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2- and 8-alkynyl-9-ethyladenines: Synthesis and biological activity at human and rat adenosine receptors

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Abstract

The synthesis of a series of 9-ethyladenine derivatives bearing alkynyl chains in 2- or 8-position was undertaken, based on the observation that replacement of the sugar moiety in adenosine derivatives with alkyl groups led to adenosine receptor antagonists. All the synthesized compounds were tested for their affinity at human and rat A_1, A_{2A}, and A_3 adenosine receptors in binding assays; the activity at the human A_{2B} receptor was determined in adenylyl cyclase experiments. Biological data showed that the 2-alkynyl derivatives possess good affinity and are slightly selective for the human A_{2A} receptor. The same compounds tested on the rat A_1 and A_{2A} subtypes showed in general lower affinity for both receptors. On the other hand, the affinity of the 8-alkynyl derivatives at the human A_1, A_{2A}, and A_{2B} receptors proved to be lower than that of the corresponding 2-alkynyl derivatives. On the contrary, the affinity of the same compounds for the human A_3 receptor was improved, resulting in A_3 selectivity. As in the case of the 2-alkynyl-substituted compounds, the 8-alkynyl derivatives showed decreased affinity for rat receptors. However, it is worthwhile to note that the 8-phenylethynyl-9-ethyladenine was the most active compound of the two series (K_i in the nanomolar range) at both thehuman and rat A_3 subtype. Docking experiments of the 2- and 8-phenylethynyl-9-ethyladenines, at a rhodopsin-based homology model, gave a rational explanation of the preference of the human A_3 receptor for the 8-substituted compound.

Introduction

Adenosine is an autacoid involved in the regulation of many aspects of cellular metabolism [1] and mediates its effects through the activation of at least four human receptors (P1), belonging to the superfamily of G protein-coupled receptors, which have been recently cloned [2] and classified as A_1, A_{2A}, A_{2B}, and A_3 [3]. All subtypes have been cloned from a variety of species including the rat and human. Species differences for the A_3 adenosine receptor (AdoR) are larger than for other AR subtypes, particularly between rodent and human receptors (only 74% sequence identity between rat and human A_3 amino acid sequence) [4]. This results in different affinities of ligands – particularly antagonists – for rat *versus* human A_3 receptors [5, 6]. During the past decades many efforts have been directed toward the discovery of potent and selective adenosine receptor ligands aimed at finding novel drugs [7].

Most adenosine agonists possess a structure very close to that of the natural ligand adenosine; in contrast a wide range of structural classes have been characterized as adenosine receptor antagonists and developed as potential therapeutic agents for CNS disorders, inflammatory diseases, asthma, kidney failure and ischaemic injuries [8].

However, only a few xanthine antagonists as caffeine and theophylline have been approved as drugs for their CNS stimulating, diuretic, and bronchodilating effects, respectively [8, 9].

In many papers it has been demonstrated that introduction of alkynyl chains in the 2-position of adenosine derivatives led to compounds endowed with high affinity at all adenosine receptors (AdoRs) [10–15], whereas the introduction of the same alkynyl chains in the 8-position resulted in 8-alkynyladenosines, which were unable to stimulate [^{35}S]GTPγS binding, and inhibited that stimulated by NECA, yielded A_3 antagonists [16, 17].

Moreover, we have published the synthesis and activities for the human AdoR subtypes of a series of 9-ethyladenines, substituted at the 2-, 6- and 8-positions, which behaved as AdoR antagonists [18–20]. In fact, replacement of the sugar moiety of adenosine with a methyl group led to a rather unselective antagonist of AdoR and adenine itself is a weak adenosine antagonist [21].

Recently, substituted adenine derivatives, prepared as hypoglycemic agents, were found to possess high potency at the A_{2B} AdoR subtype [22], whereas novel substituted

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N^6-cyclopentyladenine derivatives were characterized as neutral antagonists endowed with high affinity for the A_1 AdoR [23].

Hence, starting from these observations and in order to widen our study on 9-ethyladenine derivatives, a series of 9-ethyladenines bearing alkynyl chains at the 2- or 8-position were synthesized and tested at human and rat AdoR subtypes.

Materials and methods

Chemistry

*Synthesis of the 2- and 8-alkynyl-9-ethylpurines **2–10**, **12–19**, and **22***

The synthesis of the 2-alkynyl-9-ethyladenines **2–10** and 8-alkynyl-9-ethyladenines **12–19**, was carried out starting from 2-iodo-9-ethyladenine (**1**; [18] or 8-bromo-9-ethyladenine (**11**; [18]), respectively, and are reported in Schemes 1 and 2. In order to obtain the final products compound **1** or **11** was reacted with the suitable terminal alkynes using a modification of the palladium catalyzed cross-coupling reaction [18].

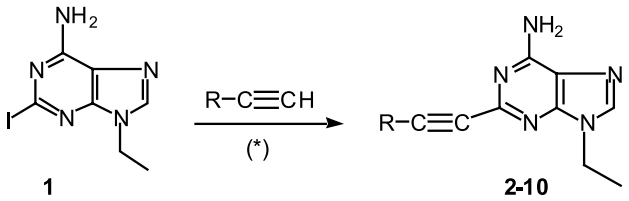
However, for the synthesis of the 8-alkynyl derivative **22**, 8-iodo-9-ethyladenine (**21**), obtained through the iodination of the 9-ethyladenine (**20**) [18] with iodine and lithiumdiisopropylamide (LDA), was used as the starting material.

This choice was due to the fact that reaction of 8-bromo-9-ethyladenine (**11**) with (*R*,*S*)-phenylhydroxypropyne failed to give the corresponding 8-phenylhydroxypropynyl analogue, while the reaction was successful when the 8-iodo analogue was used as starting material.

The synthesis of the derivatives **3**, **9**, **13**, and **19** has been already published [18].

General synthetic procedures

Melting points were determined with a Büchi apparatus and areuncorrected. ^{1}H NMR spectra were obtained with Varian Gemini 200 MHz or a Varian VXR 300-MHz spectrometer; δ in ppm, J in Hz. All exchangeable protons were confirmed by addition of D_2O. TLC were carried out on precoated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. Elemental analyses were determined on Carlo Erba model 1106 analyser and are within ±0.4% of theoretical values.



2; R = -$(CH_2)_2$-CH_3
3; R = -$(CH_2)_3$-CH_3
4; R = -$(CH_2)_4$-OH
5; R = -cyclohexyl
6; R = 1-cyclohexenyl
7; R = -C_6H_5
8; R = -C_6H_4-*p*-$COCH_3$
9; R = -$(CH_2)_2$-C_6H_5
10; R = (*R,S*) -CH(OH)-C_6H_5

(*) CuI, $[(C_6H_5)_3P]_2PdCl_2$, Et_3N

Scheme 1

*General method for the synthesis of the 2- or 8-(ar)alkynyl-9-ethyladenines **2, 4–8, 10, 12, 14–18** and **22***

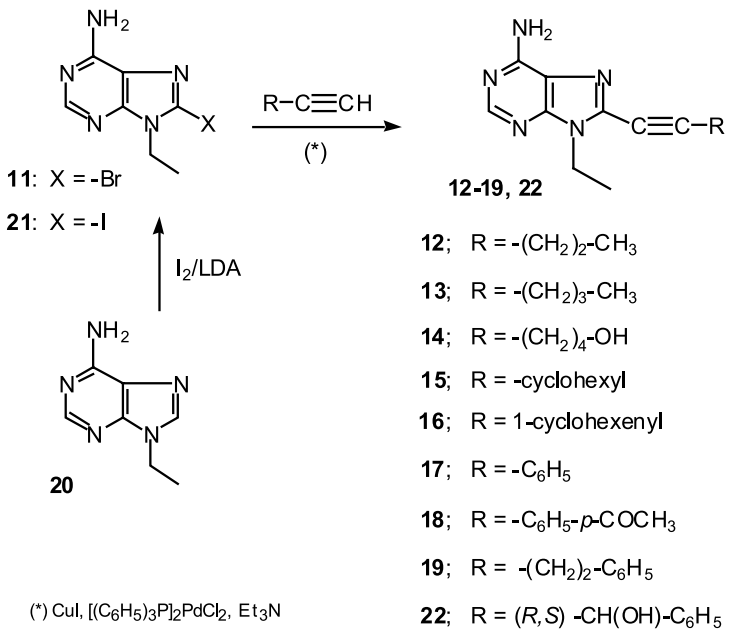
To a solution of **1**, **11** or **21** (0.84 mmol) in dry DMF and CH_3CN [15 ml (1:2)], bis(triphenylphosphine)palladium dichloride (12 mg, 0.017 mmol) and CuI (0.84 mg, 0.004 mmol), Et_3N (3.4 ml), and the appropriate terminal alkyne (4.2 mmol) were added. The reaction mixture was stirred under an atmosphere of N_2 at room temperature for the time reported for each compound. The solvent was removed *in vacuo* and the residue was chromatographed on a silica gel column or TLC plates eluting with a suitable mixture of solvents to give the desired derivatives **2**, **4–8**, **10**, **12**, **14–18** and **22**.

*9-Ethyl-2-pent-1-ynyl-9H-purin-6-ylamine (**2**).* The reaction of **1** with 1-pentyne for 24 h, followed by chromatography on a silica gel column eluting with $CHCl_3-cC_6H_{12}-CH_3OH$ (88:10:2), gave **2** as a chromatographically pure amorphous solid. Yield 51%, m.p. 105 °C (dec); ^{1}H NMR (DMSO-d_6) δ 0.97 (t, 3H, J = 7.3 Hz, CH_2CH_3); 1.35 (t, 3H, J = 7.0 Hz, NCH_2CH_3); 1.54 (m, 2H, CH_2CH_3); 2.35 (t, 2H, J = 7.0 Hz, C ≡ CCH_2); 4.11 (q, 2H, J = 7.2 Hz, NCH_2); 7.24 (s, 2H, NH_2); 8.15 (s, 1H, H-8). Anal. Calcd. for $C_{12}H_{15}N_5$ (229.3): C, 62.86; H, 6.59; N, 30.54. Found: C, 62.98; H, 6.73; N, 30.25.

*6-(6-Amino-9-ethyl-9H-purin-2-yl)-hex-5-yn-1-ol (**4**).* The reaction of **1** with 5-hexynyl-1-ol for 48 h, followed by chromatography on a silica gel column eluting with $CH_2Cl_2-cC_6H_{12}-CH_3OH$ (82:10:8), gave after crystallization from EtOH, **4**, as white crystals. Yield 83%, m.p. 138–140 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.35 (t, 3H, J = 7.0 Hz, CH_3); 1.54 (m, 4H, CH_2CH_2); 2.39 (m, 2H, C ≡ CCH_2); 3.41 (m, 2H, CH_2OH); 4.11 (q, 2H, J = 7.2 Hz, NCH_2); 4.42 (t, 1H, J = 5.3 Hz, CH_2OH); 7.26 (s, 2H, NH_2); 8.16 (s, 1H, H-8). Anal. Calcd. for $C_{13}H_{17}N_5O$ (259.3): C, 60.21; H, 6.61; N, 27.01. Found: C, 60.53; H, 6.79; N, 26.79.

*2-Cyclohexylethynyl-9-ethyl-9H-purin-6-ylamine (**5**).* The reaction of **1** with cyclohexylacetylene for 20 h, followed by chromatography on a silica gel column eluting with $CHCl_3-CH_3OH$ (99:1), gave, after crystallization from EtOH, **5**, as white crystals. Yield 62%, m.p. 203–205 °C; ^{1}H NMR (DMSO-d_6) δ 1.22–1.87 (m, 8H, CH_3 and H-cyclohexyl); 2.59 (m, 1H, C ≡ CCH); 4.12 (q, 2H, J = 7.2 Hz, NCH_2); 7.26 (s, 2H, NH_2); 8.16 (s, 1H, H-8). Anal. Calcd. for $C_{15}H_{19}N_5$ (269.3): C, 66.89; H, 7.11; N, 26.00. Found: C, 67.13; H, 7.45; N, 25.77.

*2-Cyclohex-1-enylethynyl-9-ethyl-9H-purin-6-ylamine (**6**).* The reaction of **1** with 1-ethynylcyclohexene for 20 h, followed by chromatography on a silica gel column eluting



Scheme 2

with $CHCl_3$–CH_3OH (98:2), gave, after crystallization from MeOH, **6**, as white crystals. Yield 90%, m.p. > 250 °C; ^{1}H NMR (DMSO-d_6) δ 1.39 (t, 3H, J = 7.2 Hz, CH_3); 1.62 (m, 4H, H-cyclohexenyl); 2.15 (m, 4H, H-cyclohexenyl); 4.15 (q, 2H, J = 7.5 Hz, NCH_2); 6.30 (m, 1H, CH = C); 7.35 (bs, 2H, NH_2); 8.21 (s, 1H, H-8). Anal. Calcd. for $C_{15}H_{17}N_5$ (267.3): C, 67.39; H, 6.41; N, 26.20. Found: C, 67.68; H, 6.80; N, 26.01.

9-Ethyl-2-phenylethynyl-9H-purin-6-ylamine (7). The reaction of **1** with 1-phenylacetylene for 6 h, followed by chromatography on a silica gel column eluting with CH_2Cl_2–CH_3OH (99.5:0.5), gave, after crystallization from EtOH, **7**, as white crystals. Yield 85%, m.p. 250 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.38 (t, 3H, J = 7.5 Hz, CH_3); 4.16 (q, 2H, J = 7.2 Hz, CH_2); 7.43 (m, 5H, H-Ph and NH_2); 7.58 (m, 2H, H-Ph); 8.23 (s, 1H, H-8). Anal. calcd. for $C_{15}H_{13}N_5$ (263.3): C, 68.42; H, 4.98; N, 26.60. Found: C, 68.78; H, 5.27; N, 26.35.

1-[4-(6-Amino-9-ethyl-9H-purin-2-ylethynyl)-phenyl]-ethanone (8). The reaction of **1** with 1-(4-ethynylphenyl) ethanone for 6 h, followed by chromatography on a silica gel column eluting with CH_2Cl_2–cC_6H_{12}–CH_3OH (85:10:15), gave, after crystallization from EtOH, **8**, as white crystals. Yield 67%, m.p. > 230 °C; ^{1}H NMR (DMSO-d_6) δ 1.39 (t, 3H, J = 7.3 Hz, CH_2CH_3); 2.59 (s, 3H, COCH_3); 4.17 (q, 2H, J = 7.5 Hz, CH_2CH_3); 7.46 (s, 2H, NH_2); 7.72 (d, 2H, J = 8.2 Hz, H-Ph); 7.98 (d, 2H, J = 8.2 Hz, H-Ph); 8.25 (s, 1H, H-8). Anal. Calcd. for $C_{17}H_{15}N_5O$ (305.3): C, 66.87; H, 4.95; N, 22.94. Found: C, 66.98; H, 5.24; N, 22.78.

3-(6-Amino-9-ethyl-9H-purin-2-yl)-1-phenyl-prop-2-yn-1-ol (10). The reaction of **1** with (R,S)-1-phenyl-2-propyn-1-ol for 20 h, followed by chromatography on a silica gel column eluting with $CHCl_3$–CH_3OH (96:4), gave, after crystallization from EtOH, **10**, as white crystals. Yield 57%, m.p. > 250 °C; ^{1}H NMR (DMSO-d_6) δ 1.36 (t, 3H, J = 6.9 Hz, CH_3); 4.13 (q, 2H, J = 7.2 Hz, CH_2); 5.58 (d, 1H, J = 6.0 Hz, CHOH); 6.24 (d, 1H, J = 6.0 Hz, CHOH); 7.36 (m, 5H, H-Ph and NH_2); 7.51 (d, 2H, J = 8.4 Hz, H-Ph); 8.19 (s, 1H, H-8). Anal. calcd. for $C_{16}H_{15}N_5O$ (293.3): C, 65.52; H, 5.15; N, 23.88. Found: C, 65.90; H, 5.29; N, 23.61.

9-Ethyl-8-pent-1-ynyl-9H-purin-6-ylamine (12). The reaction of **11** with 1-pentyne for 48 h, followed by chromatography on a silica gel column eluting with $CHCl_3$–CH_3OH (98:2), gave, after crystallization from MeOH, **12**, as white crystals. Yield 73%, m.p. 182–184 °C; ^{1}H NMR (DMSO-d_6) δ 1.04 (t, 3H, J = 7.2 Hz, CH_2CH_3); 1.35 (t, 3H, J = 7.2 Hz, NCH_2CH_3); 1.64 (m, 2H, CH_2CH_3); 2.57 (t, 2H, J = 6.9 Hz, C ≡ CCH_2); 4.20 (q, 2H, J = 7.2 Hz, NCH_2); 7.37 (bs, 2H, NH_2); 8.16 (s, 1H, H-2). Anal. Calcd. for $C_{12}H_{15}N_5$ (229.3): C, 62.86; H, 6.59; N, 30.54. Found: C, 63.14; H, 6.84; N, 30.27.

6-(6-Amino-9-ethyl-9H-purin-8-yl)-hex-5-yn-1-ol (14). The reaction of **11** with 5-hexynyl-1-ol for 48 h, followed by chromatography on a silica gel column eluting with $CHCl_3$–CH_3OH (95:5), gave, after crystallization from MeOH, **14**, as white crystals. Yield 73%, m.p. 162–164 °C; ^{1}H NMR (DMSO-d_6) δ 1.35 (t, 3H, J = 7.2 Hz, CH_3); 1.63 (m, 4H, CH_2CH_2); 2.60 (t, 2H, J = 6.6 Hz, C ≡ CCH_2); 3.46 (m, 2H, CH_2OH); 4.20 (q, 2H, J = 7.5 Hz, NCH_2); 4.48 (t, 1H, J = 5.1 Hz, OH); 7.36 (bs, 2H, NH_2); 8.16 (s, 1H, H-2). Anal. Calcd. for $C_{13}H_{17}N_5O$ (259.3): C, 60.21; H, 6.61; N, 27.01. Found: C, 60.49; H, 6.83; N, 26.76.

8-Cyclohexenylethynyl-9-ethyl-9H-purin-6-ylamine (15). The reaction of **11** with cyclohexylacetylene for 24 h, followed by chromatography on a silica gel column eluting with $CHCl_3-cC_6H_{12}-CH_3OH$ (55:40:5), gave after crystallization from MeOH, **15**, as white crystals. Yield 85%, m.p. 247 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.26–1.83 (m, 13H, H-cyclohexyl and CH_3); 2.79 (m, 1H, CH–C≡C); 4.15 (q, 2H, J = 7.1 Hz, N–CH_2); 7.33 (bs, 2H, NH_2); 8.11 (s, 1H, H-2). Anal. Calcd. for $C_{15}H_{19}N_5$ (269.3): C, 66.89; H, 7.11; N, 26.00. Found: C, 67.25; H, 7.41; N, 25.79.

8-Cyclohex-1-enylethynyl-9-ethyl-9H-purin-6-ylamine (16). The reaction of **11** with 1-ethynylcyclohexene for 36 h, followed by chromatography on a silica gel column eluting with $CHCl_3-CH_3OH$ (99:1), gave, after crystallization from MeOH, **16**, as white crystals. Yield 65%, m.p. 160 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.36 (t, 3H, J = 7.2 Hz, CH_3); 1.64 (m, 4H, H-cyclohexenyl); 2.21 (m, 4H, H-cyclohexenyl); 4.21 (q, 2H, J = 7.2 Hz, NCH_2); 6.47 (m, 1H, CH = C); 7.43 (bs, 2H, NH_2); 8.18 (s, 1H, H-2). Anal. Calcd. for $C_{15}H_{17}N_5$ (267.3): C, 67.39; H, 6.41; N, 26.20. Found: C, 67.74; H, 6.56; N, 26.03.

9-Ethyl-8-phenylethynyl-9H-purin-6-ylamine (17). The reaction of **11** with 1-phenylacetylene for 72 h, followed by chromatography on a silica gel column eluting with $CH_2Cl_2-cC_6H_{12}-CH_3OH$ (85:10:5), gave, after crystallization from MeOH, **17**, as white crystals. Yield 52%, m.p. 249–251 °C; ^{1}H NMR (DMSO-d_6) δ 1.43 (t, 3H, J = 7.2 Hz, CH_3); 4.33 (q, 2H, J = 7.3 Hz, NCH_2); 7.53 (m, 5H, H-Ph and NH_2); 7.72 (m, 2H, H-Ph); 8.21 (s, 1H, H-2). Anal. Calcd. for $C_{15}H_{13}N_5$ (263.3): C, 68.42; H, 4.98; N, 26.60. Found: C, 68.77; H, 5.35; N, 26.43.

1-[4-(6-Amino-9-ethyl-9H-purin-8-ylethynyl)-phenyl]-ethanone (18). The reaction of **11** with 1-(4-ethynylphenyl) ethanone for 16 h, followed by chromatography on a silica gel column eluting with $CHCl_3-CH_3OH$ (90:10), gave, after crystallization from MeOH, **18**, as white crystals. Yield 40%, m.p. > 250 °C; ^{1}H NMR (DMSO-d_6) δ 1.42 (t, 3H, J = 6.9 Hz, CH_2CH_3); 2.63 (s, 3H, $COCH_3$); 4.34 (q, 2H, J = 6.9 Hz, CH_2); 7.52 (bs, 2H, NH_2); 7.85 (d, 2H, J = 8.4 Hz, H-Ph); 8.06 (d, 2H, J = 8.4 Hz, H-Ph); 8.21(s, 1H, H-2). Anal. Calcd. for $C_{17}H_{15}N_5O$ (305.3): C, 66.87; H, 4.95; N, 22.94. Found: C, 67.23; H, 5.25; N, 22.78.

3-(6-Amino-9-ethyl-9H-purin-8-yl)-1-phenyl-prop-2-yn-1ol (22). The reaction of **21** with (*R,S*)-1-phenyl-2-propyn-1-ol for 36 h, followed by chromatography on a silica gel column eluting with $CHCl_3-CH_3OH$ (96:4), gave, after crystallization from $CH_3CN-MeOH$, **22**, as white crystals. Yield 13%, m.p. 226–228 °C; ^{1}H NMR (DMSO-d_6) δ 1.33 (t, 3H, J = 7.2 Hz, CH_3); 4.21 (q, 2H, J = 7.2 Hz, NCH_2); 5.77 (d, 1H, J = 6.1 Hz, CHOH); 6.49 (d, 1H, J = 6.1 Hz, CHOH); 7.41 (m, 5H, H-Ph and NH_2); 7.59 (m, 2H, H-Ph); 8.19 (s, 1H, H-2). Anal. Calcd. for $C_{16}H_{15}N_5O$ (293.3): C, 65.52; H, 5.15; N, 23.88. Found: C, 65.81; H, 5.27; N, 23.50.

9-Ethyl-8-iodo-9H-purin-6-ylamine (21)
To a solution of 2.2 ml (15.7 mmol) of freshly distilled diisopropylamime and 6.0 ml of dry THF, in a three necked round bottom flask, under a flux of N_2, were added dropwise 9.8 ml (15.7 mmol) of butyllithium (1.6 M in hexane). The mixture was kept under stirring at r.t. for 15 min. After that, the mixture was cooled at −70 °C and 500 mg (3.1 mmol) of **20**, dissolved in 10 ml of dry THF, were added, and after 1 h a solution of iodine (4.9 mmol) in 10 ml of dry THF was added dropwise.

After 1 h more at −70 °C 4 drops of glacial acetic acid and 3 ml of methanol were added. The mixture was allowed to warm at r. t., the solvent was removed *in vacuo* and the residue was chromatographed on a silica gel column eluting with $CHCl_3-CH_3OH$ (98:2) to give, after crystallization from MeOH, **21**, as white crystals. Yield 56%, m.p. 247–249 °C; ^{1}H NMR (DMSO-d_6) δ 1.31 (t, 3H, J = 7.2 Hz, CH_3); 4.12 (q, 2H, J = 7.2 Hz, CH_2); 7.33 (br s, 2H, NH_2); 8.08 (s, 1H, H-2). Anal. Calcd. for $C_7H_8IN_5$ (289.1): C, 29.08; H, 2.79; N, 24.23. Found: C, 29.44; H, 2.97; N, 24.02.

Biological evaluation

Binding studies and adenylyl cyclase activity at human adenosine receptors
The radioligand binding experiments were carried out exactly as described previously [24]. For A_1 adenosine receptor binding 1 nM [^{3}H]CCPA was used as a radioligand, whereas 30 and 10 nM [^{3}H]NECA was used for A_{2A} and A_3 receptors, respectively. Non specific binding was determined in the presence of 1 mM theophylline (A_1) or 100 μM R-PIA (A_{2A} and A_3). K_i values were calculated from competition curves by nonlinear curve fitting with the program SCTFIT [25]. CHO cells stably transfected with human adenosine receptors were grown adherently and maintained in Dulbecco's Modified Eagles Medium with nutrient mixture F12 (DMEM/F12) without nucleosides, containing 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM) and Geneticin (G-418, 0.2 mg/ml) at 37 °C in 5% CO_2/95% air as described earlier [24].

For radioligand binding studies and measurement of adenylyl cyclase activity crude membrane fractions were prepared from fresh or frozen cells with two different protocols which were described recently [24].

Determination of adenylyl cyclase activity followed the procedure described by [24]. IC_{50} values for the inhibition of cyclase stimulated with 5 μM NECA were calculated with the Hill equation and converted to K_i values with the Cheng and Prusoff equation [24]. The Hill slopes were near unity suggesting a competitive interaction of the antagonists tested.

Binding studies at rat adenosine receptors
A_1 and A_{2A} receptor binding: Displacement of [^{3}H]CHA (31 Ci/mmol) from A_1 adenosine receptor in rat cortical membranes and of [^{3}H]CGS 21680 (42.1 Ci/mmol) from rat striatal membranes were performed as described [26].

[^{125}I]AB-MECA binding to A_3 receptor of rat testis membranes was performed in 50 mM Tris, 10 mM $MgCl_2$ and 1 mM EDTA buffer (pH 7.4) containing 0.2 mg of proteins and 25 nM DPCPX, to selectively block A_1 receptor subtypes. Incubations were carried out for 90 min at 25 °C. Non-specific binding was determined in the presence of 50 μM R-PIA and accounted to 30% of total binding. Binding reaction was terminated by rapid filtration through a Whatman GF/C filter, washing three times with 5 ml of ice-cold buffer.

All compounds were routinely dissolved in DMSO and diluted with assay buffer to the final concentration, where the amount of DMSO never exceeded 2%. At least 6 different concentrations of each compound were used and IC_{50} values were computer-generated using non-linear regression formula on a computer program (GraphPad, San Diego, CA). IC_{50} values were converted to K_i values, knowing the K_d values of radioligands in the different tissues and using the Cheng and Prusoff equation [27]. The dissociation constant (K_d) of [^{3}H]CHA, [^{3}H]CGS 21680 and [^{125}I]AB-MECA were 1.2, 10 and 1.35 nM, respectively.

Molecular modeling studies

All the calculations were carried out utilizing the HyperChem 7.2 program (Hypercube, Gainesville, Florida, USA). For molecular mechanics calculations the AMBER 96 [28] force field was utilized with a distance-dependent dielectric constant scaled by a factor of 4. All the energy minimizations were carried out employing the Polak–Ribiere conjugate gradient. All molecular dynamics simulations were carried at a constant temperature of 298 K, using a timestep of 1 fs. Before the simulation, the system, virtually at 0 K, was slowly (1 ps) heated until it reached the simulation temperature.

The human A_3 model used for the docking experiments was the rhodopsin-based homology model previously constructed by us [17].

2-Phenylethynyl-9-ethyladenine (**7**) and 8-phenylethynyl-9-ethyladenine (**17**) were docked into the receptor helical bundle by modifying the ligands in A_3/2-phenylethynyladenosine or A_3/8-phenylethynyladenosine complex [17], respectively.

After applying a harmonic restraint of 7 kcal/mol to the backbone atoms of the protein, the receptor/ligand complexes were submitted to energy minimization (RMS < 0.5 kcal/mol/Å), 10 ps of molecular dynamics, and energy minimization (RMS < 0.001 kcal/mol/Å).

Results and discussion

Binding studies

All the compounds were evaluated at the human recombinant adenosine receptors, stably transfected into Chinese hamster ovary (CHO) cells, utilizing radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assay (A_{2B}). Receptor binding affinity was determined using [^{3}H]CCPA (2-chloro-N^6-cyclopentyladenosine) as the radioligand for A_1 receptors, whereas [^{3}H]NECA (5′-*N*-ethylcarboxamidoadenosine) was used for the A_{2A} and A_3 subtypes [24]. In the case of A_{2B} receptors K_i values were calculated from IC_{50} values determined by inhibition of NECA-stimulated adenylyl cyclase activity.

The compounds were also tested for their affinity at rat cortex A_1, rat striatum A_{2A}, and rat testis A_3 subtypes, using [^{3}H]CHA (N^6-cyclopentyladenosine), [^{3}H]CGS 21680 {2-*p*-[(2-carboxyethyl)phenylethylamino]-*N*-ethylcarboxamidoadenosine}, and [^{125}I]ABMECA [*N*^{6}-(4-amino-3-iodobenzyl)adenosine-5′-*N*-methyluronamide] as the radioligands, respectively.

K_i values are in μM with 95% confidence intervals in parentheses. The results are shown in Table 1.

Biological data showed that the 2-alkynyl derivatives possess good affinity at all human adenosine receptors and are slightly selective for the A_{2A} subtype.

Compounds **5** and **10** bearing a cyclohexylethyne and a phenylhydroxypropyne in 2-position, respectively, resulted in the most active of the series with K_i in the low nanomolar range both at human A_1 and A_{2A} receptors (**5**; K_i A_1 = 0.080 μM, K_i A_{2A} = 0.037 μM, and **10**; K_i A_1 = 0.098 μM, K_i A_{2A} = 0.035 μM).

At the A_{2B} receptor the derivatives bearing linear chains in 2-position showed functional activity in the μM range (**2**; K_i A_{2B} = 17 μM, **3**; K_i A_{2B} = 12 μM, and **4**; K_i A_{2B} = 19 μM), while the presence of sterically hindered substituents in the same position was detrimental for the activity. However, the presence of a phenylhydroxypropynyl chain seems to facilitate the interaction with the A_{2B} subtype, in fact the 2-phenylhydroxypropynyl-9-ethyladenine resulted the most active compound of the two series (**10**; K_i A_{2B} = 1.4 μM). This finding is in agreement with our previous results obtained with the corresponding adenosine derivative. In fact, the (*R*,*S*)-2-phenyhydroxypropynyl-*N*-ethylcarboxamidoadenosine [(*R*,*S*)-PHPNECA, EC_{50} A_{2B} = 1.1 μM] resulted in one of the most potent agonist with nucleoside structure at human A_{2B} receptors reported so far [14, 29].

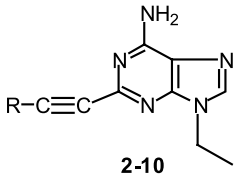
The affinity of the 2-alkynyl derivatives for the A_3 subtype was lower than that at A_1 and A_{2A} receptors.

The same compounds, tested on the rat A_1 and A_{2A} subtypes, showed in general lower affinity for the rat A_{2A} receptor, while compounds **7**–**9**, bearing an aromatic ring in the 2-position, showed slightly higher affinity at the rat A_1 receptor, compared to the corresponding human receptor, hence resulting slightly A_1 selective (**7**; K_i A_1 (r) = 0.43 μM vs K_i A_1 (h) = 0.77 μM, **8**; K_i A_1 (r) = 1.9 μM vs K_i A_1 (h) = 8.3 μM, and **9**; K_i A_1 (r) = 0.15 μM vs K_i A_1 (h) = 0.21 μM,). As in the case of human receptors, compounds **5** and **10** showed the highest affinity both at rat A_1 and A_{2A} subtypes (**5**; K_i A_1 = 0.072 μM, K_i A_{2A} = 0.069 μM, and **10**; K_i A_1 = 0.14 μM, K_i A_{2A} = 0.14 μM).

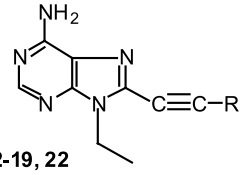
At the rat A_3 receptor these compounds showed comparable affinity to that at human one.

As far as the 8-alkynyl derivatives, their affinity at the human A_1, A_{2A}, and A_{2B} receptors proved to be lower than

Table 1. Affinities of 2- and 8-alkynyl-9-ethyladenines in radioligand binding assays at human and rat A_1, A_{2A}, and A_3 adenosine receptors and effects on adenylate cyclase activity at human A_{2B} adenosine receptor.[a]



2-10



12-19, 22

Cpd	*R*	K_i, μM or % inhibition			
		K_i (A_1)[b]	K_i (A_{2A})[c]	K_i (A_{2B})[d]	K_i (A_3)[e]
2	–$(CH_2)_2$–CH_3	1.2 (h) (1.0–1.5) 4.6 (r) (3.9–5.7)	0.76 (h) (0.43–1.3) 2.9 (r) (2.5–3.4)	17 (h) (11–27)	2.1 (h) (1.2–3.9) 8% at 10 μM (r)
3	–$(CH_2)_3$–CH_3	0.55 (h) (0.24–1.2) 0.98 (r) (0.59–1.4)	0.42 (h) (0.26–0.69) 0.72 (r) (0.63–0.81)	12 (h) (5.9–27)	2.3 (h) (1.1–4.9) 1.3 (r) (0.78–2.0)
4	–$(CH_2)_4$–OH	3.6 (h) (2.9–4.6) 12 (r) (10–14)	1.9 (h) (1.1–3.3) 4.4 (r) (3.5–5.4)	19(h) (10–35)	14 (h) (6.5–32) 17% at 10 μM (r)
5	–cyclohexyl	0.080 (h) (0.056–0.11) 0.072 (r) (0.060–0.080)	0.037 (h) (0.026–0.053) 0.069 (r) (0.063–0.081)	≥ 30 (h)	3.6 (h) (2.8–4.8) 1.0 (r) (0.46–2.2)
6	–cyclohexenyl	0.18 (h) (0.14–0.21) 0.17 (r) (0.14–0.20)	0.35 (h) (0.27–0.45) 0.80 (r) (0.75–0.86)	> 30 (h)	0.39 (h) (0.25–0.61) 3.5 (r) (3.1–3.9)
7	–C_6H_5	0.77 (h) (0.36–1.6) 0.43 (r) (0.35–0.51)	0.40 (h) (0.24–0.67) 0.81 (r) (0.67–0.96)	> 30 (h)	0.52 (h) (0.35–0.78) 3.0 (r) (0.97–6.2)
8	–C_6H_4–*p*-$COCH_3$	8.3 (h) (5.1–14) 1.9 (r) (1.5–2.4)	3.8 (h) (1.9–7.7) 5.8 (r) (4.6–7.1)	> 30 (h)	> 100 (h) 11% at 10 μM (r)
9	–$(CH_2)_2$–C_6H_5	0.21 (h) (0.12–0.39) 0.15 (r) (0.09–0.24)	0.15 (h) (0.091–0.23) 2.7 (r) (2.0–3.4)	> 30.0 (h)	4.1 (h) (2.9–5.6) 4.8 (r) (4.4–5.3)
10	(*R,S*)-CH(OH)C_6H_5	0.098 (h) (0.092–0.10) 0.14 (r) (0.11–0.17)	0.035 (h) (0.018–0.072) 0.14 (r) (0.10–0.21)	1.4 (h) (0.85–2.3)	4.3 (h) (3.0–6.2) 1.2 (r) (0.58–2.0)
12	–$(CH_2)_2$–CH_3	0.064 (h) (0.025–0.17) 0.24 (r) (0.17–0.32)	0.37 (h) (0.27–0.50) 1.4 (r) (1.1–1.8)	2.7 (h) (2.5–2.9)	0.59 (h) (0.22–1.6) 1.5 (r) (1.1–1.9)
13	–$(CH_2)_3$–CH_3	2.3 (h) (1.4–3.9) 4.6 (r) (3.6–5.7)	0.44 (h) (0.22–0.87) 0.82 (r) (0.72–0.94)	22 (h) (11–45)	0.62 (h) (0.34–1.1) 3.5 (r) (2.8–4.2)
14	–$(CH_2)_4$–OH	6.5 (h) (5.5–7.5) 12% a 10 μM (r)	1.6 (h) (0.84–3.0) 13 (r) (8.8–18)	21 (h) (19–24)	6.6 (h) (3.5–12) 23 (r) (18–29)
15	–cyclohexyl	0.60 (h) (0.48–0.69) nd (r)	0.36 (h) (0.11–0.43) nd (r)	> 100 (h)	2.2 (h) (1.5–2.7) nd (r)
16	–cyclohexenyl	1.2 (h) (0.66–2.1) 1.0 (r) (0.68–1.4)	2.0 (h) (1.2–3.4) 3.8 (r) (3.1–4.5)	> 100 (h)	0.43 (h) (0.23–0.80) 2.2 (r) (1.9–4.0)

Table 1. Continued.

Cpd	*R*	K_i, μM or % inhibition			
		K_i (A_1)[b]	K_i (A_{2A})[c]	K_i (A_{2B})[d]	K_i (A_3)[e]
17	$-C_6H_5$	1.3 (h) (0.94–1.7) 2.6 (r) (2.0–3.3)	0.60 (h) (0.64–1.1) 0.64 (r) (0.48–0.82)	≥ 30 (h)	0.086 (h) (0.067–0.11) 0.25 (r) (0.12–0.43)
18	$-C_6H_4$–*p*-$COCH_3$	> 100 (h) 10% a 10 μM (r)	25 (13–47) 5.4 (r) (4.0–7.0)	> 30 (h)	> 100 (h) 5% a 10 μM (r)
19	$-(CH_2)_2-C_6H_5$	4.6 (h) (2.6–8.1) 2.5 (r) (1.4–4.0)	1.6 (h) (0.75–3.5) 2.7 (r) (1.0–2.5)	> 100 (h)	3.2 (h) (2.2–4.7) 5.3 (r) (3.4–7.6)
22	(*R*,*S*)-$CH(OH)C_6H_5$	3.0 (h) (1.6–5.6) 4.0 (r) (2.3–6.1)	0.88 (h) (0.55–1.4) 0.94 (r) (0.68–1.2)	≥ 30.0 (h)	3.7 (h) (2.9–4.6) 2.1(r) (1.3–3.0)

[a] Species are given in brackets: h = human, r = rat. K_i values are in μM with 95% confidence intervals in parentheses.
[b] Displacement of specific [^{3}H]CCPA binding in CHO cells, stably transfected with human recombinant A_1 adenosine receptor, and displacement of specific [^{3}H]CHA binding in rat cortical membranes or percentage of inhibition of specific binding at 10 μM concentration.
[c] Displacement of specific [^{3}H]NECA binding in CHO cells, stably transfected with human recombinant A_{2A} adenosine receptor, and displacement of specific [^{3}H]CGS 21680 binding in rat striatal membranes.
[d] Measurement of receptor-stimulated adenylyl cyclase activity in CHO cells, stably transfected with human recombinant A_{2B} adenosine receptor. K_i values were calculated from IC_{50} values determined by inhibition of NECA-stimulated adenylyl cyclase activity.
[e] Displacement of specific [^{3}H]NECA binding in CHO cells, stably transfected with human recombinant A_3 adenosine receptor, and displacement of specific [^{125}I]AB-MECA binding in rat testis membranes or percentage of inhibition of specific binding at 10 μM concentration.

that of the corresponding 2-alkynyl analogues, with the exception of compound **12** (**12**; K_i A_1 = 0.064 μM, K_i A_{2A} = 0.37 μM, K_i A_{2B} = 2.7 μM, and K_i A_3 = 0.59 μM vs 2; K_i A_1 = 1.2 μM, K_i A_{2A} = 0.76 μM, K_i A_{2B} = 17 μM, and K_i A_3 = 2.1 μM).

However, it is worthwhile to note that the derivatives, which showed some activity at the A_{2B} receptor, are those bearing linear chains in the 8-position, as in the case of the 2-substituted compounds (**12**; K_i A_{2B} = 2.7 μM, **13**; K_i A_{2B} = 22 μM, and **14**; K_i A_{2B} = 21 μM).

On the contrary, the affinity of the 8-alkynyl derivatives for the human A_3 receptor, compared to the corresponding 2-substituted derivatives, was improved; hence the 8-phenylethynyl-9-ethyladenine (**17**) with a K_i A_3 (h) of 0.086 and 0.25 μM (r) resulted the most active compound of the two series at both the human and rat A_3 subtype and was A_3 selective.

As in the case of the 2-substituted compound, the 8-alkynyl derivatives showed decreased affinity for rat receptors. The selectivity of this series of derivatives for the A_1, A_{2A}, and A_3 receptor subtypes was strictly correlated to the nature of the alkynyl chain, and there is a good correlation between affinity at human and rat receptors.

Molecular modeling

In order to get an insight into the mechanism of the ligand recognition and to give a rational explanation of the preference of the human A_3 receptor for the 8-substituted compounds rather than the 2-substituted compounds in this series, we carried out docking experiments of 2-phenylethynyl-9-ethyladenine (**7**) and 8-phenylethynyl-9-ethyladenine (**17**) at a rhodopsin-based homology model of the receptor (Figure 1).

In a previous molecular modeling study [17], we proposed that 2- and 8-alkynyl derivatives of adenosine bind to the human A_3 receptor in a way that the C2 of a molecule matches the C8 of the other one, with a good steric and electrostatic overlap of the purine moieties and the alkynyl chains.

On the basis of the structure activity relationships data, we hypothesized a similar binding mode for the alkynyl derivatives of adenosine and the correspondent 9-ethyladenine analogues. The results of our docking experiments were in good agreement with the biological data, confirming the soundness of the hypothesis on which they were based.

In the case of the adenosine derivatives, the compounds substituted at the 2-position were endowed with higher affinity than the correspondent 8-substituted analogues. In fact, in the latter case the sugar moiety had unfavourable interactions with the receptor.

On the other hand, in the case of the 2- and 8-phenylethynyl-9-ethyladenine, due to the substitution of the ribose moiety with the less bulky ethyl group, the negative ligand receptor interactions were eliminated even in the case of the 8-substituted compound. According to the results of the present study, in both situations the ethyl group is accommodated in the hydrophobic

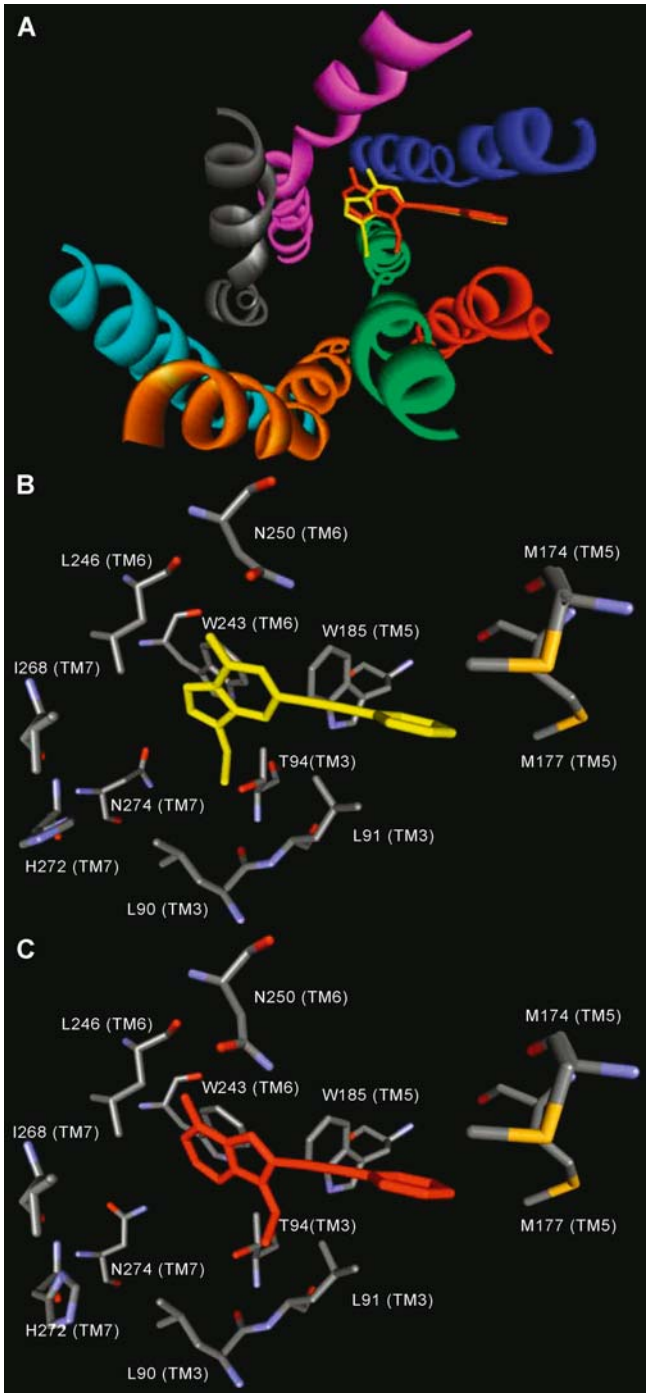


Figure 1. 2-Phenylethynyl-9-ethyladenine (**7**, yellow) and 8-phenylethynyl-9-ethyladenine (**17**, red) docked into the seven transmembrane domain of the human A_3 receptor. (A) View from outside the cell. (B and C) Detailed view of the two antagonists within the human A_3 receptor binding pocket. Color of helices: Cyan (TM1), orange (TM2), green (TM3), red (TM4), blue (TM5) magenta (TM6), gray (TM7).

pocket formed by Leu 90 and Leu 91 of the third transmembrane domain (TM3). Furthermore, the binding of the 8-alkynyl derivative to the A_3 receptor appears to be facilitated by a stronger electrostatic interaction of the crucial Asn 250 (TM6) with the adenine ring compared to the case of the 2-alkynyl derivatives (Figure 1).

Conclusions

With this study we have demonstrated that it is possible to modulate the activity at the adenosine receptor subtypes by introducing alkynyl chains in the 2- or 8-position of the 9-ethyladenine.

Although all the synthesized compounds were more active at human receptors, it is worthwhile to note that, in the series of the 2-substituted derivatives, compounds **5** and **10** are the ones endowed with the highest affinity at both human and rat A_1 and A_{2A} receptors.

The same trend has been observed with the 8-substituted derivatives: in fact the 8-phenylethynyl-9-ethyladenine (**17**) proved to be the most active and selective compound of the two series at human and rat A_3 subtype.

From these results it is possible to conclude that the selectivity of these two series of derivatives for the A_1, A_{2A}, and A_3 receptor subtypes is strictly correlated to the nature of the alkynyl chain, and that there is a good correlation between the affinity at human and rat receptors.

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