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Synthesis and Structure–Activity Relationships of a New Set of 1,2,4-Triazolo[4,3-*a*]quinoxalin-1-one Derivatives as Adenosine Receptor Antagonists

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Abstract—In a previous paper (Colotta V. et al., *J. Med. Chem*. **2000**, *43*, 1158), we reported the synthesis and the binding activity of some 4-oxo (**A**) and 4-amino (**B**) substituted 1,2,4-triazolo[4,3-*a*]quinoxalin-1-ones, bearing different substituents on the appended 2-phenyl ring (region 1), some of which were potent and selective A_1 or A_3 antagonists. To further investigate the SAR in this class of antagonists, in the present paper some 2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one derivatives of both series **A** and **B**, bearing simple substituents on the benzofused moiety (region 2), are reported. The binding data at bovine A_1 (bA_1) and A_{2A}(bA_{2A}) and at human A_3 (hA_3) adenosine receptors (ARs) show that in series **A** (compounds **1**, **4**–**11**) the presence of substituents on the benzofused moiety is, in general, not advantageous for anchoring at all three AR subtypes, while within series **B** (compounds **12**–**21**) it exerts a beneficial effect for both bA_1 and hA_3 AR affinities which span the low nanomolar range. In particular, among the 4-amino derivatives **12**–**21**, the 8-chloro-6-nitro (compound **17**) and the 6-nitro (compound **18**) substitutions afford, respectively, the highest bA_1 and hA_3 AR affinity. Moreover, compound **18**, additionally investigated in binding assays at human A_1 (hA_1) receptors, shows a 183-fold selectivity for hA_3 versus hA_1 receptors. Finally, the SAR studies provide some new insights about the steric and lipophilic requirements of the hA_3 receptor binding pocket which accommodates the benzofused moiety of our 4-amino-triazoloquinoxalin-1-one derivatives.

Introduction

Adenosine is a neuromodulator which produces many important biological functions by activation of G protein-coupled receptors that are 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]

Recent studies indicate that ARs are attractive targets for pharmacological intervention in many pathophysiological conditions[2] such as renal failure,[6] cardiac[7] and cerebral[8,9] ischemia, Parkinson's disease,[10] schizophrenia[11] and chronic inflammatory diseases.[12–14] Thus, in the last few years, much effort has been directed towards the synthesis of selective AR ligands in order to shed light on the different structural requirements of each AR subtype.

As a part of our research aimed at finding new AR selective antagonists,[15–20] we recently reported the synthesis and the binding activity at bovine A_1 (bA_1) and A_{2A} (bA_{2A}) and at human cloned A_3 (hA_3) ARs of 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-ones, that is the 4-oxo (series **A**) and the 4-amino substituted (series **B**) derivatives (Chart 1), bearing diverse substituents on the appended 2-phenyl ring (region 1).[21] The binding results showed that some of these compounds were potent and selective A_1 or A_3 antagonists. In particular, the 1,4-dione derivatives (series **A**) were, on the whole, more active at the A_3 AR than the corresponding 4-amino-1-one compounds (series **B**). On the contrary, compounds of series **B** possessed significantly higher A_1 AR affinity than those of derivatives of series **A**. Thus, these results indicated that the presence of the 4-amino proton donor group was important for high bA_1 receptor affinity,

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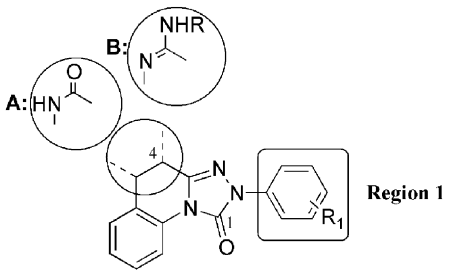
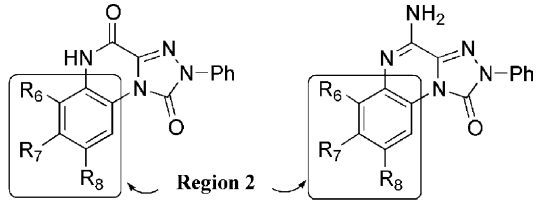


Series **A**: 1,4-dione derivatives **1A** R_1= H
Series **B**: 4-amino-1-one derivatives **1B** R=R_1=H

Chart 1. Previously reported 1,2,4-triazolo[4,3-*a*]quinoxaline derivatives.

while it was not necessary for hA_3 receptor recognition. To further investigate the SAR in this class of antagonists, in the present paper we report the synthesis and bA_1, bA_{2A} and hA_3 AR binding activities of some 2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-diones (series **A**, **1**, **4–11**) and 4-amino-1-ones (series **B**, **12–21**) (Chart 2), bearing simple substituents (chloro, nitro or amino groups) at different positions of the benzofused moiety (region 2). Taking the previously reported triazoloquinoxalin-1,4-dione **1A** and 4-amino-1-one **1B**[21] as lead compounds (Chart 1), derivatives **1–11** and **12–21** were designed, respectively.



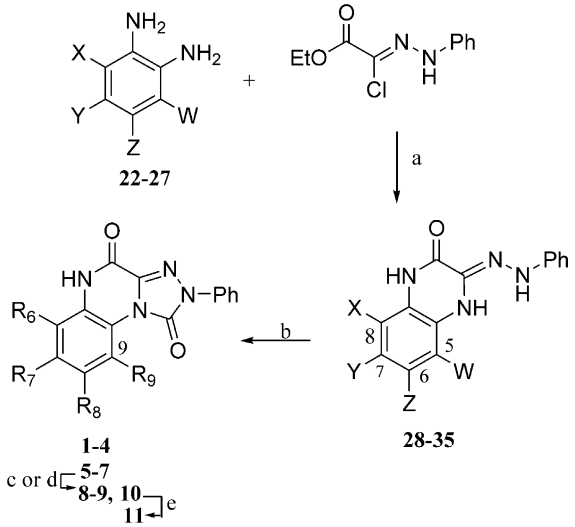
R_6, R_7, R_8= H, Cl, NO_2, NH_2, $NHCH_2Ph$

Chart 2. Currently reported 1,2,4-triazolo[4,3-*a*]quinoxaline 1-one derivatives.

Introduction of a lipophilic chlorine atom(s) at the 7- and/or 8-position of our triazoloquinoxalines was pursued due to the increased A_1 and/or A_{2A} AR affinity, observed in different tricyclic AR ligands of similar size and shape.[17,22–24] In addition, we decided also to evaluate the effect of chlorine atoms at the 6,8-positions and that of a polar or hydrophilic group (the nitro or amino group, respectively) at the 6- or 8-position, since, to our knowledge, these effects on AR affinity have never been investigated in tricyclic AR antagonists. Moreover, little was known about the influence of all these substituents on A_3 AR affinity.[20]

Chemistry

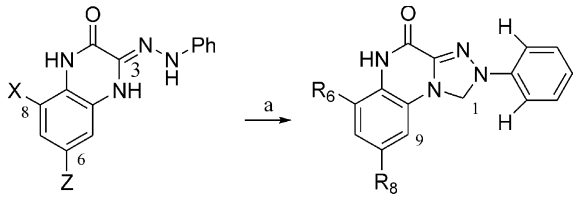
The triazoloquinoxalines **1–21** were prepared as illustrated in Schemes 1 and 3. Scheme 1 shows the synthesis



compd	X/R_6	Y/R_7	Z/R_8	W/R_9
22, 28, 1	H	Cl	Cl	H
23, 29, 2	H	Cl	H	H
30, 3	H	H	Cl	H
24, 31, 4	Cl	H	Cl	H
32	H	Cl	H	Cl
25, 33, 5	H	H	NO_2	H
26, 34, 6	NO_2	H	Cl	H
27, 35, 7	NO_2	H	H	H
8	H	H	NH_2	H
9	NH_2	H	Cl	H
10	NH_2	H	H	H
11	$NHCH_2Ph$	H	H	H

Scheme 1. (a) NEt_3, EtOH; (b) $(Cl_3CO)_2CO$, THF; (c) H_2, Pd/C, THF or AcOH; (d) $SnCl_2{\cdot}2H_2O$, EtOH/DMSO; (e) ref 19.

of the 2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-diones **1–11**. Compounds **1–6** were prepared following the reported procedure described to obtain derivative **7**.[19] Reaction of the 4,5-dichloro-1,2-phenylenediamine (**22**) with ethyl N^1-phenylhydrazono-N^2-chloroacetate afforded the 3-(6,7-dichlorophenyl)hydrazono-1,2,3,4-tetrahydroquinoxalin-2-one (**28**). Starting from the 1,2-phenylenediamines **23** and **24**,[25] two couples of regioisomers, that is compounds **29**, **30** and **31**, **32** were obtained, respectively. On the contrary, from the diamines **25**, **26**,[26] **27** only one compound was obtained, that is the corresponding 3-phenylhydrazono-1,2,3,4-tetrahydroquinoxalin-2-ones **33**, **34**, **35**.[19] The two isomers **31** and **32** were easily separated by their different solubility in the reaction medium, while the two isomers **29**, **30** were used as inseparable mixture for the next step, due to their instability on silica gel column. The structure of derivatives **31**, **33** and **34** was assigned by means of $^1H/^1H$ NOE experiments on compounds **36–38** obtained by reacting **31**, **33** and **34** with formaldehyde (Scheme 2). Pre-irradiation of the 1-methylene protons of **36–38** caused a clear enhancement of the signals of the two *ortho* protons of the 2-phenyl ring and of the diagnostic proton at position 9. Thus, the bicyclic compounds **36** and **38** were 6,8-dichloro and 6-chloro-8-nitro-substituted derivatives, respectively. The 6-nitro-structure of compound **33** was assigned on the basis of the multiplicity and the coupling constant of the H-9 signal of **37**, which appears as a doublet (J = 1.8 Hz),



	X/R_6	Z/R_8
31, 36	Cl	Cl
33, 37	H	NO_2
34, 38	NO_2	Cl

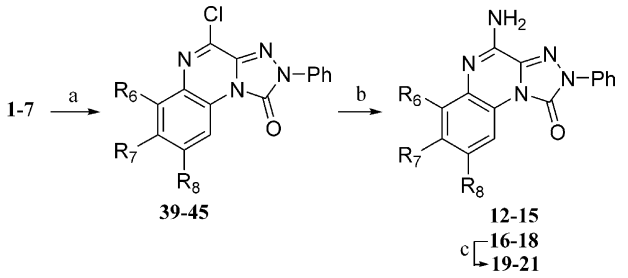
Scheme 2. (a) 40% HCHO, ethylene glycol.

due to the coupling with the H-7. Finally, on the basis of the structure of **31**, the 5,7-dichloro-structure of its regioisomer **32** was assigned.

The intermediates **28**, **31**, **33**, **34**, **35**[19] were cyclized with triphosgene to yield the 1,2,4-triazolo[4,3-*a*]quinoxaline-1,4-diones **1**, **4–6**, **7**[19] (Scheme 1). The 3-phenylhydrazono-1,2,3,4-tetrahydro-5,7-dichloroquinoxalin-2-one (**32**), obtained in small amounts, was not cyclized to the corresponding triazoloquinoxalin-1,4-dione. By reacting the mixture of the regioisomers **29** and **30** with triphosgene a mixture of the tricyclic derivatives **2** and **3** was obtained. We were unable to separate the isomers **2** and **3** with satisfactory yields, thus their inseparable mixture was used for the next step. Nevertheless, by column chromatography a few milligrams of pure **2** and **3**, only sufficient to characterize them, were obtained. The structures of **2** and **3** were assigned on the basis of their ^{1}H NMR spectra, using as key tool the coupling constant value of the H-9 signal which is easily identified in this class of tricyclic derivatives. In fact, the H-9 is in general the most deshielded aromatic proton (about 8.6–9.4 ppm) due to the paramagnetic effect of the 1-carbonyl group.[19,21,27] In both the ^{1}H NMR spectra of the isomers **2** and **3**, the H-9 signal appeared as a doublet at 8.59 ppm, but with different coupling constants: while in the spectrum of **2** the H-9 coupling constant value was 8.4 Hz, in that of **3** it was 2.3 Hz. Thus **2** and **3** were 7-chloro- and 8-chloro-substituted derivatives, respectively.

Compounds **5** and **7**[19] were catalytically reduced (Pd/C) to the corresponding amino-substituted derivatives **8** and **10**,[19] while the 6-amino-8-chloro derivative **9** was obtained by reacting **6** with tin(II) chloride (Scheme 1). The 6-benzylamino-1,4-dione derivative **11** was prepared as described in ref 19, that is by reacting **10** with benzaldehyde and then reducing the corresponding Schiff base with sodium borohydride.

By reacting the 1,4-dione derivatives **1**, **4–7** and the mixture of **2** and **3** with phosphorus pentachloride and phosphorus oxychloride the unstable 4-chloro derivatives **39**, **42–45** and the mixture of **40** and **41** were obtained (Scheme 3). The final 4-amino-triazoloquinoxalin-1-ones **12–17**, **18**[19] ensued from the reaction of **39–45** with ammonia. The mixture of the 7- and 8-chloro isomers **13** and **14** was separated by column chromatography. The structure of **13** and **14** was assigned on the



compd	R_6	R_7	R_8
39, 12	H	Cl	Cl
40, 13	H	Cl	H
41, 14	H	H	Cl
42, 15	Cl	H	Cl
43, 16	H	H	NO_2
44, 17	NO_2	H	Cl
45, 18	NO_2	H	H
19	H	H	NH_2
20	NH_2	H	Cl
21	NH_2	H	H

Scheme 3. (a) $PCl_5/POCl_3$, pyridine; (b) NH_3(g), absolute EtOH; (c) H_2, Pd/C, AcOH or THF.

basis of their ^{1}H NMR spectra, using as a key tool the coupling constant of the H-9 signal, as above illustrated for the structural attribution of their corresponding 1,4-dione derivatives **2** and **3**. In the ^{1}H NMR spectra of **13** and **14** the H-9 signal appears as a doublet at 8.59 ppm with a coupling constant of 8.4 and 2.1 Hz, respectively. Indeed, compounds **13** and **14** were 7-chloro and 8-chloro substituted derivatives, respectively.

The nitro derivatives **16–18** were catalytically reduced to afford the corresponding amino compounds **19**, **20**, **21**.[19]

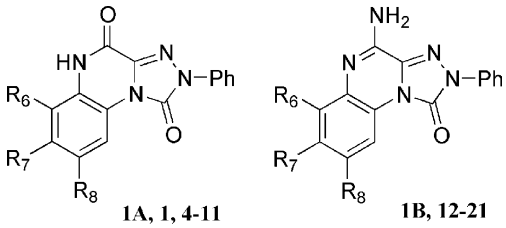
Biochemistry

Compounds **1**, **4–21** were tested for their ability to displace [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) from A_1 ARs in bovine cerebral cortical membranes, [^{3}H]-2-{4-(2-carboxyethyl)phenethyl]amino}-5′-(*N*-ethylcarbamoyl)adenosine ([^{3}H]CGS 21680) from A_{2A} ARs 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 receptors stably expressed in CHO cells. In fact, due to the high species differences in the A_3 primary amino acid sequence,[28–30] we tested our A_3 AR ligands on cloned human A_3 receptors.

The binding results of **1**, **4–11** and **12–21** are shown in Table 1 together with those of their parent compounds **1A** and **1B** (described in ref 21). Moreover, the binding data of theophylline and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), included as antagonist reference compounds, are also reported.

In addition, compound **18**, which displayed the highest hA_3 affinity, was tested for its ability to displace [^{3}H]CHA from cloned human A_1 AR (hA_1), in order to establish its A_3 versus A_1 selectivity within the same species. The hA_1 binding result of **18** together with those of theophylline and DPCPX are reported in Table 2.

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



1A, 1, 4-11 **1B, 12-21**

Compd	R_6	R_7	R_8	K_i (nM)[a] or I %		
				A_1[b]	A_{2A}[c]	A_3[d]
1A[e]	H	H	H	515±43	64%	80.0±6.3
1	H	Cl	Cl	48%	5%	936±81
4	Cl	H	Cl	36%	7%	1360±118
5	H	H	NO_2	99±8.2	28%	241±21
6	NO_2	H	Cl	3540±290	5%	856±80
7	NO_2	H	H	529±47	0%	279±23
8	H	H	NH_2	45.1±3.6	478.1±42	34%
9	NH_2	H	Cl	382±31	24%	152±11
10	NH_2	H	H	195±14	42%	418±39
11	$NHCH_2Ph$	H	H	33%	16%	399±29
1B[e]	H	H	H	11.0±0.9	49±3.7	490±41
12	H	Cl	Cl	212±32	712±63	91.5±8.3
13	H	Cl	H	47.8±3.8	113±10	10.2±0.9
14	H	H	Cl	17.1±1	102±9.3	11±1
15	Cl	H	Cl	42.7±1	150±13	1140±101
16	H	H	NO_2	8.25±0.6	49.2±4.3	40±3.2
17	NO_2	H	Cl	0.2±0.01	256±21	112±9.3
18	NO_2	H	H	82±7.4	75.8±6.9	4.75±0.3
19	H	H	NH_2	7.33±0.4	152±11	34±2.1
20	NH_2	H	Cl	28.6±1.4	56.1±4.8	23.3±1.9
21[f]	NH_2	H	H	9.2±0.8	18.7±1.4	54±4.2
Theophylline				3800±340	21,000±1800	86,000±7800
DPCPX				0.5±0.03	337±28	1300±125

[a]The K_i values are means±SEM of four separate assays, each performed in triplicate.
[b]Displacement of specific [^{3}H]CHA binding in bovine brain membranes or percentage of inhibition (I%) of specific binding at 20 μM concentration.
[c]Displacement of specific [^{3}H]CGS 21680 binding from bovine striatal membranes or percentage of inhibition (I%) of specific binding at 20 μM concentration.
[d]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.
[e]Ref 21. It is worth noting that a more careful screening of the A_3 affinity of **1B** revealed a higher affinity (K_i=490 nM) than that reported in ref 21.
[f]The A_1 and A_{2A} binding data are reported in ref 19.

Results and Discussion

The binding results reported in Table 1 show that we have produced some potent bA_1 and hA_3 AR antagonists, while only a few compounds (**16**, **18**, **20** and **21**) display good bA_{2A} receptor affinity.

In the 1,4-dione derivatives **1**, **4**–**11** (series **A**) the presence of substituent(s) on the benzofused moiety, on the whole, negatively affects A_{2A} AR affinity while it exerts different effects on A_1 binding activity. In fact, while derivatives **7**, **9** and **5**, **8**, **10** are, respectively, equi-active and 3- to 10-fold more active than **1A** at this receptor subtype, compounds **1**, **4**, **11** and **6** show, respectively, null and 7-fold lower A_1 affinity than that of the lead compound **1A**.

On the contrary, compounds **1**, **4**–**11** show A_3 AR affinities which are, on the whole, significantly lower than that of compound **1A** (K_i=80 nM) thus indicating that, in series **A**, the presence of substituents on the benzofused moiety is not well tolerated for anchoring to the A_3 AR recognition site. In fact, it should be noted that the previously reported 1,4-dione derivatives,[21] lacking substituent(s) on the benzofused moiety, showed higher A_3 affinity than those of the herein reported series **A**.

Introduction of a polar or hydrophilic substituent at the 8-position of **1A** is very advantageous for A_1 AR recognition. In fact, both the 8-nitro compound **5** and the 8-amino derivative **8** possess high A_1 AR affinity. The nanomolar A_1 affinities of the 1,4-dione derivatives **5** and **8** are noteworthy since they represent a new finding with respect to our previous results[21] which indicated that the presence of a 4-amino group on the triazoloquinoxaline framework was an important feature for obtaining high bA_1 affinity.

Displacement of the nitro and amino group of compounds **5** and **8**, respectively, from the 8- to the 6-position reduces the A_1 affinity about 5-fold (compare compounds **5** and **8** to **7** and **10**, respectively).

Introduction of a lipophilic chlorine atom at the 8-position of the 6-nitro compound **7** and of the 6-amino derivative **10** yields compounds **6** and **9**, respectively. These latter compounds show 7- and 2-fold reduced A_1 AR affinity, with respect to their 8-deschloro derivatives **7** and **10**. The negative effect of the 8-chloro substituent on A_1 affinity could be attributed to the steric bulk of the chlorine atom which may hinder the correct anchoring to the receptor recognition site. On this basis, it is possible to hypothesize that the benzofused moiety (region 2) of the 1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-diones (series **A**) interacts with a hydrophobic pocket of the bA_1 receptor that possesses strict steric requirements. The importance of the steric factors for interaction of these derivatives with the bA_1 AR is also supported by the null A_1 affinity of either the dichloro-substituted derivatives **1** and **4** or the 6-*N*-benzylamino compound **11**. Nevertheless, the significantly different A_1 AR affinity of compounds **4** and **6**, bearing at the 6-position a chlorine atom and a nitro group, respectively, indicates that not only the steric factors but also the electronic properties play an important role in the bA_1 receptor–ligand interaction. In fact, since these two substituents have comparable steric hindrance, the higher A_1 AR affinity of **6** can be explained by assuming that the negative steric effect of the 6-nitro group is compensated by its electronic properties.

The 4-amino-1-one derivatives **12**–**21** (series **B**) display, on the whole, nanomolar affinity for all three AR subtypes. Moreover, we should point out the significantly higher A_3 AR affinities of compounds **12**, **16**–**21** with respect to those of the corresponding 1,4-dione derivatives **1**, **5**–**10** (series **A**), since this is a new finding with respect to our previous results[21] which showed that the 1,4-dione derivatives were, on the whole, more active at the A_3 AR subtype than the corresponding 4-amino-1-one derivatives.

The SAR of the 4-amino-1-one derivatives **12–21** are generally different from those of the 1,4-diones (series **A**) and only a few similarities have been found.

The presence of substituent(s) on the benzofused moiety of **12–21** affects the A_1 affinity differently while it generally reduces the A_{2A} AR affinity, with respect to those of the lead compound **1B**. In particular, the 8-chloro-6-nitro derivative **17** is the most potent bA_1 AR antagonist ($K_i = 0.2$ nM) among the herein reported compounds.

In contrast, the A_3 AR affinities of compounds **12–21** are significantly enhanced, thus indicating that in series **B** the presence of substituents on the benzofused moiety is advantageous for anchoring to the hA_3 AR, in particular the 6-nitro group (compound **18**) confers the highest A_3 AR affinity ($K_i = 4.75$ nM).

Introduction of a chloro substituent either at the 7- or at the 8-position of **1B** (compounds **13** and **14**, respectively) does not elicit the beneficial effect on A_1 and A_{2A} AR affinity, which was observed in other series of tricyclic antagonists of similar size and shape.[17,22–24] The same applies to the 7,8- or 6,8-dichloro derivatives **12** and **15**, respectively.

On the contrary, the presence of a 7- or 8-chloro substituent is favorable for the binding to the A_3 AR subtype. In fact, compounds **13** and **14** show similar nanomolar A_3 affinity, which is significantly higher than that of **1B**. These results indicate that the lipophilic pocket of the A_3 AR subtype, which accommodates the benzofused moiety (region 2) of the 4-amino-triazoloquinoxalines **12–21**, well tolerates a small hydrophobic substituent, such as a chlorine atom, at the 7- or 8-position of the triazoloquinoxaline framework.

The importance of lipophilic requirements for anchoring to the A_3 AR recognition site is confirmed by the binding data of the 7,8-dichloro derivative **12** which is 5-fold more active than the parent compound **1B**. Nevertheless, compound **12** is less active at this subtype than the mono-chloro derivatives **13** and **14**. These results indicate that not only lipophilic factors, but also the steric ones are important for the correct anchoring of the 4-amino-1-one derivatives to the A_3 receptor recognition site, in accordance with recent literature data regarding tricyclic AR antagonists of similar size and shape.[31] Moreover, the important role of the steric factors is confirmed by the A_3 binding data of the 6,8-dichloro derivative **15** which exhibits the lowest A_3 AR affinity within the 4-amino derivatives **12–21**.

Introduction of a nitro or amino group at the 8-position of **1B** affords compounds **16** and **19,** respectively, which both display similar A_1 affinity to that of **1B**. The high A_1 affinity ($K_i = 7.33$ nM) of the 8-amino derivative **19** should be highlighted. This result indicates that the bA_1 AR lipophilic pocket that accomodates the benzofused moiety of the 4-amino-1-one derivatives (series **B**) well tolerates not only hydrophobic substituents, such as the 7- or 8-chloro (compounds **13** and **14**, respectively) and the 8-nitro (compound **16**), but also hydrophilic groups, such as the 8-amino substituent (compound **19**).

The presence of an 8-nitro or 8-amino group is favorable also for A_3 AR affinity, being **16** and **19**, respectively, 12- and 14-fold more active than **1B** at this AR subtype.

The beneficial effect of either a lipophilic (chloro or nitro) or a hydrophilic substituent (amino) at the 7- or 8-position on the 4-amino-triazoloquinoxalin-1-ones could be rationalized on the basis of a recent rhodopsin-based model of human A_3 AR receptor,[32] which has been docked with the 9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazolin-5-amine (CGS 15943)[33] as reference ligand. This model hypothesizes that the benzofused moiety of CGS 15943 resides in a hydrophobic pocket delimited by apolar amino acids, such as Leu90 (TM3), Phe182 (TM5) and Ile185 (TM5), which are considered important for the binding of antagonists. Also in this pocket polar amino acids, such as Thr94 (TM3) and Ser97 (TM3) are present, but are instead considered not important for the binding of antagonists. Due to their similar size and shape, it is possible to hypothesize a similar binding mode of our 4-amino-1-one derivatives (series **B**) and of CGS 15943. Thus, the significantly increased affinity of the 7- or of the 8-chloro derivatives **13** and **14**, with respect to that of **1B**, could be explained by the improvement of the hydrophobic interaction of the benzofused moiety of compounds **13** and **14** with the three above cited apolar amino acids. On the other hand, it is also possible to rationalize the increased A_3 affinity of both the 8-nitro derivative **16** and the 8-amino **19** with respect to **1B**, by hypothesizing a hydrogen bond interaction between Thr94 or Ser97 and the 8-nitro or the 8-amino group. Nevertheless, comparison of the A_3 binding data of the 8-chloro derivative **14** ($K_i = 11$ nM) with those of **16** and **19**, could confirm the major role that the hydrophobic interactions play in the anchoring of antagonists to this receptor pocket.

The human A_3 receptor model, cited above, made it possible to explain also the A_3 binding data of the 6-nitro derivative **18**. In fact, in this model, a hydrogen bond interaction between the N-6 atom of CGS 15943, corresponding to the N-5 of our triazoloquinoxalin-4-amines, and Ser247 (TM6) has been hypothesized. Moreover, the 5-NH_2 group of CGS 15943 also seems to be involved in a hydrogen bond, being surrounded by three polar amino acids: Ser242 (TM6), Ser271 (TM7) and Ser275 (TM7). Thus, although the 6-nitro group of **18** reduces the nucleophilicity and, consequently, 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 **18**, due to its electron-withdrawing properties, increases acidity of the 4-amino protons, thus enhancing the strength of interaction of this group with the receptor proton acceptor site. Similarly to the 6-nitro group of **18**, also the 8-chloro substituent of **14** could affect the N-5 nucleophilicity and the 4-amino proton acidity, even if in minor extent, due to its lower electron-withdrawing properties. The advantageous

effect of the 8-chloro substituent (compound **14**) and that of the 6-nitro group (compound **18**) for A_3 AR affinity was the rationale for the synthesis of compound **17** which, bearing both these substituents on the benzofused moiety, was expected to be more active than compounds **14** and **18**. Instead, the 8-chloro-6-nitro derivative **17** is a potent bA_1 AR antagonist. While it is difficult to rationalize the unexpected high A_1 AR affinity of **17**, its lower A_3 AR affinity, compared to those of **14** and **18**, could be attributed to the steric hindrance of the two substituents, confirming the above discussed importance of the steric factors for the A_3 receptor–ligand interaction.

It has to be noted that compound **18** possesses not only the highest A_3 AR affinity but also the highest hA_3 versus bA_1 selectivity $(A_1/A_3) = 17$, among the herein reported compounds. Thus, in order to rigorously establish the true selectivity of this compound we tested it on human A_1 (hA_1) ARs. The hA_1 binding result, reported in Table 2, indicates that **18** is about 10-fold less active at hA_1 than at bA_1 AR and consequently it possesses a 183-fold hA_1 versus hA_3 selectivity.

Table 2. Binding activity at human A_1 ARs

Compd	K_i (nM)[a,b]
18	870 ± 22
Theophylline	3.2 ± 0.2
DPCPX	6200 ± 530

[a]The K_i values are means ± SEM of four separate assays, each performed in triplicate.
[b]Displacement of specific [^{3}H]CHA binding at hA_1 receptors expressed in CHO cells.

In conclusion, the synthesis of the herein reported triazoloquinoxalin-1-one derivatives has allowed us to further investigate the SAR of the two series, **A** and **B**. The results of this study show that the presence of substituent(s) on the benzofused moiety (region 2) of the 1,4-dione derivatives **1**, **4**–**11** (series **A**) lowers the hA_3 affinity and, generally, it is also unprofitable for the bA_1 receptor binding. Nevertheless, the 8-amino substituent has been found to confer high bA_1 affinity (derivative **8**).

Introduction of substituent(s) on the benzofused moiety of the 4-amino-1-one derivatives **12**–**21** (series **B**) is advantageous for A_1 and for A_3 AR affinity to a greater extent than in series **A**. In particular, the 8-chloro-6-nitro substitution (compound **17**) achieves the highest bA_1 AR affinity while the 6-nitro group (compound **18**) produces not only the highest hA_3 affinity but also high hA_3 versus hA_1 selectivity. Taking this new and promising finding into account, together with our previous ones,[21] further modification of the 1,2,4-triazolo[4,3-*a*]quinoxalin-1-one derivatives are in progress to improve both the A_3 receptor affinity and hA_3 versus hA_1 selectivity.

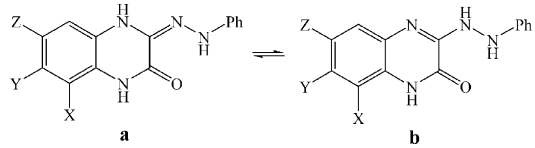
Experimental

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 (Table 3). 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 1,2,3,4-tetrahydro-3-phenylhydrazono-quinoxalin-2-ones 28–34, 35[19]

Compounds **28**–**34** were synthesized following the method described to prepare compound **35**.[19] Briefly, ethyl N^1-phenylhydrazono-N^2-chloroacetate (9 mmol), suitable *ortho*-phenylendiamines **22**, **23**, **24**,[25] **25**, **26**,[26] **27** (9 mmol) and triethylamine (10.8 mmol) were reacted in refluxing ethanol (80 mL) for 3 h. The suspension was cooled and the solid collected and washed with water (30–40 mL). The crude solid was made up of compounds **28**, **31**, **33**–**35** and of the mixture of regioisomers **29**, **30**. Compounds **29**, **30** (overall yield 95%), due to their instability on silica gel column, were used as inseparable mixture for the next step. Compound **32**, which is the regioisomer of **31**, was obtained as follows: the mother liquor of **31** was reduced to dryness by evaporation of the solvent at reduced pressure. The residue was treated with acetone (50 mL) and the solid filtered off. Evaporation of the solvent gave a residue which was chromatographed on a silica gel column, eluting system chloroform/ethyl acetate 9:1. From the central eluates compound **32** was obtained. The intermediates **28**, **31**, **33**, **34**, as the previously described **35**,[19] may exist in either one of the two tautomeric forms **a** and **b**. In fact, their ^{1}H NMR spectra revealed the existence of both tautomers **a** and **b** because there are more than three signals relative to protons that exchange with D_2O. On the contrary, the ^{1}H NMR spectrum of compound **32** showed the existence of only one tautomer.



6,7-Dichloro-1,2,3,4-tetrahydro-3-phenylhydrazono-quinoxalin-2-one (28). Yield: 60%; mp 273–275 °C dec (AcOH). ^{1}H NMR 6.64–7.49 (m, ar), 7.86 (br s, NH), 8.77 (br s, NH), 9.72 (br s, NH), 11.25 (br s, NH), 12.40 (br s, NH). Anal. ($C_{14}H_{10}Cl_2N_4O$) C, H, N.

6,8-Dichloro-1,2,3,4-tetrahydro-3-phenylhydrazono-quinoxalin-2-one (31). Yield: 45%; mp 248–249 °C dec (AcOH). ^{1}H NMR 6.73–7.42 (m, ar), 7.92 (br s, NH), 8.85 (br s, NH), 9.80 (br s, NH), 10.65 (br s, NH), 12.00 (br s, NH). Anal. ($C_{14}H_{10}Cl_2N_4O$) C, H, N.

5,7-Dichloro-1,2,3,4-tetrahydro-3-phenylhydrazono-quinoxalin-2-one (32). Yield: 2%; mp 236–238 °C dec (EtOAc). ^{1}H NMR 6.75–6.90 (m, 3H, ar), 7.10–7.30 (m, 4H, ar), 7.74 (br s, 1H, NH), 9.87 (br s, 1H, NH), 12.50 (br s, 1H, NH). Anal. ($C_{14}H_{10}Cl_2N_4O$) C, H, N.

1,2,3,4-Tetrahydro-6-nitro-3-phenylhydrazono-quinoxalin-2-one (33). Yield: 55%; mp 190–192 °C dec (AcOH). ^{1}H NMR 6.62–6.85 (m, ar), 7.05–7.38 (m, ar), 7.70–8.05 (m, ar + NH), 8.82 (br s, NH), 9.86 (br s, NH), 10.09 (br s, NH), 11.62 (br s, NH), 12.78 (br s, NH). Anal. ($C_{14}H_{11}N_5O_3$) C, H, N.

6-Chloro-1,2,3,4-tetrahydro-8-nitro-3-phenylhydrazono-quinoxalin-2-one (34). Yield: 50%; mp 172–173 °C (AcOH).^{1}H NMR 6.65–7.37 (m, ar), 7.62–7.70 (m, ar), 7.92–8.04 (m, ar + NH), 9.07 (br s, NH), 10.11 (br s, NH), 10.46 (br s, NH), 11.60 (br s, NH). Anal. ($C_{14}H_{10}ClN_5O_3$) C, H, N.

1,2,3,4-Tetrahydro-8-nitro-3-phenylhydrazono-quinoxalin-2-one (35). See ref 19.

General procedure for the synthesis of 1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-4-ones 36–38

A mixture of **31** or **33** or **34** (0.9 mmol) and aqueous formaldehyde (40%, 0.4 mL) in ethylene glycol (3 mL) was heated at reflux for 2–3 min. Dilution with water (10 mL) yielded a yellow solid which was collected and washed with water.

6,8-Dichloro-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-4-one (36). Yield: 85%; mp 289–291 °C dec (ethylene glycol). ^{1}H NMR 5.70 (s, 2H, CH_2), 6.84–6.97 (m, 4H, 3ar + H-9), 7.19 (s, 1H, H-7), 7.29–7.37 (m, 2H, ar), 11.04 (br s, 1H, NH). Anal. ($C_{15}H_{10}Cl_2N_4O$) C, H, N.

1,2,4,5-Tetrahydro-8-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-4-one (37). Yield: 80%; mp 294–296 °C dec (ethylene glycol). ^{1}H NMR 5.81 (s, 2H, CH_2), 6.91 (t, 1H, ar, J = 7.6 Hz), 7.04 (dd, 2H, J = 8.4, 1.5 Hz), 7.16 (dd, 1H, H-6, J = 8.7, 1.8 Hz), 7.35 (t, 2H, ar, J = 7.6 Hz), 7.64 (d, 1H, H-9, J = 1.8 Hz), 7.90 (dd, 1H, H-7, J = 8.7, 1.8 Hz), 11.98 (br s, 1H, NH). Anal. ($C_{15}H_{11}N_5O_3$) C, H, N.

8-Chloro-1,2,4,5-tetrahydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-4-one (38). Yield: 97%; mp 244–246 °C dec (ethylene glycol). ^{1}H NMR 5.80 (s, 2H, CH_2), 6.60–6.99 (m, 3H, ar), 7.30 (d, 1H, H-9, J = 2.4 Hz), 7.39 (t, 2H, ar, J = 7.6 Hz), 7.77 (d, 1H, H-7, J = 2.4 Hz), 10.56 (br s, 1H, NH). Anal.($C_{15}H_{10}ClN_5O_3$) C, H, N.

General procedure for the synthesis of 1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-diones 1–6, 7[19]

Compounds **1–6** were synthesized following the reported procedure to prepare **7** from **35**.[19] Briefly, compounds **28**, **31**, **33**, **34** and the mixture of regioisomers **29**, **30** (4 mmol) were reacted with triphosgene (4 mmol) in refluxing anhydrous tetrahydrofuran (40 mL) for 2–3 h. The suspension was diluted with water (40 mL) and the solid collected by filtration. The mixture of isomers **2** and **3** (overall yield 94%) was chromatographed on silica gel column, eluting system cyclohexane/ethyl acetate 6:4. Evaporation of the solvent of the first and the last eluates gave few milligrams of pure **2** and **3**, respectively. From the central eluates the inseparable mixture of **2** and **3** was obtained which was used for the next step.

7,8-Dichloro-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (1). Yield: 98%; mp > 300 °C (AcOH). ^{1}H NMR 7.35–7.46 (m, 2H, ar), 7.57 (t, 2H, ar, J = 8.1 Hz), 8.01 (d, 2H, ar J = 8.1 Hz), 8.73 (s, 1H, H-9), 12.05 (br s, 1H, NH); IR 3200, 1740, 1700. Anal. ($C_{15}H_8Cl_2N_4O_2$) C, H, N.

7-Chloro-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (2). Mp > 300 °C (AcOH). ^{1}H NMR 7.24–7.43 (m, 3H, ar), 7.57 (t, 2H, ar, J = 8.2 Hz), 7.03 (d, 2H, ar, J = 7.5 Hz), 8.61 (d, 1H, H-9, J = 8.4 Hz), 12.09 (br s, 1H, NH). Anal.($C_{15}H_9ClN_4O_2$) C, H, N.

8-Chloro-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (3). Mp > 300 °C (AcOH). ^{1}H NMR 7.28–7.63 (m, 5H, ar), 8.01 (d, 2H, ar, J = 7.8 Hz), 8.60 (d, 1H, H-9, J = 2.3 Hz), 12.11 (br s, 1H, NH). Anal. ($C_{15}H_9ClN_4O_2$) C, H, N.

6,8-Dichloro-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (4). Yield: 90%; mp 296–298 °C (AcOH). ^{1}H NMR 7.40 (t, 1H, ar, J = 7.7 Hz), 7.58 (t, 2H, ar, J = 7.7 Hz), 7.75 (d, 1H, H-7, J = 2.3 Hz), 7.99 (d, 2H, ar, J = 7.7 Hz), 8.63 (d, 1H, H-9, J = 2.3 Hz), 11.61 (br s, 1H, NH). Anal. ($C_{15}H_8Cl_2N_4O_2$) C, H, N.

1,2,4,5-Tetrahydro-8-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (5). Yield: 93%; mp 277–278 °C (EtOAc). ^{1}H NMR 7.35–7.52 (m, 2H, ar), 7.60 (t, 2H, J = 7.6 Hz), 8.02 (d, 2H, J = 8.1 Hz), 8.29 (dd, 1H, H-7, J = 8.9, 2.6 Hz), 9.40 (d, 1H, H-9, J = 2.6 Hz). Anal. ($C_{15}H_9N_5O_4$) C, H, N.

8-Chloro-1,2,4,5-tetrahydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (6). Yield: 98%; mp > 300 °C (MeOH/EtOAc). ^{1}H NMR 7.42 (t, 1H, ar, J = 7.2 Hz), 7.61 (t, 2H, ar, J = 7.2 Hz), 8.01 (d, 2H, ar, J = 8.3 Hz), 8.25 (d, 1H, H-7, J = 2.4 Hz), 8.98 (d, 1H, H-9, J = 2.4 Hz), 11.46 (br s, 1H, NH). IR 3330, 3100, 1740, 1710. Anal. ($C_{15}H_8ClN_5O_4$) C, H, N.

1,2,4,5-Tetrahydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (7). See ref 19.

General procedure for the synthesis of 8-amino-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (8) and 6-amino-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (10)[19]

Pd/C 10% (0.05 g) was added to a hot solution of **5** or **7**[19] (0.8 mmol) in tetrahydrofuran (200 mL). 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.

8. Yield: 98%; mp >300 °C (EtOH/AcOH). ^{1}H NMR 5.37 (br s, 2H, NH_2), 6.55 (dd, 1H, H-7, J = 2.1, 8.4 Hz), 6.96 (d, 1H, H-6, J = 8.4 Hz), 7.36 (t, 1H, ar, J = 7.3 Hz), 7.54 (t, 2H, ar, J = 7.7 Hz), 7.91 (d, 1H, H-9, J = 2.2 Hz), 7.98 (d, 2H, ar, J = 8.1 Hz), 11.6 (br s, 1H, NH). Anal. ($C_{15}H_{11}N_5O_2$) C, H, N.

10. See ref 19.

Synthesis of 6-amino-8-chloro-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione (9). A suspension of compound **6** (0.6 mmol) and tin(II) chloride dihydrate (2.8 mmol) in ethanol (10 mL) and dimethylsulfoxide (0.3 mL) was refluxed for 90 min and then cooled at room temperature. The solid was filtered, resuspended in water (100 mL) and stirred for 15 min at room temperature. The resulting solid was collected by filtration and washed with water (20–30 mL). Yield: 45%; mp >300 °C (AcOH).^{1}H NMR 5.93 (br s, 2H, NH_2), 6.75 (d, 1H, H-7, J = 2.3 Hz), 7.39 (t, 1H, ar, J = 7.4 Hz), 7.57 (t, 2H, ar, J = 7.3 Hz), 7.94–8.02 (m, 3H, ar). Anal. ($C_{15}H_{10}ClN_5O_2$) C, H, N.

Synthesis of 6-benzylamino-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*] quinoxalin-1,4-dione (11).[19] A mixture of **10**[19] (3.4 mmol), benzaldehyde (4.1 mmol) and anhydrous zinc chloride (1.7 mmol) in anhydrous tetrahydrofuran (50 mL) was refluxed under nitrogen atmosphere for 5 h. The solid, made up of the corresponding Schiff base, that is the 4-amino-6-(*N*-benzyliden)amino-1,2,4,5-tetrahydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1,4-dione,[19] was filtered off, washed with water and dried. Sodium borohydride (4.2 mmol) was added portionwise in 30 min to a suspension of the Schiff base (2.1 mmol), in refluxing anhydrous methanol (40 mL). The suspension was refluxed for 3 h, then it was cooled at room temperature and diluted with water (20 mL). The solid was filtered off and washed with water. Yield: 78%; mp 283–285 °C (AcOH). ^{1}H NMR 4.43 (d, 2H, CH_2, J = 4.4 Hz), 6.40 (t, 1H, NH, J = 4.4 Hz), 6.63 (d, 1H, ar, J = 8.3 Hz), 7.08 (t, 1H, ar, J = 8.1 Hz), 7.27–7.62 (m, 7H, ar), 8.00–8.10 (m, 3H, ar), 11.21 (br s, 1H, NH).

General procedure for the synthesis of 4-chloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-ones 39–45

Compounds **1**, **4–6**, **7**[19] and the mixture of **2** and **3** (2 mmol) were reacted with phosphorus pentachloride (4 mmol) in refluxing phosphorus oxychloride (40 mL) and anhydrous pyridine (0.2 mL) until the disappearance (TLC monitoring) of the starting material (12–24 h). Evaporation at reduced pressure of the excess phosphorus oxychloride afforded a solid which was treated with iced water (50 mL), collected and washed with cyclohexane. The 4-chloro derivatives **39–45**, obtained in high overall yields (85–90%), were instable, nevertheless they were pure enough to be used without further purification. The isomers **40** and **41** were used as a mixture for the next step.

4,7,8-Trichloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (39). ^{1}H NMR 7.48 (t, 1H, ar, J = 7.4 Hz), 7.62 (t, 2H, ar, J = 7.6 Hz), 8.04 (d, 2H, ar, J = 7.6 Hz), 8.29 (s, 1H, H-6), 8.85 (s, 1H, H-9).

4,6,8-Trichloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (42). ^{1}H NMR 7.46 (t, 1H, ar, J = 7.3 Hz), 7.62 (t, 2H, ar, J = 7.3 Hz), 7.98–8.08 (m, 3H, 2ar + H-7), 8.71 (d, 1H, H-9, J = 1.6 Hz).

4-Chloro-1,2-dihydro-8-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (43). ^{1}H NMR 7.45 (t, 1H, ar, J = 7.4 Hz), 7.63 (t, 2H, ar, J = 7.4 Hz), 7.98–8.18 (m, 3H, ar), 8.42 (dd, 1H, H-7, J = 8.1, 1.4 Hz), 9.46 (d, 1H, H-9, J = 1.4 Hz).

4,8-Dichloro-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (44). ^{1}H NMR 7.46 (t, 1H, ar, J = 7.4 Hz), 7.65 (t, 2H, ar, J = 7.4 Hz), 8.05 (d, 2H, ar, J = 7.4 Hz), 8.42 (d, 1H, H-7, J = 1.7 Hz), 8.88 (d, 1H, H-9, J = 1.7 Hz).

4-Chloro-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (45). ^{1}H NMR 7.42 (t, 1H, ar, J = 7.3 Hz), 7.60 (t, 2H, ar, J = 7.3 Hz), 7.85–8.16 (m, 4H, ar), 8.92 (d, 1H, H-9, J = 7.0 Hz).

General procedure for the synthesis of 4-amino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-ones 12–17, 18[19]

A suspension of **39**, **42–44** and of the mixture of isomers **40** and **41** (2 mmol) in absolute ethanol (30 mL) saturated with ammonia was heated overnight at 120 °C in a sealed tube. After cooling, the solid was collected and washed with water. The two isomers **13** and **14** were separated by column chromatography, eluting system cyclohexane/ethyl acetate 1:1. Evaporation of the solvent of the first and the second eluates gave compounds **13** and **14**, respectively.

4-Amino-7,8-dichloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (12). Yield: 90%; mp 257–258 °C (AcOH).^{1}H NMR 7.38 (t, 1H, ar, J = 7.5 Hz), 7.55–7.63 (m, 3H, 2ar + H-6), 7.88 (br s, 2H, NH_2), 8.04 (d, 2H, ar, J = 8.4 Hz), 8.72 (s, 1H, H-9). Anal. ($C_{15}H_9Cl_2N_5O$) C, H, N.

4-Amino-7-chloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (13). Yield: 40%; mp >300 °C (AcOH). ^{1}H NMR 7.24–7.40 (m, 3H, ar), 7.56 (t, 2H, ar, J = 7.8 Hz), 7.76 (br s, 2H, NH_2), 8.05 (d, 2H, ar, J = 8.1 Hz), 8.59 (d, 1H, H-9, J = 8.4 Hz); IR 3480, 3310, 3260–3020, 1740. Anal. ($C_{15}H_{10}ClN_5O$) C, H, N.

4-Amino-8-chloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (14). Yield: 40%; mp 275–277 °C (AcOH). ^{1}H NMR 7.36–7.41 (m, 3H, ar), 7.53–7.66 (m, 4H, 2ar + NH_2), 8.04 (d, 2H, ar, J = 8.4 Hz), 8.59 (d, 1H, H-9, J = 2.1 Hz). Anal. ($C_{15}H_{10}ClN_5O$) C, H, N.

4-Amino-6,8-dichloro-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (15). Yield: 70%; mp >300 °C (AcOH).^{1}H NMR 7.42 (t, 1H, ar, J = 7.32 Hz), 7.55–7.65 (m, 3H, 2ar + H-7), 7.98–8.07 (m, 4H, 2ar + NH_2), 8.61 (d, 1H, H-9, J = 2.4 Hz). Anal. ($C_{15}H_9Cl_2N_5O$) C, H, N.

4-Amino-1,2-dihydro-8-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (16). Yield: 78%; mp >300 °C (AcOH). ^{1}H NMR 7.35–7.62 (m, 4H, ar), 8.05 (d, 2H, ar, J = 7.8 Hz), 8.15–8.42 (m, 3H, 1ar + NH_2), 9.36 (d, 1H, H-9, J = 2.5 Hz). Anal. ($C_{15}H_{10}N_6O_3$) C, H, N.

4-Amino-8-chloro-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (17). Yield: 85%; mp >300 °C (AcOH). ^{1}H NMR 7.43 (t, 1H, ar, J = 7.5 Hz), 7.60 (t, 2H, ar, J = 7.5 Hz), 8.03–8.08 (m, 3H, ar), 8.15–8.40 (br s, 2H, NH_2), 8.73 (d, 1H, H-9, J = 2.3 Hz); IR 3480, 3340, 3280–3080, 1730, 1460, 1360. Anal. ($C_{15}H_9ClN_6O_3$) C, H, N.

4-Amino-1,2-dihydro-6-nitro-2-phenyl-1,2,4-triazolo[4,3-*a*]quinoxalin-1-one (18). See ref 19.

General procedure for the synthesis of 4,8-diamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-a]quinoxalin-1-one (19), 4,6-diamino-8-chloro-1,2-dihydro-2-phenyl-1,2,4-triazolo [4,3-*a*]quinoxalin-1-one (20) and 4,6-diamino-1,2-dihydro-2-phenyl-1,2,4-triazolo[4,3-*a*] quinoxalin-1-one (21)[19]

Compounds **19** and **21**[19] were prepared by hydrogenating **16** and **18**[19] (0.8 mmol), dissolved in hot glacial acetic acid (200 mL), following the experimental conditions described above to obtain compounds **8** and **10**. Similarly, compound **20** was prepared from **17** (0.8 mmol) which was dissolved in hot tetrahydrofuran (200 mL) and hydrogenated for 3 days.

19. Yield: 94%; mp >300 °C (EtOAc/AcOH).^{1}H NMR 5.40 (br s, 2H, NH_2 at the 8-position), 6.64 (dd, 1H, ar, J = 2.4, 8.6 Hz), 6.83 (s, 2H, NH_2 at 4-position), 7.16 (d, 1H, ar, J = 8.6 Hz), 7.36 (t, 1H, ar, J = 7.3 Hz), 7.57 (t, 2H, ar, J = 7.7 Hz), 7.95 (d, 1H, H-9, J = 2.4 Hz), 8.08 (d, 2H, ar, J = 7.7 Hz). Anal. ($C_{15}H_{12}N_6O$) C, H, N.

20. Yield: 92%; mp 237–238 °C (MeOH).^{1}H NMR 5.61 (br s, 2H, NH_2 at the 6-position), 6.69 (d, 1H, H-7, J = 2.5 Hz), 7.30–7.37 (m, 3H, 1ar + NH_2 at the 4-position), 7.56 (t, 2H, ar, J = 7.7 Hz), 7.85 (d, 1H, H-9, J = 1.8 Hz), 8.04 (d, 2H, ar, J = 8.4 Hz). Anal. ($C_{15}H_{11}ClN_6O$) C, H, N.

21. See ref 19.

Biochemistry

Bovine A_1 and A_{2A} receptor binding. Displacement of [^{3}H]CHA from A_1 ARs in bovine cerebral cortical membranes and [^{3}H]CGS 21680 from A_{2A} ARs in bovine striatal membranes was performed as described in ref 34.

Human A_1 and A_3 receptor binding. Binding experiments at hA_1 and hA_3 adenosine receptors were performed on crude membranes obtained from CHO cells.[35]

A_1 adenosine receptor binding assay was performed using [^{3}H]CHA as radioligand. The assay medium consisted of a buffer containing 50 mM Tris/HCl at pH 7.4. The glass incubation tubes, containing 20 μg of membrane proteins, 0.2 U/mL adenosine deaminase, 1 nM [^{3}H]CHA and 10 μL of the tested ligand, were incubated for 2 h at 25 °C. After incubation, samples were filtered under vacuum on Whatman GF/C filters and washed three times with 5 mL of ice-cold assay buffer. Nonspecific binding was determined in the presence of 50 μM NECA. Specific binding, obtained by subtracting nonspecific binding from total binding, corresponded to 90% of the total binding. Compounds were dissolved in DMSO (buffer/concentration of 2%) and added to the assay mixture. Blank experiments were carried out to determine the effect of solvent on [^{3}H]CHA binding.

Displacement of [^{125}I]AB-MECA from hA_3 ARs stably expressed in CHO cells was performed as previously described.[20]

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, USA) with five concentrations of displacer, each performed in triplicate. Inhibition constants (K_i) were calculated according to the Cheng–Prusoff equation.[36] The dissociation constant (K_d) of [^{3}H]CHA and [^{3}H]CGS 21680 in cortical and striatal bovine brain membranes were 1.2 and 14 nM, respectively. The K_d values of [^{3}H]CHA and [^{125}I]AB-MECA in hA_1 and hA_3 ARs in CHO cell membranes were 1.9 and 1.4 nM, respectively.

Table 3. Analytical data of the newly synthesized compounds

Compd	Formula	C calcd–found	H calcd–found	N calcd–found
1	$C_{15}H_8Cl_2N_4O_2$	51.89–51.71	2.33–2.32	16.14–16.10
2	$C_{15}H_9ClN_4O_2$	57.61–57.80	2.91–2.90	17.92–17.87
3	$C_{15}H_9ClN_4O_2$	57.61–57.45	2.91–2.92	17.92–17.96
4	$C_{15}H_8Cl_2N_4O_2$	51.89–51.75	2.33–2.34	16.14–16.18
5	$C_{15}H_9N_5O_4$	55.72–55.88	2.81–2.80	21.67–22.75
6	$C_{15}H_8ClN_5O_4$	50.36–50.18	2.26–2.27	19.58–19.50
8	$C_{15}H_{11}N_5O_2$	61.42–61.61	3.79–3.80	23.88–23.98
9	$C_{15}H_{10}ClN_5O_2$	54.97–55.09	3.08–3.07	21.37–21.29
12	$C_{15}H_9Cl_2N_5O$	52.04–52.27	2.63–2.64	20.23–20.29
13	$C_{15}H_{10}ClN_5O$	57.79–57.61	3.24–3.25	22.47–22.55
14	$C_{15}H_{10}ClN_5O$	57.79–57.95	3.24–3.23	22.47–22.56
15	$C_{15}H_9Cl_2N_5O$	52.04–52.21	2.63–2.61	20.63–20.56
16	$C_{15}H_{10}N_6O_3$	55.89–55.70	3.13–3.14	26.08–26.16
17	$C_{15}H_9ClN_6O_3$	50.50–50.34	2.55–2.53	23.56–23.65
19	$C_{15}H_{12}N_6O$	61.63–61.39	4.15–4.16	28.76–28.67
20	$C_{15}H_{11}ClN_6O$	55.13–55.31	3.40–3.39	25.72–25.62
28	$C_{14}H_{10}Cl_2N_4O$	52.35–52.59	3.14–3.28	17.45–17.59
31	$C_{14}H_{10}Cl_2N_4O$	52.35–52.16	3.14–3.15	17.45–17.39
32	$C_{14}H_{10}Cl_2N_4O$	52.35–52.47	3.14–3.25	17.45–17.58
33	$C_{14}H_{11}N_5O_3$	56.56–56.74	3.74–3.75	23.56–23.64
34	$C_{14}H_{10}ClN_5O_3$	50.68–50.48	3.04–3.03	21.12–21.20
36	$C_{15}H_{10}Cl_2N_4O$	54.07–54.35	3.03–3.11	16.82–16.65
37	$C_{15}H_{11}N_5O_3$	58.24–58.02	3.59–3.60	22.65–22.72
38	$C_{15}H_{10}ClN_5O_3$	52.41–52.28	2.94–2.93	20.38–20.30

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