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2-Aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amines as highly potent A_1 and A_3 adenosine receptor antagonists

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Abstract—Some 2-aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxaline derivatives **2**–**18**, obtained by introducing different substituents on either the 4-amino moiety (acyl or carbamoyl groups) or the 2-phenyl ring (4-OCH_3) of previously reported 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amine (**1**), have been synthesized and tested in radioligand binding assays at bovine A_1 and A_{2A} and at cloned human A_1 and A_3 adenosine receptors. The rationally designed 8-chloro-2-(4-methoxy-phenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-acetylamine (**14**) can be considered one of the most potent and hA_3 versus hA_1 selective AR antagonists reported till now. The structure–activity relationships of compounds **2**–**18** are in agreement with those of previously reported 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalines (series **A**) and 2-arylpyrazolo[3,4-*c*]quinolines (series **B**), thus suggesting a similar AR binding mode. In fact, the importance for the A_3 receptor–ligand interaction of both a strong acidic NH proton donor and a C=O proton acceptor at position-4, able to engage hydrogen-bonding interactions with specific sites on the A_3 AR, has been confirmed. Using our recently published hA_3 receptor model, to better elucidate our experimental results, we decided to theoretically depict the putative TM binding motif of the herein reported 1,2,4-triazolo[1,5-*a*]quinoxaline derivatives on human A_3 receptor. Structure–activity relationships have been explained analyzing the three-dimensional structure of the antagonist-receptor models obtained by molecular docking simulation.

1. Introduction

Adenosine, an important regulator of homeostasis in the brain, heart, kidney, and other organs, interacts with at least four cell surface receptor subtypes classified as A_1, A_{2A}, A_{2B}, and A_3. These adenosine receptors (ARs) belong to the superfamily of seven transmembrane G-protein-coupled receptors: A_1 and A_3 subtypes inhibit adenylate cyclase (AC) via G_i protein, whereas A_{2A} and A_{2B} activate AC via G_s protein.[1,2] In addition, coupling with other messenger systems, such as calcium and potassium channels (A_1 receptor) or phospholipase C (A_1, A_{2B}, and A_3 receptors) and D (A_3 receptor), has been described.[3–6] The A_1 and A_{2A} receptors are high affinity receptors, while A_{2B} and A_3 are low affinity ARs.[7,8]

ARs from different species show high amino acid sequence homology (82–92%) with the exception of the A_3 receptor, which exhibits significant differences in primary sequence homology (74%) between rat and human, or sheep.[9–11]

While A_1 and A_{2A} receptors have been pharmacologically characterized through the use of selective ligands,[12–15] A_{2B} and A_3 subtypes are still under study in order to better understand their pathophysiological roles. ARs are distributed in different cell types of

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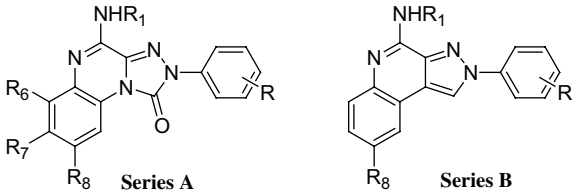
mammals and, for this reason, are involved in a great variety of physiological processes. Accordingly, in the last two decades, many efforts have been invested in the research of potent and selective AR ligands for their potential therapeutic uses.[2,12,14–16] In particular, while A_1 AR antagonists have been developed as antihypertensives and potassium-saving diuretics,[14] cognitive enhancers[2,14,15] for geriatric therapy and for the treatment of CNS disorders such as Alzheimer's disease[17] and schizophrenia,[18] A_3 AR antagonists are sought as potential anti-inflammatory and antiasthmatic agents.[16,19] Other applications of both A_1 and A_3 AR antagonists such as prevention and treatment of cerebral and cardiac ischaemia-induced injuries, have been proposed.[16,20–22]

Since most of the AR antagonists are nitrogen-containing heterocyclic compounds,[2,12–16,23,24] research in our laboratory has been directed toward the synthesis of tricyclic heteroaromatic systems as AR antagonists.[25–34]

In recent papers we reported the synthesis and binding activity at bovine A_1 (bA_1) and A_{2A} (bA_{2A}) and at human cloned A_3 (hA_3) ARs of two different classes, that is, 2-aryl-1,2,4-triazolo[4,3-*a*]quinoxalines (series **A**)[30,32–34] and 2-arylpyrazolo[3,4-*c*]quinolines (series **B**)[31] substituted at position-4 with an amino group (Chart 1). Some of these **A** and **B** compounds were potent and selective A_1 or A_3 antagonists. Structure–activity relationship (SAR) studies of these derivatives showed that in both series the nature of the substituent on the 4-amino group was important to modulate the A_1 and A_3 receptor affinity. In particular, while replacement of a hydrogen atom of the 4-amino group with cycloalkyl or aryl(alkyl) groups, in general, enhances A_1 binding affinity,[30,31,33] the presence of an acyl or a carbamoyl substituent at the same position afforded potent A_3 receptor antagonists.[30,31] Moreover, it emerged that introduction of a 4-methoxy group on the 2-phenyl ring can be used to increase A_3 potency.[30,31]

The present paper reports the synthesis and A_1, A_{2A}, and A_3 AR binding affinities of some 2-aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amines **2**–**12**, **14**–**18** bearing different substituents on the 4-amino moiety (Chart 2). In particular, the previously reported 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amine (**1**),[27] was modified by introducing suitable substituents



R = H, CH_3, OCH_3, Cl
R_1 = H, cycloalkyl, aralkyl, acyl, carbamoyl
R_6, R_7, R_8 = H, Cl, NO_2, NH_2

Chart 1. Previously reported adenosine receptor antagonists.

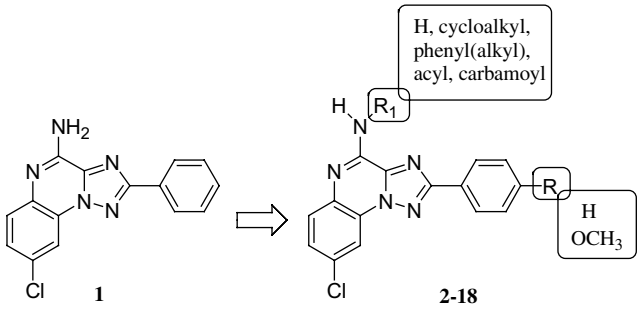


Chart 2. Previously reported and newly synthesized triazoloquinoxaline derivatives.

on the 4-amino group. Moreover, a methoxy group was introduced at position-4 on the appended 2-phenyl ring of some selected compounds in order to obtain more potent A_3 AR antagonists. These investigations were undertaken to verify whether the modifications apported on series **A** and **B** afford the same effects on tricyclic systems of similar size and shape.

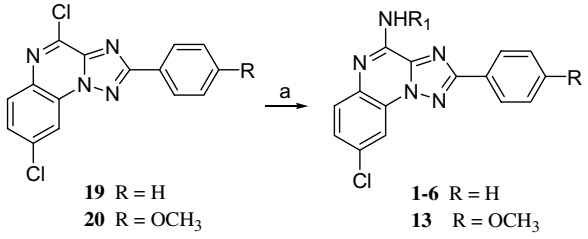
In order to elucidate our experimental results, we decided to theoretically depict the putative TM binding motif of the herein reported 1,2,4-triazolo[1,5-*a*]quinoxaline derivatives by using our recently published hA_3 receptor model.[34]

2. Chemistry

The synthetic pathways which yielded compounds **1**–**18** are illustrated in Schemes 1 and 2. The synthesis of **1** and **13** was previously reported.[27]

Compounds **1**–**6**, **13** were obtained by reacting the known 4,8-dichloro-2-aryl-1,2,4-triazolo[1,5-*a*]quinoxaline **19**, **20**[27] with ammonia or amines (Scheme 1).

Allowing the 8-chloro-2-aryl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amines **1**, **13** to react with either acyl chloride or phenyl(alkyl)isocyanates, the 4-amides **7**–**10**, **14**–**17** and the 4-ureides **11**–**12**, **18** were obtained, respectively (Scheme 2).

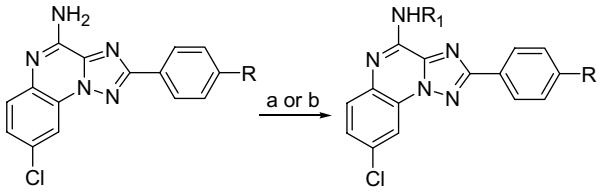


19 R = H
20 R = OCH_3

1-6 R = H
13 R = OCH_3

compd	R_1
1, 13	H
2	C_5H_9
3	C_6H_{11}
4	C_6H_5
5	$CH_2C_6H_5$
6	$CH_2CH_2C_6H_5$

Scheme 1. (a) NH_3(g), or cycloalkyl- or phenyl(alkyl)-amines in absolute EtOH.



1 : R = H
13 : R = OCH_3

7-12 : R = H
14-18 : R = OCH_3

R_1	compd
$COCH_3$	**7, 14**
COC_2H_5	**8, 15**
COC_6H_5	**9, 16**
$COCH_2C_6H_5$	**10, 17**
$CONHC_6H_5$	**11, 18**
$CONHCH_2C_6H_5$	**12**

Scheme 2. (a) acylchloride in anhydrous tetrahydrofuran or dichloromethane; (b) phenyl(alkyl)isocyanate in anhydrous tetrahydrofuran under nitrogen atmosphere.

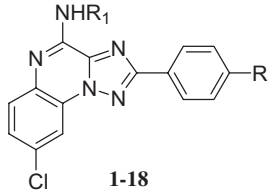
3. Biochemistry

Compounds **1**–**18** 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*-ethyl-carbamoyl)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,[9–11] we tested our A_3 AR ligands on cloned human A_3 receptors.

The binding results of **1**–**18** are shown in Table 1 together with those of their parent compounds **1** and **13** (described in Ref. 27). Moreover, the binding data of theophylline and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), included as antagonist reference compounds, are also reported.

In addition, compound **14**, which is the most potent hA_3 AR antagonist herein reported, was tested, together with the unsubstituted **13**, 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 results of **13**, **14**, and 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



1-18

Compd	R	R_1	K_i (nM)[a] or I%		
			bA_1[b]	bA_{2A}[c]	hA_3[d]
1[e]	H	H	50 ± 4.2	161 ± 14.1	91.4 ± 7.6
2	H	C_5H_9	39.5 ± 29.3	28.4 ± 2.9	38 ± 2.9
3	H	C_6H_{11}	21.8 ± 1.9	17.5%	83.8 ± 7.5
4	H	C_6H_5	635 ± 53.1	0%	20.1 ± 2.4
5	H	$CH_2C_6H_5$	3.6 ± 0.4	21,000 ± 233	12.7 ± 1.4
6	H	$CH_2CH_2C_6H_5$	45.4 ± 3.9	37.4%	53.5 ± 6.1
7	H	$COCH_3$	18.1 ± 1.9	929 ± 83.3	29.2 ± 3.1
8	H	COC_2H_5	6.5 ± 0.7	11,500 ± 1227	4.4 ± 0.5
9	H	COC_6H_5	38.7 ± 4.1	49.4%	47 ± 3.9
10	H	$COCH_2C_6H_5$	45.3 ± 3.9	34.6%	2.0 ± 0.1
11	H	$CONHC_6H_5$	181 ± 14.5	37%	6.0 ± 0.54
12	H	$CONHCH_2C_6H_5$	N.T.[f]	N.T.[f]	N.T.[f]
13[e]	OCH_3	H	150 ± 13.7	53.4%	2.9 ± 0.18
14	OCH_3	$COCH_3$	127 ± 10.4	0%	0.5 ± 0.03
15	OCH_3	COC_2H_5	77 ± 0.5	0%	10.6 ± 0.9
16	OCH_3	COC_6H_5	3250 ± 180	0%	13 ± 1.1
17	OCH_3	$COCH_2C_6H_5$	244 ± 14	20%	42.8 ± 2.7
18	OCH_3	$CONHC_6H_5$	0%	0%	81.4 ± 7.7
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] The A_1 and A_{2A} binding data are reported in Ref. 27.
[f] Not tested due to its insolubility in the medium assay.

4. Results and discussions

The binding results of compounds **2**–**18**, displayed in Table 1, show that the synthesis of these 2-aryl-8-chloro-1,2,4-triazolo[1,5-*a*]quinoxalines has produced some potent bA_1 and/or hA_3 antagonists.

With the aim of defining the SAR in the 1,2,4-triazolo[1,5-*a*]quinoxaline derivatives, we modified the previously reported 8-chloro-2-phenyl-1,2,4-triazolo-[1,5-*a*]quinoxalin-4-amine (**1**).[27] All the herein reported compounds bear, at position-8, a chlorine atom. In fact, it is well known that the AR affinity of ligands with similar size and shape can be enhanced by the presence of a chlorine atom on the benzofused moiety in a position corresponding to our 8-position.[27,35–39]

The AR binding affinities of the parent **1** indicate that this compound is nonselective, being about equiactive at all three different receptor types.

The modifications carried out on the parent structure **1** have different effects on A_1 affinities, while, in general, they positively affect A_3 receptor ligand interaction. Furthermore, A_{2A} receptor binding activities are dramatically reduced: most of the reported compounds (**2**–**18**) are inactive or active in the high micromolar range at the A_{2A} receptor, the only exception being compound **2**, which displays a K_i value in the low nanomolar range.

The 4-cycloalkylamino derivatives **2**–**3** were prepared as potential adenosine A_1 receptor antagonists since the cycloalkyl substituent yielded potent A_1 ligands in several compounds of similar size and shape.[12,14,15,30,31,33] However, this substitution does not affect A_1 binding affinities of these derivatives much: **2** exhibits A_1 binding affinity comparable to that of the parent compound **1**, and **3** is 2-fold more active. With regard to the A_3 binding, **2** is twice as active as **1**, while **3** is equipotent.

Replacement of a hydrogen atom of the 4-amino moiety of **1** with a phenyl(alkyl) group of different length (compounds **4**–**6**) has contrasting effects on A_1 affinity (depending on the nature of the 4-N-substituent), while it positively affects A_3 binding activities. In fact, the N-phenyl derivative **4** is about 13-fold less potent at the A_1 and more potent at the A_3 than **1**. Homologation of the N-phenyl chain (compound **5**) produces an increase of both A_1 and A_3 receptor affinities. Indeed, compound **5** is one of the most potent A_1 receptor antagonists of this series. Further homologation of the N-side chain gives compound **6**, which is about equiactive to **1** at both A_1 and A_3 receptors.

The differences between the binding data of compounds **4**, **5**, and **6** on A_1 receptor and, to a minor extent, on A_3 receptor, suggest that the affinity at both receptor subtypes is influenced by the length of the spacer between the phenyl moiety and the NH group at position-4.

Replacement of a hydrogen atom of the 4-amino group of **1** with an acyl (compounds **7**–**10**) or a carbamoyl (compound **11**) moiety yields, in agreement with the literature data,[23,24,30,31] an increment in A_3 potency, while it does not affect (compounds **9** and **10**) or influences little (compounds **7** and **11**) A_1 affinity, the only exception being compound **8**, which is about 8-fold more active than **1**. In fact, the 4-propionylamine **8** is the most potent toward the A_1 receptor among the 4-amido-derivatives **7**–**10**. On the other hand, the 4-phenylacetylamine **10** is the most active at the A_3 subtype, showing about 46-fold increased A_3 receptor affinity with respect to the parent compound **1**.

The phenylcarbamoylamine **12** was not tested because of its high insolubility in the medium binding assays.

In this series of triazoloquinoxalines, the importance of the presence of the C=O amide group at position-4 in the A_3 receptor ligand interaction is shown by comparison of the A_3 affinity of the 4-phenylacetylamine **10** and that of the 4-phenylethylamine **6**.

Improvement in A_3 potency of **10**, with respect to **6**, could be due to (i) the enhanced acidity of the NH proton donor because of the presence of the electron-withdrawing C=O group and/or (ii) the presence of the amide C=O proton acceptor able to engage a hydrogen bond receptor–ligand interaction with a donor site of the A_3 AR subtype. On the contrary, the C=O group is not necessary for binding at the A_1 AR since the 4-phenylacetylamino derivative **10** is equipotent to the phenylethylamino **6** at this receptor subtype.

In the present series, a ureido group at position-4 does not seem to offer a great advantage for the A_3 receptor–ligand interaction. In fact, compound **11** is about 3-fold less active than the corresponding amide **10** at the A_3 receptor subtype.

In the previously reported series **A** and **B**, the presence of a substituent on the appended 2-phenyl ring, in general, negatively affected A_1 and A_{2A} potency, while A_3 affinity was influenced as a function of the nature and position of the substituent. In particular, introduction of a 4-methoxy group enhanced A_3 receptor affinity and was not advantageous for A_1 receptor–ligand interaction. However, the previously reported 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amine **13**[27] is only 3-fold less active at the A_1 receptor, but about 30-fold more potent at the A_3 subtype with respect to the 2-phenyl derivative **1**. The same can be applied, with some exceptions, to the 2-(4-methoxyphenyl)-derivatives (compounds **14**–**18**) with respect to the 2-phenyl-substituted ones (compounds **7**–**11**). In fact, introduction of the 4-methoxy group generally produces a 5- to 10-fold reduction of A_1 affinity, the only exception being compounds **16** and **18**, which are 80- and more than 100-fold less active than the corresponding unsubstituted derivatives **9** and **11**, respectively. Furthermore, this modification affects the A_3 binding activity differently depending on the hindrance of the N-acyl substituent: the A_3 receptor–ligand interaction of these derivatives strongly increases only when the acyl substituent R_1 is represented by the small acetyl group (compare **14** to **7**). On the contrary, the presence of

Table 2. Binding activity at human A_1 ARs

Compd	K_i (nM)[a] or I%[b]
13	36 ± 2.8
14	49%
Theophylline	6200 ± 530
DPCPX	3.2 ± 0.2

[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 cloned hA_1 receptors expressed in CHO cells or percentage of inhibition (I%) of specific binding at 10 μM concentration.

the 4-methoxy group on the 2-phenyl ring, when combined with either the propionyl or the benzoyl group on the 4-NH_2 moiety, has different and modest effects on A_3 affinity (compare **15** and **16** to **8** and **9**, respectively), while it negatively affects A_3 binding activity when the acyl substituent is represented by the more hindered phenylacetyl or phenylcarbamoyl moiety (compare **17** and **18** to **10** and **11**, respectively).

However, it is worth noting that the 4-acetylamino-derivative **14** possesses the highest A_3 AR affinity among the herein reported compounds. Thus, in order to evaluate the A_3 versus A_1 selectivity among the same species, we tested it on human A_1 (hA_1) AR together with the corresponding 4-amino-derivatives **13** as reference compound. The hA_1 binding result, reported in Table 2, indicates that **14** is inactive at the hA_1 AR and consequently it possesses a high (>20,000) hA_3 versus hA_1 selectivity. On the contrary, **13** is an hA_3 versus hA_1 nonselective AR antagonist, thus suggesting the importance of the 4-N-acetyl group for receptor selectivity.

In conclusion, the SARs of these triazoloquinoxaline derivatives **1**–**18** are in accordance with those of the previously reported series **A** and **B**, suggesting a similar AR binding mode. In particular, the results herein obtained confirm the importance for A_3 receptor–ligand interaction of the presence at position-4 of both a strong acidic NH proton donor and a C=O proton acceptor able to engage hydrogen bonds with specific sites on the A_3AR.

Introduction of a methoxy group in *para*-position on the 2-phenyl ring seems to be important for shifting the selectivity toward the A_3 AR, but produces a great increase of A_3 binding activity only when the 4-NH_2 group is unsubstituted or replaced by an acetylamino moiety.

Thus, the synthesis of these triazoloquinoxalines afforded the 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-acetylamine (**14**), which can be considered one of the most potent and hA_3 versus hA_1 selective AR antagonist reported till now.[15,16,23,24,30,31] Moreover, it has emerged that our 1,2,4-triazolo[1,5-*a*]quinoxaline ring system could be a versatile scaffold to obtain potent and selective AR antagonists. In fact, taking into account these preliminary and promising results, further modifications of these derivatives are in progress to obtain potent and selective AR antagonists.

5. Building an antagonist-bound model of human A_3 adenosine receptor

We recently depicted, by using a computational strategy, the putative TM binding motif of 1,2,4-triazolo[4,3-*a*]quinoxaline analogs (series **A**) on hA_3 receptor.[34] Briefly, we have built an improved model of the hA_3 receptor, using the bovine rhodopsin crystal structure as template, which can be considered a further refinement in building the hypothetical binding site of the A_3 receptor antagonists already proposed.[40–43] Special attention had to be given to the second extracellular (E2) loop, which has been described in bovine rhodopsin as folding back over transmembrane helices, and, therefore, limiting the size of the active site. Details of the building model are given in Experimental. As previously reported, the recognition of classic hA_3 AR antagonists seems to occur in the upper region of the TM helical bundle. TMs 3, 5, 6, and 7 seem to be crucial for the recognition of both agonists and antagonists. As described for previously published 1,2,4-triazolo[4,3-*a*]quinoxaline derivatives[34] (series **A**), also this new 8-chloro-substituted 1,2,4-triazolo[1,5-*a*]quinoxaline family (compounds **1**–**18**) can nicely fit inside the TM region of the hA_3 receptor model. Molecular modeling studies have been carried out for all the compounds reported in Table 1. Consistent with our previously published docking studies, the binding of triazoloquinoxaline moiety seems to occur in the upper region of the helical bundle. In particular, a very clear steric and electrostatic complementarity has been found between derivative **14** and the hypothetical binding cavity on hA_3 receptor model. At least four stabilizing hydrogen-bonding interactions have been described using the most energetically stable docked conformation. His95 (TM3), Ser165 (EL2), Ser170 (EL2), and Asn250 (TM6) seem to characterize the ligand recognition region on the receptor, as shown in Figure 1. Accordingly, the triazoloquinoxaline nucleus should be most favorably oriented perpendicular to the plane of the lipid bilayer, with the 2-aryl substituent in proximity to TM2 and TM7 and the 8-chloro-benzene moiety close to TM5 and TM6. The peculiarity of this new 8-chloro-substituted family consists in a stabilizing dipolar interaction between the chlorine substituent and the hydroxy group of Ser170 (EL2).

In accordance with the SAR on 1,2,4-triazolo[4,3-*a*]quinoxaline derivatives,[30] the 2-(*p*-methoxyphenyl) substituent on compound **14** is crucial to give high potency and selectivity. In our model, the 2-aryl substituent is positioned in a small cleft between TM2 and TM7, and the methoxy group is able to strongly accept a hydrogen bond from Ser165 (EL2). The replacement of the –OCH_3 with a hydrogen (compound **7**) reduces the antagonist activity toward A_3 receptor without, in any case, abolishing it.

The chemical nature of the substituent on the 4-amino group seems to be crucial for differentiating both potency and affinity among this series of new adenosine antagonists. Analyzing our structure–activity data in details, the 4-amino position is surrounded by two polar amino acids: His95 (TM3) and Ser247 (TM6). This

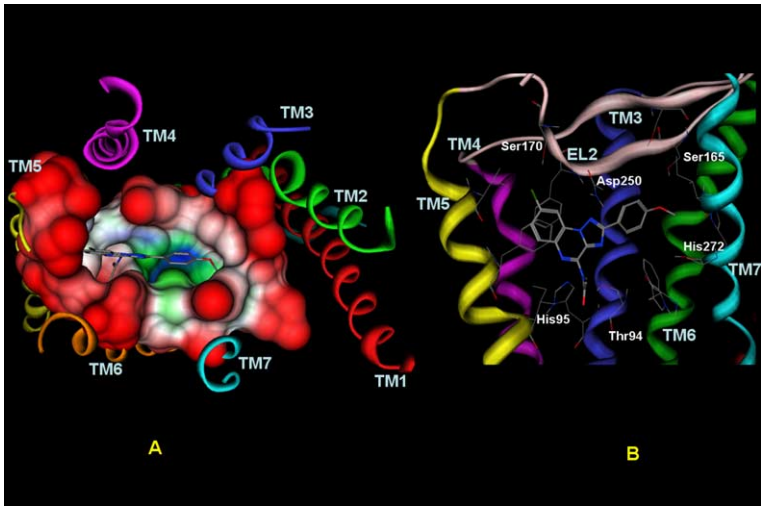


Figure 1. Panel A: The hA_3 receptor model viewed from the extracellular side. E2 loop is not shown facilitating the TM visual inspection. Putative binding sites, suggested by site-directed mutagenesis studies, is delimited by the docked derivative **14**. Panel B: 8-chloro-triazoloquinoxaline derivatives binding site in the hA_3 receptor. Derivative **14** docked into the ligand binding crevice of the hA_3 receptor viewed from the membrane side facing TM helices 5 and 6. Nonpolar hydrogen atoms are not displayed.

region appears to be very critical for the recognition of the antagonist structures. In fact, a major structural difference between the hypothetical binding sites in these receptor subtypes is that the A_3 receptor does not contain the histidine residue in TM6 common to all A_1 (His251 in hA_1) and A_2 (His250 in hA_{2A}) receptors. This histidine has been shown to participate in both agonist and antagonist binding to A_{2A} receptors.[44] In the A_3 receptor this histidine in TM6 is replaced with a serine residue (Ser247 in hA_3). It is assumed that these two polar amino acids (His95 and Ser247) give strong interactions with the amide moiety of **14** (Fig. 1). In particular, the NH of the 4-amino group seems to interact through a hydrogen bond with His95 (TM3) whereas the CO amide group at position-4 is involved in a strong hydrogen bond with Ser247 (TM6). However, evaluation of the ligand binding pocket of the receptor in this region reveals that very limited empty space is present between TM5 and TM6 and, consequently, a steric control seems to be taking place around the *para*-position of the phenyl ring. Thus, derivatives **16** and **17** reduce their antagonist activity toward A_3 receptor.

In conclusion, the receptor-based SAR analysis provides us with new useful insights about the steric and electrostatic requirements, which are important for the optimal anchoring of the 1,2,4-triazolo[1,5-*a*]quinoxaline derivatives to the hA_3 receptor recognition site.

6. Experimental

6.1. 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 values except where stated otherwise (Table 4). The IR spectra were recorded with a Perkin–Elmer 1420 spectrometer in Nujol mulls and are expressed in cm^{-1}. The ^1H 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. All the exchangeable protons were confirmed by addition of D_2O. The following abbreviations are used: s = singlet, d = doublet, dd = double doublet, t = triplet, q = quartet, m = multiplet, br = broad, and ar = aromatic protons. The physical data of the newly synthesized compounds are shown in Table 3.

Table 3. Physical data of the newly synthesized compounds

Compd	Mp (°C)	Solvent[a]	Yield (%)
2	175–177	A	30
3	180–182	B	72
4	237–239	B	95
5	170–172	B	85
6	198–200	C	94
7	246–248	B	15
8	252–253	B	95
9	257–260	D	20
10	264–266	E	60
11	240–242	B	94
12	259–261	B	19
14	235–239	F	16
15	213–216	F	21
16	266–268	G	25
17	252–254	H	37
18	232–234	A	25

[a] Recrystallization solvents: A = ethanol; B = glacial acetic acid; C = cyclohexane, D = ethanol/glacial acetic acid; E = dimethylformamide; F = ethyl acetate; G = ethanol/ethyl acetate; H = chloroform.

6.1.1. Synthesis of 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amine (1). The title compound was synthesized starting from 4,8-dichloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline (**19**) as previously reported.[27]

6.1.2. General procedure to prepare 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amines (2–6). A mixture of **19**[27] (1.2 mmol), triethylamine (2.8 mmol), and the suitable amine (1.4 mmol) in absolute ethanol (15 mL) was heated overnight at 120 °C in a sealed tube. Upon cooling a solid was obtained, which was collected, washed with water and recrystallized.

6.1.2.1. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-cyclopentylamine (2). ^{1}H NMR (DMSO-d_6) δ: 1.60–1.80 (m, 6H, cyclopentyl protons), 2.00–2.11 (m, 2H, cyclopentyl protons), 4.61–4.66 (m, 1H, cyclopentyl proton), 7.51–7.71 (m, 5H, ar), 8.15–8.33 (m, 4H, ar + NH). IR (cm^{-1}) 3440.

6.1.2.2. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-cyclohexylamine (3). ^{1}H NMR (DMSO-d_6) δ: 1.22–2.02 (m, 10H, cyclohexyl protons), 4.18–4.23 (m, 1H, cyclohexyl proton), 7.50–7.70 (m, 5H, ar), 8.04 (d, 1H, NH, J = 8.35 Hz), 8.15 (d, 1H, ar, J = 2.20 Hz), 8.28–8.31 (m, 2H, ar). IR (cm^{-1}) 3300.

6.1.2.3. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenylamine (4). ^{1}H NMR (DMSO-d_6) δ: 7.13 (t, 1H, ar, J = 7.68 Hz), 7.42 (t, 2H, ar, J = 7.68 Hz), 7.58–7.65 (m, 4H, ar), 7.83 (d, 1H, ar, J = 8.79 Hz), 8.19–8.25 (m, 3H, ar), 8.36 (dd, 2H, ar, J = 7.68, 2.2 Hz), 10.19 (s, 1H, NH). IR (cm^{-1}) 3390.

6.1.2.4. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-benzylamine (5). ^{1}H NMR (DMSO-d_6) δ: 4.80 (d, 2H, CH_2, J = 6.05 Hz), 7.33–7.36 (m, 3H, ar), 7.46–7.70 (m, 7H, ar), 8.15 (d, 1H, ar, J = 2.20 Hz), 8.29 (dd, 2H, ar, J = 7.89, 2.2 Hz), 8.87 (t, 1H, NH, J = 6.05 Hz). IR (cm^{-1}) 3330.

6.1.2.5. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenylethylamine (6). ^{1}H NMR (DMSO-d_6) δ: 3.04 (t, 2H, CH_2, J = 8.06 Hz), 3.79–3.83 (m, 2H, CH_2), 7.20–7.33 (m, 5H, ar), 7.52–7.75 (m, 5H, ar), 8.17 (d, 1H, ar, J = 2.19 Hz), 8.24–8.29 (m, 2H, ar), 8.40 (t, 1H, NH, J = 7.99 Hz). IR (cm^{-1}) 3420.

6.1.3. Synthesis of 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-acetylamine (7). A solution of acetylchloride (6.8 mL) in anhydrous tetrahydrofuran (7 mL) was slowly added to a solution of **1**[27] (1.9 mmol) in anhydrous tetrahydrofuran (70 mL) and anhydrous pyridine (18.9 mmol). The mixture was stirred at room temperature overnight and then was refluxed for 20 h. Evaporation at reduced pressure of the solvent yielded a solid, which was treated with ethanol (10 mL), collected, and purified by silica gel column chromatography, eluting system chloroform/ethyl acetate 9:1.

^{1}H NMR (DMSO-d_6) δ: 2.39 (s, 3H, CH_3), 7.59–7.63 (m, 3H, ar), 7.78 (dd, 1H, ar, J = 8.80, 2.40 Hz), 8.02 (dd, 1H, ar, J = 8.80, 1.47 Hz), 8.32–8.40 (m, 3H, ar), 10.89 (s, 1H, NH). IR (cm^{-1}) 3180, 1710.

6.1.4. General procedure to prepare 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-acylamines 8–10. A solution of the suitable acyl chloride (3.0 mmol) in anhydrous dichloromethane (3 mL) was slowly added at 0 °C to a suspension of **1**[27] (1.0 mmol) in anhydrous dichloromethane (30 mL) and anhydrous pyridine (10 mmol). In the case of compound **8** the mixture was stirred at room temperature for 3 days. For the preparation of compounds **9** and **10**, after stirring at room temperature overnight, the mixture was refluxed until the disappearance of the starting material (TLC monitoring, eluting system chloroform/ethyl acetate 9:1, on silica gel plates). Evaporation at reduced pressure of the solvent yielded a residue, which was treated with ethanol (10 mL), collected, and purified by crystallization (compounds **8** and **10**) or by silica gel column chromatography (eluting system chloroform/cyclohexane 9:3) (compound **9**).

6.1.4.1. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-propionylamine (8). ^{1}H NMR (DMSO-d_6) δ: 1.16 (t, 3H, CH_3, J = 7.45 Hz), 2.74 (q, 2H, CH_2, J = 7.45 Hz), 7.58–7.66 (m, 2H, ar), 7.77 (dd, 2H, ar, J = 8.78, 2.34 Hz), 8.01 (d, 1H, ar, J = 8.78 Hz), 8.31–8.40 (m, 3H, ar), 10.82 (s, 1H, NH). IR (cm^{-1}) 3280, 1700.

6.1.4.2. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-benzoylamine (9). ^{1}H NMR (DMSO-d_6) δ: 7.55–7.72 (m, 6H, ar), 7.83 (dd, 1H, ar, J = 8.81, 2.25 Hz), 8.10–8.14 (m, 3H, ar), 8.27–8.32 (m, 2H, ar), 8.49 (d, 1H, ar, J = 2.25 Hz), 11.50 (s, 1H, NH). IR (cm^{-1}) 3270, 1720.

6.1.4.3. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenylacetylamine (10). ^{1}H NMR (DMSO-d_6) δ: 4.06 (s, 2H, CH_2), 7.28–7.47 (m, 5H, ar), 7.59–7.66 (m, 3H, ar), 7.77 (dd, 1H, ar, J = 8.80, 2.34 Hz), 8.01 (d, 1H, ar, J = 8.80 Hz), 8.32–8.41 (m, 3H, ar), 11.15 (s, 1H, NH). IR (cm^{-1}) 3290, 1695.

6.1.5. General procedure to prepare 8-chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenyl(methyl)carbamoylamines 11 and 12. Phenyl(methyl)isocyanate (1.5 mmol) was dropwise added to a suspension of **1**[27] (1.0 mmol) in anhydrous tetrahydrofuran (50 mL). The mixture was refluxed under nitrogen atmosphere until the disappearance of the starting material (TLC monitoring, eluting system chloroform/methanol 9:1, on silica gel plates). Evaporation at reduced pressure of the solvent yielded a solid, which was treated with petroleum ether 40–60 °C (10 mL) and collected. Purification of compound **11** was achieved by recrystallization, while **12** was purified by silica gel column chromatography (eluting system chloroform/methanol 9.7:0.3) and then recrystallized.

6.1.5.1. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenylcarbamoylamine (11). ^{1}H NMR (DMSO-d_6) δ: 7.12 (t, 1H, ar, J = 7.33 Hz), 7.40 (t, 2H, ar,

J = 7.70 Hz), 7.59–7.62 (m, 3H, ar), 7.71–7.78 (m, 3H, ar), 8.18 (d, 1H, ar, J = 8.79 Hz), 8.32–8.35 (m, 3H, ar), 10.3 (br s, 1H, NH), 11.46 (s, 1H, NH). IR (cm^{-1}) 3220, 1665.

6.1.5.2. 8-Chloro-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxaline-4-benzylcarbamoylamine (12). ^{1}H NMR (DMSO-d_6) δ: 4.57 (d, 2H, CH_2, J = 5.86 Hz), 7.27–7.44 (m, 5H, ar), 7.59–7.64 (m, 3H, ar), 7.73 (dd, 1H, ar, J = 8.79, 2.3 Hz), 8.05 (d, 1H, ar, J = 8.79 Hz) 8.31–8.35 (m, 3H, ar), 9.71–9.77 (m, 2H, NH). IR (cm^{-1}) 3220, 3130, 1690.

6.1.6. Synthesis of 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-amine (13). The title compound was synthesized starting from 4,8-dichloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxaline (**20**) as previously reported.[27]

6.1.7. General procedure to prepare 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-acylamines 14 and 15. The suitable acylchloride (5.1 mmol) was added to a suspension of **13**[27] (1.3 mmol) in anhydrous tetrahydrofuran (50 mL) and anhydrous pyridine (1.3 mL). The reaction mixture was heated under reflux until the disappearance of the starting material (TLC monitoring, eluting system $CHCl_3$/AcOEt 9:1 on silica gel plates). Evaporation at reduced pressure of the solvent gave a solid, which was treated with water (100 mL), collected by filtration, and recrystallized.

6.1.7.1. 8-Chloro-2-(4-methoxyphenyl)-1,2,4-triazolo-[1,5-*a*]quinoxalin-4-acetylamine (14). ^{1}H NMR (DMSO-d_6) δ: 2.30 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 7.12 (d, 2H, ar, J = 8.79 Hz), 7.71 (dd, 1H, ar, J = 8.79, 2.56 Hz), 7.96 (d, 1H, ar, J = 8.79 Hz), 8.21 (d, 2H, ar, J = 8.79 Hz), 8.31 (d, 1H, ar, J = 2.56 Hz), 10.8 (br s, 1H, NH). IR (cm^{-1}) 3350, 1700.

6.1.7.2. 8-Chloro-2-(4-methoxyphenyl)-1,2,4-triazolo-[1,5-*a*]quinoxaline-4-propionylamine (15). ^{1}H NMR (DMSO-d_6) δ: 1.13 (t, 3H, CH_3, J = 7.69 Hz), 2.70 (q, 2H, CH_2, J = 7.69 Hz), 3.85 (s, 3H, OCH_3), 7.13 (d, 2H, ar, J = 8.79 Hz), 7.72 (dd, 1H, ar, J = 8.79, 2.56 Hz), 7.96 (d, 1H, ar, J = 8.79 Hz), 8.23 (d, 2H, ar, J = 8.79 Hz), 8.33 (d, 1H, ar, J = 2.56 Hz), 10.8 (br s, 1H, NH). IR (cm^{-1}) 3350, 1700.

6.1.8. Synthesis of 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxaline-4-benzoylamine (16). Benzoylchloride (2 mmol) was added to a suspension of **13** (1 mmol) in anhydrous tetrahydrofuran (50 mL) and anhydrous pyridine (50 mL). The reaction mixture was heated under reflux for 16 h and then, an excess of benzoylchloride (1 mmol) was added. After 8 h, another portion of benzoylchloride (1 mmol) was added to the reaction mixture, which was heated for another 8 h. The solvent was removed by distillation under reduced pressure and the resulting solid was treated with water (100 mL) and collected by filtration. The crude product was purified by silica gel column chromatography (eluting system chloroform/ethyl acetate 9:1). Evaporation of the second eluates yielded a solid, which was recrystallized.

^{1}H NMR (DMSO-d_6) δ: 3.82 (s, 3H, OCH_3), 7.10 (d, 2H, ar, J = 7.32 Hz), 7.54–7.68 (m, 3H, ar), 7.79 (d, 1H, ar, J = 8.79 Hz), 8.05–8.10 (m, 3H, ar), 8.18 (d, 2H, ar, J = 7.32 Hz), 8.43 (s, 1H, ar), 11.47 (s, 1H, NH). IR (cm^{-1}) 3257, 1663.

6.1.9. Synthesis of 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenylacetyl-amine (17). A solution of phenylacetylchloride (3.0 mmol) in anhydrous tetrahydrofuran (3 mL) was dropwise added to a suspension of **13** (0.92 mmol) in anhydrous tetrahydrofuran (50 mL) and anhydrous pyridine (10 mL) at room temperature. The reaction mixture was heated under reflux for 6 h. Evaporation of the solvent at reduced pressure afforded a solid, which was treated with ethanol (10 mL), collected by filtration, and then recrystallized.

^{1}H NMR (DMSO-d_6) δ: 3.85 (s, 3H, OCH_3), 4.02 (s, 2H, CH_2), 7.15 (d, 2H, ar, J = 8.06 Hz), 7.25–7.40 (m, 5H, ar), 7.72 (dd, 1H, ar, J = 8.79, 2.56 Hz), 7.97 (d, 1H, ar, J = 8.79 Hz), 8.24 (d, 2H, ar, J = 8.46 Hz), 8.35 (d, 1H, ar, J = 2.56 Hz), 10.9 (br s, 1H, NH). IR (cm^{-1}) 3245, 1690.

6.1.10. Synthesis of 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxaline-4-phenyl-carbamoylamine (18). Phenylisocyanate (2 mmol) was added to a solution of **13** (1 mmol) in anhydrous tetrahydrofuran (100 mL) under nitrogen atmosphere. The reaction mixture was refluxed for 8 h and then an excess of phenylisocyanate (2 mmol) was added. Three other portions of phenylisocyanate (2 mmol × 3) were added at 8 hour intervals. After an overall time of 32 h, the solvent was evaporated under reduced pressure and the resulting solid was treated with a small amount of ethanol (3 mL) and collected by filtration. The crude product was purified by silica gel column chromatography (eluting system chloroform/methanol 10:0.25). Evaporation of the first eluates gave a solid, which was recrystallized.

^{1}H NMR (DMSO-d_6) δ: 3.83 (s, 3H, OCH_3), 7.21 (d, 2H, ar, J = 8.42 Hz), 7.37 (t, 2H, ar, J = 8.42 Hz), 7.67–7.72 (m, 3H, ar), 8.10–8.29 (m, 5H, ar), 10.1 (br s, 1H, NH), 11.42 (s, 1H, NH). IR (cm^{-1}) 3370, 1700.

6.2. Biochemistry

6.2.1. 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. 45.

6.2.2. 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.[46]

Table 4. Analytical data of the newly synthesized compounds

Compd	Formula	C calcd–found	H calcd–found	N calcd–found
2	$C_{20}H_{18}ClN_5$	66.01–66.31	5.00–5.20	19.25–19.39
3	$C_{21}H_{20}ClN_5$	66.74–66.96	5.35–5.28	18.54–18.32
4	$C_{21}H_{14}ClN_5$	67.83–67.48	3.80–3.92	18.84–19.03
5	$C_{22}H_{16}ClN_5$	68.47–68.18	4.19–3.98	18.15–17.98
6	$C_{23}H_{18}ClN_5$	69.07–68.92	4.55–4.78	17.52–17.74
7	$C_{17}H_{12}ClN_5O$	60.44–60.00	3.59–3.38	20.74–20.46
8	$C_{18}H_{14}ClN_5O$	61.45–61.83	4.02–3.87	19.91–20.12
9	$C_{22}H_{14}ClN_5O$	66.08–66.37	3.54–3.78	17.52–17.22
10	$C_{23}H_{16}ClN_5O$	66.74–66.59	3.90–4.10	16.92–17.13
11	$C_{22}H_{15}ClN_6O$	63.69–63.78	3.65–3.82	20.26–20.37
12	$C_{23}H_{17}ClN_6O$	64.40–64.19	4.00–4.22	19.60–19.38
14	$C_{18}H_{14}ClN_5O_2$	58.77–58.45	3.84–3.67	19.04–18.88
15	$C_{19}H_{16}ClN_5O_2$	59.76–59.63	4.23–4.52	18.34–18.68
16	$C_{23}H_{16}ClN_5O_2$	64.26–64.13	3.76–4.00	16.29–16.38
17	$C_{24}H_{18}ClN_5O_2$	64.93–65.18	4.10–4.29	15.78–15.99
18	$C_{23}H_{17}ClN_6O_2$	62.09–62.39	3.86–3.57	18.89–18.68

Displacement of either [^{3}H]CHA or [^{125}I]AB-MECA from hA_1 or hA_3 ARs, respectively, was performed as previously described.[32]

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 nonlinear regression method implemented in the InPlot program (Graph-Pad, San Diego, CA) with five concentrations of displacer, each performed in triplicate. Inhibition constants (K_i) were calculated according to the Cheng–Prusoff equation.[47] 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.

6.3. Computational methodologies

All molecular modeling studies were carried out on a 6 CPU (PIV 2.0–3.0 GHz) linux cluster running under openMosix architecture.[48]

Homology modeling, energy calculation, and docking studies were performed using Molecular Operating Environment (MOE, version 2004.03) suite.[49]

The ground state geometry of all, charged and uncharged, docked structures was fully optimized without geometry constraints using RHF/AM1 semiempirical calculations. Vibrational frequency analysis was used to characterize the minima stationary points (zero imaginary frequencies). The software package Spartan O2 was utilized for all quantum mechanical calculations.[50]

6.3.1. Homology model of the hA_3 AR. Based on the assumption that GPCRs share similar TM boundaries and overall topology,[46] a homology model of the hA_3 receptor was constructed. First, the amino acid sequences of TM helices of the A_3 receptor were aligned with those of bovine rhodopsin, guided by the highly conserved amino acid residues, including the DRY motif (D3.49, R3.50, and Y3.51) and three Pro residues (P4.60, P6.50, and P7.50) in the TM segments of GPCRs. The same boundaries were applied for the TM helices of the A_3 receptor as they were identified from the X-ray crystal structure for the corresponding sequences of bovine rhodopsin, the C_α coordinates of which were used to construct the seven TM helices for the human A_3 receptor. The loop domains of the hA_3 receptor were constructed by the loop search method implemented in MOE. In particular, loops are modeled first, in random order. For each loop, a contact energy function analyzes the list of candidates collected in the segment searching stage, taking into account all atoms already modeled and any atoms specified by the user as belonging to the model environment. These energies are then used to make a Boltzmann-weighted choice from the candidates, the coordinates of which are then copied to the model. Any missing side chain atoms are modeled using the same procedure. Side chains belonging to residues whose backbone coordinates were copied from a template are modeled first, followed by side chains of modeled loops. Outgaps and their side chains are modeled last. Special caution had to be given to the second extracellular (E2) loop, which has been described in bovine rhodopsin as folding back over transmembrane helices,[46] and, therefore, limiting the size of the active site. Hence, amino acids of this loop could be involved in direct interactions with the ligands. A driving force to this peculiar fold of the E2 loop might be the presence of a disulfide bridge between cysteines in TM3 and E2. Since this covalent link is conserved in all receptors modeled in the current study, the E2 loop was modeled using a rhodopsin-like constrained geometry around the E2-TM3 disulfide bridge. After the heavy atoms were modeled, all hydrogen atoms were added, and the protein coordinates were then minimized with MOE using the AMBER94 force field.[51] The minimizations were carried out by the 1000 steps of steepest descent followed by conjugate gradient minimization until the *rms* gradient of the potential energy was less than 0.1 kcal/mol Å.

6.3.2. Molecular docking of the hA_3 AR antagonists. All antagonist structures were docked into the hypothetical TMs binding site by using the DOCK docking program, part of the MOE suite. Searching is conducted within a user-specified 3D docking box, using Tabù Search protocol[52] and MMFF94 force field.[53–59] MOE-Dock performs a user-specified number of independent docking runs (50 in our specific case) and writes the resulting conformations and their energies in a molecular database file. The resulting docked complexes were subjected to MMFF94 energy minimization until the *rms* of conjugate gradient was $<0.1\,kcal\,mol^{-1}\,Å^{-1}$. Charges for the ligands were imported from the Spartan output files.

The interaction energy values were calculated as follows: $\Delta E_{binding} = E_{complex} - (E_{ligand} + E_{receptor})$. These energies are not rigorous thermodynamic quantities, but can only be used to compare the relative stabilities of the complexes. Consequently, these interaction energy values cannot be used to calculate binding affinities since changes in entropy and solvation effects are not taken into account.

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