Vittoria Colotta[a], Daniela Catarzi[a], Flavia Varano[a], Guido Filacchioni[a], Claudia Martini[b], Letizia Trincavelli[b], Antonio Lucacchini[b]

[a] Dipartimento di Scienze Farmaceutiche, Universita' di Firenze, Firenze, Italy
[b] Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, Università di Pisa, Pisa, Italy

Synthesis and Structure-Activity Relationships of 4-Cycloalkylamino-1,2,4-triazolo[4,3-a]quinoxalin-1-one Derivatives as A_1 and A_3 Adenosine Receptor Antagonists

In a previous paper (Colotta V. et al., *J. Med. Chem.* **2000**, *43*, 1158–1164) we reported the synthesis and binding activity of 4-cycloalkylamino-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives, differently substituted on the appended 2-phenyl ring, some of which were potent and selective A_1 adenosine receptor (AR) antagonists. In the present paper several 4-cycloalkylamino-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives (**1**–**11**), bearing simple substituents on the benzofused moiety, are reported. The binding data of bovine A_1 and A_{2A} and human A_3 AR show that we have obtained highly potent A_1 AR antagonists. In particular, the 4-cyclohexylamino derivatives **1**–**5** show higher A_1 *vs* A_{2A} selectivity than the parent compound **A**, which lacks substituents on the benzofused moiety. Moreover, compounds **1**–**11** display, in general, good A_3 AR affinity. Finally, SAR studies provide some new insights about the steric requirements of the A_3 receptor pocket, which accommodates the benzofused moiety of our 4-amino-triazoloquinoxalin-1-one derivatives.

Keywords: G Protein-coupled receptors; Adenosine receptor antagonists; Triazoloquinoxalines; Tricyclic heteroaromatic systems

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Introduction

Adenosine is a neuromodulator, which produces many important biological functions by activation of G protein-coupled receptors classified into A_1, A_{2A}, A_{2B} and A_3 subtypes [1, 2]. Adenosine receptors (ARs) from different species show 82–93 % amino acid sequence homology, the only exception being the A_3 subtype, which exibits 74 % primary sequence homology between rat and human [3–5]. In the last few years, much effort has been directed towards the synthesis of selective AR antagonists since they are attractive tools for pharmacological intervention in many pathophysiological conditions [6]. In particular, A_1 AR selective antagonists are developed as antihypertensives and potassium-saving diuretics [7], cognition enhancers [6] and useful therapeutics in alleviation of the symptoms of Alzheimer's disease [6, 7], while A_3 AR antagonists are considered potential anti-inflammatory and anti-asthmatic agents [8].

As a part of our research aimed at finding new AR selective antagonists [9–15] we recently reported the synthesis and binding activity of bovine (b) A_1 and A_{2A} and human (h) cloned A_3 ARs of 2-aryl-4-amino-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives bearing some substituents on the 2-phenyl ring and the 4-amino group (Figure 1) [16]. Structure-Activity Relationship (SAR) studies indicated that introduction of a substituent on the 2-phenyl moiety did not increase A_1 AR affinity and selectivity while the presence of a cycloalkyl ring on the 4-amino group was an important feature to obtain highly potent A_1 and A_1 *vs* A_{2A} selective antagonists. The 4-cycloalkylamino derivatives also displayed A_3 AR affinity in the nanomolar range [16]. Continuing our studies of this class of AR antagonists, in the present paper we have focused our attention on A_1 selective ligands. Therefore, taking the potent and selective A_1 antagonists 2-phenyl-4-cycloalkylamino derivatives **A** and **B** [16] as lead compounds (Figure 1), new 4-cycloalkylamino-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives (**1**–**11**) were prepared and tested at bA_1, bA_{2A} and hA_3 ARs. Compounds **1**–**11** were designed to evaluate the influence on A_1 affinity and selectivity of some simple substituents (chloro, nitro or amino groups) at different positions of the benzofused moiety. Moreover, we were interested in investigating the effect of all these substituents on hA_3 AR affinity.

Introduction of lipophilic chlorine atoms at the 7,8-position of our triazoloquinoxalines was pursued since this substitution was profitable for A_1 and/or A_{2A} AR affinity of

Correspondence: Prof. Vittoria Colotta, Dipartimento di Scienze Farmaceutiche, Università di Firenze, Polo Scientifico, Via Ugo Schiff, 6, 50016 Sesto Fiorentino (FI), Italy. Phone: +39 55 4573731, Fax: +39 55 4573671; e-mail vittoria.colotta@unifi.it

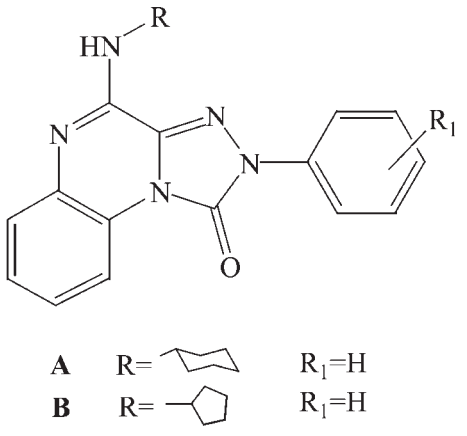
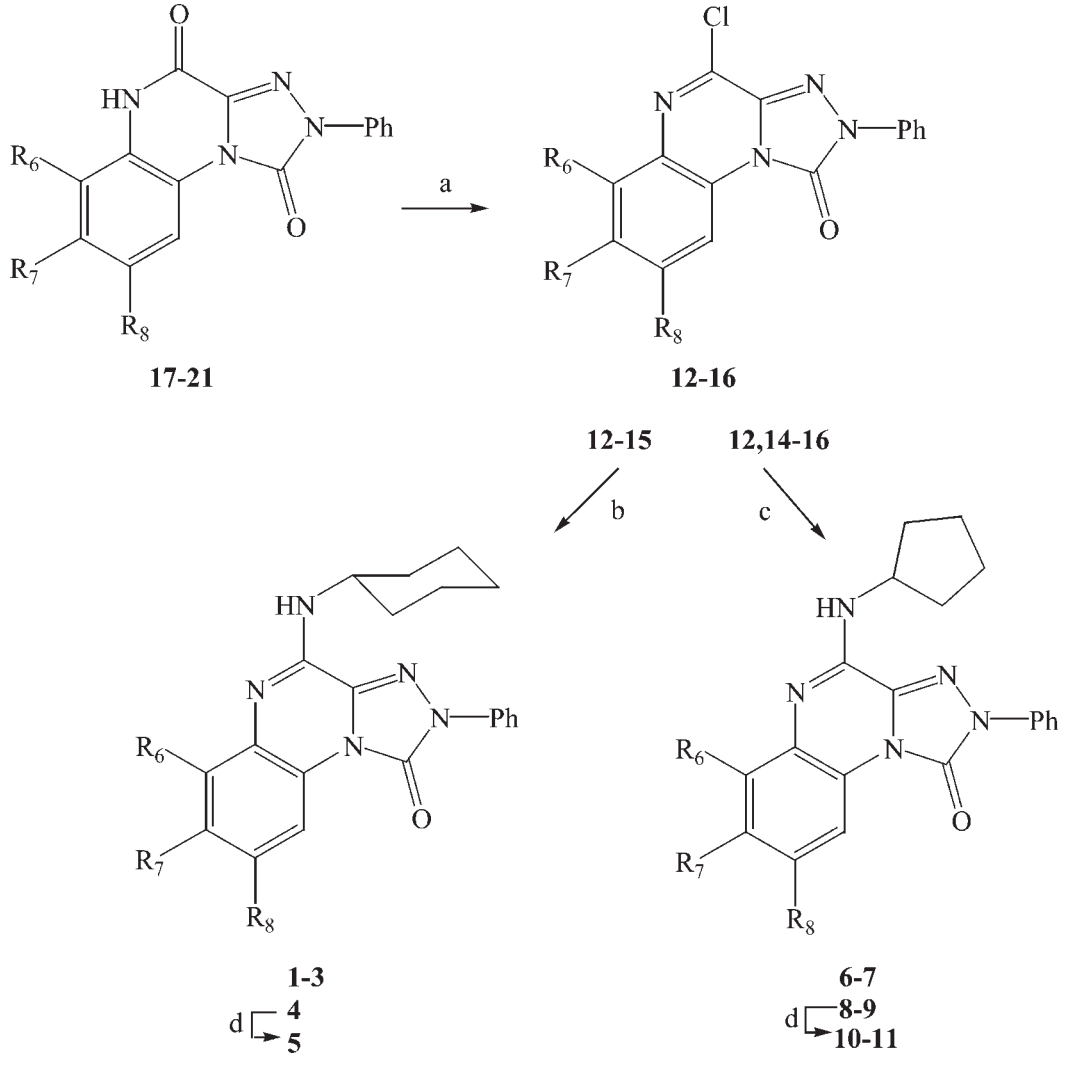


Figure 1. Previously reported 4-amino-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-ones **A** and **B.**

some tricyclic AR ligands of similar size and shape [17, 18]. In addition, we also decided to evaluate the effect of chlorine atoms at the 6,8-positions and of substituents (the nitro or amino group) at the 6- or 8-position, able to engage hydrogen bonds with the binding site. It has to be noted that, to our knowledge, the effects of these substituents on AR affinity have never been investigated in tricyclic AR antagonists.

Chemistry

The novel 4-cycloalkylamino-triazoloquinoxalin-1-one derivatives **1**–**11** were prepared as illustrated in Scheme 1. Briefly, the key intermediates 4-chloro-triazoloquinoxalin-1-ones **12**–**16** were obtained by treatment of the corresponding 1,4-dione derivatives **17**–**21** [13, 15] with



Scheme 1. Reagents: (a) $PCl_5/POCl_3$, pyridine; (b) Cyclohexylamine, Et_3N, absolute EtOH; (c) Cyclopentylamine, Et_3N, absolute EtOH; (d) H_2, Pd/C, AcOH or THF.

phosphorous pentachloride and phosphorous oxychloride. Reaction of the 4-chloro derivatives **12**–**15** with cyclohexylamine yielded compounds **1**–**4**, while derivatives **6**–**9** were obtained by reacting **12**, **14**–**16** with cyclopentylamine. Finally, catalytic reduction (Pd/C) of the nitro derivatives **4**, **8** and **9** afforded the corresponding amino-compounds **5**, **10** and **11**.

Biochemistry

Compounds **1**–**11** were tested for their ability to displace [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) from A_1 AR in bovine cerebral cortical membranes, [^{3}H]-2-[[4-(2-carboxyethyl)phenethyl]amino]-5′-(*N*-ethyl-carbamoyl)-adenosine ([^{3}H]CGS 21680) from A_{2A} AR in bovine striatal membranes, and [^{125}I]N^6-(4-amino-3-iodobenzyl)-5′-*N*-methylcarbamoyladenosine ([^{125}I]AB-MECA) from human cloned A_3 receptor stably expressed in CHO cells. In fact, due to the high species differences in the A_3 primary amino acid sequence [3, 19, 20], we tested our A_3 AR ligands on cloned human A_3 receptors.

Results and discussion

The binding results of **1**–**11** are shown in Table 1. In this table the binding affinities of compounds **A** and **B** [16] are also reported, together with those of theophylline and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), included as antagonist reference compounds.

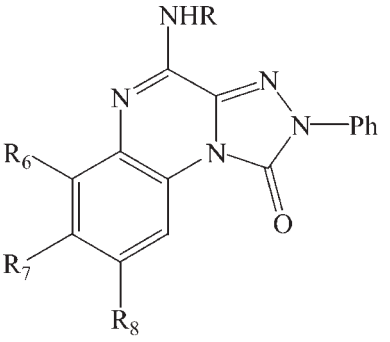
Due to the presence of the 4-cycloalkyl moiety, which is well-known to yield A_1 *vs* A_{2A} selective ligand [7, 11, 14, 16–18], compounds **1**–**11** possess nanomolar or subnanomolar A_1 affinity, while they are on the whole scarcely active or inactive at the A_{2A} receptor subtype. Furthermore, in accordance with our previous data [16], the 4-cyclohexylamino derivatives **3**–**5** show higher A_1 *vs* A_{2A} selectivity than the corresponding 4-cyclopentylamino derivatives **7, 8, 10**. Compounds **1**–**11** are also active at the A_3 AR since they display, generally, A_3 AR affinities in the nanomolar range. Moreover, confirming our previous findings [16], most of the 4-cyclohexylamino derivatives show lower A_3 AR affinity than the corresponding 4-cyclopentylamino compounds (compare **1**, **3**, **4** and **5** with **6**, **7**, **8** and **10**, respectively).

Introduction of substituents on the benzofused moiety of the 4-cyclohexylamino derivative **A** generally reduces the A_1 and, especially, the A_{2A} AR affinity, thus improving the A_1 *vs* A_{2A} selectivity. In fact, compounds **1**–**5** are more A_1 *vs* A_{2A}-selective than compound **A**. Among the 4-cyclopentylamino derivatives, compounds **6**–**8** and **9**–**11** show, respectively, lower and similar A_1 affinity than their parent compound **B**, while compounds **6** and **9** display higher A_1 *vs* A_{2A} selectivity than **B**. The presence of substituents on the benzofused moiety of compounds **A** and **B** affects A_3 AR affinity differently. In fact, compounds **2**, **3** are significantly less active at this subtype than **A** and the same can be said for **7** with respect to **B**. On the contrary, compounds **10**–**11** display higher A_3 affinity than **B**. In particular, the 4-cyclopentylamino derivative **11**, bearing an 8-amino group, possesses the highest A_3 AR affinity (K_i = 2.7 nM) among the herein reported ligands.

The presence of two chlorine atoms, at the 7,8- position (compounds **1**, **6**), did not elicit the beneficial effects for A_1 and A_{2A} affinity exerted in other tricyclic derivatives [17, 18] and was not profitable for A_3 AR affinity either. The same applies to the 6,8-dichloro (compound **2**) and to the 6-nitro-8-chloro (compounds **3** and **7**) substitutions, with particular regard to A_3 AR affinity. The negative effects of the presence of two substituents (chloro and nitro) on the benzofused moiety of compounds **1**–**3**, **6**–**7** could be attributed to their steric bulk, which may hinder the correct anchoring to the AR recognition site. This hypothesis can be confirmed by comparison of the A_1 and A_3 AR affinities of the 8-chloro-6-nitro derivatives **3** and **7** with those of compounds **4** and **8**, lacking the bulky chlorine atom at the 8-position. In fact, **4** and **8** are significantly more active than **3** and **7**. On the other hand, the importance of steric requirements for interaction with the A_3 AR binding pocket has already been shown in previous studies about this class of AR antagonists [14] and other tricyclic antagonists of similar size and shape [21].

Comparison of the A_3 AR affinity of compound **4** with that of compound **A** confirms that the presence of a 6-nitro group is profitable for anchoring of 4-amino-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one derivatives to the A_3 AR recognition site [15]. The advantageous effect of this group has been previously rationalized [15] on the basis of a recent rhodopsin-based model of human A_3 AR [22] which has been docked with the 9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-c]quinazolin-5-amine (CGS 15943) [23] as reference ligand. Due to their similar size and shape, we have hypothesized [15] a similar binding mode of our 4-amino-triazolo[4,3-a]quinoxalin-1-one derivatives and CGS 15943 [14]. This model assumes a hydrogen bond interaction between the N-6 atom of CGS 15943, corresponding to the N-5 of our ligands, and Ser247 (TM6). Moreover, the 5-NH_2 group of CGS 15943 also seems to give a hydrogen bond being surrounded by Ser 242(TM6), Ser271 (TM7) and Ser275 (TM7). Thus, although the 6-nitro group of **4** reduces the capability of the N-5 to act as hydrogen bond acceptor, it could reinforce the hydrogen bond with Ser247, being able itself to interact with this residue. Moreover, the 6-nitro group of **4**, due to its electron-withdrawing properties, increases acidity of the 4-amino protons, thus enhancing the strenght of interaction of this group with the receptor

Table 1. Binding activity at bovine A_1 and A_{2A} and human A_3 ARs.



A, B, 1-11

Compound	R_6	R_7	R_8	R	K_i (nM)† or %-inhibition A_1#	$A_{2A}$$	A_3‡
A∫	H	H	H		1.43 ± 0.1	1370 ± 118	506 ± 43
1	H	Cl	Cl		90 ± 7.2	15 %	1360 ± 120
2	Cl	H	Cl		48 ± 3.8	0 %	16 %
3	NO_2	H	Cl		17 ± 0.9	54 %	3 %
4	NO_2	H	H		1.98 ± 0.15	32 %	281 ± 24
5	NH_2	H	H		0.39 ± 0.02	2000 ± 170	316 ± 29
B∫	H	H	H		0.42 ± 0.03	986 ± 82	55.4 ± 4.2
6	H	Cl	Cl		1.07 ± 0.1	55 %	144 ± 11
7	NO_2	H	Cl		11.9 ± 0.9	8800 ± 410	53 %
8	NO_2	H	H		2.36 ± 0.11	5000 ± 290	116 ± 24
9	H	H	NO_2		0.35 ± 0.02	25 %	212 ± 18
10	NH_2	H	H		0.55 ± 0.04	310 ± 28	18 ± 1.5
11	H	H	NH_2		0.83 ± 0.05	1142 ± 109	2.7 ± 0.15
Theophylline					3800 ± 340	21000 ± 1800	86000 ± 7800
DPCPX					0.5 ± 0.03	337 ± 28	1300 ± 125

† The K_i values are means ± SEM of 4 separate assays, each performed in triplicate.

Displacement of specific [^{3}H]CHA binding in bovine brain membranes or percentage of inhibition (I%) of specific binding at 20 μM concentration.

$ Displacement of specific [^{3}H]CGS 21680 binding from bovine striatal membranes or percentage of inhibition (I%) of specific binding at 20 μM concentration.

‡ Displacement of specific [^{125}I]AB-MECA binding at human A_3 receptors expressed in CHO cells or percentage of inhibition (I%) of specific binding at 1 μM concentration.

∫ Reference [16].

proton acceptor site. Nevertheless, we must admit that this hypothesis is in contrast with the 2-fold lower A_3 affinity of the 6-nitro derivative **8** with respect to compound **B**.

The A_3 receptor model, cited above, could also justify the beneficial effect of the 6-amino group for A_3 affinity of compounds **5**, **10** and of some previuosly reported triazolo[4,3-a]quinoxalin-1-one derivatives [15]. In fact, the increased A_3 receptor affinity of **5** and **10** with respect to **A** and **B**, could be due to the capability of the 6-amino group to form a hydrogen bond with Ser247, thus reinforcing the interaction of this serine residue with the N-5 area of **5** and **10**.

In contrast, the influence of the hydrophilic 6-amino group on A_1 and A_{2A} affinity is difficult to rationalize since this substituent elicits opposite effects on the 4-cyclohexylamino derivative **A** and on the 4-cyclopentylamino derivative **B**. In fact, while compound **5** possesses an increased A_1 affinity and A_1 *vs* A_{2A} selectivity, with respect to **A**, compound **10** shows similar A_1 affinity and reduced A_1 *vs* A_{2A} selectivity compared to **B**.

Also the beneficial effect of the 8-amino group on compound **B** (compound **11**) could be rationalized on the basis of the previously cited A_3 receptor model which hypothesizes that the benzofused moiety of CGS 15943 resides in a hydrophobic pocket in which polar amino acids, such as Thr94 (TM3) and Ser97 (TM3), are present. Thus, the significantly increased affinity of **11**, compared to **B**, could be explained by hypothesizing a hydrogen bond interaction between Thr94 or Ser97 and the 8-amino group.

In conclusion, introduction of simple substituents on the benzofused moiety of the previously reported triazoloquinoxalin-1-one derivatives **A** and **B** was advantageous since it afforded some highly potent A_1 and A_1 *vs* A_{2A} selective antagonists. In particular, all the 4-cyclohexylamino derivatives (**1**–**5**) showed higher A_1 *vs* A_{2A} selectivity than the parent compound **A**. Furthermore, on the basis of the SAR studies, we confirmed the important role played by the steric factors for the A_3 receptor-ligand interaction. In addition, we highlighted the importance of the presence on the triazoloquinoxalin-1-one framework of a group (6-nitro, 6- or 8-amino) able to form hydrogen bonds with a proton donor or acceptor site of the A_3 receptor-binding pocket.

Acknowledgment

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Experimental

Chemistry

Silica gel plates (Merck F_{254}) and silica gel 60 (Merck, 70–230 mesh) were used for analytical and column chromatography, respectively. All melting points were determined on a Gallenkamp melting point apparatus. Microanalyses were performed with a Perkin-Elmer 260 elemental analyzer for C, H, N, and the results were within ± 0.4 % of the theoretical. The IR spectra were recorded with a Perkin-Elmer 1420 spectrometer in Nujol mulls and are expressed in cm^{-1}. The ^{1}H-NMR spectra were obtained with a Varian Gemini 200 instrument at 200 MHz. The chemical shifts are reported in δ (ppm) and are relative to the central peak of the solvent that is always DMSO-d_6. The following abbreviations are used: s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet, br = broad, and ar = aromatic protons.

General Procedure for the Synthesis of 4-Cycloalkylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-ones **1–4, 6–9**

The title compounds were obtained starting from the triazoloquinoxaline-1,4-dione derivatives **17**–**21** [13, 15] (2 mmol) which were reacted with phosphorus pentachloride (4 mmol) by refluxing phosphorus oxychloride (40 mL) and anhydrous pyridine (0.2 mL) until the disappearance of the starting material (12–24 h) could be observed by TLC monitoring. Evaporation at reduced pressure of the excess phosphorus oxychloride afforded a solid, which was treated with ice water (50 mL), collected and washed with cyclohexane. The 4-chloro derivatives **12**–**16** [16], obtained in high overall yields (85–90 %), were unstable, nevertheless they were pure enough to be used without further purification.

The 4-cyclohexylamino derivatives **1**–**4** were prepared from the 4-chloro derivatives **12**–**15** (1 mmol) which were reacted overnight at 120 °C, in a sealed tube with cyclohexylamine (1.2 mmol) in absolute ethanol (5 mL) and triethylamine (2 mmol). Upon cooling, a solid was obtained which was collected and washed with water.

7,8-Dichloro-4-cyclohexylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**1**)

Yield: 95 %; mp 223–225 °C (AcOH). ^{1}H-NMR 1.06–2.07 (m, 10 H, aliphatic protons), 4.02–4.29 (m, 1 H, aliphatic proton), 7.42 (t, 1 H, ar, J = 7.0 Hz), 7.58 (t, 2 H, ar, J = 7.4 Hz), 7.67 (s, 1 H, H-6), 8.05–8.15 (m, 3 H, 2ar + NH), 8.71 (s, 1 H, H-9). Anal. ($C_{21}H_{19}Cl_2N_5O$) C, H, N.

6,8-Dichloro-4-cyclohexylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**2**)

Yield: 98 %; mp 222–224 °C (AcOH). ^{1}H-NMR 1.09–2.10 (m, 10 H, aliphatic protons), 4.08–4.30 (m, 1 H, aliphatic proton), 7.37 (t, 1 H, ar, J = 7.3 Hz), 7.50–7.62 (m, 3 H, ar), 8.06 (d, 2 H, ar, J = 7.7 Hz), 8.22 (d, 1 H, NH, J = 7.9 Hz), 8.56 (d, 1 H, H-9, J = 2.4 Hz). Anal. ($C_{21}H_{19}Cl_2N_5O$) C, H, N.

8-Chloro-4-cyclohexylamino-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**3**)

Yield: 88 %; mp 214–215 °C (Cyclohexane/EtOAc). ^{1}H-NMR 1.04–2.08 (m, 10 H, aliphatic protons), 3.79–4.09 (m, 1 H, aliphatic proton), 7.39 (t, 1 H, ar, J = 7.6 Hz), 7.56 (t, 2 H, ar, J = 7.6 Hz), 7.99–8.06 (m, 3 H, ar), 8.55 (d, 1 H, NH, J = 7.7 Hz), 8.70 (d, 1 H, H-9, J = 2.2 Hz). Anal. ($C_{21}H_{19}ClN_6O_3$) C, H, N.

4-Cyclohexylamino-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**4**)

Yield: 90 %; mp 184–185 °C (EtOH). ^{1}H-NMR 1.05–2.11 (m, 10 H, aliphatic protons), 3.87–4.13 (m, 1 H, aliphatic proton), 7.33–7.42 (m, 2 H, ar), 7.59 (t, 2 H, ar, J = 7.6 Hz), 7.80 (d, 1 H, J = 7.9 Hz), 8.09 (d, 2 H, ar, J = 7.8 Hz), 8.60 (d, 1 H, NH, J = 7.3 Hz), 8.79 (d, 1 H, H-9, J = 7.0 Hz). Anal. ($C_{21}H_{20}N_6O_3$) C, H, N.

The 4-cyclopentylamino derivatives **6**–**9** were obtained from **12**, **14**–**16** (1 mmol) and cyclopentylamine (1.2 mmol) following the experimental conditions described above to obtain **1**–**4**.

7,8-Dichloro-4-cyclopentylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**6**)

Yield: 88 %; mp 220–222 °C (EtOAc). ^{1}H-NMR 1.52–2.15 (m, 8 H, aliphatic protons), 4.42–4.63 (m, 1 H, aliphatic proton), 7.40 (t, 1 H, ar, J = 7.6 Hz), 7.54–7.66 (m, 3 H, ar), 8.07 (d, 2 H, ar, J = 7.3 Hz), 8.22 (d, 1 H, NH, J = 7.3 Hz), 8.71 (s, 1 H, H-9). Anal. ($C_{20}H_{17}Cl_2N_5O$) C, H, N.

8-Chloro-4-cyclopentylamino-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**7**)

Yield: 86 %; mp 212–214 °C (Cyclohexane/EtOAc). ^{1}H-NMR 1.43–2.12 (m, 8 H, aliphatic protons), 4.21–4.40 (m, 1 H, aliphatic proton), 7.40 (t, 1 H, ar, J = 7.6 Hz), 7.56 (t, 2 H, ar, J = 7.6 Hz), 7.99–8.06 (m, 3 H, ar), 8.60–8.69 (m, 2 H, H-9 + NH). Anal. ($C_{20}H_{17}ClN_6O_3$) C, H, N.

4-Cyclopentylamino-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**8**)

Yield: 70 %; mp 216–217 °C (EtOAc). ^{1}H-NMR 1.45–2.16 (m, 8 H, aliphatic protons), 4.42–4.50 (m, 1 H, aliphatic proton), 7.38 (t, 2 H, ar, J = 8.1 Hz), 7.59 (t, 2 H, ar, J = 7.7 Hz), 7.77 (d, 1 H, ar, J = 8.2 Hz), 8.09 (d, 2 H, ar, J = 7.7 Hz), 8.52 (d, 1 H, NH, J = 6.9 Hz), 8.78 (d, 1 H, H-9, J = 8.2 Hz). Anal. ($C_{20}H_{18}N_6O_3$) C, H, N.

4-Cyclopentylamino-1,2-dihydro-8-nitro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**9**)

Yield: 80 %; mp 276–277 °C (EtOAc). ^{1}H-NMR 1.58–1.9 (m, 6 H, aliphatic protons), 2.04–2.18 (m, 2 H, aliphatic proton), 4.52–4.62 (m, 1 H, aliphatic proton), 7.42 (t, 1 H, ar, J = 7.2 Hz), 7.51–7.64 (m, 3 H, ar), 8.03–8.23 (m, 3 H, ar), 8.72 (d, 1 H, NH, J = 7.3 Hz), 9.38 (d, 1 H, H-9, J = 2,7 Hz); IR 3380, 1740. Anal. ($C_{20}H_{18}N_6O_3$) C, H, N.

General Procedure for the Synthesis of 6-Amino-4-cyclohexylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (**5**), *6-Amino-4-cyclopentylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one* (**10**) *and 8-Amino-4-cyclopentylamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]-quinoxalin-1-one* (**11**)

Compounds **4, 8** or **9** (0.8 mmol) were dissolved in hot glacial acetic acid (200 mL). Pd/C 10 % (0.05 g) was added to the solution and the mixture was hydrogenated overnight in a Parr apparatus at 30 psi. The suspension was heated and the catalyst filtered off. Evaporation of the solvent at reduced pressure gave a residue, which was suspended in diethyl ether (5–10 mL) and filtered.

5: Yield: 82 %; mp 191–192 °C (EtOAc). ^{1}H-NMR 1.04–2.08 (m, 10 H, aliphatic protons), 4.07–4.33 (m, 1 H, aliphatic proton), 5.44 (br s, 2 H, NH_2), 6.69 (d, 1 H, ar, J = 7.9 Hz), 6.99 (t, 1 H, ar, J = 7.8 Hz), 7.30–7.61 (m, 4 H, 3 ar + NH), 7.86 (d, 1 H, ar, J = 8.3 Hz), 8.10 (d, 2 H, ar, J = 8.5 Hz). Anal. ($C_{21}H_{22}N_6O$) C, H, N.

10: Yield: 75 %; mp 212–213 °C (Cyclohexane/EtOAc). ^{1}H-NMR 1.50–2.10 (m, 8 H, aliphatic protons), 4.35–4.52 (m, 1 H, aliphatic proton), 5.50 (br s, 2 H, NH_2), 6.67 (d, 1 H, ar, J = 7.8 Hz), 7.10–7.47 (m, 3 H, 2 ar + NH), 7.51 (t, 2 H, ar, J = 7.7 Hz), 7.95 (d, 1 H, H-9, J = 3.1 Hz), 8.08 (d, 2 H, ar, J = 8.1 Hz); IR 3480, 3400–3200, 1700. Anal. ($C_{20}H_{20}N_6O$) C, H, N.

11: Yield: 80 %; mp 178–180 °C (Cyclohexane/EtOAc). ^{1}H-NMR 1.49–2.23 (m, 8 H, aliphatic protons), 4.50–4.60 (m, 1 H, aliphatic proton), 5.46 (br s, 2 H, NH_2), 6.69 (d, 1 H, J = 7.7 Hz), 6.99 (t, 1 H, ar, J = 8.3 Hz), 7.36 (t, 1 H, ar, J = 7.2 Hz), 7.50–7.60 (m, 3 H, 2 ar + NH), 7.86 (d, 1 H, ar, J = 7.9 Hz), 8.10 (d, 2 H, ar, J = 7.8 Hz). Anal. ($C_{20}H_{20}N_6O$) C, H, N.

Biochemistry

Bovine A_1 and A_{2A} Receptor binding: Displacement of [^{3}H]CHA from A_1 AR in bovine cerebral cortical membranes and [^{3}H]CGS 21680 from A_{2A} AR in bovine striatal membranes was performed as described in [24].

Human A_3 Receptor binding: Binding experiments at human A_3 adenosine receptors were performed on crude membranes obtained from CHO cells [25], using [^{125}I]AB-MECA according to a procedure previously described [16].

The concentration of the tested compounds that produced 50 % inhibition of specific [^{3}H]CHA, [^{3}H]CGS 21680 or [^{125}I]AB-MECA binding (IC_{50}) was calculated using a non-linear regression method implemented in the InPlot program (Graph-Pad, San Diego, CA) with 5 concentrations of the displacer, each performed in triplicate. Inhibition constants (K_i) were calculated according to the Cheng-Prusoff equation [26]. The dissociation constant (K_d) of [^{3}H]CHA and [^{3}H]CGS 21680 in cortical and striatal bovine brain membranes were 1.2 nM and 14 nM, respectively. The K_d value of [^{125}I]AB-MECA in human A_3 AR in CHO cell membranes was 1.4 nM.

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