5)

[a]All tested compounds are full agonists at the A_1, A_{2A}, and A_{2B} receptors (efficacy ≥90%, unless stated otherwise). All tested compounds are antagonists at the A_3 receptor. pag, partial agonist (% efficacy).

Adenylyl Cyclase Activity. All novel compounds were tested in a functional A_{2B} receptor assay and some showed a moderate potency in stimulation of adenylyl cyclase activity. The most active was **1** (EC_{50} = 347 nM), while the least active was **8** (EC_{50} >30000 nM). It is noteworthy that **4** and **13** behaved as a partial A_{2B} agonist, whereas all other compounds showing measurable potency presented as full agonists (Table 2). All compounds were additionally tested for their functional effect on human A_1, A_{2A}, and A_3 receptors by determination of adenylyl cyclase activity. As expected, all tested compounds were found to be agonists at A_1, and A_{2A} receptors, whereas they were antagonists at the A_3 subtype (Table 2, Figure 1). Interestingly, only compound **13** was found to be a partial agonist at A_{2A}AR. **5**−**11** showed EC_{50} values at hA_1AR ranging from 3.62 to 10.2 nM, whereas the most potent hA_3AR antagonists **3**, **4**, and **13** displayed EC_{50} values at hA_3AR ranging from 6.38 to 13.3 nM. Among the tested compounds, the best EC_{50} values at A_{2A}AR were displayed by **1** (2.36 nM), **2** (3.84 nM), **6** (5.31 nM), **7** (7.71 nM), and **11** (8.04 nM).

Molecular Modeling. To explain the observed binding data from a molecular point of view, we performed a receptor-based molecular modeling study of the potent dual acting hA_1AR and hA_3AR ligands **6** and **13**. Previously reported homology models of the hA_1-[22] and hA_3ARs,[23] built using the agonist-bound A_{2A}AR (PDB: 3QAK)[24] and the antagonist-bound A_{2A}AR (PDB: 3UZC)[25] crystallographic structures as templates, were used to perform docking simulations of each compound. Docking was carried out using the GOLD 5.2.2

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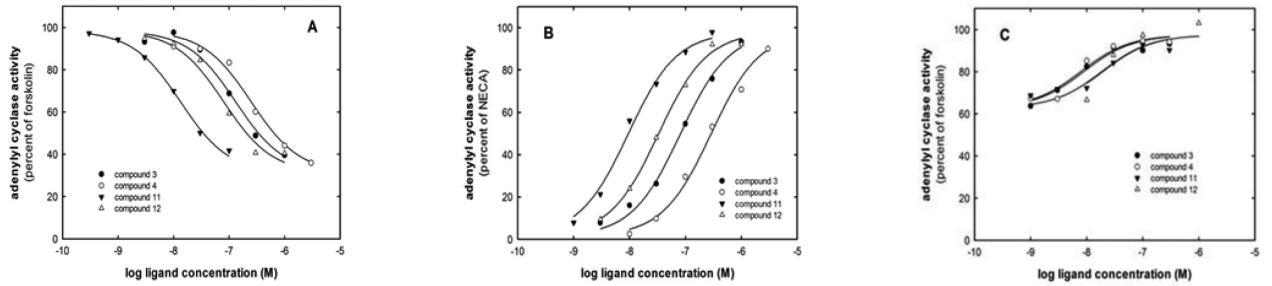

Figure 1. Effect of selected compounds on activity of adenylyl cyclase. **3**, **4**, **11**, and **12** mediate an inhibition of forskolin-stimulated adenylyl cyclase activity via A_1 adenosine receptors (A). They show the same inhibition as the full agonist CCPA (not shown). The A_{2A}-mediated stimulation by these four compounds shown in (B) reaches the level of stimulation with NECA (100%) and thus documents that they act as full agonists at the A_{2A} receptor as well. In contrast, all four compounds present as antagonists at the A_3 receptor. They fully reverse the NECA-induced inhibition of forskolin-stimulated adenylyl cyclase activity (C).

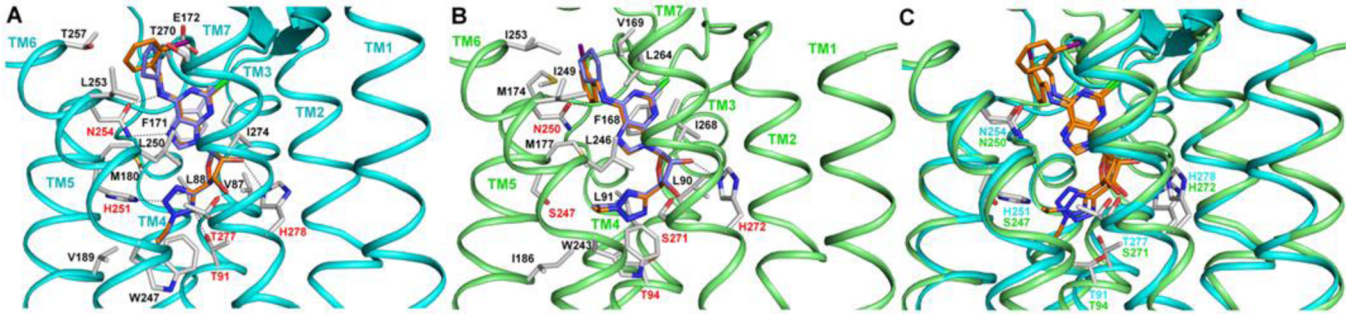

Figure 2. Putative binding modes of selected 5′-*C*-ethyl-tetrazolyl derivatives **6** (slate carbons) and **13** (orange carbons) obtained after docking simulations at the hA_1AR (A, cyan ribbons) and hA_3AR (B, green ribbons) models. Poses are viewed from the membrane side. Ligands and interacting key residues (gray) are represented as stick models. The critical amino acids important for ligand recognition are labeled in red. H-bonding interactions are pictured as dotted black lines. (C) Superimposition of the binding modes of **13** into both hA_1AR and hA_3AR binding pockets. For clarity, only side chain atoms of some residues are shown. Amino acid variations in the ligand binding pocket between hA_1AR and hA_3AR subtypes are shown with cyan and green coloring, respectively.

program[26] in combination with the ChemPLP[27] scoring function (rescoring with ChemScore).[28] A docking pose of **6** and **13** inside both the hA_1AR and hA_3AR binding sites (Figure 2) featured most of the main receptor−ligand interactions observed in the agonist-bound $hA_{2A}AR$ crystal structures.[24,29] These interactions, shown as 2D diagrams (see SI, Figure S1), involved both the adenine core and the ribose ring. In particular, the 3′- and 2′-OH groups formed H-bonds with residues (numbers in parentheses follow the Ballesteros−Weinstein notation)[30] at positions 7.42 (T277 in hA_1AR and S271 in hA_3AR) and 7.43 (H278 in hA_1AR and H272 in hA3AR), respectively. The side chain of N6.55 (N254 in hA1AR and N250 in hA3AR) strongly interacted with these compounds through two H-bonds, including the 6-NH group and the N7 atom of the adenine ring. It is noteworthy that in hA_3AR, only the 6-NH group and not the N7 atom of the adenine ring was in contact with the N250 side chain. Moreover, the adenine ring was anchored inside the binding site by a $\pi-\pi$ stacking interaction with a phenylalanine in EL2 (F171 in hA_1AR and F168 in hA_3AR) and strong hydrophobic contacts with leucine 6.51 (L250 in hA_1AR and L246 in hA_3AR) and isoleucine 7.39 (I274 in hA_1AR and I268 in hA_3AR). Two key interactions observed in the $hA_{2A}AR$ X-ray structures between T88 (3.36) and H250 (6.52) and the 5′-CO-NH-alkyl groups of the cocrystallized agonists NECA and UK-432097[24,29] were maintained at the hA_1AR binding site by these 5′-*C*-ethyl-tetrazolyl nucleosides, while they were absent in the hA_3AR binding site. In particular, in the hA_1AR, the N1 and N3 nitrogens of the 5′-*C*-tetrazole ring of ligands were able to accept H-bonds by $T91^{3.36}$ OH and $H251^{6.52}$ NH hydrogens, respectively. In the case of hA_3AR, the $H^{6.52}$ residue is replaced with a shorter serine ($S247^{6.52}$), which allows the 5′-*C*-tetrazole ring to adopt a 180° flipped-ring orientation with the 2-ethyl substituent pointing toward S247. Consequently, the N3 and N4 sp^2 lone pairs lying in the plane of tetrazole ring were oriented too far away from the $T94^{3.36}$ OH group to accept an effective H-bond. Therefore, it can be hypothesized that the replacement of $H^{6.52}$ in hA_1AR with a serine in hA_3AR and the lack of H-bonds with $T94^{3.36}$ is related to the different efficacy profile of the 5′-*C*-ethyl-tetrazolyl nucleosides at hA_1 and hA_3ARs. This is in line with the previous results of Tosh et al.,[22] who demonstrated that the missing interaction between 4′-truncated nucleosides and $T94^{3.36}$ was related to the lack of receptor activation, indicative of antagonist behavior. Furthermore, the hydrophilic ribose-binding region is critical for activation that likely entails essential residues of TM3, TM6, and TM7 throughout the AR family, as in the $hA_{2A}AR$.[24,29] Multiple H-bonding groups in this region promote agonism at the hA_3AR,[31] such as the interactions of the 5′-CH_2OH of the ribose moiety or the corresponding 5′-CO-NH-alkyl in NECA-like analogues. Thus, loss of the crucial interaction between the 5′-*C*-ethyl-tetrazolyl substituent and $T94^{3.36}$ is expected to reduce AR efficacy, which it clearly does at the hA_3AR. It should also be noted that the residue at position 3.32 (L90) in hA_3AR is a nonconserved hydrophobic and bulky leucine, whereas a smaller valine (V87) is present in the other AR subtypes. Residue 3.32 was near the ribose ring of the docked compounds in both hA_1AR and hA_3AR binding cavities. The

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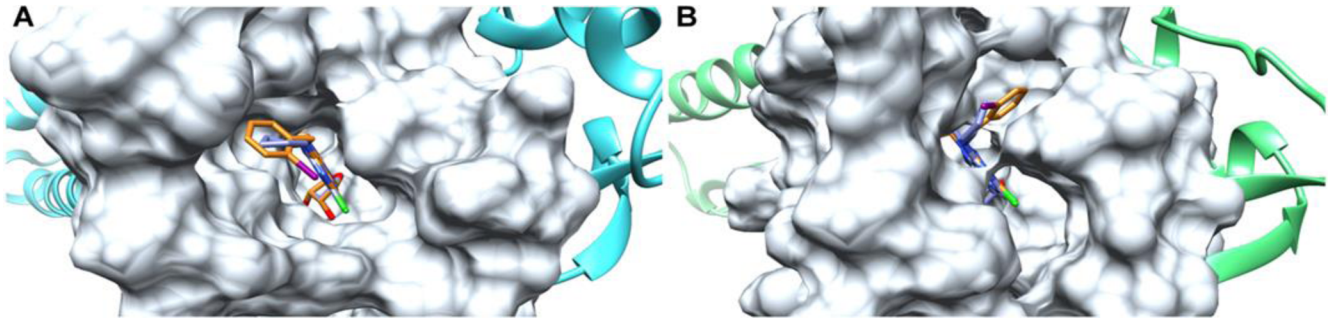

Figure 3. Top view of the docking pose of **6** (slate carbons) and **13** (orange carbons) inside the binding site of the hA_1AR (A, cyan ribbons) and hA_3AR (B, green ribbons) models. A gray Connolly surface of amino acids at the entrance of the binding site is displayed.

longer side chain of $L90^{3.32}$ in hA_3AR compared to $V87^{3.32}$ of hA_1AR, together with the lack of interactions with $T94^{3.36}$, could negatively affect the ability to fully activate the receptor through conformational affects at TM3, TM6, and TM7 caused by the binding of the 5′-*C*-ethyl-tetrazolyl derivatives. The structural basis for the full agonism of **6** at hA_1AR might be related to some interactions formed by the N^6 group at the entrance of the binding site that are important in orienting and stabilizing the compound within the pocket. As shown in Figure 3A, the N^6-cyclopentyl substituent of **6** and the 2-iodo-benzyl substituent of **13** occupied a small hydrophobic pocket, designated "subpocket B" by Tosh et al.,[32] which is located between TM6 and TM7 and lined by residues L253 (6.54), T257 (6.58), T270 (7.35), and at the bottom by L250 (6.51). An optimal occupancy of this pocket by compounds with a sterically more bulky substituent at the N^6-position seems to be required for increased affinity at this receptor subtype (compare **5**−**12** and methyl-substituted compounds **3** and **4** in Table 1). The hA_1AR and hA_3AR differ in the nature of the residues delimiting this pocket, which could explain the different affinity profiles of the 5′-*C*-ethyl-tetrazolyl derivatives at these receptors. In particular, the three residues lining the hydrophobic pocket in the hA_1AR are replaced in the hA_3AR by bulkier side chains I249 (6.54), I253 (6.58), and L264 (7.35). Thus, the steric restriction of this pocket between TM6 and TM7 (Figure 3B) can be the reason for the decreased affinity at hA_3AR of **5**−**12** bearing larger N^6-substituents, as they are too bulky to fit in the pocket. On the other hand, if an extended and more flexible group (e.g., 2-iodobenzyl) is present at the N^6-position, then the substituent slightly shifts within the hA_3AR binding site pointing toward TM5, TM6, and EL2 (Figure 3B), and so it establishes hydrophobic interactions with V169 (EL2), M172 (EL2), M174 (5.35), M177 (5.38), and I253 (6.58). This finding can explain the enhanced hA_3AR affinity of **13**. As shown in Table 1, the N^6-unsubstituted compounds (**1**, **2**) have greater affinity at the hA_1AR than the N^6-methyl compounds (**3**, **4**), while the converse is true at the hA_3AR. The difference in the behavior of these compounds between the two receptor subtypes seems to be related to the fact that at the hA_1AR the exocyclic amino groups of **1** and **2** make two polar interactions with residues $N254^{6.55}$ (H6) and $E172^{5.30}$ (EL2). These polar interactions contribute to the binding energy and seem to be a major selectivity factor that distinguishes hA_1AR and $hA_{2A}AR$ from the hA_3AR subtype. In fact, in this last subtype, a valine residue (V169) takes the place of $E172^{5.30}$ and the above-mentioned stabilizing interaction is lost, thus decreasing the binding affinity of **1** and **2** for hA_3AR. The interaction of $E^{5.30}$ toward the adenine primary amine has been confirmed by the recently published structure of the $A_{2A}AR$ cocrystallized with the two agonists adenosine and NECA.[29] Obviously, a small alkyl chain at the N^6-position, such as a methyl group, prevents the secondary amine from interacting with the $E172^{5.30}$ side chain because the only available hydrogen atom is already engaged in an H-bonding interaction with $N254^{6.55}$, and this explains the lower affinity of **3** and **4** at hA_1AR. In contrast, at hA_3AR, the N^6-methyl groups of **3** and **4** were involved in a stabilizing hydrophobic interaction with V169 (EL2), which is missing at hA_1AR because this amino acid is replaced by the polar residue E172. It appears that this is why **3** and **4** maintain their binding affinity at hA_3AR but reduce it at hA_1AR.

CONCLUSIONS

In summary, a series of N^6-substituted-5′-*C*-(2-ethyl-2*H*-tetrazol-5-yl)-adenosine and 2-chloro-adenosine derivatives were synthesized in order to study the structure−activity relationships of this class of nucleosides. We reported for the first time that N^6-substituted-5′-*C*-(2-ethyl-2*H*-tetrazol-5-yl)-adenosine derivatives acted as potent dual hA_1AR full agonists and hA_3AR antagonists. The combination of 5′-*C*-ethyl-tetrazolyl with the appropriate N^6-substitution in adenosine derivatives provides improved affinity for both hA_1AR and hA_3AR. A methyl or a 3-iodo-benzyl group at the N^6 position of 5′-*C*-2-ethyl-2*H*-tetrazolyl-adenosine derivatives were beneficial for high binding affinity at the hA_3AR, whereas a cycloalkyl-, bicycloalkyl- or an aryl group conferred subnanomolar affinity for hA_1AR. Unexpectedly, an unsubstituted amino group at the N^6 position of 5′-*C*-2-ethyl-2*H*-tetrazolyl-adenosine derivatives (**1**, **2**), maintains subnanomolar A_1AR and low nanomolar A_3AR affinities. Additionally, **1** and **2** displayed significant binding affinity also at the $hA_{2A}AR$, resulting in nonselective ligands. A molecular modeling study fully rationalized the mixed activity of these novel dual hA_1AR agonists and hA_3AR antagonists. This feature might be advantageous for the treatment of glaucoma and other diseases (e.g., epilepsy).

EXPERIMENTAL SECTION

Chemistry. All compounds were analyzed by ^1H and ^{13}C NMR, and MASS. The purity of final compounds was ≥95% as measured by combustion analysis and HPLC. Full experimental procedures can be found in the SI.

*Analytical Data for (2R,3S,4R,5R)-2-(2-Ethyl-2H-tetrazol-5-yl)-5-(6-(methylamino)-9H-purin-9-yl)tetrahydrofuran-3,4-diol (**3**).* Yield 86%. ^1H NMR (400 MHz, DMSO-d_6): δ 1.47 (t, J = 7.3 Hz, 3H, CH_2CH_3), 2.91 (brs, 3H, CH_3), 4.53−4.59 (m, 1H, H-4′), 4.72 (q, J = 7.3 Hz, 2H, CH_2CH_3), 4.81 (q, J = 5.3 Hz, 1H, H-3′), 5.18 (d, J = 4.3 Hz, 1H, H-5′), 5.75 (d, J = 5.5 Hz, 1H, OH), 5.83 (d, J = 5.6 Hz, 1H,

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OH), 6.10 (d, J = 4.7 Hz, 1H, H-2′), 7.78 (brs, 1H, NH), 8.11 (s, 1H, H-2), 8.38 (s, 1H, H-8). ^{13}C NMR (400 MHz, DMSO-d_6): δ 14.21, 28.32, 48.38, 73.55, 73.79, 77.49, 87.84, 118.31, 138.92, 151.05, 153.74, 156.78, 164.26 ppm. MS (API-ESI): m/z 334.13 $[M + H]^+$. Anal. Calcd for ($C_{13}H_{17}N_9O_3$) C, 44.95; H, 4.93; N, 36.29. Found: C, 44.92; H, 4.89; N, 36.31. HPLC purity: t_R = 3.37, 99.81%

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details of chemical synthesis, computational and biological tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

A_1AR, A_1 adenosine receptor; A_{2A}AR, A_{2A} adenosine receptor; A_{2B}AR, A_{2B} adenosine receptor; A_3AR, A_3 adenosine receptor

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