NEW 9-METHYL-8-(4-HYDROXYPHENYL)ADENINE DERIVATIVES AS A_1 ADENOSINE RECEPTOR ANTAGONISTS

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Dedicated to Professor Antonín Holý on the occasion of his 75th birthday.

A new series of 9-methyladenines, bearing different bulky groups at the 8-position, were prepared and their affinity for the four human adenosine receptor subtypes were evaluated. All the synthesized compounds showed affinities at the A_1, A_{2A}, and A_3AR subtypes ranging from nanomolar to micromolar levels with different degrees of A_1 selectivity, while they resulted nearly inactive at A_{2B}AR. In particular, 9-methyl-8-[4-(4-methylbenzyloxy)phenyl]-adenine showed A_1AR affinity in the nanomolar range and good levels of selectivity versus the other receptor subtypes. Furthermore, a functional assay at mouse ileum allowed to assess the potency of selected compounds at A_1AR subtype. Results showed that all the tested derivatives are neutral antagonists and their K_b values are in good agreement with the K_i values from radioligand binding assay at human A_1AR, confirming that the effect is due to inhibition of this subtype.
Keywords: Adenosine receptors; Adenosine receptor antagonists; Adenine derivatives; A_1AR functional studies; Receptors; Nucleobases; Heterocycles; Ligand design; Ligand effects.

Adenosine is a naturally occurring nucleoside that mediates numerous physiological and physio-pathological processes[1] through the activation of

four G protein-coupled receptors, which have been cloned[2] and classified as A_1, A_{2A}, A_{2B}, and A_3 adenosine receptor (AR) subtypes[3] on the bases of coupling to second messengers and pharmacological profiles for agonists and antagonists. Clarifying the physiological functions of the different subtypes has been useful to detect possible targets of therapeutic intervention. At the same time, lack of potent and selective ligands for a specific subtype could be a drawback to the attribution of specific activity.

In the case of A_{2B}AR, this lack precluded for many years a deep exploration of its therapeutic prospective but the discovery of antagonists with xanthine structure such as MRS 1754 (**1**) and CVT-6883 (**2**), and with non xanthine structure such as OSIP 339391 (**3**) led to preclinical studies mainly on inflammatory and angiogenic diseases (Fig. 1)[4]. Moreover, it is known that in general adenosine antagonists could be promising therapeutic targets in a wide range of conditions, including cerebral and cardiac ischaemic diseases, sleep disorders, immune and inflammatory disorders and cancer[5].

MRS 1754 (1)
hA_1 = 403 nM
hA_{2A} = 503 nM
hA_{2B} = 2.0 nM
hA_3 = 570 nM

CVT-6883 (2)
hA_1 = 990 nM
hA_{2A} = 690 nM
hA_{2B} = 1.0 nM
hA_3 = 1,000 nM

OSIP 339391 (3)
hA_{2B} KD = 20 nM
selectivities > 70

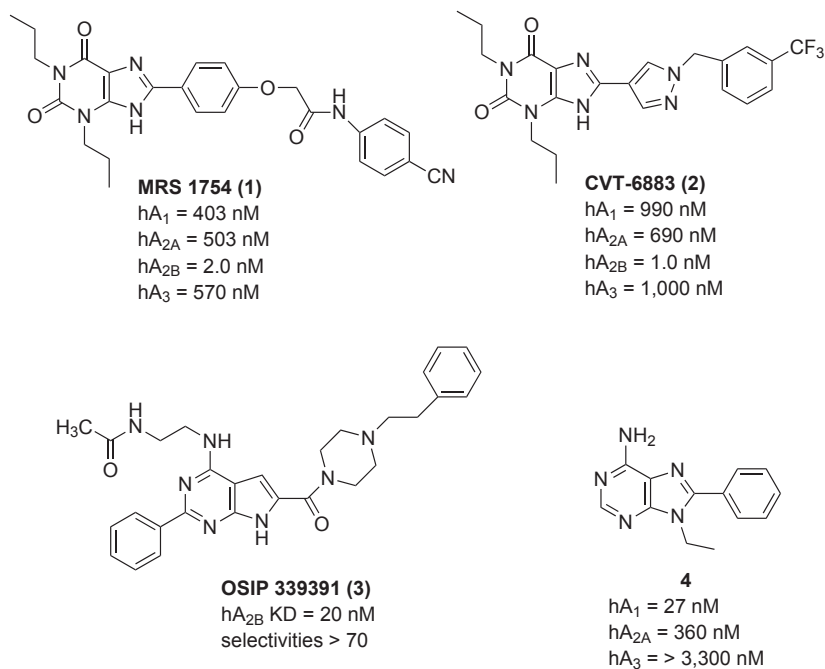**4**
hA_1 = 27 nM
hA_{2A} = 360 nM
hA_3 = > 3,300 nM

FIG. 1
Structures and binding profile of some AR antagonists (K_i, nM)

A structural analysis of the xanthine derivatives **1** and **2** clearly points out the importance for A$_{2B}$ affinity of bulky substituents containing phenyl and heteroaromatic groups at the 8-position. On the other hand, we reported that the introduction of different substituents at the 2-, 8-, and 9-position of the adenine moiety resulted in high-affinity antagonists presenting distinct receptor selectivity profile[6–10], with the derivative 9-ethyl-8-phenyladenine (**4**) endowed with good affinity and selectivity for the A$_1$AR subtype[11]. Furthermore, Harada et al.[12] some years ago found that 2-alkynyl-9-methyladenines bearing a substituted phenyl group in 8-position inhibited agonist-induced glucose production in rat hepatocytes and showed hypoglycemic activity in an animal model of noninsulin-dependent diabetes mellitus, effects that are considered to be mediated by the interaction with the A$_{2B}$AR subtype.

Taking into account all the above reported observations and to check the effect of introducing bulky substituents in 8-position of the purine core, we designed and synthesized new 9-methyladenine derivatives **5–17** bearing in 8-position a para phenoxy moiety with an attached benzyl (Scheme 1) or *N*-phenylacetamido group (Scheme 2, Fig. 2). The synthesized compounds were tested at human AR subtypes expressed in Chinese hamster ovary

MRS like molecules

Harada Compounds

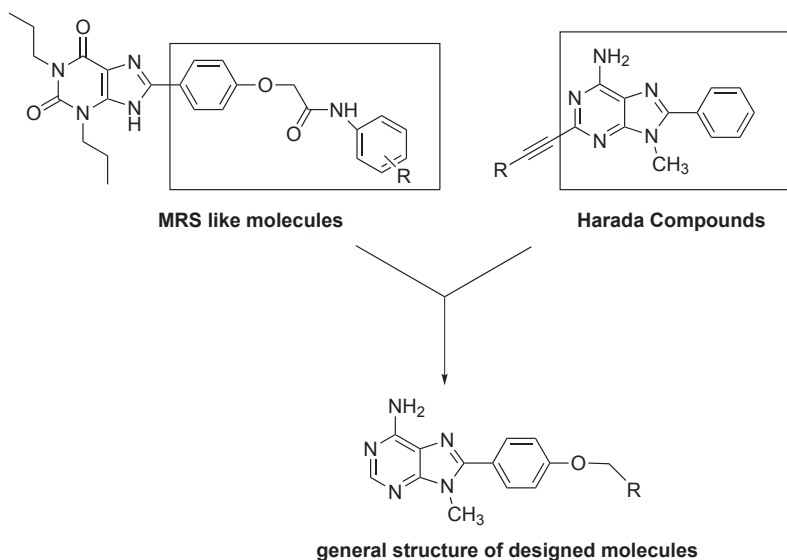**general structure of designed molecules**

Fig. 2
Rational design of synthesized compounds

(CHO) cells in radioligand binding assay with the aim at evaluating the effect of the introduced substituents on A_1 and A_{2B}ARs binding affinity.

Introduction of alkynyl groups at the 2-position, suggested by Harada, was avoided for two reasons. First of all, adenine derivatives with big lipophilic substituents in 2- and 8-positions would be rather insoluble and, secondly, we already proved that the introduction of an alkynyl group in 2-position of adenine brought about an improvement of affinity at all the other receptor subtypes higher than that at the A_{2B} subtype[7,10].

RESULTS AND DISCUSSION

Chemistry

The designed compounds **5–17** were synthesized as summarized in Schemes 1 and 2. Reaction of commercially available 5-amino-4,6-dichloro-pyrimidine (**18**) with methylamine at 55 °C afforded derivative **19** as major product[13], which after a two-step coupling with 4-hydroxybenzadehyde gave the purine derivative **20** in a good yield. The latter was then reacted with liquid ammonia in a sealed tube at 70 °C to afford the desired 8-substituted adenine **5** (Scheme 1). Reaction of compound **5** with the ap-
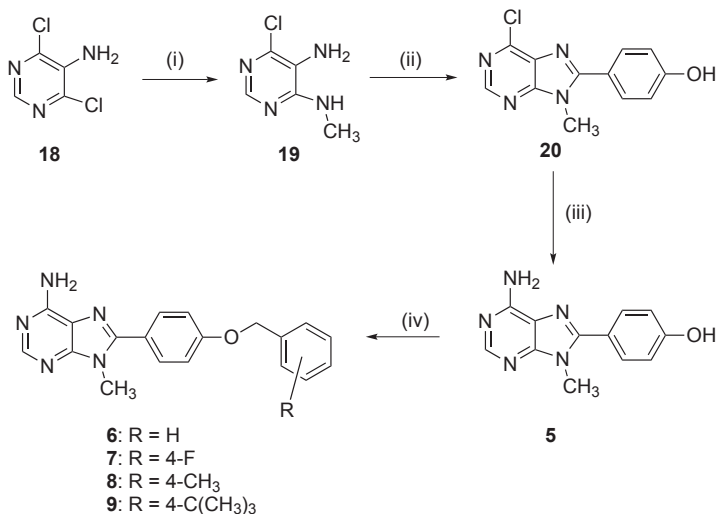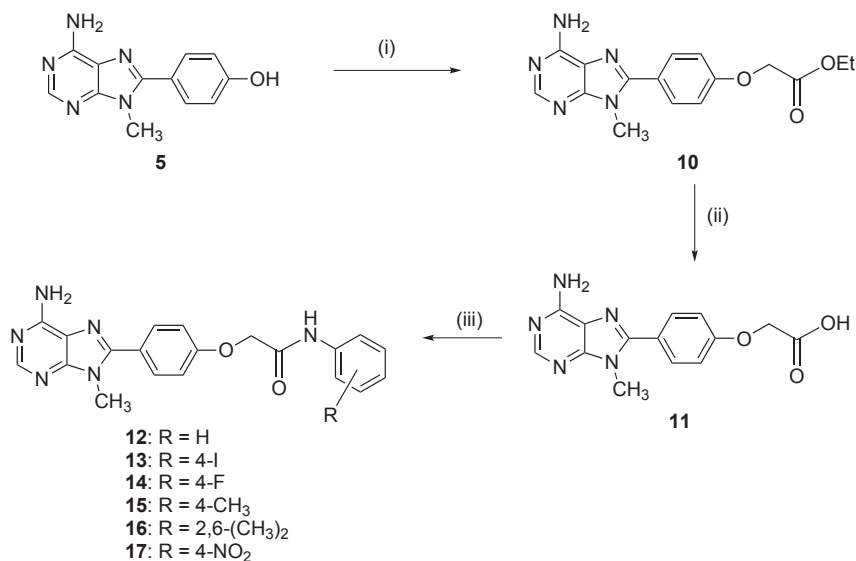

SCHEME 1
(i) CH_3NH_2, Et_3N, 55 °C; (ii) 4-hydroxybenzaldehyde, CH_3CO_2H, r.t. then EtOH, $FeCl_3$, 85 °C; (iii) NH_3, 70 °C, 24 h; (iv) substituted benzyl halide, K_2CO_3, dry DMF, r.t., 4 h

propriate substituted benzyl halides in dry dimethylformamide (DMF) in the presence of potassium carbonate gave the corresponding *O*-benzyl derivatives **6–9** (Scheme 1).

On the other hand, by reaction of **5** with α-bromo ethyl acetate under the same conditions, the corresponding phenoxyacetate ethyl ester **10** was obtained, which was converted into the corresponding acid derivative **11** by saponification with 5% NaOH. The final amido compounds **12–17** were obtained by reaction of **11** with the suitable aniline derivatives in dry DMF in the presence of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI; Scheme 2).

The newly synthesized compounds **5–17** were tested at the human A$_1$, A$_{2A}$, and A$_3$AR subtypes expressed in CHO cells in radioligand binding using 2-chloro-N^6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA) for A$_1$AR, [^{3}H]5′-*N*-ethylcarboxamidoadenosine ([^{3}H]NECA) for A$_{2A}$, and [^{3}H]2-hexyn-2-yl-N^6-methyladenosine ([^{3}H]HEMADO) for A$_3$ARs [14]. In the case of A$_{2B}$AR, K_i values were calculated from IC$_{50}$ values determined by inhibition of NECA-stimulated adenylyl cyclase activity[15]. K_i values are expressed in nM, with 95% confidence intervals in parentheses. The results of binding and cyclase activity studies are reported in Table I.

SCHEME 2
(i) α-bromoethyl acetate, K$_2$CO$_3$, dry DMF, r.t., 4 h; (ii) 5% NaOH, dioxane, r.t., 3 h; (iii) substituted aniline, EDCI, dry DMF, r.t., 24 h

TABLE I
Biological profile of synthesized compounds **5–17**
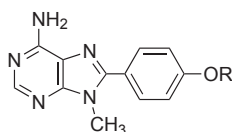
5-17

Cpd	R	K_i, nM			
		hA_1AR^a	$hA_{2A}AR^b$	$hA_{2B}AR^c$	hA_3AR^d
5	H	164 (136–198)	772 (652-914)	>30000	13600 (10300–18000)
6	CH_2Ph	831 (727–950)	24500 (23200–25800)	>30000	>40000
7	CH_2Ph-4-F	215 (183–252)	36100 (25800–50600)	>30000	>40000
8	CH_2Ph-4-CH_3	89 (62–127)	16800 (13400–21130)	>30000	>40000
9	CH_2Ph-4-$C(CH_3)_3$	176 (104–300)	>100000	>30000	>40000
10	CH_2CO_2Et	2990 (2150–4170)	4190 (3380–5200)	>30000	6630 (5590–7340)
11	CH_2CO_2H	26700 (24000–34300)	>100000	>30000	>40000
12	$CH_2CONHPh$	812 (777–849)	3380 (2420–4730)	>30000	>40000
13	$CH_2CONHPh$-4-I	145 (101–208)	1120 (911–1390)	>30000	2180 (1750–2720)
14	$CH_2CONHPh$-4-F	462 (294–727)	2600 (2270–2970)	>30000	>40000
15	$CH_2CONHPh$-4-CH_3	222 (184–268)	1250 (1020–1520)	>30000	>40000
16	$CH_2CONHPh$-2,6-$(CH_3)_2$	4070 (2380–7030)	12000 (9260–15500)	>30000	>40000
17	$CH_2CONHPh$-4-NO_2	1150 (1090–1200)	9550 (7950–11500)	>30000	12600 (10100–15900)

[a] Displacement of specific [^{3}H]CCPA binding at human A_1AR expressed in CHO cells, (n = 3–6). [b] Displacement of specific [^{3}H]NECA binding at human $A_{2A}AR$ expressed in CHO cells. [c] K_i values of the inhibition of NECA-stimulated adenylyl cyclase activity in CHO cells expressing human $A_{2B}AR$. [d] Displacement of specific [^{3}H]NECA binding at human A_3AR expressed in CHO cells. Data are expressed as geometric means with 95% confidence limits in parentheses.

All the synthesized compounds **5**–**17** showed affinities at the A$_1$, A$_{2A}$, and A$_3$AR subtypes ranging from nanomolar to micromolar levels with different degrees of A$_1$ selectivity, while they resulted nearly inactive at A$_{2B}$AR. In particular, the 8-(4-hydroxy)phenyl derivative **5** presented an A$_1$AR affinity of 164 nM with good selectivity vs A$_{2B}$ and A$_3$ARs, while resulted to be poorly selective vs the A$_{2A}$AR (A$_{2A}$/A$_1$ = 4.7). Alkylation of the phenolic function with benzyl groups (compounds **6**–**9**) did not modify significantly the potency at the A$_1$AR but an increased selectivity could be observed, since the interaction of these compounds with the other receptor subtypes seems to be less favourable. More in detail, the unsubstituted benzyl derivative **6** resulted to be five-fold less potent at the A$_1$AR but the affinity versus the other receptor subtypes resulted dramatically reduced if compared with the parent compound **5**, with a significant increase of selectivity as a consequence.

A general increase in affinity could be detected when a substitution at the *para*-position of the phenyl ring was introduced (in **7**–**9**). In particular, the presence of a methyl group afforded the best compound of the series (derivative **8**) with an A$_1$AR affinity of 89 nM and good levels of selectivity versus the other receptor subtypes (A$_{2A}$/A$_1$ = 191, A$_3$/A$_1$ > 454). Other substituents at the *para*-position, such as fluorine (in **7**) or *tert*-butyl (in **9**) permitted retention of affinity at the A$_1$AR, comparable to that of the non alkylated derivative **5**, but the levels of selectivity versus the other receptors resulted to increase (e.g. **7** A$_{2A}$/A$_1$ = 168, A$_3$/A$_1$ > 186 and **5** A$_{2A}$/A$_1$ = 4.7, A$_3$/A$_1$ > 83).

Alkylation of the phenolic function of compound **5** with an ethyl acetate **10** significantly reduced the affinity at the A$_1$ and A$_{2A}$ARs, while a slight improvement was detected at the A$_3$AR if compared with derivative **5**. In contrast, the corresponding acid derivative **11** showed a complete loss of affinity at the four human AR subtypes.

Introduction of an amido group between the methylene and the phenyl ring did not modify substantially the A$_1$ binding profile, derivatives **12**–**17** affinity being only slightly lower than that of the corresponding parent compounds (compare **6**, **7**, and **8** with **12**, **14**, and **15**, respectively). However, the amido derivatives presented a reduced selectivity, in particular vs the A$_{2A}$AR subtype since their A$_{2A}$ affinity was ten-fold higher than that of the corresponding compounds **6**–**8**. Interestingly, the presence of an iodine at the 4-position of the phenyl ring (compound **13**) induced affinity in the μM range at the A$_3$AR (K_i = 2180 nM) while all the other derivatives resulted to be almost inactive at this subtype. The presence of a strong electron-withdrawing group such as *para*-NO$_2$ (in **17**) or double substitution of the

phenyl ring (in **16**) led to a reduction of affinity at all the four human AR subtypes.

Functional Studies

Since some adenine derivatives presented good affinity for the A_1AR, a functional assay at mouse ileum was performed to assess their potency at this AR subtype. This assay was chosen since it is known that the inhibition of rhythmic spontaneous contraction of ileum tissue is due to the activation of A_1ARs [16].

Concentration–response curves of NECA (Fig. 3) alone and in the presence of compounds **5**, **8**, **9**, **13**, and **15** at 10^{-6} M are reported in Fig. 4. In the same figure, the NECA curve in the presence of the A_1AR reference an-
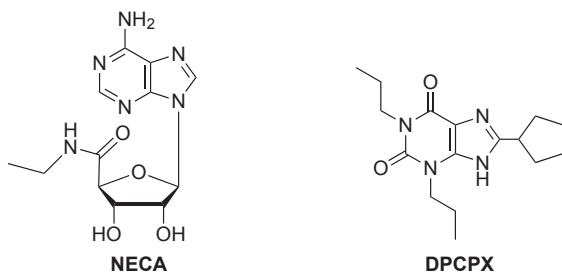

Fig. 3
AR agonist (NECA) and antagonist (DPCPX) compounds used for biological evaluation analysis
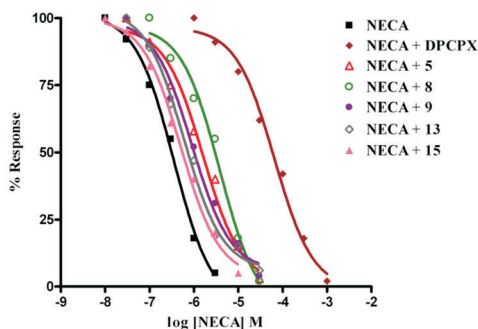

Fig. 4
Concentration–response curves of NECA in the presence of compounds **5**, **8**, **9**, **13**, and **15** at 10^{-6} M at mouse ileum (A_1AR) in comparison with DPCPX at the same concentration. Each point represents the mean of four or five experiments with a maximum SEM lower than ± 10

tagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX; Fig. 3), tested at the same concentration, is shown. The obtained K_b values of the same compounds with the addition of ligand **11** are reported in Table II.

TABLE II
Antagonist potency of selected compounds, expressed as K_b values, in the isolated mouse ileum (A$_1$AR) in comparison with DPCPX (K_i values from binding experiments at A$_1$AR are reported for comparison)

Compounds[a]	K_b, nM[b]	K_i, nM (A$_1$AR)
DPCPX	5 (4.9–5.1)	3.9
5	204 (181–227)	164
8	102 (90–114)	89
9	309 (274–344)	176
11	22900[c] (22600–23200)	26700
13	371 (340–402)	145
15	562 (524–600)	222

[a] Compounds (except **11**) were tested at 10^{-6} M concentration. [b] K_b values in functional tests are geometric means of four or five experiments with 95% confidence limits in parentheses. [c] Values obtained at 10^{-4} M concentration.
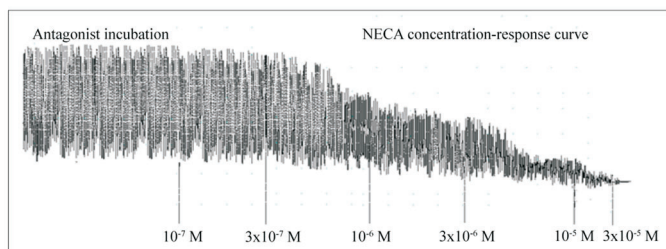

FIG. 5
NECA effect on rhythmic spontaneous contractions of mouse ileum in the presence of an antagonist

Results showed that all the selected compounds are neutral antagonists as they were able to shift the NECA concentration–response curve (Fig. 5) without affecting the rhythmic spontaneous contraction of ileum (data not shown).

It is worthwhile to note that the K_b values obtained with this functional assay at mouse ileum are in good agreement with the K_i values from radioligand binding assay at human A_1AR, confirming that the effect is due to inhibition of the A_1AR subtype (Table II).

CONCLUSIONS

In conclusion, the present study clearly demonstrated that introduction of bulky substituents, similar to those present at the 8-position of A_{2B} antagonists with xanthine structure, in the adenine nucleus does not induce any $A_{2B}AR$ activity but good A_1AR affinity and selectivity. Furthermore, a functional study at mouse ileum showed that all the selected compounds are neutral antagonists and their K_b values are in good agreement with the K_i values from radioligand binding assay at human A_1AR, confirming that the effect is due to inhibition of this subtype. It is worthwhile to note that A_1AR antagonists may have therapeutic applications[5] and some of them are in clinical trials for the treatment of acute decompensated heart failure (ADHF) with renal impairment[4].

EXPERIMENTAL

Chemical Synthesis

Reactions were routinely monitored by thin-layer silica gel chromatography (TLC; precoated F_{254} Merck plates) and products visualized by UV light and iodine or potassium permanganate solution. IR spectra (v, cm^{-1}) were recorded on a Jasco FT-IR 200 spectrometer. ^1H NMR spectra were determined in DMSO-d_6 solutions on a Bruker AC 200 spectrometer downfield from tetramethylsilane as internal standard. Chemical shifts are given in ppm (δ-scale) and coupling constants (J) in Hz. Light petroleum ether refers to the fractions boiling at 40–60 °C. Melting points were determined on a Buchi–Tottoli instrument and are uncorrected. Column chromatography was performed using Merck 60–200 mesh silica gel. All products reported showed ^1H NMR spectra in agreement with the assigned structures. Organic solutions were dried over anhydrous magnesium sulfate.

6-Chloro-9-methyl-8-(4-hydroxyphenyl)purine (**20**)

A methanolic (8 ml) solution of compound **19** (300 mg, 1.89 mmol), acetic acid (0.25 ml), and 4-hydroxybenzaldehyde (231 mg, 1.89 mmol) was stirred at room temperature for 24 h. Then the solvent was removed under reduced pressure and to the residue dissolved in abso-

lute EtOH (8 ml), FeCl$_3$ (307 mg) was added. The resulting mixture was heated at 85 °C for 6 h. After cooling to room temperature, the pure product **20** was obtained after filtration as a white solid (yield 62%); m.p. 206 °C. IR (KBr): 3450–3090, 1610, 1515, 1430. ^{1}H NMR (DMSO-d_6): 3.92 (s, 3 H, CH$_3$); 6.98 (d, 2 H, H-Ph, *J* = 8.4); 7.84 (d, 2 H, H-Ph, *J* = 8.4); 8.75 (s, 1 H, H-2); 10.23 (s, 1 H, OH). For C$_{12}$H$_9$ClN$_4$O (260.68) calculated: 55.29% C, 3.48% H, 21.49% N; found: 55.41% C, 3.52% H, 21.34% N.

9-Methyl-8-(4-hydroxyphenyl)adenine (**5**)

In a steel vial cooled to –80 °C, compound **20** (1.55 g, 5.95 mmol) and an excess of liquid ammonia were poured. The vial was sealed and heated at 70 °C for 24 h. Then the mixture was cooled to room temperature and, after evaporation of ammonia, the crude product was purified by flash chromatography (CHCl$_3$–cyclohexane–MeOH 75:25:5) to give compound **5** as a white solid (yield 91%); m.p. > 250 °C. IR (KBr): 3430–3055, 1615, 1510, 1425. ^{1}H NMR (DMSO-d_6): 3.75 (s, 3 H, CH$_3$); 6.92 (d, 2 H, H-Ph, *J* = 8.0); 7.18 (s, 2 H, NH$_2$); 7.68 (d, 2 H, H-Ph, *J* = 8.4); 8.13 (s, 1 H, H-2); 9.97 (s, 1 H, OH). For C$_{12}$H$_{11}$N$_5$O (241.25) calculated: 59.74% C, 4.60% H, 29.03% N; found: 59.82% C, 4.57% H, 29.07% N.

Synthesis of *O*-Alkylated Compounds **6–10**. General Procedure

A mixture of the adenine derivative **5** (50 mg, 0.207 mmol), K$_2$CO$_3$ (31.5 mg, 0.228 mmol), and the appropriate halide (1.2 eq) in dry DMF (4 ml) was stirred at room temperature for 6 h. The solvent was removed under reduced pressure and the residue was poured into water (10 ml) and extracted with EtOAc (3 × 15 ml). The combined organic layers were dried and concentrated under vacuum. The crude product was purified by flash chromatography (EtOAc–light petroleum 1:1) to yield the final compounds **6–10** as solids.

9-Methyl-8-(4-benzyloxyphenyl)adenine (**6**). Yield 75%, white solid, m.p. 225 °C. IR (KBr): 3330–3120, 1615, 1510, 1425. ^{1}H NMR: 4.05 (s, 3 H, CH$_3$); 5.19 (s, 2 H, O-CH$_2$); 7.18 (d, 2 H, H-Ph, *J* = 9.0); 7.22–7.26 (m, 2 H, H-Ph); 7.30 (bs, 2 H, NH$_2$); 7.38–7.45 (m, 3 H, H-Ph); 7.83 (d, 2 H, H-Ph, *J* = 9.0); 8.37 (s, 1 H, H-2). For C$_{19}$H$_{17}$N$_5$O (331.37) calculated: 68.87% C, 5.17% H, 21.13% N; found: 69.01% C, 5.21% H, 21.07% N.

9-Methyl-8-[4-(4-fluorobenzyloxy)phenyl]adenine (**7**). Yield 66%, white solid, m.p. 237 °C. IR (KBr): 3335–3120, 1605, 1515, 1420. ^{1}H NMR: 4.07 (s, 3 H, CH$_3$); 5.16 (s, 2 H, O-CH$_2$); 7.15 (d, 2 H, H-Ph, *J* = 9.0); 7.21 (d, 2 H, H-Ph, *J* = 9.0); 7.27 (bs, 2 H, NH$_2$); 7.42 (d, 2 H, H-Ph, *J* = 9.0); 7.89 (d, 2 H, H-Ph, *J* = 9.0); 8.40 (s, 1 H, H-2). For C$_{19}$H$_{16}$FN$_5$O (349.36) calculated: 65.32% C, 4.62% H, 20.05% N; found: 65.21% C, 4.55% H, 20.07% N.

9-Methyl-8-[4-(4-methylbenzyloxy)phenyl]adenine (**8**). Yield 72%, white solid, m.p. 225 °C. IR (KBr): 3340–3110, 1610, 1515, 1410. ^{1}H NMR: 2.40 (s, 3 H, Ph-CH$_3$); 4.09 (s, 3 H, N9-CH$_3$); 5.16 (s, 2 H, O-CH$_2$); 7.18 (d, 2 H, H-Ph, *J* = 9.0); 7.20 (d, 2 H, H-Ph, *J* = 9.0); 7.25 (bs, 2 H, NH$_2$); 7.38 (d, 2 H, H-Ph, *J* = 9.0); 7.87 (d, 2 H, H-Ph, *J* = 9.0); 8.42 (s, 1 H, H-2). For C$_{20}$H$_{19}$N$_5$O (345.40) calculated: 69.55% C, 5.54% H, 20.28% N; found: 69.61% C, 5.51% H, 20.22% N.

9-Methyl-8-[4-(4-tert-butylbenzyloxy)phenyl]adenine (**9**). Yield 69%, white solid, m.p. 238 °C. IR (KBr): 3335–3110, 1600, 1505, 1410. ^{1}H NMR: 1.36 (s, 9 H, C(CH$_3$)$_3$); 4.01 (s, 3 H, CH$_3$); 5.15 (s, 2 H, O-CH$_2$); 7.20 (d, 2 H, H-Ph, *J* = 9.0); 7.24 (d, 2 H, H-Ph, *J* = 9.0); 7.33 (bs, 2 H, NH$_2$); 7.43 (d, 2 H, H-Ph, *J* = 9.0); 7.78 (d, 2 H, H-Ph, *J* = 9.0); 8.35 (s, 1 H, H-2). For C$_{23}$H$_{25}$N$_5$O (387.48) calculated: 71.29% C, 6.50% H, 18.07% N; found: 71.23% C, 6.42% H, 18.02% N.

Ethyl[4-(9-methyladenin-8-yl)phenoxy]acetate (**10**). Yield 83%, white solid, m.p. 155 °C. IR (KBr): 3350–3120, 1725, 1605, 1515, 1420. ^{1}H NMR: 1.2 (t, 3 H, CH$_2$CH$_3$, *J* = 7.0); 4.00 (s, 3 H, N9-CH$_3$); 4.34 (q, 2 H, CH$_2$CH$_3$, *J* = 7.0); 4.75 (s, 2 H, O-CH$_2$); 7.11 (d, 2 H, H-Ph, *J* = 9.0); 7.26 (bs, 2 H, NH$_2$); 7.79 (d, 2 H, H-Ph, *J* = 9.0); 8.35 (s, 1 H, H-2). For C$_{16}$H$_{17}$N$_5$O$_3$ (327.34) calculated: 58.71% C, 5.23% H, 21.39% N; found: 58.83% C, 5.28% H, 21.33% N.

[4-(9-Methyladenin-8-yl)phenoxy]acetic Acid (**11**)

Ester **10** (500 mg, 1.53 mmol) was dissolved in dioxane (3 ml) and 5% aqueous NaOH (3 ml). The resulting mixture was stirred at room temperature for 4 h. The solution was diluted with water (5 ml) and acidified with 10% aqueous HCl solution to pH 2. The precipitated acid **11** was collected by filtration. Yield 93%, grey solid, m.p. > 300 °C. IR (KBr): 3550–3020, 1715, 1610, 1505, 1410. ^{1}H NMR: 3.78 (s, 3 H, CH$_3$); 4.76 (s, 2 H, O-CH$_2$); 7.09 (d, 2 H, H-Ph, *J* = 9.0); 7.25 (bs, 2 H, NH$_2$); 7.81 (d, 2 H, H-Ph, *J* = 9.0); 8.16 (s, 1 H, H-2); 12.3 (bs, 1 H, COOH). For C$_{14}$H$_{13}$N$_5$O$_3$ (299.28) calculated: 56.18% C, 4.38% H, 23.40% N; found: 56.26% C, 4.32% H, 23.47% N.

Preparation of Phenylacetamido Derivatives **12–17**. General Procedure

A mixture of acid **11** (50 mg, 0.167 mmol), appropriate aniline (1.1 eq.), and EDCI (64 mg, 0.338 mmol) in dry DMF (4 ml) was stirred at room temperature for 24 h. Then the solvent was removed under reduced pressure and the residue was suspended in water (5 ml) and extracted with EtOAc (3 × 5 ml). The combined organic layers were dried and concentrated at reduced pressure. The crude product was then purified by flash chromatography (EtOAc–light petroleum 7:3) to yield the desired final compounds **12–17** as solids.

2-[4-(9-Methyladenin-8-yl)phenoxy]-N-phenylacetamide (**12**). Yield 74%, pale yellow solid, m.p. 195 °C. IR (KBr): 3330–3120, 1700, 1615, 1525, 1430. ^{1}H NMR: 3.79 (s, 3 H, CH$_3$); 4.82 (s, 2 H, O-CH$_2$); 7.17–7.28 (m, 5 H, H-Ph); 7.32 (bs, 2 H, NH$_2$); 7.65 (d, 2 H, H-Ph, *J* = 9.0); 7.85 (d, 2 H, H-Ph, *J* = 9.0); 8.17 (s, 1 H, H-2); 10.20 (bs, 1 H, CONH). For C$_{20}$H$_{18}$N$_6$O$_2$ (374.40) calculated: 64.16% C, 4.85% H, 22.45% N; found: 64.23% C, 4.82% H, 22.49% N.

2-[4-(9-Methyladenin-8-yl)phenoxy]-N-(4-iodophenyl)acetamide (**13**). Yield 67%, pale yellow solid, m.p. 192 °C. IR (KBr): 3350–3150, 1695, 1620, 1522, 1410. ^{1}H NMR: 3.78 (s, 3 H, CH$_3$); 4.82 (s, 2 H, O-CH$_2$); 7.18 (d, 2 H, H-Ph, *J* = 9.0); 7.23 (bs, 2 H, NH$_2$); 7.51 (d, 2 H, H-Ph, *J* = 9.0); 7.68 (d, 2 H, H-Ph, *J* = 9.0); 7.84 (d, 2 H, H-Ph, *J* = 9.0); 8.16 (s, 1 H, H-2); 10.29 (bs, 1 H, CONH). For C$_{20}$H$_{17}$IN$_6$O$_2$ (500.29) calculated: 48.01% C, 3.42% H, 16.80% N; found: 47.88% C, 3.37% H, 16.75% N.

2-[4-(9-Methyladenin-8-yl)phenoxy]-N-(4-fluorophenyl)acetamide (**14**). Yield 70%, grey solid, m.p. 260 °C. IR (KBr): 3340–3135, 1695, 1615, 1500, 1420. ^{1}H NMR: 3.79 (s, 3 H, CH$_3$); 4.81 (s, 2 H, O-CH$_2$); 7.16 (d, 2 H, H-Ph, *J* = 9.0); 7.21 (d, 2 H, H-Ph, *J* = 9.0); 7.27 (bs, 2 H, NH$_2$); 7.66 (d, 2 H, H-Ph, *J* = 9.0); 7.85 (d, 2 H, H-Ph, *J* = 9.0); 8.17 (s, 1 H, H-2); 10.25 (bs, 1 H, CONH). For C$_{20}$H$_{17}$FN$_6$O$_2$ (392.39) calculated: 61.22% C, 4.37% H, 21.42% N; found: 61.33% C, 4.42% H, 21.37% N.

2-[4-(9-Methyladenin-8-yl)phenoxy]-N-(4-tolyl)acetamide (**15**). Yield 56%, pale yellow solid, m.p. 218 °C. IR (KBr): 3340–3125, 1692, 1615, 1510, 1410. ^{1}H NMR: 2.26 (s, 3 H, Ph-CH$_3$); 3.79 (s, 3 H, N9-CH$_3$); 4.80 (s, 2 H, O-CH$_2$); 7.12 (d, 2 H, H-Ph, *J* = 9.0); 7.19 (d, 2 H, H-Ph, *J* = 9.0); 7.25 (bs, 2 H, NH$_2$); 7.54 (d, 2 H, H-Ph, *J* = 9.0); 7.84 (d, 2 H, H-Ph, *J* = 9.0); 8.16 (s, 1 H, H-2); 10.10 (bs, 1 H, CONH). For C$_{21}$H$_{20}$N$_6$O$_2$ (388.42) calculated: 64.94% C, 5.19% H, 21.64% N; found: 65.03% C, 5.22% H, 21.75% N.

2-[4-(9-Methyladenin-8-yl)phenoxy]-N-(2,6-dimethylphenyl)acetamide (**16**). Yield 74%, pale yellow solid, m.p. 236 °C. IR (KBr): 3330–3100, 1694, 1620, 1500, 1430. ^{1}H NMR: 2.13 (s, 6 H, PH-(CH$_3$)$_2$); 3.79 (s, 3 H, N9-CH$_3$); 4.85 (s, 2 H, O-CH$_2$); 7.07–7.09 (m, 2 H, H-Ph); 7.20–7.22 (m, 1 H, H-Ph); 7.25 (d, 2 H, H-Ph, J = 9.0); 7.28 (bs, 2 H, NH$_2$); 7.65 (d, 2 H, H-Ph, J = 9.0); 8.17 (s, 1 H, H-2); 9.59 (bs, 1 H, CONH). For C$_{22}$H$_{22}$N$_6$O$_2$ (402.45) calculated: 65.66% C, 5.51% H, 20.88% N; found: 65.59% C, 5.43% H, 21.00% N.

2-[4-(9-Methyladenin-8-yl)phenoxy]-N-(4-nitrophenyl)acetamide (**17**). Yield 73%, yellow solid, m.p. 150 °C. IR (KBr): 3345–3120, 1697, 1620, 1550, 1510, 1430, 1360. ^{1}H NMR: 3.81 (s, 3 H, CH$_3$); 4.80 (s, 2 H, O-CH$_2$); 7.18 (d, 2 H, H-Ph, J = 9.0); 7.25 (d, 2 H, H-Ph, J = 9.0); 7.33 (bs, 2 H, NH$_2$); 7.71 (d, 2 H, H-Ph, J = 9.0); 7.88 (d, 2 H, H-Ph, J = 9.0); 8.19 (s, 1 H, H-2); 10.02 (bs, 1 H, CONH). For C$_{20}$H$_{17}$N$_7$O$_4$ (419.39) calculated: 57.28% C, 4.09% H, 23.38% N; found: 57.19% C, 4.13% H, 23.29% N.

Biology

All pharmacological methods followed the procedures as described earlier[14]. In brief, membranes for radioligand binding were prepared from CHO cells stably transfected with human adenosine receptor subtypes in a two-step procedure. In a first low-speed step (1000 × g) cell fragments and nuclei were removed. The crude membrane fraction was sedimented from the supernatant at 100,000 × g. The membrane pellet was resuspended in the buffer used for the respective binding experiments, frozen in liquid nitrogen and stored at –80 °C. For the measurement of adenylyl cyclase activity, only one high speed centrifugation of the homogenate was used. The resulting crude membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4 and immediately used for the cyclase assay.

For radioligand binding at A$_1$AR 1 nM [^{3}H]CCPA, at A$_{2A}$AR 10 nM [^{3}H]NECA and at A$_3$AR 1 nM [^{3}H]HEMADO were used, respectively. Non specific binding of [^{3}H]CCPA was determined in the presence of 1 mM theophylline, in the case of [^{3}H]NECA 100 pM N^6-(R)-phenylisopropyladenosine (R-PIA) was used. K_i values from competition experiments were calculated with the program SCTFIT [17]. Radioligand binding at A$_{2B}$AR is problematic as no high-affinity ligand is available for this subtype. Therefore, inhibition of NECA-stimulated adenylyl cyclase activity was determined as a measurement of affinity of compounds. EC$_{50}$ values from these experiments were converted to K_i values with the Cheng and Prusoff equation[15].

Functional antagonism in mouse ileum. Animal testing was carried out according to the European Communities Council Directive of 24 November 1986 (86/609/EEC). Experiments were performed on male mice (BALB/C; 25.5 ± 0.5 g body weight, 8 weeks old) reared at the School of Pharmacy building B, University of Camerino (Camerino, Italy). They were housed in a room on a reverse 12-h light/dark cycle, temperature of 20–22 °C, and humidity of 45–55% with free access to food and water. Animals were sacrificed by cervical dislocation, ileum was isolated, freed from adhering connective tissue and placed in Krebs solution of the following composition (in mM): NaCl 119, KCl 4.5, MgSO$_4$ 2.5, NaHCO$_3$ 25, KH$_2$PO$_4$ 1.2, CaCl$_2$ 2.5, glucose 11.1. Segments (20 mm in length) were suspended in organ baths containing 10 ml of Krebs solution kept at 37 °C and aerated with 5% CO$_2$:95% O$_2$.

Recording of mechanical activity. The experiments were performed according to a reported procedure[16]. Ileum segments were connected to isometric force transducers (Ugo Basile, Biological Research Apparatus, VA, Italy) and mechanical activity was visualized, recorded and analyzed on a personal computer using the PowerLab/400 system (Ugo Basile, Italy).

Longitudinal preparations were subjected to an initial tension of 200 mg and were allowed to equilibrate for at least 30 min. Rhythmic spontaneous contractions of varying amplitude developed in all preparations. Concentration–response curve for NECA was constructed by cumulative addition of the drug. All compounds were solubilized in DMSO to prepare 10^{-2} M stock solutions, which were then diluted with the suitable buffer. The concentration of agonist in the organ bath was increased approximately 3-fold at each step, with each addition being made only after the response to the previous addition had attained a maximal level and remained steady.

Antagonism study was carried out incubating tissues for 1 h with a dose of compound under study before repeating the curve of NECA. Time control experiments showed that a second curve to the agonist was reproducible. Parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

Statistical analysis. Values of concentration–response curves are given as mean ± standard error of four or five independent observations. K_b values are expressed as geometric means with 95% confidence limits. Student's t-test was used to assess the statistical significance of the difference between two means. Statistical significance was set at $p < 0.05$.

Inhibitory effects induced by NECA were estimated as the decrease in the amplitude of the spontaneous contraction where 100% corresponding to maximum amplitude. Curves were fitted by a non-linear regression using a Prism 4.0 program (GraphPAD Software, San Diego (CA), USA).

To quantify agonist (NECA) potency, EC_{50} values were calculated. EC_{50} is the concentration of agonist required to produce 50% of the maximum effect.

To quantify antagonist potency, K_b values were calculated from the equation $K_b = (DR - 1) - [B]$, where DR is the ratio of EC_{50} values of agonist after and before treatment with antagonist concentration [B][18].

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