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Original article

N^6-1,3-Diphenylurea derivatives of 2-phenyl-9-benzyladenines and 8-azaadenines: Synthesis and biological evaluation as allosteric modulators of A_{2A} adenosine receptors

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Abstract

Some 1-[4-(9-benzyl-2-phenyl-9*H*-purin-6-ylamino)-phenyl]-3-phenyl-urea derivatives and some 1-[4-(9-benzyl-2-phenyl-9*H*-8-azapurin-6-ylamino)-phenyl]-3-phenyl-urea derivatives were synthesised and evaluated for their interaction with adenosine receptors. It was found that some of these compounds can act as positive enhancers of agonist and antagonist radioligands for the A_{2A} adenosine receptors. This evidence was also strengthened by functional data. Other compounds can act as negative modulators.

Furthermore these compounds show inhibitory properties for A_1 and A_3 adenosine receptors.

Keywords: 2-Phenyl-9-benzyladenines; 2-Phenyl-9-benzyl-8-azaadenines; Adenosine receptor ligands; Allosteric modulators; 1,3-Diphenylureas

1. Introduction

Allosteric modulation of G protein-coupled receptors is a relatively novel and unexplored pharmacological concept. Classically, the mechanism of action for receptor ligands consists either of mimicking or inhibiting the action of the endogenous signalling molecules, leading to the traditional classification of agonists as well as antagonists/inverse agonists, respectively. The desired effect is exerted through competition at the binding site for the endogenous neurotransmitter or hormone. Another kind of action elicited on the receptors by chemical compounds is the allosteric modulation. Allosteric modulators act at sites distinct from the agonist binding site, and their effect is evident only in the presence of an exogenously added agonist because they do not have an action ''per se'' but modulate the action of the naturally occurring hormone or neurotransmitter.

The presence of allosteric sites on a receptor provided a new target for drug discovery [1] and, in particular, allosteric modulators for adenosine receptors have potential therapeutic advantages over orthosteric ligands [2]. Two of the four subtypes of adenosine receptors have been reported to be allosterically regulated (A_1, A_3). For both A_1 and A_3 receptors, some allosteric modulators that are relatively selective have been developed and characterized [2]. For example, PD 81,723 showed selectivity toward the A_1 [3], while 3-(2-pyridinyl)isoquinoline derivatives [4] and a group of 1*H*-imidazo-[4,5-*c*]quinolines showed selectivity toward the A_3 subtype [5]. No A_{2A} receptor selective allosteric modulators have been reported [2]. Only very few compounds can be found in the literature that act as allosteric modulators of A_{2A} adenosine receptors: {4-methyl-7-[(methylamino)carbonyl]oxy}-2*H*-1-benzopyran-2-one (PD 120,918) [6] was reported to enhance agonist radioligand binding to the rat striatal A_{2A} adenosine receptor, but without functional enhancement. Like other G protein-coupled receptors, A_{2A} adenosine receptors are allosterically modulated by sodium ions, and by the potassium sparing diuretic, amiloride [7].

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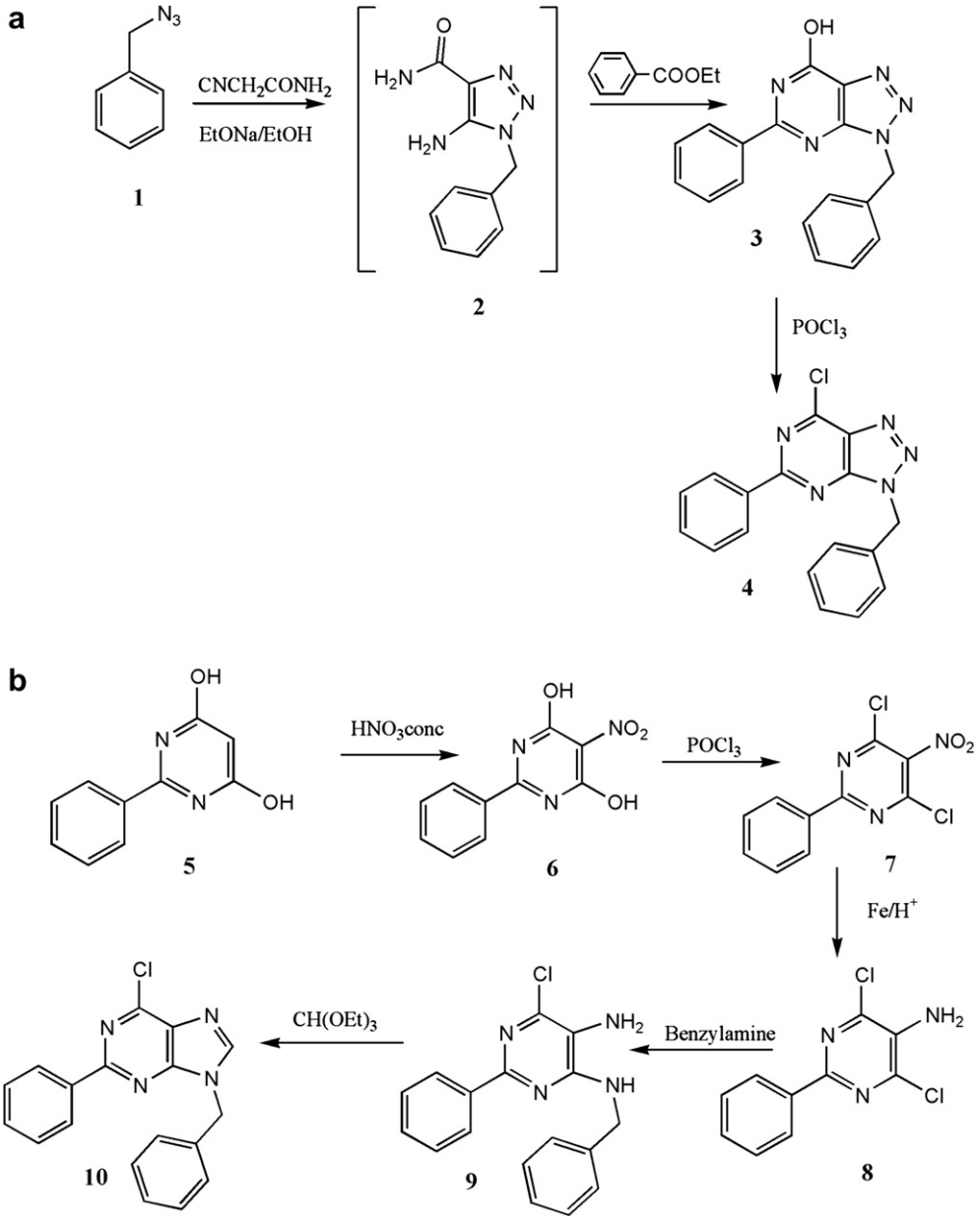
Also the compound SCH-202676 and other 2,3,5-substituted-[1,2,4]-thiadiazoles displayed peculiar displacement characteristics of both radiolabelled agonist and antagonist binding to A_{2A} receptors [8], but further studies have suggested that thiadiazoles act rather as sulfhydryl modifying agents than as allosteric modulators [9].

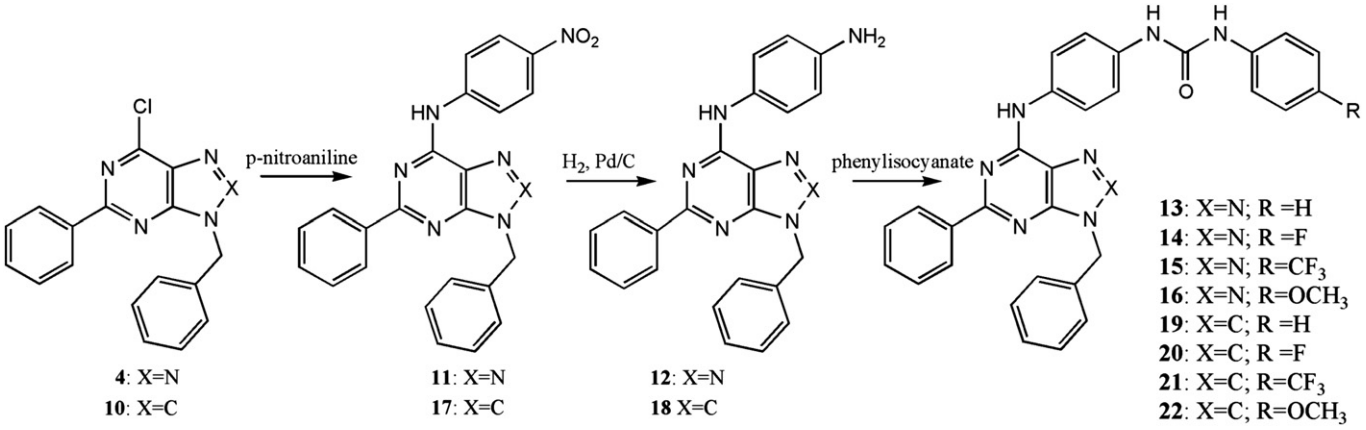
In this study, we present a synthesis of new purine- and 8-azapurine-N^6-1,3-diphenylurea derivatives and their biological characterization at the adenosine receptors with particular regard to allosteric modulation of the A_{2A} subtype.

2. Chemistry

The novel substituted 8-azaadenine derivatives **11**–**16** and substituted adenine derivatives **17**–**22** were synthesised as shown in Schemes 1a,b and 2. The 9-benzyl-8-azahypoxanthine **3** was prepared following a known two-step reaction (Scheme 1a) in the presence of sodium ethoxide: the first step was the 1,3 dipolar addition reaction of benzylazide **1** and cyanoacetamide to give 1-benzyl-4-carbamoyl-5-amino-1*H*-1,2,3-triazole **2**, which was not isolated; then, in the same flask, ethyl benzoate was added to obtain **3** [10] by annulation reaction. Hypoxanthine **3** was then treated with phosphorous oxychloride to give **4** [11]. Instead, to obtain the corresponding purine derivative **10** [12], a multi-step procedure is required, starting from 4,6-dihydroxy-2-phenylpyrimidine **5**, obtained by reaction of benzamidine and diethylmalonate, that was transformed to 5-amino-4,6-dichloro-2-phenylpyrimidine **8** by the reactions described in Scheme 1b and reported in the literature [12]. Compound **8** was treated with benzylamine to give **9**, that was cyclised to purine **10** by reaction with triethylorthoformate.



Scheme 1. Synthesis of compounds **4** (a) and **10** (b).



Scheme 2. Synthesis of compounds **11**–**22**.

8-Azapurine **4** and purine **10** were submitted to the same reaction sequence to obtain the final products, as shown in Scheme 2. The first step was a reaction with *p*-nitroaniline in ethanol at reflux temperature, to give **11** and **17** which, by catalytic hydrogenation, were transformed to **12** and **18**, respectively. By reaction with the suitable isocyanates in ethyl acetate at reflux temperature, from **12** were obtained compounds **13**–**16** and from **18** were obtained compounds **19**–**22**.

3. Biochemistry

Compounds **11**–**22** (see Table 1) were initially tested on adenosine A_1, A_{2A} and A_3 receptors in equilibrium radioligand displacement experiments. For tests involving the A_1 receptors both a radiolabelled agonist ([^{3}H]CCPA) and a radiolabelled antagonist ([^{3}H]DPCPX) were used, while the agonist radioligand [^{3}H]CGS21680 and the antagonist radioligand [^{3}H]ZM241385 were used in the experiments involving A_{2A} receptors. In the case of A_3 receptors only the agonist radioligand [^{3}H]NECA was used as a probe. The results are given as $K_i \pm$ SEM (nM) and/or percentage of specific binding of the remaining radioligand, where control binding is 100% and non-specific binding is 0%. The two compounds **12** and **14** were selected for closer analysis, since they presented intriguing behaviour, as shown in detail below.

4. Pharmacology

Since the activation of adenosine A_{2A} receptors in ''endothelium intact'' rat isolated aorta induces vasorelaxing effects, mediated through the release of endothelial nitric oxide, this experimental model is widely used as a functional tool for the pharmacological characterization of drugs acting on this receptor subtype. In this study, the influence of the selected compound **14** on the vasorelaxing responses evoked by the adenosine agonist CGS21680 was evaluated on rat aortic rings with intact endothelium, in order to identify a pharmacodynamic profile consistent with the feature of positive enhancer.

5. Results and discussion

All compounds assayed on the A_1 adenosine receptor at a final concentration of 10 μM inhibited [^{3}H]CCPA binding, in particular, in a significant way, compounds **11**, **12**, **17** and

Table 1
Biological results

Compound	% Specific binding of remaining radioligand[a] and $K_i \pm$ SEM (nM) of selected compounds				
	r-A_1		h-A_{2A}		h-A_3
	[^{3}H]DPCPX	[^{3}H]CCPA	[^{3}H]ZM241385	[^{3}H]CGS21680	[^{3}H]NECA
11	105	33	98	96	58
12	33 (K_i 132 ± 26)	4 (K_i 103 ± 18)	77 (K_i 1300 ± 349)	0 (K_i 280 ± 44)	49
13	110	79	133	57	78
14	107	72	167	176	92
15	94	74	127	180	72
16	105	74	142	59	58
17	84 (K_i 699 ± 105)	2 (K_i 571 ± 58)	84 (K_i 1900 ± 500)	114	51
18	46 (K_i 396 ± 52)	7 (K_i 294 ± 17)	59 (K_i 5737 ± 688)	44 (K_i 4170 ± 375)	56
19	101	73	107	74	53
20	105	85	111	83	70
21	106	67	149	57	37 (K_i 5608 ± 841)
22	107	68	166	120	56

[a] Data are expressed as means from 2 to 3 independent experiments performed in duplicate; individual values varied less than 15%. The results are given as $K_i \pm$ SEM (nM) and/or percentage of specific binding of radioligand remaining, where control binding is 100% and non-specific binding is 0%.

18 which have *p*-NO_2 or *p*-NH_2 phenylamine as substituent on the N^6 of the adenine or the 8-azaadenine nucleus; the other compounds, having a bulkier substituent in the same position (**13**–**16** and **19**–**22**), showed much lower activity. It may be worth specifying here that, with the term ''inhibition'' we mean a decrease of signal associated with the receptor bound to the radioligand in the presence of the tested compound, with regard to the value measured in the absence of the tested compound itself. However, when the antagonist radioligand [^{3}H]DPCPX was used, the inhibition of the measured binding was lower. In fact, only compounds **12**, **17** and **18** (also in this case these compounds are three of those having less bulky substituent in the N^6 position) showed significant inhibition, while all the other compounds did not seem to modulate the [^{3}H]DPCPX binding. This may be explained because compounds **11**–**22** show higher structural similarity with regard to the agonist radioligand [^{3}H]CCPA, which contains the adenine moiety, in comparison to the antagonist radioligand [^{3}H]DPCPX which contains a xanthine nucleus. This implies that compounds **11**–**22** show higher affinity for the binding sub-site that accommodates [^{3}H]CCPA than for the binding sub-site that accommodates [^{3}H]DPCPX. Further a bulky substituent in position N^6 seems to lower affinity for A_1 receptors. With regard to the assays involving A_{2A} receptors, for which the radioligand [^{3}H]ZM241385 was used, the observations reported below were made. Some of the assayed compounds (again **12**, **17** and **18**) inhibited the binding (percentage of specific binding lower than 100), while others (**11**, **19** and **20**) did not seem to affect it. Other compounds (**13**, **14**, **15**, **16**, **21** and **22**), all N^6-1,3-diphenylurea derivatives, caused an increase in the percentage of specific binding, significantly higher than 100. This last effect cannot be attributed to a competitive mechanism of interaction with the receptors, because both agonist and antagonist compounds, which act through a displacement mechanism at the orthosteric site, are only capable of inhibiting radioligand binding. Analogous behaviour in binding modulation at A_{2A} receptors was observed when the assays were performed by using the agonist radioligand. Some compounds (**12**, **13**, **16**, **18**–**21**) inhibited binding (percentage of specific binding lower than 100), while others (**11** and **17**) did not seem to affect binding. Other compounds (**14**, **15** and **22**) caused an increase in the percentage of specific binding, significantly higher than 100. It is worthy of note that compounds **14**, **15** and **22** are N^6-1,3-diphenylurea derivatives.

From comparison of the modulating behaviour of the binding of [^{3}H]ZM241385 and [^{3}H]CGS21680 at the adenosine A_{2A} receptors, it can be reasonably hypothesized that compounds **12**, **17** and **18** present an inhibitory action on these receptors, while compounds **14**, **15** and **22** enhance the binding of a ligand bound to the orthosteric site.

In the case of the A_3 receptors, compounds **11**–**22** showed weak inhibitory properties, when the agonist radioligand [^{3}H]NECA was used.

The two compounds **12** and **14** were selected for closer analysis in order to look in more detail at a compound showing inhibitory properties on all the adenosine receptors, and a compound showing enhancer properties to the A_{2A} receptor subtype.

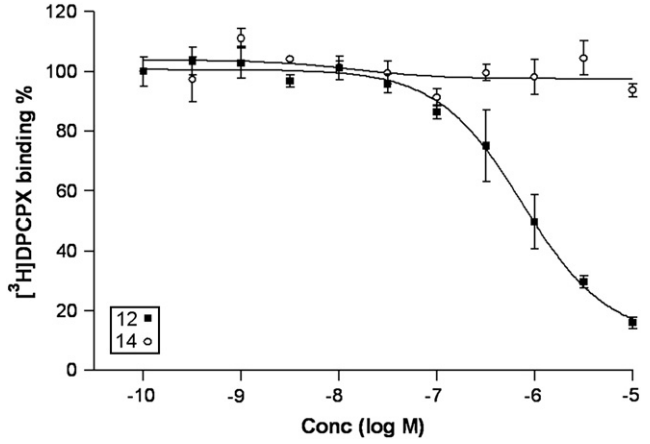


Fig. 1. Displacement of [^{3}H]DPCPX binding at human adenosine A_1 receptors due to compounds **12** and **14**. Data were taken from a representative experiment performed in duplicate. Radioligand binding is expressed as a percentage of specific binding.

In the classical experiment involving the adenosine A_1 receptor subtype, performed under displacement conditions, it can be observed that **12** shows the typical sigmoid curve of competitive displacement of [^{3}H]DPCPX, with $EC_{50} = 0.75$ μM (Fig. 1). In the case of **14**, the inhibition found is very weak, in practice negligible; the observed curve is nearly a straight line, which makes the EC_{50} undetectable. In the experiment involving the A_{2A} subtype, also performed under displacement conditions, by using [^{3}H]ZM241385 as a probe, **12** showed inhibitory behaviour, described by the decreasing curve and characterized by $EC_{50} = 9.74$ μM (Fig. 2).

Moreover, **14** shows receptor enhancer behaviour on the A_{2A} subtype, characterized by an increasing curve when both [^{3}H]ZM241385 and [^{3}H]CGS21680 are used (Fig. 3). $EC_{50} = 16$ μM, in the presence of [^{3}H]ZM241385, and $EC_{50} = 9.95$ μM, in the presence of [^{3}H]CGS21680, were measured.

Further investigations on the mechanism by which **12** and **14** interact with the A_{2A} receptor subtype were performed

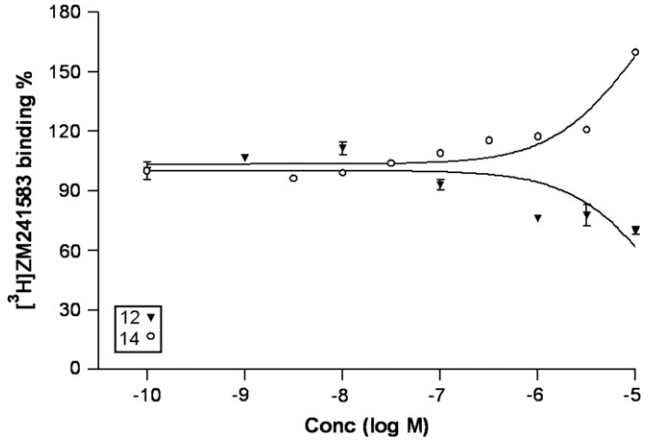


Fig. 2. Displacement of [^{3}H]ZM241385 binding at human adenosine A_{2A} receptors due to compounds **12** and **14**. Data were taken from a representative experiment performed in duplicate. Radioligand binding is expressed as a percentage of specific binding.

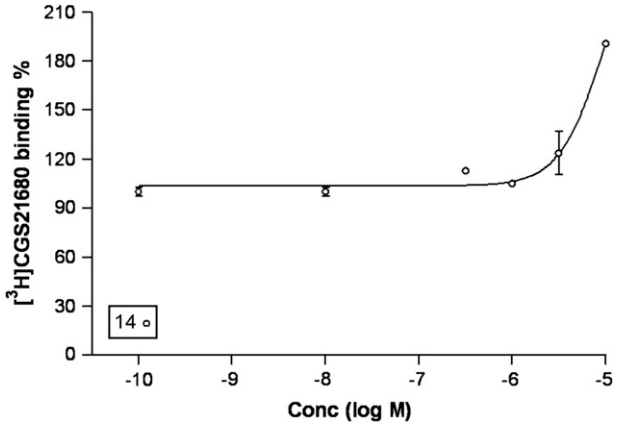


Fig. 3. Displacement of [^{3}H]CGS21680 binding at human adenosine A_{2A} receptors due to compound **14**. Data were taken from a representative experiment performed in duplicate. Radioligand binding is expressed as a percentage of specific binding.

by estimating the change in the maximum dissociation rate of radioligand [^{3}H]ZM241385 caused by an excess (at 10 μM concentration) of the same unlabelled molecule (the corresponding cold ligand ZM241385, well-known for its competitive action), in the presence and absence of compounds **12** and **14**. In fact, only a compound that interacts with a receptor in a noncompetitive way can give rise to a change in the maximum dissociation rate of the radioligand from the receptor, when the dissociation is induced by an excess of a competitive agent. The known A_{2A} agonist NECA, at 10 μM concentration, was used as an internal standard.

The results showed that, while both 10 μM NECA and 10 μM **12** do not influence the dissociation of 10 μM [^{3}H]ZM241385 with respect to the control, **14** causes a decrease of the dissociation rate (Fig. 4) (Table 2).

In order to evaluate the pharmacodynamic feature by a functional experimental approach, compound **14** was selected as a representative of those derivatives which exhibited enhancer

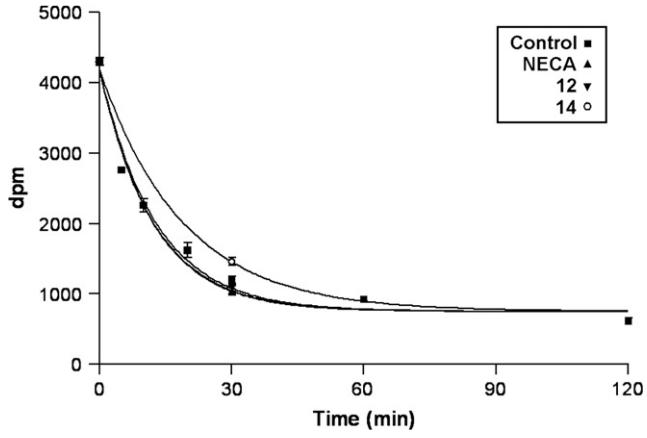


Fig. 4. Dissociation curves of [^{3}H]ZM241385 from the human adenosine A_{2A} receptors at 0 °C in the presence and in the absence of NECA, **12** and **14** (10 μM). Data were taken from a representative experiment performed in quadruplicate. Radioligand binding is expressed as a percentage of specific binding.

Table 2
Dissociation kinetic parameters for [^{3}H]ZM241385 binding at human adenosine A_{2A} receptors in the presence or absence of NECA, **12** or **14** (10 μM)

	K_{diss} (s^{-1})	$t_{1/2}$ (s)
Control	0.082	8.42
NECA	0.081	8.52
12	0.78	8.89
14	0.052	13.2

behaviour in the biochemical study. The agonist CGS21680 induced A_{2A}-mediated concentration-dependent vasorelaxing responses in rat aortic rings pre-contracted by the adrenergic agonist noradrenaline, with values of potency (pIC50) and efficacy (E_{max}) of 6.58 ± 0.074 and 76 ± 7, respectively. In the presence of compound **14** (10 μM), the vasorelaxing effects of CGS21680 were enhanced (Fig. 5), with a significant ($P < 0.0001$) increase of the potency parameter ($pIC50 = 7.16 \pm 0.061$), significant increases of the responses evoked by CGS21680 0.1, 0.3 and 1 μM, and a marked (albeit non-statistically significant) increase of the efficacy parameter ($E_{max} = 91 \pm 5$).

6. Conclusions

A quite diverse library of potential ligands for adenosine receptors was synthesised and assayed in both equilibrium radioligand binding assays and dissociation kinetic experiments with the aim of explaining in detail the interaction mechanisms by which the above ligands exert their actions. On the basis of analysis of the initial experiments, it may be concluded that the new compounds **11–22** bind to the adenosine A_1, A_{2A} and A_3 receptors. Their behaviour at A_1 and A_3 receptors is an inhibitory one: they probably act as antagonists by a competitive mechanism. Additional focused experiments showed that some compounds also act as inhibitors of the

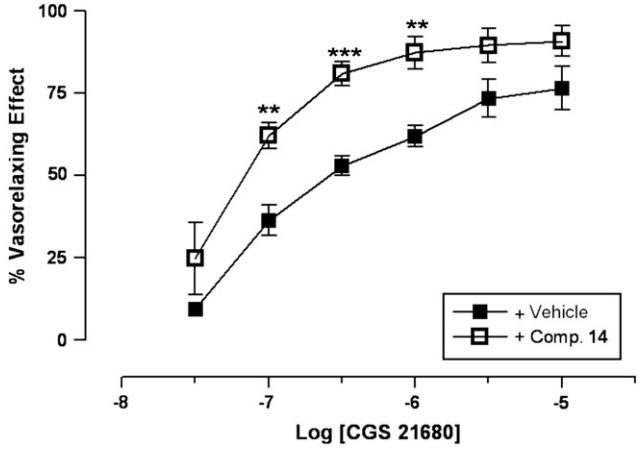


Fig. 5. Concentration–vasorelaxing effect curves for CGS21680, obtained in rat aortic rings with intact endothelium, in the presence of compound **14** (10 μM; white squares) or its vehicle (black squares). Vertical bars indicate the standard errors. The asterisks indicate statistically significant differences between the responses evoked by the same concentration of CGS21680 (**$P < 0.005$; ***$P < 0.0001$).

A_{2A} receptors, while others possess noncompetitive enhancer properties, that could be accounted for by a mechanism of allosteric modulation. In particular, the hypothesis of such a possible pharmacodynamic property seems to be well supported by the evidence deriving from the functional tests, which showed that the A_{2A}-mediated pharmacological effects of CGS21680 were significantly improved by compound **14**.

7. Experimental

7.1. Chemistry

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. IR spectra in Nujol mulls were recorded on a Mattson Genesis series FTIR spectrometer. ^{1}H NMR spectra were recorded on a Bruker AC 200 spectrometer in δ units from TMS as an internal standard; the compounds were dissolved in DMSO-d_6. Mass spectra data were obtained with a Hewlett–Packard GC/MS system 5988. TLC was performed on precoated silica gel F_{254} plates (Merck). Microanalyses (C H N) were carried out on a Carlo Erba elemental analyser (Model 1106) and were within ±0.4% of the theoretical values.

7.1.1. (3-Benzyl-5-phenyl-3H-[1,2,3]triazolo[4,5-d]-pyrimidin-7-yl)-(4-nitrophenyl)-amine (**11**)

A solution of 6-chloro-9-benzyl-2-phenyl-8-azapurine **4** [11] (2.0 g, 6.2 mmol) and *p*-nitroaniline (2.6 g, 18.6 mmol) in absolute ethanol (30 ml) was heated in a well stopped flask at 85 °C for 48 h. After cooling, the precipitate was filtered off and crystallized from ethanol to give **11** as a yellow solid, 1.52 g, 58% yield; m.p. 263–264 °C. Anal. $C_{23}H_{17}N_7O_2$ (C, H, N); ^{1}H NMR: 11.61 (s, 1H, Exch), 8.46–8.34 (m, 6H, Arom), 7.59–7.35 (m, 8H, Arom), 5.93 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3337 (NH). MS: *m/z* 423 [M^+]. TLC: $R_f = 0.13$ ($CHCl_3$).

7.1.2. N-(3-Benzyl-5-phenyl-3H-[1,2,3]triazolo[4,5-d]-pyrimidin-7yl-)-(4-aminophenyl)-amine (**12**)

A solution of compound **11** (150 mg, 0.33 mmol) in absolute ethanol (125 ml) was stirred under hydrogen atmosphere in the presence of 5% Pd/C (150 mg) for 12 h at room temperature and pressure. The catalyst was removed by filtration, and the filtrate evaporated under reduced pressure to give **12**, 124 mg, 95% yield; m.p. 257–259 °C. Anal. $C_{23}H_{19}N_7$ (C, H, N); ^{1}H NMR: 10.59 (s, 1H, Exch), 8.39 (m, 2H, Arom), 7.59–7.30 (m, 10H, Arom), 6.64 (d, $J = 8.4$ Hz, 2H, Arom), 5.86 (s, 2H, CH_2), 5.09 (br s, 2H, Exch) δ, ppm. IR (cm^{-1}): 3404 (NH), 3325 (NH), 3183 (NH). MS: *m/z* 393 [M^+]. TLC: $R_f = 0.30$ ($CHCl_3$/CH_3OH 9.8:0.2).

7.1.3. {1-[4-(3-Benzyl-5-phenyl-3H-[1,2,3]triazolo[4,5-d]-pyrimidin-7-ylamino)-phenyl]-3-phenyl}-urea (**13**)

To a solution of **12** (50 mg, 0.127 mmol) and 0.1 ml of *N*,*N*-diethylaniline in anhydrous acetonitrile (15 ml), phenyl isocyanate (75 mg, 0.635 mmol) was added and the solution stirred at reflux for 12 h under nitrogen atmosphere. After cooling, the precipitate was filtered off and crystallized from methanol to give **13** as a white solid, 43 mg, 66% yield; m.p. >300 °C. Anal. $C_{30}H_{24}N_8O$ (C, H, N); ^{1}H NMR: 10.88 (s, 1H, Exch), 8.70 (m, 2H, Exch), 8.44 (m, 2H, Arom), 7.90 (m, 2H, Arom) 7.55–7.24 (m, 14H, Arom), 7.00 (t, $J = 7.4$ Hz, 1H, Arom) 5.89 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3390 (NH), 3375 (NH), 3322 (NH), 1678 (C=O). MS: *m/z* 512 [M^+]. TLC: $R_f = 0.32$ ($CHCl_3$/CH_3COOEt 8:2).

7.1.4. 1-[4-(3-Benzyl-5-phenyl-3H-[1,2,3]triazolo[4,5-d]-pyrimidin-7-ylamino)-phenyl]-3-(4-fluorophenyl)-urea (**14**)

Compound **14** was prepared by reaction of **12** (50 mg, 0.127 mmol) and 4-fluorophenyl isocyanate (87 mg, 0.635 mmol), following the procedure described for **13**. By crystallization from ethyl acetate the title compound was obtained as a white solid, 21 mg, 32% yield; m.p. >300 °C. Anal. $C_{30}H_{23}FN_8O$ (C, H, N); ^{1}H NMR: 10.88 (s, 1H, Exch), 8.73 (s, 1H, Exch), 8.70 (s, 1H, Exch), 8.42 (m, 2H, Arom), 7.92 (m, 2H, Arom), 7.54–7.08 (m, 14H, Arom), 5.89 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3391 (NH), 3379 (NH), 3315 (NH), 1671 (C=O). MS: *m/z* 530 [M^+]. TLC: $R_f = 0.32$ ($CHCl_3$/CH_3COOEt 8:2).

7.1.5. 1-[4-(3-Benzyl-5-phenyl-3H-[1,2,3]triazolo[4,5-d]-pyrimidin-7-ylamino)-phenyl]-3-(4-trifluoromethylphenyl)-urea (**15**)

Compound **15** was prepared by reaction of **12** (50 mg, 0.127 mmol) and 4-trifluoromethylphenyl isocyanate (118 mg, 0.635 mmol), following the procedure described for **13**. By crystallization from ethyl acetate the title compound was obtained as a white solid, 35 mg, 48% yield; m.p. >300 °C. Anal. $C_{31}H_{23}F_3N_8O$ (C, H, N); ^{1}H NMR: 10.90 (s, 1H, Exch), 9.16 (s, 1H, Exch), 8.87 (s, 1H, Exch), 8.43 (m, 2H, Arom), 7.95–7.31 (m, 16H, Arom), 5.89 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3395 (NH), 3311 (NH), 1676 (C=O). MS: *m/z* 580 [M^+]. TLC: $R_f = 0.32$ ($CHCl_3$/CH_3COOEt 8:2).

7.1.6. 1-[4-(3-Benzyl-5-phenyl-3H-[1,2,3]triazolo[4,5-d]-pyrimidin-7-ylamino)-phenyl]-3-(4-methoxyphenyl)-urea (**16**)

Compound **16** was prepared by reaction of **12** (50 mg, 0.127 mmol) and 4-methoxyphenyl isocyanate (95 mg, 0.635 mmol), following the procedure described for **13**. By crystallization from ethyl acetate the title compound was obtained as a white solid, 25 mg, 37% yield; m.p. 270 °C. Anal. $C_{31}H_{26}N_8O_2$ (C, H, N); ^{1}H NMR: 10.87 (s, 1H, Exch), 8.70 (s, 1H, Exch), 8.58 (s, 1H, Exch), 8.43 (m, 2H, Arom), 7.87 (m, 4H, Arom), 7.54–7.30 (m, 8H, Arom), 6.88 (m, 4H, Arom), 5.89 (s, 2H, CH_2), 3.71 (s, 3H, CH_3) δ, ppm. IR (cm^{-1}): 3390 (NH), 3319 (NH), 3273 (NH), 1645 (C=O). MS: *m/z* 542 [M^+]. TLC: $R_f = 0.39$ ($CHCl_3$/CH_3COOEt 8:2).

7.1.7. N^6-[(4-Nitro)-phenyl]-9-benzyl-2-phenyladenine (**17**)

A solution of 9-benzyl-6-chloro-2-phenylpurine **10** [12] (2.0 g, 6.2 mmol) and *p*-nitroaniline (2.6 g, 18.6 mmol) in absolute ethanol (30 ml) was heated in a well stopped flask at

85 °C for 48 h. After cooling, the precipitate was filtered off and crystallized from ethanol to give **17** as a yellow solid, 1.59 g, 61% yield; m.p. 250–251 °C. Anal. $C_{24}H_{18}N_6O_2$ (C, H, N); ^{1}H NMR: 11.59 (s, 1H, Exch), 8.46–8.32 (m, 7H, Arom), 7.58–7.33 (m, 8H, Arom), 5.93 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3334 (NH). MS: *m/z* 422 [M^+]. TLC: $R_f = 0.17$ ($CHCl_3$).

*7.1.8. N^6-[(4-Amino)-phenyl]-9-benzyl-2-phenyladenine (**18**)*

A solution of compound **17** (150 mg, 0.35 mmol) in ethanol (125 ml) was stirred under hydrogen atmosphere in the presence of 5% Pd/C (150 mg) for 12 h at room pressure and temperature. The catalyst was removed by filtration, and the filtrate evaporated under reduced pressure to give **18,** 116 mg, 84% yield; m.p. 220 °C (Dec.). Anal. $C_{24}H_{20}N_6$ (C, H, N); ^{1}H NMR: 10.59 (s, 1H, Exch), 8.39 (m, 3H, Arom), 7.58–7.33 (m, 10H, Arom), 6.63 (m, 2H, Arom), 5.90 (s, 2H, CH_2), 5.07 (s, 2H, Exch) δ, ppm. IR (cm^{-1}): 3395 (NH), 3318 (NH), 3175 (NH). MS: *m/z* 392 [M^+]. TLC: $R_f = 0.27$ ($CHCl_3$/CH_3OH 9.8:0.2).

*7.1.9. 1-[4-(9-Benzyl-2-phenyl-9H-purin-6-ylamino)-phenyl]-3-phenyl-urea (**19**)*

To a solution of **18** (50 mg, 0.127 mmol) and 0.1 ml of *N*,*N*-diethylaniline in anhydrous acetonitrile (15 ml), phenyl isocyanate (75 mg, 0.635 mmol) was added and the solution stirred at reflux for 12 h under nitrogen atmosphere. After cooling, the precipitate was filtered off and crystallized from methanol to give **19** as a white solid, 26 mg, 40% yield; m.p. >300 °C. Anal. $C_{31}H_{25}N_7O$ (C, H, N); ^{1}H NMR: 10.88 (s, 1H, Exch), 8.73 (m, 2H, Exch), 8.43 (m, 2H, Arom), 7.90 (m, 2H, Arom), 7.55–7.24 (m, 15H, Arom), 6.97 (t, 1H, $J = 7$ Hz, Arom), 5.89 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3385 (NH), 3376 (NH), 3324 (NH), 1679 (C═O). MS: *m/z* 511 [M^+]. TLC: $R_f = 0.20$ ($CHCl_3$).

*7.1.10. 1-[4-(9-Benzyl-2-phenyl-9H-purin-6-ylamino)-phenyl]-3-(4-fluorophenyl)-urea (**20**)*

Compound **20** was prepared by reaction of **18** (50 mg, 0.127 mmol) and 4-fluorophenyl isocyanate (87 mg, 0.635 mmol), following the procedure described for **13**. By crystallization from methanol the title compound was obtained as a white solid, 35 mg, 52% yield; m.p. >300 °C. Anal. $C_{31}H_{24}FN_7O$ (C, H, N); ^{1}H NMR: 10.85 (s, 1H, Exch), 9.14 (m, 2H, Exch); 8.43 (m, 2H, Arom), 7.92 (m, 2H, Arom), 7.55–7.15 (m, 15H, Arom), 5.93 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3345 (NH), 3295 (NH), 3241 (NH), 1650 (C═O). MS: *m/z* 529 [M^+]. TLC: $R_f = 0.29$ ($CHCl_3$).

*7.1.11. 1-[4-(9-Benzyl-2-phenyl-9H-purin-6-ylamino)-phenyl]-3-(4-trifluoromethylphenyl)-urea (**21**)*

Compound **21** was prepared by reaction of **18** (50 mg, 0.127 mmol) and 4-trifluoromethylphenyl isocyanate (118 mg, 0.635 mmol), following the procedure described for **13.** By crystallization from methanol the title compound was obtained as a white solid, 23 mg, 31% yield; m.p. >300 °C. Anal. $C_{32}H_{24}F_3N_7O$ (C, H, N); ^{1}H NMR 10.90 (s, 1H, Exch), 9.05 (s, 2H, Exch), 8.43 (m, 2H, Arom), 7.90 (m, 2H, Arom); 7.60–7.12 (m, 15H, Arom), 5.89 (s, 2H, CH_2) δ, ppm. IR (cm^{-1}): 3395 (s, NH), 3310 (s, NH), 3292 (s, NH), 1676 (s, C═O). MS: *m/z* 578 [$M^+ - 1$]. TLC: $R_f = 0.30$ ($CHCl_3$).

*7.1.12. 1-[4-(9-Benzyl-2-phenyl-9H-purin-6-ylamino)-phenyl]-3-(4-methoxyphenyl-urea (**22**)*

Compound **22** was prepared by reaction of **18** (50 mg, 0.127 mmol) and 4-methoxyphenyl isocyanate (95 mg, 0.635 mmol), following the procedure described for **13.** By crystallization from methanol the title compound was obtained as a white solid, 49 mg, 71% yield; m.p. >300 °C. Anal. $C_{32}H_{27}N_7O_2$ (C, H, N); ^{1}H NMR: 10.87 (br s, 1H, Exch), 8.62 (s, 1H Exch), 8.50 (s, 1H Exch), 8.45 (m, 3H, Arom), 7.92 (m, 2H, Arom), 7.46 (m, 11H, Arom), 6.88 (m, 2H, Arom), 5.89 (s, 2H, CH_2), 3.72 (s, 3H, CH_3) δ, ppm. IR (cm^{-1}): 3383 (NH), 3354 (NH), 3274 (NH), 1643 (C═O). MS: *m/z* 541 [M^+]. TLC: $R_f = 0.23$ ($CHCl_3$).

7.2. Biological assays

7.2.1. Materials

[^{3}H]DPCPX (120 Ci/mmol), [^{3}H]NECA (20.6 Ci/mmol), [^{3}H]CGS21680 (47 Ci/mmol) and [^{3}H]CCPA (42.6 Ci/mmol) were purchased from Amersham Pharmacia Biotech. [^{3}H]ZM241385 (27.4 Ci/mmol) was from Tocris Cookson. DPCPX, NECA and CPA were from Sigma–Aldrich. All other chemicals used, at analytical grade, were from standard commercial sources.

7.2.2. Radioligand binding assays

Membranes of rat cerebral cortex which express adenosine A_1 receptors were prepared by using the method described by Lohse et al. [13] with slight modifications. Male Wistar rat brain cortex was homogenised in 10 volumes of ice-cold 0.32 M sucrose, 20 mM Tris–HCl buffer pH 7.4 with 30 strokes in Dounce homogeniser. The homogenate was centrifuged at 1000*g* for 10 min to remove the nuclear fraction, and the resulting supernatant centrifuged at 30,000*g* for 30 min. The pellet was re-suspended using 10 strokes in Dounce homogeniser in 10 volumes of ice-cold 5 mM Tris–HCl buffer pH 7.4 for 30 min. After 60 strokes in Dounce homogeniser, the resulting synaptosomal membranes were pre-incubated for 30 min at 37 °C with 2 U/ml of adenosine deaminase to remove endogenous adenosine. The membrane suspension was then centrifuged at 48,000*g* for 30 min, and the resulting pellet was re-suspended in 10 volumes of 50 mM Tris–HCl buffer pH 7.4, and stored at −80 °C until binding assays were made.

For displacement experiments involving adenosine A_1 receptors, rat cortex membranes (40 μg of protein) were incubated at 25 °C for 60 min with [^{3}H]DPCPX 0.5 nM ($K_d = 0.4$ nM) or 0.3 nM [^{3}H]CCPA ($K_d = 0.2$ nM), and fixed concentration (10 μM) or increasing concentrations of the compounds, in a final volume of 0.4 ml of Tris–HCl buffer. Non-specific binding was measured in the presence of

100 μM CPA (when [^{3}H]DPCPX was used) or 10 μM DPCPX (when [^{3}H]CCPA was used). Binding reactions were terminated by dilution with ice-cold 50 mM Tris–HCl buffer pH 7.4. Samples were then filtered through Whatman GF/C glass–fiber filters using a Brandel cell harvester. Filters were washed three times with 2–3 ml of the same buffer. Bound radioactivity was measured in a liquid scintillation counter (1600 TR Packard) after the addition of 4 ml of scintillation liquid (Emulsifier-Safe, Packard).

Slightly different conditions were set in the case of binding displacement experiments regarding adenosine A_{2A} and A_3 receptors. Membranes of CHO cells expressing recombinant human A_{2A} or A_3 receptors were prepared as previously described [14]. Membranes (40 μg of protein) were incubated with [^{3}H]ZM241385 6 nM ($K_d = 4$ nM) or [^{3}H]CGS21680 13 nM ($K_d = 13$ nM) in the experiments involving the A_{2A} subtype, and [^{3}H]NECA 7 nM ($K_d = 150$ nM) in the ones involving the A_3 one, and the compounds to be assayed, at fixed concentration (10 μM) or at increasing concentrations of the compounds in duplicate, in a final volume of 0.4 ml of Tris–HCl buffer for 120 min at 25 °C. Non-specific binding was measured in the presence of 100 μM NECA in the case of A_{2A}, and 100 μM R-PIA in the case of A_3 binding assay. Samples were handled as mentioned before.

For the dissociation kinetic studies from adenosine A_{2A} receptors, a single concentration of [^{3}H]ZM241385 (4 nM) was used. Membranes (40 μg of protein) were pre-incubated at 0 °C for 180 min. The dissociation experiment started by adding 10 μM ZM241385 with or without 100 μM test compounds in quadruplicate at appropriate time intervals. The time course of dissociation of total binding was measured by rapid filtration through Whatman GF/B glass–fiber filters, washing three times with 2 ml of ice-cold buffer. Samples were treated as described above.

Binding parameters were calculated by GraphPAD Prism software (GraphPAD, San Diego, CA, USA).

7.3. Pharmacological functional assay

All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609. Endothelium dependent, adenosine A2A receptor-mediated vasorelaxing responses were obtained in rat aortic rings with intact endothelium, following widely used experimental approaches [15].

Six male Wistar rats (250–350 g) were sacrificed by cervical dislocation under light ether anaesthesia and bled. The aortae were immediately excised and freed of extraneous tissues. The endothelium was preserved. Five millimeter wide aortic rings were suspended, under a preload of 2 g, in 20 ml organ baths, containing Tyrode solution (composition of saline in mM: NaCl 136.8; KCl 2.95; $CaCl_2$ 1.80; $MgSO_4$ $7H_2O$ 1.05; NaH_2PO_4 0.41; $NaHCO_3$ 11.9; Glucose 5.5), thermostated at 37 °C and continuously gassed with a mixture of O_2 (95%) and CO_2 (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with a preamplifier (Buxco Electronics) and with a software of data acquisition (BIOPAC Systems Inc., MP 100).

After an equilibration period of 60 min, the endothelial integrity was confirmed by the administration of acetylcholine (ACh) (10 μM) to noradrenaline (NA; 1 μM)-pre-contracted vascular rings. A relaxation ≥80% of the NA-induced contraction was considered representative of an acceptable integrity of the endothelium. Forty-five minutes after confirmation of the endothelium integrity, the selected compound **14** or its vehicle were added in the organ bath. After 20 min of incubation, the aortic preparations were contracted by treatment with a single concentration of NA (10 μM) and when the contraction reached a stable plateau, threefold increasing concentrations of CGS21680 (30 nM–10 μM) were added cumulatively.

Preliminary experiments showed that the NA (1 μM)-induced contractions remained in a stable tonic state for at least 40 min and that the pre-incubation with compound **14** did not produce any significant influence on the contractile effect of NA.

Both CGS21680 and compound **14** were dissolved in dimethylsulphoxide at the concentration of 10 mM and further diluted in bi-distilled water. All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicle.

The vasorelaxing efficacy was evaluated as vasorelaxing response evoked by CGS21680 10 μM, expressed as a percentage (%) of the contractile tone induced by NA (1 μM). The parameter of potency was expressed as pIC_{50}, calculated as a negative logarithm of the molar concentration of CGS21680, evoking a half reduction of the contractile tone induced by NA (1 μM). The parameters of efficacy and potency were expressed as mean ± standard error, for six experiments. Student *t* test was selected as statistical analysis, $P < 0.05$ was considered representative of a significant statistical difference. Experimental data were analysed by a computer fitting procedure (software: GraphPad Prism 4.0).

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