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New Substituted 9-Alkylpurines as Adenosine Receptor Ligands

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Abstract—In the present study an investigation of the structure–activity relationships in 9-ethylpurine derivatives, aimed at preparing A_1, A_{2A}, A_{2B}, and A_3 selective adenosine receptor antagonists, was undertaken. Our synthetic approach was to introduce various substituents (amino, alkoxy and alkynyl groups) into the 2-, 6-, or 8-positions of the purine ring. The starting compounds for each series of derivatives were respectively: 2-iodo-9-ethyladenine (**9**), obtained from 2-amino-6-chloropurine (**5**); 9-ethyl-6-iodo-9*H*-purine (**11**), 8-bromo-9-ethyl-adenine (**3**) and 8-bromo-9-ethyl-6-iodo-9*H*-purine (**13**), obtained from 9-ethyl-adenine (**2**). The synthesized compounds were tested in in vitro radioligand binding assays at A_1, A_{2A}, and A_3 human adenosine receptor subtypes. Due to the lack of a suitable radioligand the affinity of the 9-ethyladenine derivatives at A_{2B} adenosine receptors was determined in adenylyl cyclase experiments. In general, the series of 9-ethylpurine derivatives exhibited a similar pharmacological profile at A_1 and A_{2A} receptors whereas some differences were found for the A_3 and the A_{2B} subtypes. 8-Bromo-9-ethyladenine (**3**) showed higher affinity for all receptors in comparison to the parent compound **2**, and the highest affinity in the series for the A_{2A} and A_{2B} subtypes ($K_i = 0.052$ and $0.84\,\mu M$, respectively). Analyzing the different substituents, a phenethoxy group in 2-position (**10a**) gave the highest A_{2A} versus A_{2B} selectivity (near 400-fold), whereas a phenethylamino group in 2- and 6-position (**10b** and **12b**, respectively) improved the affinity at A_{2B} receptors, compared to the parent compound **2**. The presence of a hexynyl substituent in 8-position led to a compound with good affinity at the A_3 receptor (**4d**, $K_i = 0.62\,\mu M$), whereas (ar)alkynyl groups are detrimental for the potency at the A_{2B} subtype. These differences give raise to the hope that further modifications will result in the development of currently unavailable leads with good affinity and selectivity for A_{2B} adenosine receptors.

Introduction

The purine nucleoside adenosine specifically modulates neurotransmission through the interaction with four surface receptors designated as A_1, A_{2A}, A_{2B} and A_3 on the basis of biological experiments and receptor cloning.[1,2]

The first compounds that were identified as adenosine receptor antagonists were the naturally occurring xanthines, caffeine and theophylline.[3] These xanthines are non-selective and of moderate potency. Substitution at appropriate sites of xanthine may enhance affinity and selectivity for A_1 and A_{2A} subtypes.[4–6] The in vivo use of these compounds is however hampered by their poor solubility: hence considerable efforts have been devoted to the discovery of non-xanthine antagonists[7] to be used as pharmacological probes and potential therapeutic agents.[8] More recently, it has been demonstrated that the cloned rat A_3 receptor subtype is quite insensitive to xanthines.[9a] However Linden et al. found that certain xanthines bind appreciably to sheep and human A_3 receptors but generally with less affinity than at A_1 and A_{2A} receptors in a variety of species.[9b,c] Furthermore, no selective high affinity ligands or radioligands are currently available for the A_{2B} subtype.

A ribose or ribose equivalent at N-9 is present in all currently known adenosine receptor agonists. Replacement of ribose with a methyl group led to a relatively

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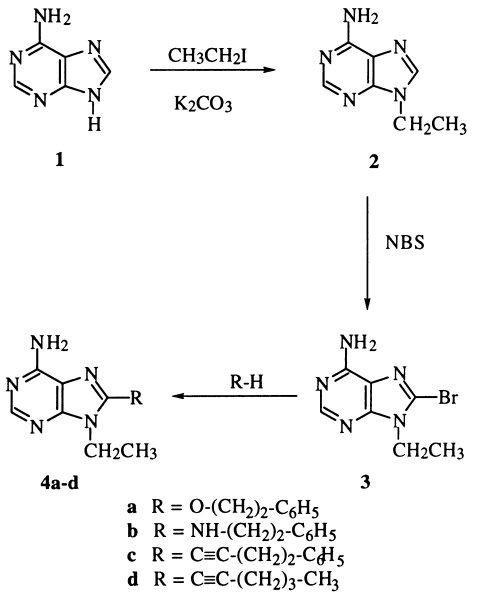


Scheme 1.

unselective antagonist and adenine itself is a weak adenosine antagonist.[10] As in the case of adenosine derivatives, alkyl and cycloalkyl substituents in N^6 increase the affinity of 9-methyladenine for the A_1 receptors.[11,12] Moreover, the presence of a 3-iodobenzyl group at the 6-amino position of 9-methyladenine resulted in compounds with properties indicating an A_3 versus A_1 selectivity. However, a 9-methyl adenine substituted in the 2-position with a phenylethoxy group has been reported not to discriminate between A_2 receptors in the coronary vessels and A_1 receptors in the atria of guinea pig.[13]

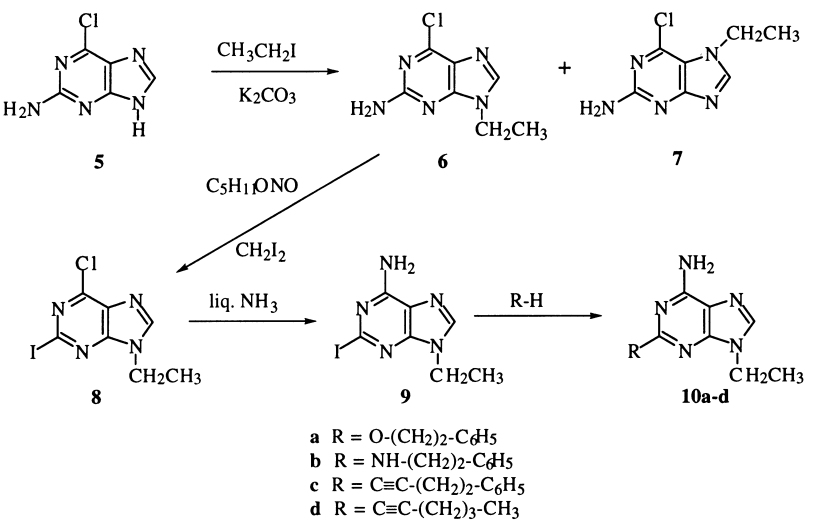
In the present study an investigation on the structure-activity relationships of 9-ethyladenine derivatives, aimed at finding selective adenosine receptor antagonists, was undertaken. This molecule was selected on the basis that preliminary studies showed 9-ethyladenine to be the most potent ligand at A_1 and A_{2A} rececceptor subtypes in a series of 9-alkyladenines.[14]

The synthetic program included the introduction of phenethylamino, phenethoxy, phenylbutynyl and hexynyl substituents in the 2-, 6-, or 8-positions of 9-ethyladenine, respectively. Furthermore, a few 6,8-disubstituted compounds bearing a bromine atom in position 8 were prepared. These substituents have been selected on the basis of previous findings on adenosine receptor agonist affinity.

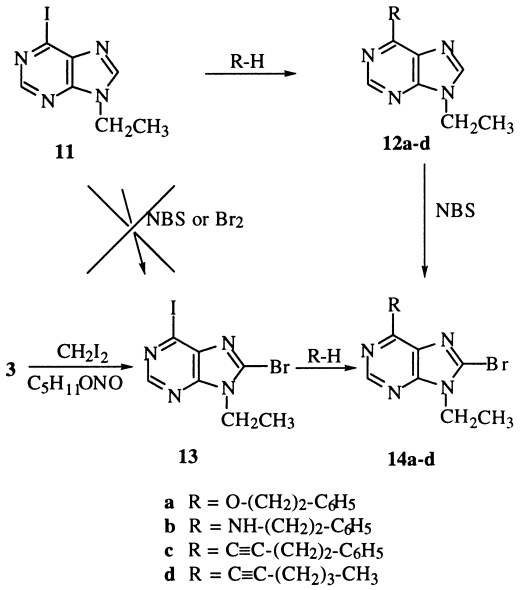
Chemistry

9-Ethyladenine (**2**)[15] was selected as the parent compound and the synthesis of its derivatives, substituted in turn in 8-, 2- or 6-position with amino, alkoxy and alkynyl groups was designed as reported in Schemes 1–3. The starting compounds for each series of derivatives were respectively: 8-bromo-9-ethyladenine (**3**),[16] 9-ethyl-2-iodoadenine (**9**), 9-ethyl-6-iodo-9*H*-purine (**11**), and 8-bromo-9-ethyl-6-iodo-9*H*-purine (**13**).

The commercially available adenine (**1**) was reacted with ethyl iodide in dry dimethylformamide, employing potassium carbonate as base, to give 9-ethyladenine (**2**) in 60% yield. The yield of **2** is higher than that reported earlier (40%), obtained by using sodium hydride as base.[15] The reaction of 9-ethyladenine with *N*-bromo-



Scheme 2.



Scheme 3.

succinimide gave the 8-bromo-9-ethyladenine (**3**) in 55% yield (Scheme 1).

Reaction of the commercially available 2-amino-6-chloropurine (**5**) with ethyl iodide gave the desired N-9 derivative **6**, together with the 7-alkylated isomer **7**, in an about 3:1 ratio. The isomers were isolated in 67% and 20% yields, respectively. The isomeric structure was assigned on the basis of ^{1}H NMR and UV spectra, according to data reported in the literature for similar alkylated 2-amino-6-chloropurines,[17] and by means of an unambiguous chemical proof reported below. The iodide in 2-position was introduced by the classical diazotization/halogenation procedure using *iso*-pentyl nitrite as the nitrosating agent. From 6-chloro-9-ethyl-2-iodo-9*H*-purine (**8**) the desired 9-ethyl-2-iodoadenine (**9**) was obtained in 65% yield by treatment with liquid ammonia at room temperature in a sealed tube. It is well documented that the selective substitution of the halogen in 6-position can be easily achieved, since nucleophilic displacements at this position are more feasible at room temperature than those at 2-position. However, catalytic deiodination of **9** was carried out to give 9-ethyladenine (**2**). This reaction confirmed both the assignment of the alkylation site of 2-amino-6-chloro-9-ethyl-9*H*-purine (**6**) and, at the same time, the selective substitution of the chlorine atom in 6-position of compound **8** (Scheme 2).

Starting from **2**, 9-ethyl-6-iodopurine (**11**) was obtained in 45% yield by modification of the method previously reported.[15] Attempt to obtain 8-bromo-9-ethyl-6-iodo-9H-purine (**13**) from **11** by treating it with *N*-bromosuccinimide or bromine in dry DMF at 60 °C was unsuccessful. Alternatively, the desired compound was obtained from the 6-amino derivative **3**, by introducing the iodide in 6-position through the classical diazotization/halogenation procedure using *iso*-pentyl nitrite as the nitrosating agent.

Substitution of halogens (iodine or bromine) in compounds **3**, **9**, **11**, and **13** with different groups was carried out following the general methods A–C, described in the experimental section, and according to the reaction conditions reported in Table 1. Briefly:

Table 1. Preparation of compounds in Schemes 1–3

Compd	Method	Base	*t* (°C)	Time (h)	Chromatography solvent[a]	Yield (%)
4a	A	K_2CO_3	85	16	$CHCl_3$:cC_6H_{12}:MeOH(80:13:7); TLC	35
4b	B		100	36	$CHCl_3$:MeOH(95:5)	70
4c	C	Et_3N	rt	60	$CHCl_3$:cC_6H_{12}:MeOH (86:10:4)	75
4d	C	Et_3N	rt	60	$CHCl_3$:MeOH (99:1);f	73
10a	A	NaOH	85	3	EtOAc:cC_6H_{12}:MeOH (60:33:7)	84
10b	B		130	24	EtOAc:cC_6H_{12}:MeOH (60:36:4)	80
10c	C	Et_3N	rt	24	$CHCl_3$:cC_6H_{12}:MeOH (86:10:4)	62
10d	C	Et_3N	rt	20	CH_2Cl_2:MeOH (97:3)	83
12a	A	NaOH	85	16	CH_3CN:H_2O:MeOH(40:40:20); rp	60
12b	B		60	6	$CHCl_3$:MeOH (99:1);f	60
12c	C	Et_3N	rt	3	$CHCl_3$:MeOH (98:2)	70
12d	C	Et_3N	rt	3	CHCl3:MeOH (98:2); TLC	82
14a	A	K_2CO_3	rt	72	cC_6H_{12}:EtOAc:MeOH (50:49:1); TLC	65
14b	B	Et_3N	60	16	$CHCl_3$:MeOH (99:1); TLC	45
14c	C	Et_3N	rt	16	$CHCl_3$; TLC	64
14d	C	Et_3N	rt	20	$CHCl_3$; TLC	45

[a]TLC: preparative thin-layer chromatography; f: flash chromatography; rp: reverse-phase (C18).

Method A: The alkoxy derivatives **4a**, **10a**, **12a**, and **14a** were obtained by reaction of the suitable synthon with 2-phenethyl alcohol in dry acetonitrile in the presence of K_2CO_3 or NaOH at the temperature and for the time reported in Table 1.

Method B: The amino derivatives **4b**, **10b**, **12b**, and **14b** were obtained by reacting the suitable synthon with 2-phenethylamine at the temperature and for the time reported in Table 1.

Method C: The introduction of the alkynyl chain to give compounds **4c** and **d**, **10c** and **d**, **12c** and **d**, and **14c** and **d** was carried out by modification of the classical cross-coupling reaction in the presence of CuI, Et_3N and $(Ph_3P)_2PdCl_2$ at room temperature for the time reported in Table 1.

Biological activity

All the synthesized compounds were tested in radioligand competition at membranes from stably transfected CHO cells expressing the human A_1, A_{2A}, and A_3 adenosine receptor subtypes,[18] using DPCPX and theophylline as reference compounds. [^{3}H]DPCPX[19] on A_1, [^{3}H]CGS 21680[20] on A_{2A}, and [^{125}I]ABMECA[21] on A_3 have been used as radioligands. Due to the lack of a suitable radioligand the affinity (K_B, K_i) of the 9-ethyladenine derivatives at A_{2B} adenosine receptors was determined by inhibition of NECA-stimulated adenylyl cyclase activity. The results are reported in Table 2.

All the compounds tested showed higher affinity at A_{2A} receptor in comparison with other subtypes except for **12d** and **14a**. This finding is in agreement with preliminary results indicating that the presence of an ethyl group in 9-position increased the A_{2A} versus A_1 selectivity.[14]

8-Bromo-9-ethyladenine (**3**) showed higher affinity for all receptors in comparison to the parent compound **2**, and the highest affinity in the series for the A_{2A} and A_{2B} subtypes ($K_i = 0.052$ and $0.84\,\mu M$, respectively). The 8-phenethoxy-, 8-phenethylamino-, and 8-(ar)alkynyl-compounds **4a**–**d** showed in general decreased potency at all receptors in comparison with compound **3**, with the exception of the alkynyl derivatives **4c** and **4d** which showed higher affinity at the A_3 subtype ($K_i = 3.20$ and $0.62\,\mu M$, respectively versus $27.8\,\mu M$). It is worthwhile to note that the hexynyl derivative **4d** exibited the higher affinity at the A_3 subtype in the series. The introduction of the same substituents in 2-position of compound **2** (**10a**–**d**) enhanced the affinity for all receptors in comparison with 9-ethyladenine itself. The 2-phenethoxy derivative **10a** resulted to be the most potent in the series in respect to the A_1 receptor suptype ($K_i = 0.17\,\mu M$).

The same modifications in 6-position (**12a**–**d**) did not have major effects on the affinity at the four subtypes, with the exception of the phenethylamino group in compound **12b**, which improved the affinity for the A_{2B} and A_3 receptors to $K_i = 8.13$ and $10.8\,\mu M$, respectively. The combined presence of a phenethylamino group in 6- and a bromine in the 8-position led to compound **14b** with even higher affinity for the A_{2B} and A_3 receptors ($K_i = 2.21$ and $2.44\,\mu M$, respectively).

Analyzing the different substituents, a phenethoxy group in 2-position (**10a**) gave the highest A_{2A} versus A_{2B} selectivity (near 400-fold). On the other hand, a phenethylamino group in 2- or 6-position (**10b** and **12b**, respectively) was the most effective in improving the affinity at A_{2B} receptors, compared to the parent compound **2**. The presence of a hexynyl substituent in 2- or 8-position (**10d** and **4d**, respectively) led to compounds with good affinity at A_3 receptor, whereas (ar)alkynyl groups are detrimental for the potency at A_{2B} subtype.

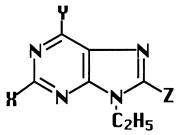
It is worthwhile to note that in both the N^6-substituted series, **12a**–**d** and **14a**–**d**, the most active compounds bear the phenethylamino group. Replacing the 6-amino by alkoxy or alkynyl groups greatly diminished affinity at the four adenosine receptor subtypes, suggesting that a N–H group is required to provide a H-bond donor for a good interaction with all the adenosine receptors.

In conclusion, most compounds with higher affinity at A_{2A} receptors were also more potent at A_1 versus A_3 subtype, with the exception of 8-hexynyl-9-ethyladenine (**4d**), which was the most potent compound at A_3 receptors ($K_i = 0.62\,\mu M$), with a fourfold selectivity for this subtype versus A_1.

In general, the series of 9-ethylpurine derivatives exhibited a similar pharmacological profile at A_1 and A_{2A} receptors (Figure 1(a)) whereas some differences in the profile were found for the A_3 and the A_{2B} subtypes, as shown in Figure 1(b). In fact the diagram in Figure 1(b) clearly illustrates the A_{2B} versus A_3 selectivity of compound **3** and conversely the A_3 versus A_{2B} selectivity of compound **4d**. On the other hand, compounds **10b**, **12b**, and **14b** exhibit no preference for A_{2B} or A_3 receptors.

The differences detected in this study will serve as a basis for further modifications, including a selection of different 9-(ar)alkylpurine derivatives, for the development of new ligands with currently unavailable affinity and selectivity for the A_{2B} subtype. This will help to identify relevant in vivo functions of A_{2B} adenosine receptors, in particular in cardiovascular regulation.

Table 2. Affinity of 9-ethylpurine derivatives in radioligand binding and NECA-stimulated adenylyl cyclase assays at membranes from stably transfected CHO cells expressing human adenosine receptor subtypes



Compd	Substituent			Binding (K_i, μM)[a]			
	X	Y	Z	A_1	A_{2A}	A_{2B}	A_3
2	H	NH_2	H	**7.44** (4.22–13.12)	**2.20** (1.40–3.53)	**> 100**	**> 100**
3	H	NH_2	Br	**0.28** (0.25–0.32)	**0.052** (0.024–0.113)	**0.84** (0.63–1.12)	**27.8** (22.3–34.7)
4a	H	NH_2	O-$(CH_2)_2$-C_6H_5	**0.89** (0.59–1.33)	**0.15** (0.096–0.23)	**7.59** (4.35–13.25)	**36.2** (16.9–77.6)
4b	H	NH_2	NH-$(CH_2)_2$-C_6H_5	**14.2** (5.99–33.7)	**3.01** (1.48–6.11)	**> 100**	**49.4** (26.0–93.7)
4c	H	NH_2	C≡C-$(CH_2)_2$-C_6H_5	**4.60** (2.62–8.06)	**1.62** (0.75–3.46)	**> 100**	**3.20** (2.19–4.65)
4d	H	NH_2	C≡C-$(CH_2)_3$-CH_3	**2.34** (1.40–3.89)	**0.44** (0.22–0.87)	**22.4** (11.1–45.1)	**0.62** (0.34–1.14)
10a	O-$(CH_2)_2$-C_6H_5	NH_2	H	**0.17** (0.13–0.23)	**0.12** (0.07–0.22)	**45.8** (29.8–70.5)	**7.15** (2.95–17.3)
10b	NH-$(CH_2)_2$-C_6H_5	NH_2	H	**0.33** (0.21–0.51)	**0.15** (0.11–0.21)	**2.41** (1.44–4.03)	**3.15** (2.44–4.06)
10c	C≡C-$(CH_2)_2$-C_6H_5	NH_2	H	**0.21** (0.12–0.39)	**0.15** (0.091–0.23)	**> 100**	**4.05** (2.93–5.62)
10d	C≡C-$(CH_2)_3$-CH_3	NH_2	H	**0.55** (0.24–1.23)	**0.42** (0.26–0.69)	**12.5** (5.89–26.7)	**2.30** (1.08–4.90)
12a	H	O-$(CH_2)_2$-C_6H_5	H	**19.2** (7.32–50.5)	**17.1** (7.37–39.7)	**> 100**	**22.1** (17.8–27.6)
12b	H	NH-$(CH_2)_2$-C_6H_5	H	**8.73** (3.79–20.1)	**1.34** (0.67–2.68)	**8.13** (5.98–11.06)	**10.8** (8.66–13.4)
12c	H	C≡C-$(CH_2)_2$-C_6H_5	H	**14.1** (11.1–17.8)	**4.90** (4.27–5.62)	**74.9** (42.7–131.7)	**39.3** (31.1–49.6)
12d	H	C≡C-$(CH_2)_3$-CH_3	H	**12.1** (8.75–16.7)	**17.6** (8.03–38.6)	**> 100**	**39.2** (13.8–111.3)
14a	H	O-$(CH_2)_2$-C_6H_5	Br	**11.7** (9.41–14.5)	**19.3** (13.9–26.9)	**> 100**	**60.9** (29.9–124.2)
14b	H	NH-$(CH_2)_2$-C_6H_5	Br	**1.54** (1.30–1.81)	**0.28** (0.12–0.68)	**2.21** (1.46–3.35)	**2.44** (1.52–3.90)
14c	H	C≡C-$(CH_2)_2$-C_6H_5	Br	**16.7** (9.66–28.8)	**10.8** (5.29–21.9)	**> 100**	**44.7** (20.4–97.3)
14d	H	C≡C-$(CH_2)_3$-CH_3	Br	**23.6** (10.7–52.1)	**6.09** 1.94–19.1	**> 100**	**19.0** (12.6–29.6)
DPCPX[b]				**0.0039** (0.0035–0.0042)	**0.13** (0.064–0.26)	**1.00** (0.60–1.70)	**4.00** (2.60–6.00)
Theophylline[b]				**6.8** (4.10–11.3)	**1.70** (1.00–2.90)	**40.0** (36.0–44.0)	**86.0** (74.0–101.0)

[a]Receptor binding affinity at A_1, A_{2A}, and A_3 receptors was determined using [^{3}H]DPCPX,[19] [^{3}H]CGS 21680,[20] and [^{125}I]ABMECA[21] as radioligands, respectively. Affinity of the 9-ethylpurine derivatives at A_{2B} adenosine receptors was determined in adenylyl cyclase experiments. Data are geometrical means from at least three separate experiments; 95% confidence limits in parenthesis.
[b]See Ref. 25.

Experimental

Chemistry

Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were obtained with Varian VX 300 and 200 MHz spectrometers. All exchangeable protons were confirmed by addition of D_2O. TLC was carried out on precoated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. Microanalytical results are indicated by atomic symbols and are within $\pm 0.4\%$ of theoretical values.

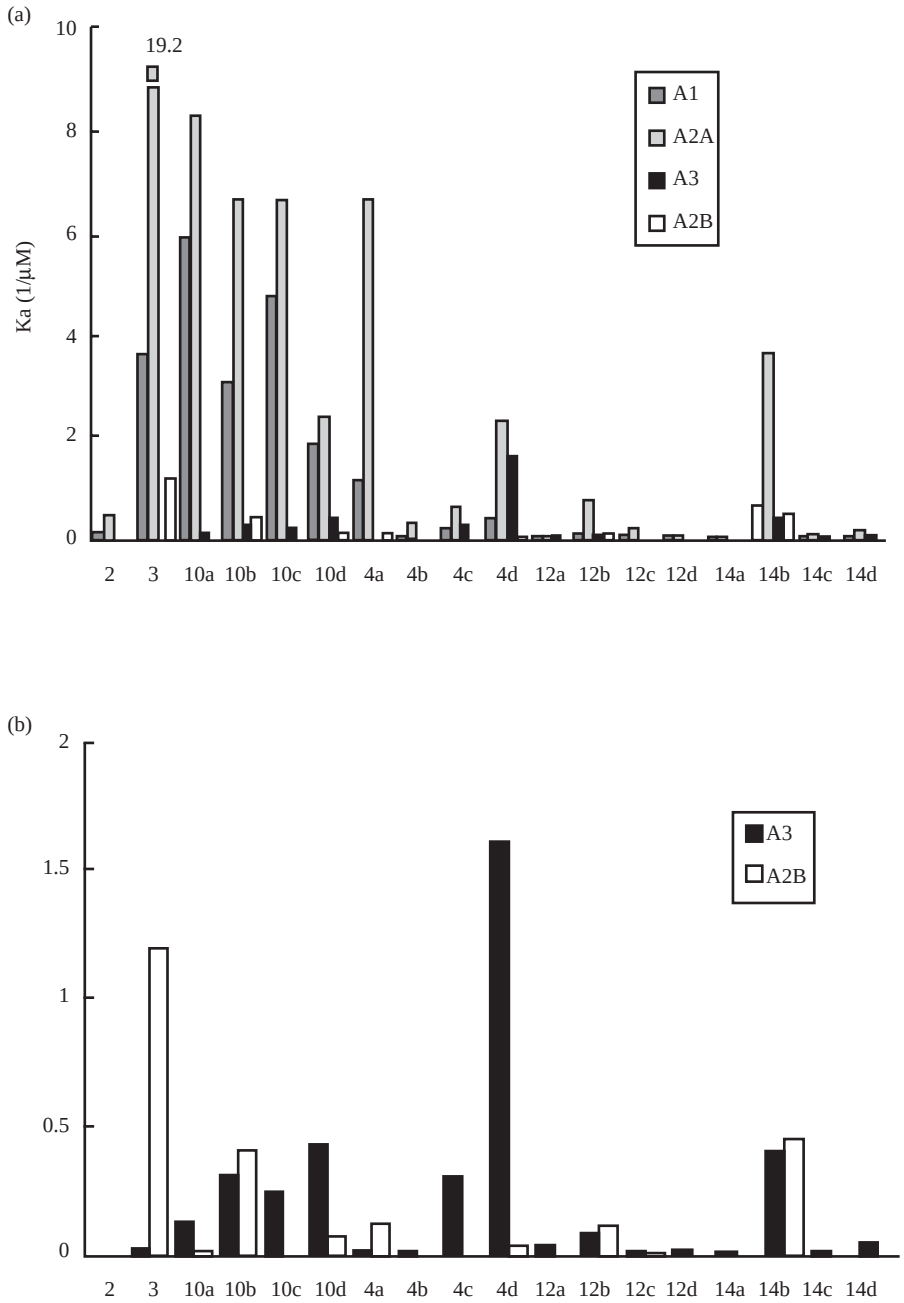


Figure 1. (a) Affinity of 9-ethylpurine derivatives at adenosine receptors. The association constants (K_a) of 9-ethylpurine derivatives are shown as an indication of their affinity to adenosine receptor subtypes. Values given as 1/μM and were calculated from K_i-values in Table 2. (b) Comparison of A_{2B} and A_3 adenosine receptor binding of 9-ethylpurine derivatives. Association constants (K_a) of 9-ethylpurine derivatives at A_{2B} and A_3 receptors are derived from K_i-values in Table 2. For further details see text.

General method A

To a suspension of iodo or bromo 9-ethyl-9*H*-purine derivative (1 mmol) and dry K_2CO_3 (2 mmol) or NaOH (5 mmol) in dry CH_3CN (25 mL) was added 2-phenethyl alcohol (5 mL, 40 mmol). This mixture was heated at 85 °C for the time reported in the Table 1. The solvent was removed under reduced pressure and the residue was neutralized with 2 N HCl and extracted with $CHCl_3$. The organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was chromatographed on a silica gel column eluting with a suitable mixture of solvents (Table 1) to give the desired derivatives as chromatographically pure solids.

General method B

To iodo or bromo 9-ethyl-9*H*-purine derivative (1 mmol) was added 2-phenethylamine (5 mL) and the mixture was heated at the temperature and for the time listed in Table 1. In the case of compound **14b**, 2-phenethylamine was added in equimolar amount in dry CH_3CN (20 mL) and triethylamine (2 mL). The solvent was removed in vacuo and the residue was chromatographed on a silica gel column eluting with a suitable mixture of solvents (Table 1) to give the desired derivatives as chromatographically pure solids.

General method C

To a solution of iodo or bromo 9-ethyl-9*H*-purine derivative (0.84 mmol), dry DMF (10 mL), dry CH_3CN (25 mL), and Et_3N (3.4 mL) under an atmosphere of N_2 was added bis(triphenylphosphine)palladium dichloride (12 mg) and CuI (0.84 mg). 4-Phenyl-1-butyne or 1-hexyne (4.2 mmol) was added and the reaction mixture was stirred under an atmosphere of N_2 at room temperature for the time reported in Table 1. In the case of compounds **14c** and **14d**, the terminal alkyne was added in equimolar amount. The solvent was removed in vacuo and the residue was chromatographed on a silica gel column eluting with a suitable mixture of solvents (Table 1) to give the desired derivatives as chromatographically pure solids.

9-Ethyladenine (2). To a solution of adenine (**1**) (3.0 g, 22.2 mmol) in dry DMF (10 mL) was added iodoethane (2.3 mL, 28.8 mmol) and dried K_2CO_3 (3.5 g, 25.3 mmol), and the suspension was stirred at room temperature for 16 h. The reaction mixture was filtered to remove the insoluble material and the filtrate was coevaporated three times with toluene, and then concentrated to a residue which was flash chromatographed on a silica gel column. Elution with CH_2Cl_2:MeOH (97:3) gave compound **2** (2.2 g, 60%) as a white solid: mp 185–187 °C [lit.[15] mp 192–193 °C]; ^{1}H NMR (Me_2SO-d_6) δ 1.41 (t, 3H, CH_2CH_3), 4.29 (q, 2H, CH_2CH_3), 7.19 (br s, 2H, NH_2), 8.13 (s, 1H, H-2) 8.16 (s, 1H, H-8). Anal. calcd for $C_7H_9N_5$: C, 51.52, H, 5.56, N, 42.92; found: C, 51.37, H, 5.82, N, 42.73.

8-Bromo-9-ethyladenine (3). To a solution of compound **2** (1.6 g, 9.8 mmol) in dry DMF (25 mL) was added *N*-bromosuccinimide (3.5 g, 19.6 mmol) and the reaction mixture was stirred at room temperature for 20 h. The solvent was removed in vacuo and the residue was flash chromatographed on a silica gel column using a gradient from $CHCl_3$ to $CHCl_3$:MeOH (98:2) to give compound **3** (1.42 g, 60%) as a chromatographically pure solid: mp 218–220 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.33 (t, 3H, J = 7.3 Hz, CH_2CH_3), 4.17 (q, 2H, J = 7.1 Hz, CH_2CH_3), 7.39 (br s, 2H, NH_2), 8.15 (s, 1H, H-2). Anal. calcd for $C_7H_8BrN_5$: C, 34.73, H, 3.33, N, 28.93; found: C, 35.02, H, 3.41, N, 28.67.

9-Ethyl-8-(2-phenylethyloxy)-9*H*-adenine (4a). This compound was synthesized following general method A: mp 176–178 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.17 (t, 3H, J = 7.0 Hz, CH_2CH_3), 3.13 (t, 2H, J = 6.6 Hz, CH_2Ph), 3.89 (q, 2H, J = 7.0 Hz, CH_2CH_3) 4.68 (t, 2H, J = 6.5 Hz, CH_2O), 6.78 (br s, 2H, NH_2), 7.30 (br m, 5H, H-Ph), 8.03 (s, 1H, H-2). Anal. calcd for $C_{15}H_{17}N_5O$: C, 63.59, H, 6.05, N, 24.72; found: C, 63.88, H, 6.20, N, 24.61.

9-Ethyl-8-(2-phenylethylamino)-9*H*-adenine (4b). This compound was synthesized following general method B: mp 213–215 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.17 (t, 3H, J = 7.0 Hz, CH_2CH_3), 2.96 (ps t, 2H, J = 7.7 Hz, CH_2CH_2NH), 3.58 (q, 2H, J = 7.6 Hz, CH_2NH) 3.97 (q, 2H, CH_2CH_3), 6.40 (s, 2H, NH_2), 6.85 (m, 1H, NH), 7.28 (br m, 5H, H-Ph), 7.92 (s, 1H, H-2). Anal. calcd for $C_{15}H_{18}N_6$: C, 63.81, H, 6.43, N, 29.76; found: C, 64.03, H, 6.67, N, 29.48.

9-Ethyl-8-(4-phenyl-1-butyn-1-yl)-9*H*-adenine (4c). This compound was synthesized following general method C: mp 180–182 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.22 (t, 3H, J = 7.2 Hz, CH_2CH_3), 2.94 (s, 4H, C≡CCH_2CH_2), 4.04 (q, 2H, J = 7.0 Hz, CH_2CH_3), 7.34 (br m, 7H, H-Ph and NH_2), 8.15 (s, 1H, H-2). Anal. calcd for $C_{17}H_{17}N_5$: C, 70.08, H, 5.88, N, 24.04; found: C, 70.39, H, 6.01, N, 23.89.

9-Ethyl-8-(1-hexyn-1-yl)-9*H*-adenine (4d). This compound was synthesized following general method C: mp 173–175 °C; ^{1}H NMR (Me_2SO-d_6) δ 0.95 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.35 (t, 3H, J = 7.3 Hz, NCH_2CH_3), 1.38–1.61 (br m, 4H, $CH_2CH_2CH_3$), 2.60 (t, 2H, J = 6.8 Hz, C≡CCH_2), 4.20 (q, 2H, J = 7.1 Hz, NCH_2CH_3), 7.39 (br s, 2H, NH_2) 8.19 (s, 1H, H-2). Anal. calcd for $C_{13}H_{17}N_5$: C, 64.17, H, 7.04, N, 28.78; found: C, 64.28, H, 7.27, N, 28.51.

2-Amino-6-chloro-9-ethyl-9*H*-purine (6) and 2-amino-6-chloro-7-ethyl-7*H*-purine (7). To a solution of 2-amino-6-chloropurine (**5**) (2.5 g, 14.7 mmol) in dry DMF (20 mL) under an atmosphere of N_2 was added dried K_2CO_3 (3.0 g, 21.7 mmol) and iodoethane (1.5 mL, 18.7 mmol) and the suspension was stirred at room temperature for 16 h. The reaction mixture was filtered to remove the insoluble material and the filtrate was coevaporated three times with toluene, and then concentrated to a residue which was flash chromatographed on a silica gel column. Elution with $CHCl_3$ gave 2-amino-6-chloro-9-ethyl-9*H*-purine (**6**) (1.95 g, 67%), and then elution with $CHCl_3$:MeOH (98:2) gave 2-amino-6-chloro-7-ethyl-7*H*-purine (**7**) (0.59 g , 20%) as white solids.

Compound **6**: Mp 151–153 °C; UV λ_{max} (MeOH) 221 nm (ε 23700), 246 (ε 4800), 307 (ε 5800), (pH 1) 216 nm (ε 24700), 240 (ε 5800), 321 (ε 5400); ^{1}H NMR (Me_2SO-d_6) δ 1.34 (t, 3H, CH_2CH_3), 4.05 (q, 2H, CH_2CH_3), 6.90 (s, 2H, NH_2), 8.14 (s, 1H, H-8). Anal. calcd for $C_7H_8ClN_5$: C, 42.54, H, 4.08, N, 35.44; found: C, 42.71, H, 4.25, N, 35.27.

Compound **7**: Mp 185 °C (dec); UV λ_{max} (MeOH) 221 nm (ε 18800), 253 (sh), 321 (ε 4400), (pH 1) 216 nm (ε 22900), 240 (sh), 322 (ε 5300); ^{1}H NMR (Me_2SO-d_6) δ 1.38 (t, 3H, CH_2CH_3), 4.30 (q, 2H, CH_2CH_3), 6.61 (s, 2H, NH_2), 8.38 (s, 1H, H-8).Anal. calcd for $C_7H_8ClN_5$: C, 42.54, H, 4.08, N, 35.44; found: C, 42.77, H, 4.28, N, 35.15.

6-Chloro-9-ethyl-2-iodo-9*H*-purine (8). A mixture of compound **6** (2.9 g, 14.7 mmol) in dry THF (75 mL), diiodomethane (12.6 mL), and *iso*-pentyl nitrite (6.3 mL) was heated at 85 °C for 1 h. The solvent was removed under reduced pressure (oil pump, 60 °C) and the residue was purified by flash chromatography on a silica gel column eluting with $CHCl_3$:*n*-C_6H_{14} (70:30) to give compound **8** (2.8 g, 62%) as a pure solid: mp 117–119 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.42 (t, 3H, CH_2CH_3), 4.24 (q, 2H, CH_2CH_3), 8.65 (s, 1H, H-8). Anal. calcd for $C_7H_6ClIN_4$: C, 27.25, H, 1.96, N, 18.16; found: C, 27.38, H, 2.06, N, 17.98.

9-Ethyl-2-iodo-9*H*-adenine (9). A solution of compound **8** (3.0 g, 9.7 mmol) in liquid ammonia (20 mL) was sealed in a stainless steel tube and set aside at room temperature for 24 h. The solvent was removed in vacuo and the residue was purified by flash chromatography on a silica gel column eluting with a gradient of CH_2Cl_2:MeOH from (99.5:0.5) to (99:1) to give compound **9** (1.85 g, 66%) as a chromatographically pure solid: mp 235–237 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.35 (t, 3H, CH_2CH_3), 4.09 (q, 2H, CH_2CH_3), 7.59 (s, 2H, NH_2), 8.07 (s, 1H, H-8). Anal. calcd for $C_7H_8IN_5$: C, 29.08, H, 2.79, N, 24.23; found: C, 29.39, H, 2.95, N, 23.95.

9-Ethyl-2-(2-phenylethyloxy)-9*H*-adenine (10a). This compound was synthesized following general method A: mp 181–183 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.38 (t, 3H, J = 7.2 Hz, CH_2CH_3), 3.02 (t, 2H, J = 6.9 Hz, CH_2-Ph), 4.08 (q, 2H, J = 7.3 Hz, CH_2CH_3) 4.42 (t, 2H, J = 7.0 Hz, CH_2O), 7.21 (s, 2H, NH_2), 7.32 (br m, 5H, H-Ph), 7.96 (s, 1H, H-8). Anal. calcd for $C_{15}H_{17}N_5O$: C, 63.59, H, 6.05, N, 24.72; found: C, 63.81, H, 6.17, N, 24.40.

9-Ethyl-2-(2-phenylethylamino)-9*H*-adenine (10b). This compound was synthesized following general method B: mp 139–141 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.37 (t, 3H, CH_2CH_3), 2.84 (q, 2H, CH_2CH_2NH), 3.44 (m, 2H, CH_2NH) 4.01 (q, 2H, CH_2CH_3), 6.23 (t, 1H, NH), 6.62 (s, 2H, NH_2), 7.24 (br m, 5H, H-Ph), 7.71 (s, 1H, H-8). Anal. calcd for $C_{15}H_{18}N_6$: C, 63.81, H, 6.43, N, 29.76; found: C, 63.89, H, 6.49, N, 29.54.

9-Ethyl-2-(4-phenyl-1-butyn-1-yl)-9*H*-adenine (10c). This compound was synthesized following general method C: mp 117–120 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.39 (t, 3H, J = 7.3 Hz, NCH_2CH_3), 2.72 (ps t, 2H, $C{\equiv}CCH_2$), 2.88 (ps t, 2H, CH_2Ph) 4.14 (q, 2H, J = 7.3 Hz, NCH_2), 7.32 (br m, 7H, H-Ph and NH_2), 8.20 (s, 1H, H-8). Anal. calcd for $C_{17}H_{17}N_5$: C, 70.08, H, 5.88, N, 24.04; found: C, 70.18, H, 5.92, N, 23.77.

9-Ethyl-2-(1-hexyn-1-yl)-9*H*-adenine (10d). This compound was synthesized following general method C: mp 165–167 °C; ^{1}H NMR (Me_2SO-d_6) δ 0.89 (t, 3H, J = 7.2 Hz, CH_2CH_3), 1.35 (t, 3H, J = 7.2 Hz, NCH_2CH_3), 1.41 and 1.49 (m, 2H each, CH_2), 2.38 (t, 2H, J = 7.2 Hz, $C{\equiv}CCH_2$), 4.10 (q, 2H, J = 7.2 Hz, NCH_2), 7.24 (s, 2H, NH_2) 8.15 (s, 1H, H-8). Anal. calcd for $C_{13}H_{17}N_5$: C, 64.17, H, 7.04, N, 28.78; found: C, 64.23, H, 7.16, N, 28.52.

9-Ethyl-6-(2-phenylethyloxy)-9*H*-purine (12a). This compound was synthesized following general method A: mp 61–63 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.43 (t, 3H, J = 7.3 Hz, CH_2CH_3), 3.14 (t, 2H, J = 7.0 Hz, CH_2-Ph), 4.26 (q, 2H, J = 7.3 Hz, CH_2CH_3), 4.76 (t, 2H, J = 7.0 Hz, CH_2O), 7.12–7.40 (br m, 5H, H-Ph), 8.41 (s, 1H, H-8), 8.52 (s, 1H, H-2). Anal. calcd for $C_{15}H_{16}N_4O$: C, 67.15, H, 6.01, N, 20.88; found: C, 67.33, H, 6.22, N, 20.67.

9-Ethyl-6-(2-phenylethylamino)-9*H*-purine (12b). This compound was synthesized following general method B: mp 56–56 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.40 (t, 3H, J = 7.3 Hz, CH_2CH_3), 2.92 (t, 2H, J = 7.2 Hz, CH_2-Ph), 3.71 (br m, 2H, CH_2NH) 4.18 (q, 2H, J = 7.3 Hz, CH_2CH_3), 7.27 (br m, 5H, H-Ph), 7.74 (br s, 1H, NH), 8.16 (s, 1H, H-8), 8.22 (s, 1H, H-2). Anal. calcd for $C_{15}H_{17}N_5$: C, 67.39, H, 6.41, N, 26.20; found: C, 67.51, H, 6.47, N, 25.93.

9-Ethyl-6-(4-phenyl-1-butyn-1-yl)-9*H*-purine (12c). This compound was synthesized following general method C: mp 110–112 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.46 (t, 3H, J = 7.3 Hz, CH_2CH_3), 2.94 (ps t, 4H, C≡CCH_2CH_2), 4.30 (q, 2H, J = 7.3 Hz, CH_2CH_3), 7.34 (br m, 5H, H-Ph), 8.67 (s, 1H, H-8), 8.84 (s, 1H, H-2). Anal. calcd for $C_{17}H_{16}N_4$: C, 73.89, H, 5.84, N, 20.27; found: C, 73.96, H, 6.02, N, 19.88.

9-Ethyl-6-(1-hexyn-1-yl)-9*H*-purine (12d). This compound was synthesized following general method C to give a chromatographically pure oil; ^{1}H NMR (Me_2SO-d_6) δ 0.94 (t, 3H, J = 7.1 Hz, CH_2CH_3), 1.45 (t, 3H, J = 7.2 Hz, NCH_2CH_3), 1.38–1.61 (br m, 4H, $CH_2CH_2CH_3$), 2.62 (t, 2H, J = 6.7 Hz, C≡CCH_2), 4.30 (q, 2H, J = 7.2 Hz, NCH_2CH_3), 8.66 (s, 1H, H-8), 8.85 (s, 1H, H-2). Anal. calcd for $C_{13}H_{16}N_4$: C, 68.39, H, 7.06, N, 24.54; found: C, 68.23, H, 7.25, N, 24.32.

8-Bromo-9-ethyl-6-iodo-9*H*-purine (13). A mixture of compound **3** (0.31 g, 1.3 mmol) in dry CH_3CN (25 mL), CH_2I_2 (8 mL), and *iso*-pentyl nitrite (0.8 mL, 5.9 mmol) was heated at 85 °C for 1 h. The solvent was removed under reduced pressure (oil pump, 60 °C) and the residue was purified by chromatography on a silica gel column eluting with $CHCl_3$ to give compound **13** (0.33 g, 72%) as a pure solid: mp 178–180 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.37 (t, 3H, J = 7.3 Hz, CH_2CH_3), 4.27 (q, 2H, J = 7.3 Hz, CH_2CH_3), 8.64 (s, 1H, H-2). Anal. calcd for $C_7H_6BrN_4$: C, 23.82, H, 1.71, N, 15.87; found: C, 24.14, H, 1.95, N, 15.63.

8-Bromo-9-ethyl-6-(2-phenylethyloxy)-9*H*-purine (14a). This compound was synthesized following general method A: mp 102–104 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.21 (t, 3H, J = 7.3 Hz, CH_2CH_3), 3.17 (t, 2H, J = 6.4 Hz, CH_2-Ph), 3.98 (q, 2H, J = 7.2 Hz, CH_2CH_3) 4.81 (t, 2H, J = 6.5 Hz, CH_2O), 7.31 (br m, 5H, H-Ph), 8.43 (s, 1H, H-2). Anal. calcd for $C_{15}H_{15}BrN_4O$: C, 51.89, H, 4.35, N, 16.14; found: C, 52.16, H, 4.56, N, 15.89.

8-Bromo-9-ethyl-6-(2-phenylethylamino)-9*H*-purine (14b). This compound was synthesized following general method B: mp 114–117 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.33 (t, 3H, J = 7.3 Hz, CH_2CH_3), 2.92 (t, 2H, J = 7.3 Hz, CH_2-Ph), 3.72 (br m, 2H, CH_2NH) 4.18 (q, 2H, J = 7.1 Hz, CH_2CH_3), 7.28 (br m, 5H, H-Ph), 8.02 (m, 1H, NH), 8.23 (s, 1H, H-2). Anal. calcd for $C_{15}H_{16}BrN_5$: C, 52.04, H, 4.36, N, 20.23; found: C, 52.35, H, 4.91, N, 20.02.

8-Bromo-9-ethyl-6-(4-phenyl-1-butyn-1-yl)-9*H*-purine (14c). This compound was synthesized following general method C: mp 105–106 °C; ^{1}H NMR (Me_2SO-d_6) δ 1.37 (t, 3H, J = 7.2 Hz, NCH_2CH_3), 2.94 (m, 4H, C≡CCH_2CH_2), 4.29 (q, 2H, J = 7.2 Hz, NCH_2), 7.33 (br m, 5H, H-Ph), 8.85 (s, 1H, H-2). Anal. calcd for $C_{17}H_{15}BrN_4$: C, 57.48, H, 4.26, N, 15.77; found: C, 57.81, H, 4.38, N, 15.61.

8-Bromo-9-ethyl-6-(1-hexyn-1-yl)-9*H*-purine (14d). This compound was synthesized following general method C to give a chromatographically pure oil; ^{1}H NMR (Me_2SO-d_6) δ 0.95 (t, 3H, J = 7.3 Hz, CH_2CH_3), 1.37 (t, 3H, J = 7.3 Hz, NCH_2CH_3), 1.45–1.60 (br m, 4H, $CH_2CH_2CH_3$), 2.63 (t, 2H, J = 6.9 Hz, C≡CCH_2), 4.29 (q, 2H, J = 7.2 Hz, NCH_2), 8.85 (s, 1H, H-2). Anal. calcd for $C_{13}H_{15}BrN_4$: C, 58.83, H, 4.92, N, 18.24; found: C, 59.06, H, 5.27, N, 17.97.

Biological studies

[^{3}H]DPCPX, [^{3}H]NECA, [^{3}H]CGS21680, and [^{32}P]ATP were from Du Pont NEN, Dreieich, Germany, and [^{125}I]AB-MECA from Amersham Buchler, Braunschweig, Germany. The 96-well microplate filtration system (MultiScreen MAFC) was obtained from Millipore, Eschborn, Germany. All other materials were from sources as described earlier.[6,22] Of all substances under investigation 10 mM stock solutions in DMSO were prepared which were diluted with binding buffer to 0.4 mM. All other concentrations needed were prepared by further dilution of the 0.4 mM solution with binding buffer.

Cells and membranes

CHO cells were stably transfected with all the human adenosine receptor subtypes as described for β-adrenergic receptors.[18] After growing cells on Petri dishes (Ø 140 mm) they were washed and frozen in the dishes until preparation of membranes. Details have been published elsewhere. Crude membranes were prepared by thawing frozen cells followed by scraping them off the Petri dishes in hypotonic buffer (5 mM Tris/HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized (Ultra–Turrax, 2×15 sec at full speed) and the homogenate was spun for 10 min at 1,000 *g*. The supernatant was then centrifuged for 40 min at 50,000 *g*. The membrane pellet was resuspended in 50 mM Tris/HCl buffer pH 7.4 (for A_3 adenosine receptors: 50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.25), frozen in liquid nitrogen at a protein concentration of 1–3 mg/mL and stored at −80 °C.

Radioligand binding

Dissociation constants (K_i-values) of the 9-ethyladenine derivatives were determined in radioligand competition experiments. K_i-values were calculated from IC_{50}-values according to Cheng and Prusoff.[23]

For A_1 adenosine receptors [^{3}H]DPCPX was used as an antagonist radioligand. Binding was carried out as described previously[23] in a total volume of 200 μL in 96-well microplate filtration system (Millipore MultiScreen MAFC) containing the compound to be tested at different concentrations, 1 nM [^{3}H]DPCPX, 0.2 U/mL adenosine deaminase and 20 μg of membrane protein in 50 mM Tris/HCl pH 7.4. Samples were incubated for 3 h at 25 °C, filtered and washed three times with 200 μL of ice-cold binding buffer. After addition of 20 μL of scintillator to the dried filterplates samples were counted in a Wallac MicroBeta counter.

Conditions for A_{2A} adenosine receptor binding with [^{3}H]CGS21680 or [^{3}H]NECA (30 nM) as radioligand were essentially the same as for A_1 receptor binding. Samples with a protein concentration of 50–80 μg were incubated for 2 h at 25 °C and filtered individually as described for conventional radioligand binding.[6]

A_3 adenosine receptor binding was also performed by incubation and filtration of single samples with ^{125}I-AB-MECA (0.2–0.3 nM) at conditions adopted from Ji et al.[24] As a binding buffer 50 mM Tris/HCl, 1 mM EDTA, 10 mM $MgCl_2$, pH 8.25 was used and samples were incubated for 2 h at 25 °C.

Adenylyl cyclase activity

Due to the lack of a suitable radioligand the affinity (K_i, K_B) of the 9-ethyladenine derivatives at A_{2B} adenosine receptors was determined in adenylyl cyclase experiments. The procedure was carried out as previously published with minor modifications.[25] About 100,000 cpm of [α-^{32}P]ATP were used and EGTA and NaCl were omitted from the incubation mixture. IC_{50}-values for the concentration-dependent inhibition of NECA-stimulated (5 μM) adenylyl cyclase were determined and converted to K_i-values according to Cheng and Prusoff.[23] For this conversion the actual EC_{50}-value of NECA (2.4 μM) was used instead of the K_D. In selected experiments the concentration-dependent shift of NECA-stimulated adenylyl cyclase caused by the 9-ethyladenine derivatives was used as a second method to determine the dissociation constants by Schild plots. Comparison of the methods confirmed that the two approaches yielded identical results.[25]

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