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Article

1,2,4-Triazolo[1,5-*a*]quinoxaline as a Versatile Tool for the Design of Selective Human A Adenosine Receptor Antagonists: Synthesis, Biological Evaluation, and Molecular Modeling Studies of 2-(Hetero)aryl- and 2-Carboxy-Substitued Derivatives

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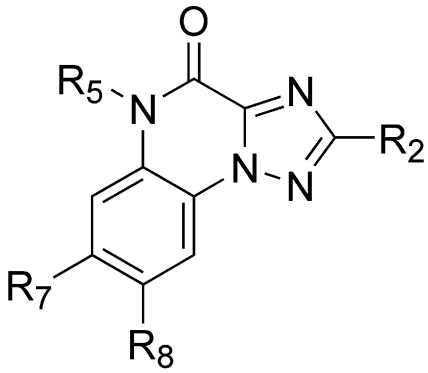

R_2 = (hetero)aryl, H, COOR, CONHR
R_5 = H, CH_3, C_2H_5, OH, COOEt
R_7, R_8 = H, Cl, CH_3, NO_2, NH_2, $NHCOCH_3$

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1,2,4-Triazolo[1,5-*a*]quinoxaline as a Versatile Tool for the Design of Selective Human A_3 Adenosine Receptor Antagonists: Synthesis, Biological Evaluation, and Molecular Modeling Studies of 2-(Hetero)aryl- and 2-Carboxy-Substitued Derivatives

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A number of 4-oxo-substituted 1,2,4-triazolo[1,5-*a*]quinoxaline derivatives bearing at position-2 the claimed (hetero)aryl moiety (compounds **1**−**15**) but also a carboxylate group (**16**−**28**, **32**−**36**) or a hydrogen atom (**29**−**31**) were designed as human A_3 (hA_3) adenosine receptor (AR) antagonists. This study produced some interesting compounds and among them the 2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (**8**), which can be considered one of the most potent and selective hA_3 adenosine receptor antagonists reported till now. Moreover, as a new finding, replacement of the classical 2-(hetero)aryl moiety with a 2-carboxylate function (compounds **16**−**28** and **32**−**36**) maintained good hA_3 AR binding activity but, most importantly and interestingly, produced a large increase in hA_3 versus hA_1 selectivity. A receptor-based SAR analysis provided new interesting insights about the steric and electrostatic requirements that are important for the anchoring of these derivatives at the hA_3 receptor recognition site, thus highlighting the versatility of the triazoloquinoxaline scaffold for obtaining potent and selective hA_3 AR antagonists.

Introduction

Adenosine, a regulatory autacoid that is generated as a result of cellular injury or stress, interacts with at least four specific G-protein-coupled receptor subtypes (GPCRs) classified as A_1, A_{2A}, A_{2B}, and A_3.[1,2] Interaction of adenosine with its receptors mediates signal transduction pathways, which utilize different effector systems including adenylate cyclase (A_1, A_{2A}, A_{2B}, and A_3 receptors), potassium and calcium channels (A_1 receptor), and phospholipase C (A_1, A_{2B}, and A_3 receptors) and D (A_3 receptor).[3−7]

Among the four adenosine receptors (ARs), the A_1 and A_{2A} subtypes have been well pharmacologically characterized, while both A_{2B} and A_3 receptors are the most recently identified and, for this reason, are still under study to better understand their physiological functions. In fact, adenosine plays an important role in many diseases of the central nervous system (CNS), as well as in peripheral organs and tissues.[8,9] Thus, the use of AR antagonists as potential therapeutic agents has been proposed.[10,11] In particular, while A_1 AR antagonists have been developed as antihypertensives, potassium-saving diuretics,[12] and cognitive enhancers[8,11−13] for geriatric therapy and for the treatment of CNS disorders such as Alzheimer's disease,[8] A_3 AR antagonists are sought as potential antiinflammatory agents,[14] but they are also useful in the treatment of cancer[15,16] and glaucoma.[17] Other applications of both A_1 and A_3 AR antagonists, such as prevention and treatment of cerebral and cardiac ischemic injuries, have been proposed.[14,18−20] Accordingly, taking into account the potential of A_3 adenosine receptor antagonists in the development of prospective therapeutic agents,[10,11] in the past few years many attempts in our laboratory have been made to explore structural requirements of different classes of tricyclic heteroaromatic compounds that could be important for the binding at each receptor subtype.[21−31]

In recent papers, we reported the synthesis and binding activities at bovine A_1 (bA_1) and A_{2A} (bA_{2A}) and human cloned A_1 (hA_1), A_{2A} (hA_{2A}), and A_3 (hA_3) ARs of some 4-amino-2-aryl-1,2,4-triazolo[1,5-*a*]quinoxaline derivatives[31] bearing different substituents on the 4-amino group (Chart 1, Series A). Structure−activity relationship studies (SAR) of these derivatives showed that, in general, the presence of substituents on the 4-amino group was important to increase A_1 or A_3 potency or both. In particular, the nature of the substituent on the 4-amino group was essential to modulate the A_1 and A_3 receptor affinity. In fact, the presence of an acyl substituent on the 4-amino group afforded potent A_3 AR antagonists such as the 8-chloro-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxaline-4-acetylamine,[31] which can be considered one of the most potent and hA_3 versus hA_1 selective AR antagonists reported till now.

Therefore, to evaluate the importance of the presence of a C=O proton acceptor at the level of position-4 of

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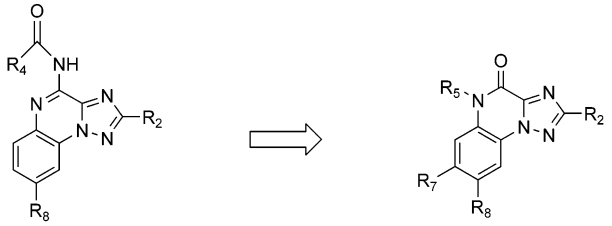
Chart 1. Previously and Currently Reported 1,2,4-Triazolo[1,5-*a*]quinoxaline Derivatives



Series A : R_2 = Aryl
Series B : R_2 = COOEt, $CONH_2$, H

R_4 = alkyl, (alkyl)aryl
R_8 = H, Cl

1-15 : R_2 = (Hetero)Aryl
16-36 : R_2 = H, COOR, CONHR

R = H, C_2H_5, $CH(CH_3)_2$, $CH_2C_6H_5$
R_5 = H, CH_3, C_2H_5, OH, COOEt
R_7, R_8 = H, Cl, CH_3, NO_2, NH_2, $NHCOCH_3$

the triazoloquinoxaline ring system, in the present work we studied a series of 2-(hetero)aryl-1,2,4-triazolo[1,5-*a*]quinoxaline derivatives (compounds **1**−**15**, Chart 1) bearing a 4-oxo function replacing the 4-amino group of previously reported Series A.[23,31] Moreover, with the aim of shedding light on the SAR of this triazoloquinoxaline class also a series of 2-carboxy-substituted compounds (**16**−**28**, **32**−**36**), together with some triazoloquinoxaline derivatives lacking a substituent at position-2 (compounds **29**−**31**) were designed. Both these series can be considered the 4-oxo analogues of previously described 4-amino derivatives[29] (series B). The modifications carried out on the triazoloquinoxaline scaffold could be useful to further investigate position-2 of this tricyclic system. In fact, the diverse groups introduced at this position are endowed with different lipophilic and electronic properties and, consequently, different capabilities to engage interactions with the receptor site when compared to the 2-(hetero)aryl groups. To our knowledge, an aryl moiety is always present at position-2 in most of the structurally related tricyclic AR antagonists reported in the literature[10−12,14,32−34] with the exception of only a few examples.[11]

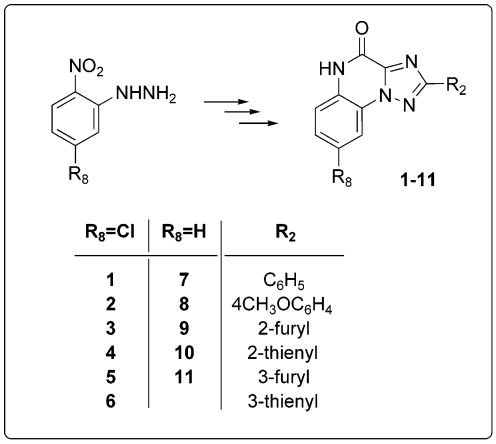
To elucidate our binding results, we decided to theoretically depict the putative transmembrane (TM) binding motif of the herein reported 1,2,4-triazolo[1,5-*a*]quinoxalin-4-one derivatives on hA_3 receptor. SARs have been explained by analyzing the three-dimensional structure of the antagonist−receptor models obtained by molecular docking simulation.

Chemistry

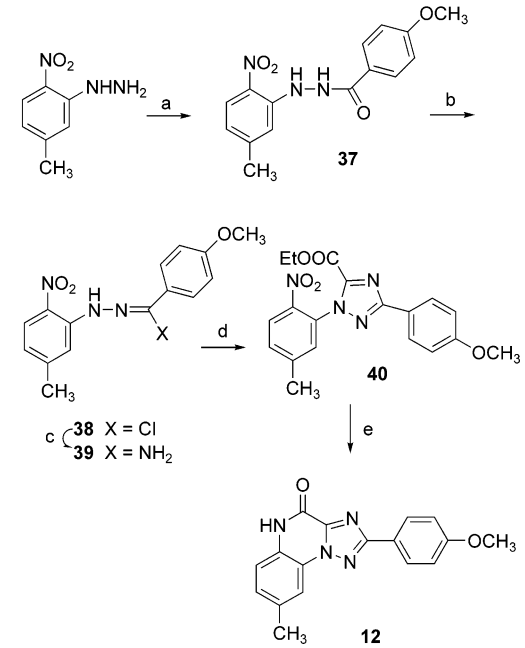
The triazoloquinoxaline derivatives herein reported were synthesized as illustrated in Schemes 1−5. Compounds **26**, **27**,[35,38] **29**,[29] **30**,[38] and **32**[38] (Tables 1 and 2) were prepared following synthetic pathways previously reported.

Scheme 1 shows the synthesis of 4,5-dihydro-2-(4-methoxyphenyl)-8-methyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one **12**, obtained following the procedures that yielded the already described compounds **1**−**11**.[35,36] Thus, by reacting the known 5-methyl-2-nitrophenylhydrazine[35,37] with *para*-anisoyl chloride, we prepared the hydrazide **37**. The latter was transformed into the corresponding chloroimine **38**, which, when treated with ammonia, gave the amidrazone **39**. Reaction of compound **39** with ethyloxalyl chloride directly yielded the

Scheme 1[a]



R_8=Cl	R_8=H	R_2
1	**7**	C_6H_5
2	**8**	$4CH_3OC_6H_4$
3	**9**	2-furyl
4	**10**	2-thienyl
5	**11**	3-furyl
6		3-thienyl

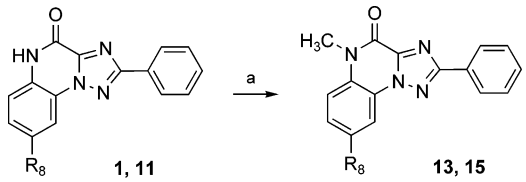


[a] Reagents: (a) *para*-anisoylchloride, pyridine; (b) $POCl_3$; (c) NH_3 (gas), anhydrous dioxane; (d) ClCOCOOEt, anhydrous toluene; (e) iron powder, glacial acetic acid.

ethyl 1-(5-methyl-2-nitrophenyl)-3-(4-methoxyphenyl)-1,2,4-triazole-5-carboxylate **40**, which, after reduction and contemporary cyclization, afforded the tricyclic derivative **12**.

By a method previously reported for obtaining compound **14**[35] from **7**, reaction of compounds **1** and **11** with

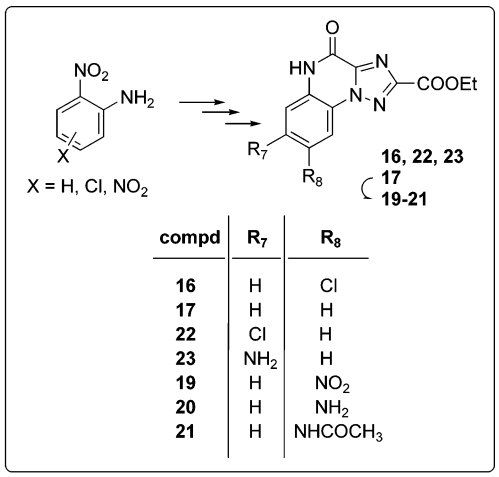
Scheme 2[a]



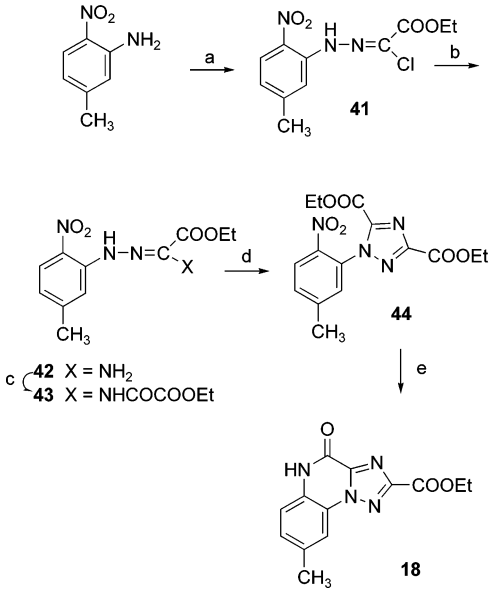
compd	R_8
1, 13	Cl
7, 14	H
11, 15	CH_3

[a] Reagents: (a) CH_3I, NaH, DMF.

Scheme 3[a]



compd	R_7	R_8
16	H	Cl
17	H	H
22	Cl	H
23	NH_2	H
19	H	NO_2
20	H	NH_2
21	H	$NHCOCH_3$

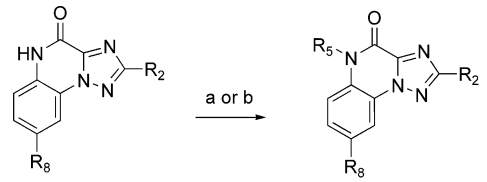


[a] Reagents: (a) $NaNO_2$/HCl, $CH_3COCHClCOOEt$; (b) NH_3 (gas), anhydrous dioxane; (c) ClCOCOOEt, anhydrous toluene, diethyl ether; (d) 180 °C; (e) iron powder, glacial acetic acid.

methyl iodide produced the 5-*N*-methyl-substituted derivatives **13** and **15** (Scheme 2).

Scheme 3 depicts the synthesis of the ethyl 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate **18**, which was prepared following reported procedures described to obtain derivatives **16**,[35] **17**,[35,38] **22**, and **23**.[39] Thus, the diazonium salt of the commercially available 5-methyl-2-nitroaniline was reacted with ethyl 2-chloro-3-oxobutanoate to yield the N^2-chloroacetate **41**, which was transformed with ammonia into the corresponding N^2-oxamidrazonate **42**. Reaction of **42** with ethyloxalyl chloride afforded the N^3-ethoxalyl derivative **43**, which was cyclized to the ethyl triazole-3,5-dicarboxylate **44** by heating over its melting point. By reduction of **44** with iron in glacial acetic acid, the tricyclic derivative **18** was obtained. Compounds **19**–**21** (Scheme 3) were obtained starting from the corresponding benzo-unsubstituted derivatives **17** following reported pathways.[40,41]

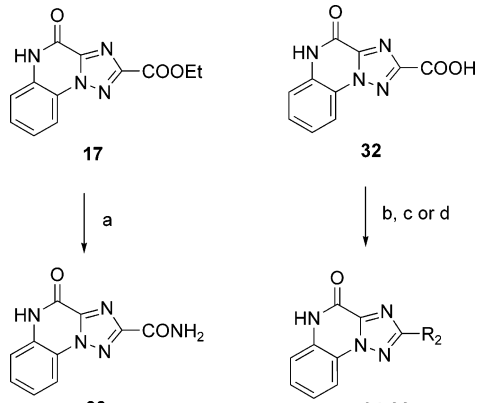
Scheme 4[a]



compd	R_2	R_8
16, 24	COOEt	Cl
17, 25, 28	COOEt	H
29, 31	H	Cl

[a] Reagents: (a) CH_3I, NaH, DMF; (b) ClCOOEt, K_2CO_3, acetone.

Scheme 5[a]



compd	R_2
34	$COOCH(CH_3)_2$
35	$COOCH_2C_6H_5$
36	$CONHCH_2C_6H_5$

[a] Reagents: (a) NH_3 (gas), EtOH; (b) $SOCl_2$; (c) $HOCH(CH_3)_2$ or $C_6H_5CH_2OH$, anhydrous tetrahydrofuran; (d) $C_6H_5CH_2NH_2$, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, 1-hydroxybenzotriazole.

The synthetic procedures that yielded compounds **24**, **25**, **28**, and **31** are illustrated in Scheme 4. By reacting compounds **16** and **17** with methyl iodide, we obtained compounds **24** and **25**, respectively, while reaction of **17** with ethyl chlorocarbonate in alkaline condition

yielded the corresponding 5-*N*-ethoxycarbonyl-derivative **28**. N-Methylation of the 2-unsubstituted compound **29** under the same conditions used to obtain **24** and **25** yielded **31**.

Transformations of the 2-carboxylate function of **17** (derivatives **32**−**36**) are reported in Scheme 5. By treating **17**[35,38] with ammonia, we obtained the corresponding 2-carboxamide compound **33** in good yield. Reaction of **32**[38] with thionyl chloride gave the key intermediate 4,5-dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carbonyl chloride (not isolated), which was treated with different reactants and conditions to give the 2-carboxamide derivatives **34**−**36**.

Biochemistry

Compounds **1**−**36** were tested for their ability to displace [^{3}H]N^6-cyclohexyladenosine ([^{3}H]CHA) from A_1 AR in bovine cerebral cortical membranes, [^{3}H]2-[4-(2-carboxyethyl)phenethyl]amino-5′-(*N*-ethylcarbamoyl)adenosine ([^{3}H]CGS 21680) from A_{2A} AR in bovine striatal membranes, and [^{125}I]N^6-(4-amino-3-iodobenzyl)-5′-(*N*-methylcarbamoyl)adenosine ([^{125}I]AB-MECA) from cloned hA_3 receptor stably expressed in CHO cells. In fact, due to the high species differences in A_3 primary amino acid sequence,[42−44] we tested our A_3 AR ligands on cloned hA_3 receptors.

Subsequently, some selected compounds (**2**, **3**, **5**, **8**, **9**, **10**, **12**, **17**, **20**, **28**, **35**, and **36**) showing high hA_3 AR affinity (K_i < 100 nM) were tested for their ability to displace [^{3}H]CHA from cloned hA_1 AR to establish their A_3 vs A_1 selectivity within the same species. Moreover, the most hA_3 versus hA_1 selective compounds (**3**, **8**, **17**, **20**, **28**, and **35**) were also tested for their ability to displace [^{3}H]-5′-(*N*-ethylcarboxamido)adenosine ([^{3}H]-NECA) from cloned hA_{2A} ARs stably expressed in CHO cells. The binding results of **1**−**36**, together with those of compounds **1A**, **2A**, and **1B** as comparison, are shown in Tables 1−4. Moreover, the binding data of theophylline and 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), included as antagonist reference compounds, are also reported.

Results and Discussion

The binding results reported in Tables 1−4 indicate that we have produced some new potent and selective A_3 receptor antagonists belonging to the 1,2,4-triazolo-[1,5-*a*]quinoxaline series. In particular, when the 8-chloro substitution pattern is held constant as in the lead compound CGS 15943 (9-chloro-2-furyl-1,2,4-triazolo-[1,5-*c*]quinazolin-5-amine),[45] replacement of the 4-amino group of compounds **1A** and **2A**[23,31] with a 4-oxo function (Table 1) abolishes the bA_{2A} binding, while it reduces to different degrees the bA_1 and the hA_3 affinities depending on the nature of the 2-substituent (compare compounds **1A** and **2A** to **1** and **2**, respectively).

In the 4-amino series (compounds **1A** and **2A**), introduction of the 4-methoxy substituent on the 2-phenyl ring has little effect on both bA_1 (3-fold decrease) and bA_{2A} binding activity (3-fold increase), while it positively influences (30-fold increase) the hA_3 affinity (compare compound **2A** to **1A**).[31] To a minor extent, the same applies in the 4-oxo series: while this modification has little or no effect on both bA_1 and bA_{2A} binding activity, the hA_3 affinity increases about 6-fold (compare **2** to **1**).

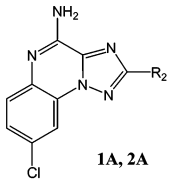
In general, all the 4-oxo derivatives, **1**−**15**, are inactive or active in the micromolar range at the bA_{2A} receptor. For this reason, the 4-amino group seems to be essential for the interaction of these derivatives with the bA_{2A} subtype.[26] Moreover, while most of the compounds are active at the hA_3 receptor in the low micromolar range, the bA_1 binding activities have, in general, values higher than 1 μM. Among the 8-chloro-substituted series (derivatives **1**−**6**), all the compounds follow this trend for the bA_1 binding activity with the exception of the 2-thienyl derivative **4**, which is inactive. These data suggest that the nature of the substituent at position-2 of the triazoloquinoxaline ring system influences just a little the bA_1 receptor−ligand interaction. In contrast, the presence of either the 2-furyl or 2-thienyl moiety at the same position positively affects the bA_{2A} binding affinity: in fact, **3** and **4** are two among the only three compounds herein reported that are endowed with a micromolar bA_{2A} binding activity. Moreover, the nature of the 2-substituent seems to be essential in affecting markedly the activity at the hA_3 receptor subtype; in fact, while the 2-(4-methoxyphenyl) derivative **2** is 6-fold more active than the 2-phenyl-substituted **1**, introduction of the 2-furyl group at the 2-position (compound **3**) produces a 30-fold ameliorated hA_3 binding affinity. A possible explanation for this trend is that the oxygen of the furane ring engages a hydrogen bond with a specific proton donor site on the receptor; this hydrogen bond is less effective in the corresponding 2-(3-furyl) derivative **5** showing a K_i value comparable to that of the 2-phenyl-substituted derivative **1**.

It is difficult to explain the low hA_3 binding activity of the 2-(2-thienyl) derivative **4** when compared to the corresponding 2-(2-furyl) compound **3**. In fact, the binding result seems to indicate that the sulfur atom of the 2-(2-thienyl) moiety, in contrast to the oxygen of the 2-(2-furyl) group, is not capable of engaging a hydrogen bond receptor−ligand interaction. The hA_3 affinity of the 2-(3-thienyl) derivative **6** is in accordance with that of compound **5**.

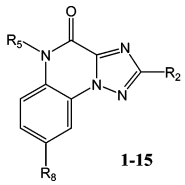
Elimination of the 8-chloro atom, in general, positively affects the bA_1 affinity (compare compounds **1**, **3**, and **4** to **7**, **9**, and **10**, respectively) the only exception being the 2-(4-methoxyphenyl) derivative **8**, which is completely inactive. In contrast, this modification does not influence the bA_{2A} binding activity, while it has diverse effects on the hA_3 binding depending on the nature of the 2-substituent. In particular, when a phenyl or 2-furyl group is present at position 2, a 3-fold decrease of the hA_3 binding affinity is observed (compare compounds **1** and **3** to **7** and **9**, respectively), while an ameliorated interaction is obtained when the 2-substituent is represented by a 4-methoxy-phenyl or a 2-thienyl group (compare derivatives **2** and **4** to **8** and **10**, respectively). It is worth noting that elimination of the 8-chloro substituent gives rise to the most potent hA_3 receptor antagonists herein reported, that is, compounds **8** and **10**.

Replacement of the 8-chlorine atom of **2** with an 8-methyl group produced a 10-fold increase of hA_3 AR affinity. In fact, the 2-(4-methoxyphenyl)-8-methyl-substituted derivative **12** is one of the most active compounds at the hA_3 subtype among the 1,2,4-triazolo-

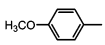
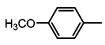
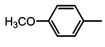
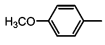
Table 1. Binding Activity of 2-(Hetero)aryl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one Derivatives at Bovine A_1 and A_{2A} and Human A_3 ARs



1A, 2A



1-15

				Ki (nM) [a] or I %		
compd	**R_2**	**R_5**	**R_8**	**bA_1 [b]**	**bA_{2A} [c]**	**hA_3 [d]**
1A [e]		H	Cl	50 ± 4.2	161 ± 14.1	91.4 ± 7.6
2A [e]		H	Cl	150 ± 13.7	53.4%	2.9 ± 0.18
1		H	Cl	2400 ± 180	0%	163 ± 13
2		H	Cl	1060 ± 89	12.1% [f]	25.8 ± 1.9
3		H	Cl	3660 ± 250	5160 ± 320	6 ± 0.48
4		H	Cl	54.2% [f]	6530 ± 386	257 ± 17
5		H	Cl	1950 ± 170	25.3% [f]	78 ± 6.3
6		H	Cl	1000 ± 99	54.2% [f]	126 ± 11
7		H	H	268 ± 22	7%	396 ± 29
8		H	H	0% [f]	8.7% [f]	2.1 ± 0.10
9		H	H	1130 ± 107	4750 ± 290	33 ± 2.7
10		H	H	2940 ± 256	54% [f]	2 ± 0.11
11		H	CH_3	525 ± 49	25%	111 ± 9
12		H	CH_3	1440 ± 133	5% [f]	2.5 ± 0.09
13		CH_3	Cl	18%	0%	19%
14		CH_3	H	1080 ± 98	8.8%	30%
15		CH_3	CH_3	508 ± 48	11.5%	308 ± 28
Theophylline	-	-	-	3800 ± 340	21000 ± 1800	86000 ± 7800
DPCPX	-	-	-	0.5 ± 0.03	337 ± 28	1300 ± 125

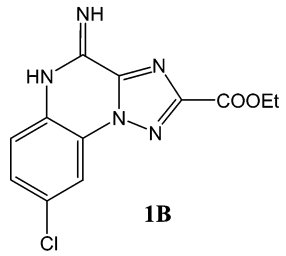
[a] The K_i values are means ± SEM of four separate assays, each performed in triplicate. [b] Displacement of specific [^{3}H]CHA binding in bovine brain membranes or percentage of inhibition (*I*%) of specific binding at 20 μM concentration except where stated otherwise. [c] Displacement of specific [^{3}H]CGS 21680 binding from bovine striatal membranes or percentage of inhibition (*I*%) of specific binding at 20 μM concentration except where stated otherwise. [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] Reference 31. [f] Percentage of inhibition (*I*%) of specific binding at 34 μM concentration.

[1,5-*a*]quinoxaline series. In contrast, this modification maintained both bA_1 and bA_{2A} binding activity unchanged.

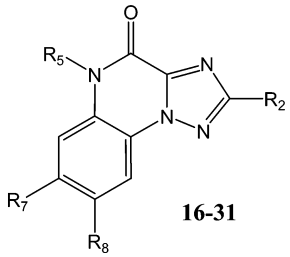
When the 2-phenyl substituent on the triazoloquinoxaline nucleus is kept constant, introduction of a methyl group at position 5 (derivatives **13**−**15**) does not influence the bA_{2A} binding activity, while it reduces both the bA_1 and hA_3 affinities to different extents; the only exception is represented by compound **15**, which is equiactive to the corresponding des-methyl derivative **11** at the bA_1 receptor subtype.

In pursuing our studies on the 1,2,4-triazolo[1,5-*a*]-quinoxaline ring system, we have replaced the 2-(hetero)aryl moiety of compounds **1**−**15** (Table 1) with an ethyl carboxylate group (Table 2). The improved hA_3 binding activity of the 2-(2-furyl) derivatives **3** and **9** compared to the corresponding 2-phenyl ones **1** and **7**, respectively, has suggested this modification: in fact, an ethyl carboxylate substituent could be capable of giving the hydrogen bond interaction with a receptor site hypothesized above for the 2-furyl moiety. Thus, replacing the 2-phenyl group of **1** with an ethyl carboxylate group yields compound **16**, which is, without any doubt, less active at the hA_3 receptor than **1** (Table 2). Nevertheless, it has to be noted that while the 2-carboxyethyl-4-imino derivative **1B**[29] is completely

Table 2. Binding Activity of Ethyl 2-Carboxy-Substituted Trizoloquinoxaline Derivatives at Bovine A_1 and A_{2A} and Human A_3 ARs



1B



16-31

					K_i (nM)[a] or *I*%		
compd	R_2	R_5	R_7	R_8	bA_1[b]	bA_{2A}[c]	hA_3[d]
1B[e]					5 ± 0.7	4900 ± 396	15%
16	COOEt	H	H	Cl	20%	7%[f]	557 ± 38
17	COOEt	H	H	H	26%	3.6%	70.8 ± 4.5
18	COOEt	H	H	CH_3	41%	6.5%	108 ± 8.9
19	COOEt	H	H	NO_2	58%[g]	7.4%	0%
20	COOEt	H	H	NH_2	64%	32.7%	25 ± 11
21	COOEt	H	H	$NHCOCH_3$	14170 ± 902	8.8%	673 ± 58
22	COOEt	H	Cl	H	48%	15.2%	340 ±26
23	COOEt	H	NH_2	H	43%	9%	354 ± 19
24	COOEt	CH_3	H	Cl	8%	0%	60%
25	COOEt	CH_3	H	H	6.4%	9.2%	820 ± 54
26	COOEt	C_2H_5	H	H	13.3%[f]	20.5%[f]	0%
27	COOEt	OH	H	H	6.9%[f]	2%[f]	20%
28	COOEt	COOEt	H	H	14%	4%	28.2
29	H	H	H	Cl	0%	23%	18.8%
30	H	H	H	H	29%	20%	53%
31	H	CH_3	H	Cl	11%	8.9%	0%

[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 except where stated otherwise. [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 except where stated otherwise. [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] Reference 29. [f] Percentage of inhibition (*I*%) of specific binding at 34 μM concentration. [g] Percentage of inhibition (*I*%) of specific binding at 10 μM concentration.

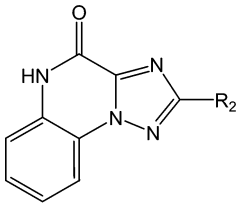
inactive at the hA_3 receptor, the corresponding 4-oxo compound **16** showed a binding activity in the micromolar range. Moreover, the latter shows no affinity for either bA_1 or bA_{2A} AR; this is the trend of all the ethyl 2-carboxylate derivatives **16**−**28**, which are inactive or very poorly active (compound **21)** at both these receptor subtypes. In contrast to derivative **16**, some of the herein reported compounds have a good hA_3 binding activity, in general depending on the nature of the substituent on the benzo-fused moiety. Elimination of the chlorine atom at position 8 increases the hA_3 activity (compare **16** to **17**), while movement from position 8 to 7 maintains the K_i value unchanged (compare compound **16** to **22**), thus suggesting that the presence of an electron-withdrawing substituent is not profitable for anchoring at the hA_3 receptor site. This hypothesis may find confirmation in the total absence of hA_3 activity of compound **19**, which bears an 8-nitro group. In contrast, introduction at position 8 of electron-donating substituents does not affect (methyl group) or 3-fold increases (amino group) the hA_3 binding affinity (compare compound **17** to **18** and **20**). It is possible that the 8-amino group plays a role in the receptor−ligand interaction by engaging a hydrogen bond with a receptor site. This hypothesis can be supported by the 14-fold reduction in potency that can be observed when the amino group is moved from position 8 to 7 (compound **23**). Introduction of an acetyl group on the 8-amino moiety (compound **21**), though increasing the 8-NH acidity, strongly decreases the hA_3 binding activity; this result could suggest the existence of a receptor pocket with a limited steric tolerance.

As observed above for the 2-aryl-substituted series (Table 1), alkylation of the NH at position 5 causes a dramatic reduction in hA_3 potency (compounds **24**−**26**), which however falls into the micromolar range for compound **25**. Also introduction of the hydrophilic hydroxy group at the same position (compound **27)** completely abolishes the hA_3 receptor affinity. In contrast, introduction of a 5-ethyl carboxylate group (compound **28**) is profitable for hA_3 receptor−ligand interaction: in fact, compound **28** is one of the most active hA_3 receptor antagonists among the 2-carboxy-substituted derivatives, **16**−**28** and **32**−**36**.

The importance of the presence of a (hetero)aryl or an ethyl carboxylate group at position 2 for the interaction of these 1,2,4-triazolo[1,5-*a*]quinoxaline derivatives with all three ARs is suggested by the total inactivity of compounds **29** and **30**, which are devoid of the 2-substituent.

Further results regarding investigation of the 2-position of the triazoloquinoxaline moiety are reported in Table 3. Transformation of the ethyl ester (compound **17**) into the corresponding carboxylic acid (compound **32**) or carboxamide (compound **33**) produces a total collapse of the hA_3 binding affinity, while trans-esterification to the isopropyl or benzyl ester (derivatives **34** and **35**) yields, respectively, a 6-fold reduction or a preservation of potency. A good hA_3 affinity is also observed for the benzylamide **36**, which can be considered a bioisoster of the ester **35**. These data may indicate that the pocket that binds the substituent at position 2 has precise steric requirements, the isopropyl

Table 3. Binding Activity of 2-Carboxy-Substituted Triazoloquinoxaline Derivatives at Bovine A_1 and A_{2A} and Human A_3 ARs



17, 32-36

		K_i (nM)[a] or *I*%		
compd	R_2	bA_1[b]	bA_{2A}[c]	hA_3[d]
17	COOEt	26%	3.6%	70.8 ± 5.8
32	COOH	10%[e]	0%[e]	6.4%
33	$CONH_2$	15%	17%	32%
34	$COOCH(CH_3)_2$	0%	2.9%	420 ± 23
35	$COOCH_2C_6H_5$	3220 ± 250	47%	51.6 ± 3.9
36	$CONHCH_2C_6H_5$	578 ± 71	27%	82 ± 6.3

[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] Percentage of inhibition (*I*%) of specific binding at 34 μM concentration.

Table 4. Binding Activity of Some Selected Triazoloquinoxaline Derivatives at Human A_1 and A_{2A} ARs

compd	K_i (nM)[a] or *I*% hA_1[b]	hA_3 vs hA_1 selectivity ratio	K_i (nM)[a] or *I*% hA_{2A}[c]	hA_3 vs hA_{2A} selectivity ratio
2A[d]	36 ± 2.8	12.4	21%	≫3400
2	3.29 ± 0.22	0.13		
3	2740 ± 128	456.7	33%	≫1700
5	124.7 ± 9.8	1.6		
8	32%	≫4700	49%	>4700
9	1926 ± 13	58		
10	2 ± 0.1	1		
12	2.86 ± 0.17	1.1		
17	5%	≫150	0%	≫150
20	33%	≫400	22%	≫400
28	12%	≫350	9%	≫350
35	33%	≫200	27%	≫200
36	205 ± 16	2.5		
theophylline	6200 ± 530	0.07	7900 ± 630	0.092
DPCPX	3.2 ± 0.2	0.0025	260 ± 18	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. [c] Displacement of specific [^{3}H]NECA binding in cloned hA_{2A} receptors expressed in CHO cells or percentage of inhibition (*I*%) of specific binding at 10 μM concentration. [d] Reference 31.

ester **34** being 6-fold less active than the corresponding benzylic one **35**.

Introduction of hindering substituents at position 2 (compounds **35** and **36**) also produces an increase in the bA_1 binding activity, while other modifications yield derivatives **32**−**34**, which are completely inactive. All the compounds reported in Table 3 do not bind at the bA_{2A} receptor.

To evaluate the A_3 versus A_1 and A_{2A} selectivity among the same species, we tested some selected compounds on both hA_1 and hA_{2A} AR, together with the corresponding 4-amino-derivative **2A** as reference compound.[31] It is particularly evident (Table 4) that all the compounds tested on hA_{2A} receptors (**3**, **8**, **17**, **20**, **28**, and **35**) were completely inactive at this AR subtype and thus highly hA_3 versus hA_{2A} selective. In contrast, the hA_1 binding results indicate that most of the 2-(hetero)-aryl compounds (**2**, **5**, **10**, and **12**) are nonselective or scarcely selective hA_3 versus hA_1 receptor antagonists. The only exceptions are represented by the 8-chloro-2-furyl-derivative **3** and the 2-(4-methoxyphenyl) compound **8**. In particular, the latter is inactive at both the hA_1 and hA_{2A} AR, and consequently, it possesses a high hA_3 versus hA_1 and hA_{2A} selectivity. Thus, the 2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (**8**) can be considered one of the most potent selective AR antagonists reported till now.

Moreover, it has to be noted that all the tested 2-carboxy-substituted compounds, **17**, **20**, **28**, and **35**, are highly hA_3 versus hA_1 selective with the exception of the 2-benzyl-carboxamide **36**. This result is particularly important considering that introduction of a 2-carboxylate function on the tricyclic heteroaromatic system of similar size and shape is an original approach to obtain AR antagonists.[29]

In conclusion, the SARs of the 2-heteroaryl-triazoloquinoxalin-4-one derivatives studied here are in accordance with those of previously reported series.[26−28,30] In particular, the importance of a methoxy group in the para position on the 2-phenyl ring for increasing potency and, in some cases, selectivity toward the hA_3 AR was confirmed.[26,27,31] Most important and intriguing, replacement of the 2-(hetero)aryl moiety with a 2-carboxylate function produces a large increase of hA_3 versus hA_1 selectivity by maintaining a good hA_3 AR binding activity. Moreover, it has to be noted that the triazoloquinoxaline framework was utilized for the synthesis of a large number of previously reported Gly/*N*-methyl-D-aspartate (NMDA) or α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonists.[39−41] Thus, we can consider this tricyclic ring system as a potential multitarget drug scaffold.

Structure−activity relationships of these triazoloquinoxaline derivatives were explained by analyzing the three-dimensional structure of the antagonist−receptor models obtained by molecular docking simulation.

We recently proposed, using a computational approach, the putative TM binding motif of 1,2,4-triazolo-[4,3-*a*]quinoxaline analogues[30] and 8-chloro-substituted 1,2,4-triazolo[1,5-*a*]quinoxaline family[31] on hA_3 receptor. As previously reported, the recognition of classic hA_3 AR antagonists seems to occur in the upper region of the TM helical bundle. In particular, we described that TM domains 3, 5, 6, and 7 seem to be crucial for the recognition of both agonists and antagonists.[46,47] As found for other classes of hA_3 receptor antagonists, both quinoxaline families[30,31] fit nicely inside the TM region of our proposed hA_3 receptor model.[30] To complete the validation of our receptor model, molecular modeling studies have been carried out for all the compounds reported in Tables 1−3. Consistent with our previously published docking studies on 4-amino-1,2,4-triazolo[1,5-*a*]quinoxaline derivatives, the binding of the corresponding 1,2,4-triazolo[1,5-*a*]quinoxalin-4-one moiety seems to occur in the same region of the helical bundle. A reasonable steric and electrostatic complementarity

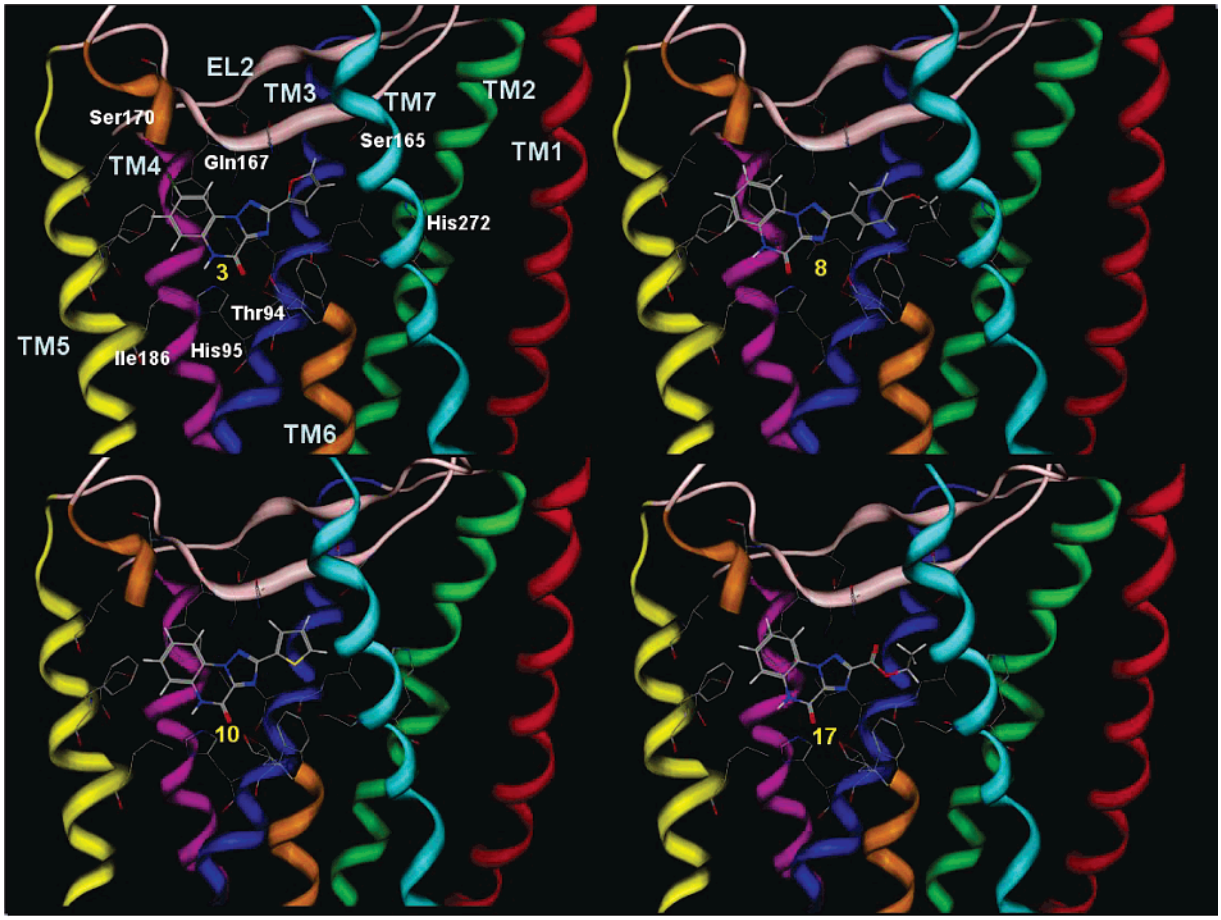


Figure 1. Binding site of the new triazoloquinoxaline derivatives in the human A_3 receptor: derivatives **3** (upper left), **8** (upper right), **10** (bottom left), and **17** (bottom right) docked into the ligand binding crevice of the human A_3 receptor viewed from the membrane side facing TM helices 5 and 6. TM hydrogen atoms are not displayed. Receptor domains and TM amino acids are labeled only for derivative **3** (upper left).

has been found among all analyzed derivatives and the hypothetical binding cavity on hA_3 receptor model. However, based on our model, derivatives **3**, **8 10**, and **17** present the best fitting into the hA_3 binding cleft (Figure 1). It is particularly interesting that also in the new 1,2,4-triazolo[1,5-*a*]quinoxaline-4-one series at least four stabilizing hydrogen-bonding interactions can be observed in all energetically stable docked conformations. Thr94 (TM3), His95 (TM3), Ser170 (EL2), and Asn250 (TM6) seem to characterize the ligand recognition region in the receptor cavity, as shown in Figure 1. In particular, the novel replacement of the 4-amino with the 4-oxo moiety seems to be generally accepted even if it is crucial for differentiating both potency and affinity among this series of new A_3 AR antagonists. Analyzing our structure−activity data in detail, we find that the 4-oxo position is surrounded by two polar amino acids, Thr94 (TM3) and His95 (TM3). It is assumed that these two polar amino acids give stabilizing interactions with the 4-oxo moiety of all new synthesized compounds through hydrogen bonding.

Considering the other general structural binding requirements, the triazoloquinoxaline nucleus should be most favorably oriented perpendicular to the plane of the lipid bilayer with the 2-aryl (or 2-heteroaryl) substituent in proximity to TM2 and TM7 and the 8-chlorobenzene moiety close to TM5 and TM6. Depending on the presence and, consequently, chemical nature of the substituents in position 7 and 8, the triazoloquinoxaline scaffold slightly shifts its position inside the TM cleft to optimize both steric and electrostatic complementarity. As previously described,[30,31] the 2-aryl and 2-heteroaryl substituents are always positioned in a relatively small cleft between TM2 and TM7. In our model, the furan ring of derivative **3** sits nicely in this receptor cavity forming a stabilizing interaction between the oxygen atom of the furan ring and the NH_2 of Gln167 (EL2).

However, the corresponding analogue with the *p*-methoxy group at the 2-aryl position (derivative **2** in Table 1) has slightly reduced activity because it loses its steric complementarity with the TM receptor cavity. Accordingly, the removal of the chlorine atom from position 8 (derivative **8** in Table 1) restores the good antagonist−receptor complementarity. Moreover, considering the 2-aryl family, an additional hydrogen-bonding interaction seems occur between the 4-methoxy group of the 2-aryl substituent and the hydroxy group of Ser165 positioned on the EL2 domain, as previously reported.[30,31] This supplementary interaction could explain the increase of affinity experimentally observed when the para position of the 2-aryl substituent is replaced with a methoxy group, when other unfavorable steric or electrostatic interactions or both do not occur (compare, for example, derivatives **1A** and **2A**, **1** and **2**, and **7** and **8** in Table 1).

Depending on the nature and on the position of the substituents (R_2, R_5, and R_8 in Table 1), the balancing

of steric and electrostatic control can also influence the activity of the 2-heteroaryl derivatives. The fine structural control imposed by the receptor binding cleft can be observed when, in the presence of a chlorine atom at position-8, the 2-furan moiety was replaced with a phenyl or thienyl substituent: the stabilizing interaction with Gln167 disappears or drastically reduces its importance. In the case of the 2-(2-furyl)-derivatives 3 and 9, we identified a favorable dipole−dipole interaction between the furan ring and the amide group of the Gln167 side chain. This stabilizing interaction is missing in both 2-(2-thienyl) (compounds **4** and **10**) and 2-phenyl (derivatives **1** and **7**) modified antagonists. This similar chemical control can operate also when we consider the 2-(3-furyl)-substituted compound. In fact, depending on the link position of the furan ring to the triazoloquinoxaline moiety (position 2 or 3), the total dipole moment of the molecule can change in both intensity and orientation.

Another interesting observation is that 2-carboxy-substituited triazoloquinoxalines are still tolerated by the TM receptor cavity, even if we always detected a reduction of their activities compared with the corresponding 2-furan or 2-(4-methoxyphenyl) derivatives (see Tables 2 and 3). As shown in Figure 1, following our docking simulations, the ethyl ester moiety roughly maintains the 2-aryl (or 2-heteroaryl) substituent position inside the TM recognition cavity. The most notable structural differences among these 2-carboxy-substituited antagonists with respect to all the other 2-aryl or 2-heteroaryl derivatives is the loss of substituent planarity at position 2. This fact inevitably introduces an unfavorable entropic control.

Moreover, also in this case depending on the nature and on the position of the substituents (R_2, R_5, R_7, and R_8 in Tables 2 and 3), the balancing of steric and electrostatic control can profoundly influence the activity of these new 2-carboxy-substituited derivatives. Fine-tuning of the observed activity differences among the new 2-carboxy-substituited antagonists seems to be effected by the presence of a substituent at position-8. Indeed, the activity of all 8-chlororinated, or vice versa of all 8-deschlorinated, antagonists is dependent on the chemical nature of the substituent at position 2. In fact, the triazoloquinoxaline scaffold can slightly shift its position inside the TM cleft to optimize both steric and electrostatic complementarity, depending on both the presence and the chemical nature of the substituents at positions 2 and 8. In particular, the case of the 8-chloro-substituted derivatives has been analyzed. In the 8-chloro-2-(hetero)aryl family, a stabilizing dipolar interaction between the chlorine substituent and the hydroxy group of Ser170 (EL2) occurs. To preserve this interaction, the 8-chloro derivatives have to shift their position inside the receptor cleft, reducing the stabilizing effect of the dipole interaction between the Gln167 (EL2) and the substituent at position 2. However, this supplementary interaction makes up for this destabilizing effect and explains the increase of affinity observed in the 8-chloro-2-(hetero)aryl derivatives **1** and **3** with respect to the corresponding deschloro compounds **7** and **9**. In contrast, in the 2-carboxy-substituted series removal of the 8-chlorine atom produces an increase of binding affinitiy (compare **16** to **17**). The loss of 2-substituent planarity and the corresponding increase of its steric hindrance does not guarantee that the ethyl ester preserves the stabilizing interaction with Gln167 (EL2). In fact, the best docked 8-chloro compound **16** shifts its position significantly inside the binding cleft, thus reducing its steric and electrostatic complementarity.

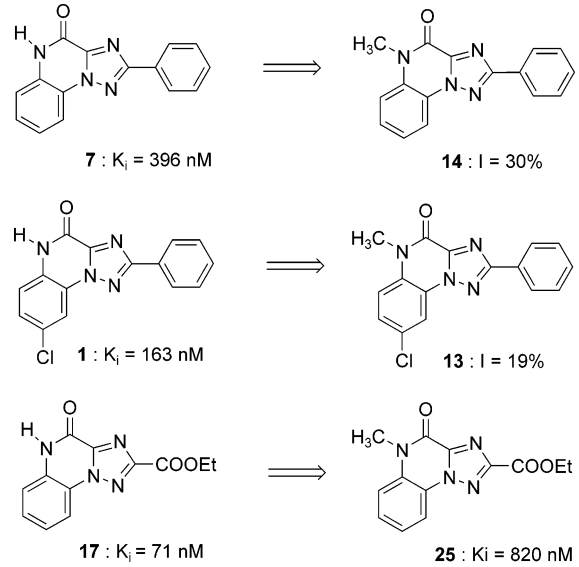


Figure 2. Triazoloquinoxaline derivatives **7**, **1**, and **17** and their corresponding *N*-methyl derivatives, **14**, **13**, and **25**. Binding activity at hA_3 receptor is also shown.

As illustrated in Table 3, the chlorine atom at position 8 can be substituted only with other relatively small polar substituents (compare derivatives **16**−**21**, Table 3) without total loss of their binding activities. In effect, looking inside the receptor cleft, position 8 is accommodated in a tiny cavity near TM4 and TM5 (see Figure 1).

Interestingly, derivative **28** (R_5 = COOEt, Table 3) presents an increased A_3 receptor affinity compared with derivatives **25** (R_5 = CH_3, Table 3) or **17** (R_5 = H, Table 3). In this case, our docking studies have shown two additional stabilizing interactions among the carboxylate moiety at position-5 and the two polar amino acids, Thr94 (TM3) and His95 (TM3).

Finally, a very peculiar effect was observed when methylation of the N-5 position occurred. As described in Tables 1 and 2, alkylation of the NH at position 5 causes a dramatic reduction in hA_3 potency as summarized in Figure 2. This peculiar effect is very ambiguous, and two reasonable explanations can be proposed. The first hypothesis is a severe steric hindrance control in proximity of the NH at position 5. In fact, Ile186 (TM5) becomes closer to the van der Waals contact distance with the methyl group of all 5-N-methylated derivatives. The second intriguing hypothesis is related to a possible "tautomeric" selection brought about by the receptor binding site. All 4-oxo compounds can formally exit in two different tautomers: the keto and the enol forms. Considering their chemical stability, in water solution all keto forms are 8−10 kcal/mol more stable with respect to the corresponding enol forms (data not shown; see Experimental Section for details). However, as shown by docking simulations, enol forms fit inside the TM binding cavity equally well or better than keto forms (see Figure 3,

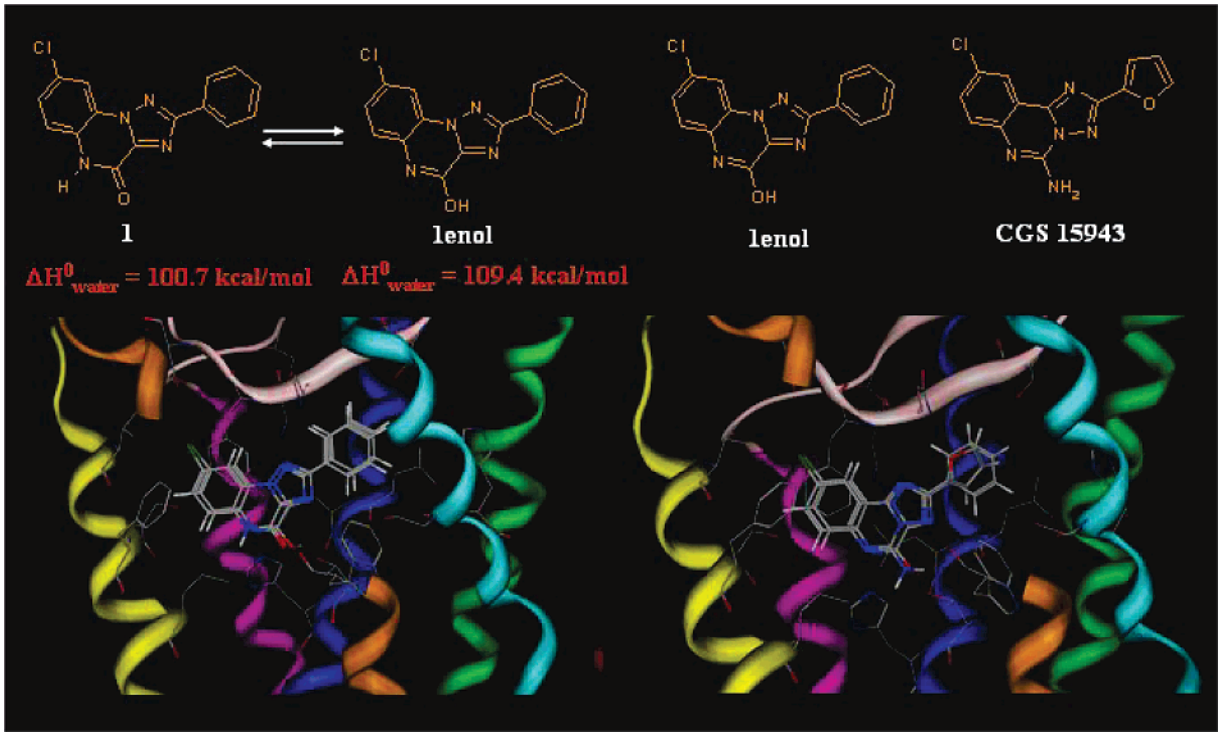


Figure 3. The left panel shows triazoloquinoxaline derivative **1** and its possible enolic form docked into the ligand binding crevice of the human A_3 receptor viewed from the membrane side facing TM helices 5 and 6. The right panel shows the best docking conformation of the enolic form of triazoloquinoxaline derivative **1** compared to CGS 15943 docked into the ligand binding crevice of the human A_3 receptor.

derivative **1** and its enol form). The best docked enol form binding arrangement is superimposable on the keto form conformation, but a stronger interaction among the 4-enol moiety and the two polar amino acids, Thr94 (TM3) and His95 (TM3), occurrs. The enol form of the 4-oxo family is chemically equivalent to the 4-amino derivatives, such as CGS 15943 or **2A**. In fact, as reported in Figure 3, the 4-OH group of the docked enol form maintains the 4-NH_2 substituent position and also forms similar interactions inside the TM recognition cavity. Obviously the 5-N-methylated derivatives do not engage in a tautomeric equilibrium. Further investigations are in progress in our laboratories to support this tautomer selection.

Conclusions

The present study has pointed out that the 1,2,4-triazolo[1,5-*a*]quinoxaline moiety is a versatile tool for the design of selective hA_3 AR antagonists. The classical SAR analysis, supported by molecular docking simulation, confirmed previous findings and also provides new interesting insights about the steric and electrostatic requirements that are important for the optimal anchoring of these triazoloquinoxaline derivatives at the hA_3 receptor recognition site. In fact, the importance of the 2-(hetero)aryl group was confirmed, and as a new finding, the 2-carboxylate function has emerged as a possible alternative to the classical 2-(hetero)aryl moiety for obtaining potent tricyclic hA_3 AR antagonists of similar size and shape. For this reason, further modifications of these derivatives are planned.

Experimental Section

Chemistry. Silica gel plates (Merck F254) 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, and N, and the results were within ±0.4% of the theoretical values except where stated otherwise (Supporting Information). 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.

***N*¹-(5-Methyl-2-nitrophenyl)-*N*²-(4-methoxybenzoyl)-hydrazide (37).** A solution of *para*-anisoyl chloride (7.77 mmol) in anhydrous pyridine (3 mL) was added dropwise at room temperature to a solution of 5-methyl-2-nitrophenylhydrazine[35,37] (5.98 mmol) in anhydrous pyridine (18 mL). The reaction mixture was heated at reflux for 1 h. Distillation of the solvent under reduced pressure gave an orange solid, which was treated with 2 M HCl (8 mL), collected under vacuum, and washed with water. Yield 89%; mp 182−184°C (ethanol); H^1 NMR (DMSO-d_6) δ 2.28 (s, 3H, CH_3), 3.83 (s, 3H, OCH_3), 6.68 (d, 1H, ar, J = 8.79 Hz), 6.93 (s, 1H, ar), 7.06 (d, 2H, ar, J = 8.79 Hz), 7.94 (d, 2H, J = 8.79 Hz), 8.02 (d, 1H, ar, J = 8.79 Hz), 9.41 (s, 1H, NH), 10.63 (s, 1H, NH); IR 3340, 3250, 1645 cm^{-1}. Anal. ($C_{15}H_{15}N_3O_4$) C, H, N.

***N*¹-(5-Methyl-2-nitrophenyl)-*N*²-[α-chloro-(4-methoxybenzyliden)]hydrazine (38).** A suspension of **37** (4.31 mmol) in $POCl_3$ (12 mL) was heated at reflux for 1 h. The excess $POCl_3$ was removed under reduced pressure, and the red residue was treated with cyclohexane (30 mL). Evaporation of the solvent under reduced pressure gave a mull, which was treated with cyclohexane/ethyl acetate 1:1. The orange solid obtained was collected by filtration and recrystallized. Yield 60%; mp 125−126°C (cyclohexane/ethyl acetate); H^1 NMR (DMSO-d_6) δ 2.42 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 6.85 (d, 1H, ar, J = 8.79 Hz), 7.06 (d, 2H, ar, J = 8.79 Hz), 7.71 (s, 1H,

ar) 7.94 (d, 2H, J = 8.79 Hz), 8.06 (d, 1H, ar, J = 8.79 Hz), 11.16 (s, 1H, NH); IR 3290, 1610 cm^{-1}. Anal. ($C_{15}H_{14}ClN_3O_3$) C, H, N.

***N*^{1}-(5-Methyl-2-nitrophenyl)-*N*^{2}-[α-amino-(4-methoxybenzylidene)]hydrazine (39).** Ammonia was bubbled until saturation into a stirred solution of **38** (1.69 mmol) in anhydrous dioxane (20 mL). The mixture was stirred at room temperature for 1 h and then was diluted with water (30 mL) to yield a dark red solid, which was collected and washed with water. Yield 81%; mp 130−132 °C (ethanol/water); ^{1}H NMR (DMSO-d_6) δ 2.32 (s, 3H, CH_3), 3.80 (s, 3H, OCH_3), 6.52−6.65 (m, 3H, NH_2 + ar), 6.99 (d, 2H, ar, J = 8.79 Hz), 7.48 (s, 1H, ar), 7.85 (d, 2H, ar, J = 8.79 Hz), 7.94 (d, 1H ar, J = 8.79 Hz), 10.27 (s, 1H, NH); IR 3440, 3360, 3300 cm^{-1}. Anal. ($C_{15}H_{16}N_4O_3$) C, H, N.

Ethyl 1-(5-Methyl-2-nitrophenyl)-3-(4-methoxyphenyl)-1,2,4-triazolo-5-carboxylate (40). The amidrazonate **39** (0.94 mmol) was slowly added in small portions to a solution of ethyloxalyl chloride (2.97 mmol) in anhydrous toluene (20 mL) at 80 °C. The reaction mixture was heated at reflux for 3 h. Distillation of the solvent under reduced pressure gave an oil, which was treated with a little ethyl acetate; a yellow solid precipitates, which was collected and washed with diethyl ether. Yield 48%; mp 160−162 °C (toluene); ^{1}H NMR (DMSO-d_6) δ 1.15 (t, 3H, CH_3, J = 7.11 Hz), 2.49 (s, 3H, CH_3), 3.81 (s, 3H, CH_3), 4.24 (q, 2H, CH_2, J = 7.11 Hz), 7.06 (d, 2H, ar, J = 8.79 Hz), 7.68 (d, 1H, ar, J = 8.42 Hz), 7.76 (s, 1H, ar), 7.98 (d, 2H, ar, J = 8.79 Hz), 8.23 (d, 1H, ar, J = 8.42 Hz); IR 1730, 1620 cm^{-1}. Anal. ($C_{19}H_{18}N_4O_5$) C, H, N.

4,5-Dihydro-8-methyl-2-(4-methoxyphenyl)-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (12). Iron powder (1.0 g) was added to a suspension of **40** (1.0 mmol) in glacial acetic acid (6 mL). The mixture was heated at 90 °C for 10 min. Evaporation at reduced pressure of the solvent yielded a residue, which was suspended in water (50 mL) and then bleached with 6 N HCl. The solid was collected by filtration, washed with water, dried, and extracted in soxlhet with acetone (250 mL). Evaporation of the solvent under reduced pressure yielded a solid, which was worked up with diethyl ether (10 mL) and collected by filtration. Yield 70%; mp > 300 °C (acetone); ^{1}H NMR (DMSO-d_6) δ 2.07 (s, 3H, CH_3), 3.84 (s, 3H, OCH_3), 7.12 (d, 2H, ar, J = 8.79 Hz), 7.34 (s, 2H, ar), 7.95 (s, 1H, ar), 8.15 (d, 2H, J = 8.42 Hz), 12.30 (s, 1H, NH); IR 3160, 1680 cm^{-1}. Anal. ($C_{17}H_{14}N_4O_2$) C, H, N.

General Procedure To Prepare 8-Chloro-4,5-dihydro-5-methyl-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (13) and 4,5-Dihydro-5,8-dimethyl-2-phenyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (15). Methyl iodide (1.5 mmol) was added to a suspension of **1** or **11**[35] (1.0 mmol) and sodium hydride (60% dispersion in mineral oil, 2.0 mmol) in anhydrous dimethylformamide (15 mL). The reaction mixture was stirred at room temperature for 2 h and then quenched with ice (30 g). The resulting solid was collected and washed with water.

13: yield 96%; mp > 300 (acetic acid); ^{1}H NMR (DMSO-d_6) δ 3.70 (s, 3H, CH_3), 7.57−7.76 (m, 5H, ar), 8.21−8.25 (m, 3H, ar); IR 1680 cm^{-1}. Anal. ($C_{16}H_{11}ClN_4O$) C, H, N.

15: yield 86%; mp 274−276 °C (acetic acid); ^{1}H NMR ($CDCl_3$) δ 2.56 (s, 3H, CH_3), 3.84 (s, 3H, N−CH_3), 7.38−7.39 (m, 2H, ar), 7.50−7.54 (m, 3H, ar), 8.21 (s, 1H, ar), 8.39−8.44 (m, 2H, ar); IR 1675 cm^{-1}. Anal. ($C_{17}H_{14}N_4O$) C, H, N.

Ethyl *N*^{1}-(5-Methyl-2-nitrophenyl)hydrazono-*N*^{2}-chloroacetate (41). A solution of 36% HCl (1.5 mL) was added to solution of 5-methyl-2-nitroaniline (2.63 mmol) in methanol (4 mL). A solution of natrium nitrite (2.89 mmol) in water (1 mL) was added dropwise to the cooled (0−5 °C) mixture in an overall time of 15 min. The resulting natrium chloride was eliminated by quick filtration under vacuum, and ethyl 2-chloro-3-oxobutanoate (2.89 mmol) was added to the clear solution. The reaction mixture was kept at room temperature for 2 h. The yellow precipitate was collected by filtration and washed with water. Yield 70%; mp 152−154 °C (ethanol); ^{1}H NMR (DMSO-d_6) δ 1.30 (t, 3H, CH_3, J = 7.00 Hz), 2.42 (s, 3H, CH_3), 4.33 (q, 2H, CH_2, J = 7.00 Hz), 7.02 (d, 1H, ar, J = 8.35 Hz), 7.56 (s, 1H, ar), 8.10 (d, 1H, ar, J = 8.35), 11.17 (s, 1H, NH); IR 3260, 1720 cm^{-1}. Anal. ($C_{11}H_{12}ClN_3O_4$) C, H, N.

Ethyl *N*^{1}-(5-Methyl-2-nitrophenyl)-*N*^{2}-oxamidrazonate (42). Ammonia was bubbled until saturation into a stirred solution of **41** (1.40 mmol) in anhydrous dioxane (18 mL). The mixture was stirred at room temperature until the disappearance of the starting material and then was diluted with water (30 mL) to yield a red solid, which was collected and washed with water. Yield 80%; mp 153−155 °C (cyclohexane); ^{1}H NMR (DMSO-d_6) δ 1.28 (t, 3H, CH_3, J = 6.90 Hz), 2.33 (s, 3H, CH_3), 4.28 (q, 2H, CH_2, J = 6.90 Hz), 6.62 (s, 2H, NH_2), 6.70 (d, 1H, ar, J = 8.40 Hz), 7.51 (s, 1H, ar), 7.98 (d, 1H, ar, J = 8.40 Hz), 10.07 (s, 1H, NH); IR 3470, 3380, 3300, 1700 cm^{-1}. Anal. ($C_{11}H_{14}N_4O_4$) C, H, N.

Ethyl *N*^{1}-(5-Methyl-2-nitrophenyl)-*N*^{3}-ethoxalyl-*N*^{2}-oxamidrazonate (43). A solution of ethyloxalyl chloride (2.02 mmol) in anhydrous diethyl ether (2 mL) was slowly added to a suspension of the amidrazonate **42** (1.01 mmol) in anhydrous diethyl ether (2 mL). The reaction mixture was diluted with anhydrous toluene (4 mL) and then heated at reflux for 45 min. Upon cooling a solid precipitated, which was collected and washed with petroleum ether. Yield 73%; mp 150−152 °C (toluene); ^{1}H NMR (DMSO-d_6) δ 1.21−1.33 (m, 6H, $2CH_3$), 2.39 (s, 3H, CH_3), 4.19−4.37 (m, 4H, $2CH_2$), 6.92 (d, 1H, ar, J = 8.20 Hz), 7.97 (s, 1H, ar), 7.98 (d, 1H, ar, J = 8.20 Hz), 10.90 (s, 1H, NH); 13.14 (s, 1H, NH); IR 3370, 3310, 1700 cm^{-1}. Anal. ($C_{15}H_{18}N_4O_7$) C, H, N.

Diethyl 1-(5-Methyl-2-nitrophenyl)-1,2,4-triazolo-3,5-dicarboxylate (44). The finely powdered ethoxalyl derivative **43** (0.68 mmol) was heated at 180 °C for 45 min. The cooled mass was dissolved in chloroform (20 mL), and the organic solvent washed with a diluted (0.5 N) solution of potassium hydroxide (15 mL × 3) and then with water (15 mL). Evaporation under reduced pressure of the dried (anhydrous sodium sulfate) organic solvent afforded a residue, which was treated with a little cyclohexane and collected by filtration. Yield 53%; mp 134−136 °C (cyclohexane); ^{1}H NMR (DMSO-d_6) δ 1.14 (t, 3H, CH_3, J = 7.00 Hz), 1.31 (t, 3H, CH_3, J = 7.00 Hz), 2.47(s, 3H, CH_3), 4.22 (q, 2H, CH_2, J = 7.00 Hz), 4.38 (q, 2H, CH_2, J = 7.00 Hz), 7.72−7.74 (m, 2H, ar), 8.27 (d, 1H, ar, J = 9.60 Hz); IR 1750 cm^{-1}. Anal. ($C_{15}H_{16}N_4O_6$) C, H, N.

Ethyl 4,5-Dihydro-8-methyl-4-oxo-1,2,4-triazolo[1,5-*a*]-quinoxaline-2-carboxylate (18). Iron powder (0.32 g) was added to a solution of **44** (0.32 mmol) in glacial acetic acid (2 mL). The mixture was heated at 90 °C for 1 h. Evaporation under reduced pressure of the solvent yielded a residue, which was treated with water (150 mL), and the resulting suspension was bleached with 6 N HCl. The solid was collected by filtration, dried, and extracted in soxlhet with acetone (250 mL). Evaporation of the solvent at small volume (about 5 mL) under reduced pressure yielded a solid, which was collected by filtration. Yield 58%; mp 270−272 °C (acetone); ^{1}H NMR (DMSO-d_6) δ 1.36 (t, 3H, CH_3, J = 7.10 Hz), 2.42 (s, 3H, CH_3), 4.40 (q, 2H, CH_2, J = 7.10 Hz), 7.35−7.42 (m, 2H, ar), 7.93 (s, 1H, ar), 12.40 (s, 1H, NH); IR 3300, 1745 cm^{-1}. Anal. ($C_{13}H_{12}N_4O_3$) C, H, N.

General Procedure To Prepare Ethyl 8-Chloro-4,5-dihydro-5-methyl-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate (24) and Ethyl 4,5-Dihydro-5-methyl-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate (25). The title compounds were synthesized from **16**[35] and **17**,[38] respectively, according to the procedure reported to obtain derivatives **13** and **15**.

24: yield 85%; mp 278−280 °C (acetic acid); ^{1}H NMR (DMSO-d_6) δ 1.38 (t, 3H, CH_3, J = 6.96 Hz), 3.69 (s, 3H, CH_3), 4.44 (q, 2H, CH_2, J = 6.96 Hz), 7.71−7.83 (m, 2H, ar), 8.20 (d, 1H, ar, J = 2.20 Hz); IR 1750, 1685 cm^{-1}. Anal. ($C_{13}H_{11}ClN_4O_3$) C, H, N.

25: yield 53%; mp 252−254 °C (acetone); ^{1}H NMR (DMSO-d_6) δ 1.36 (t, 3H, CH_3, J = 6.96 Hz), 3.69 (s, 3H, CH_3), 4.44 (q, 2H, CH_2, J = 6.96 Hz), 7.48 (t, 1H, ar, J = 7.69 Hz), 7.63−7.78 (m, 2H, ar), 8.22 (d, 1H, ar, J = 8.06 Hz); IR 1740, 1680 cm^{-1}. Anal. ($C_{13}H_{12}N_4O_3$) C, H, N.

Diethyl 4,5-Dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2,5-dicarboxylate (28). A suspension of **17**[38] (1.15 mmol) and potassium carbonate (2.30 mmol) in acetone (50 mL) was stirred at room temperature for 10 min, and then an excess of ethyl chlorocarbonate (2.30 mmol) was added. The reaction mixture was heated at reflux for 8 h. After elimination by filtration of the resulting solid, the clear solution was evaporated at small volume under reduced pressure; a solid precipitated, which was collected by filtration. Yield 95%; mp 135−137 °C (ethanol); ^{1}H NMR (DMSO-d_6) δ 1.31−1.41 (m, 6H, $2CH_3$), 4.36−4.48 (m, 4H, $2CH_2$), 7.86−7.95 (m, 2H, ar), 8.15 (d, 1H, ar, J = 7.69 Hz), 8.48 (d, 1H, ar, J = 7.69 Hz); IR 1800, 1750, 1690 cm^{-1}. Anal. ($C_{15}H_{14}N_4O_5$) C, H, N.

8-Chloro-4,5-dihydro-5-methyl-1,2,4-triazolo[1,5-*a*]quinoxalin-4-one (31). The title compound was synthesized from **29**[29] (1.0 mmol) according to the procedure described above to obtain derivatives **13** and **15**. Yield 53%; mp 243−245 °C (acetic acid); ^{1}H NMR (DMSO-d_6) δ 3.67 (s, 3H, CH_3), 7.67 (dd, 1H, ar, J = 8.97, 2.20 Hz), 7.76 (d, 1H, ar, J = 8.97 Hz), 8.16 (d, 1H, ar, J = 2.20 Hz), 8.66 (s, 1H, H-2); IR 1690 cm^{-1}. Anal. ($C_{10}H_7ClN_4O$) C, H, N.

4,5-Dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxamide (33). A suspension of **17**[38](0.77 mmol) in absolute ethanol (15 mL) saturated with ammonia was heated in a sealed tube at 120 °C for 15 h. The resulting solid was collected by filtration and washed with water. Yield 68%; mp > 300 °C (2-methoxyethanol); ^{1}H NMR (DMSO-d_6) δ 7.36−7.54 (m, 3H, ar), 7.86 (s, 1H, NH), 8.10 (d, 1H, ar, J = 7.05 Hz), 8.27 (s, 1H, NH), 12.3 (br s, 1H, NH); IR 3570, 3400, 3320, 1700, 1680 cm^{-1}. Anal. ($C_{10}H_7N_5O_2$) C, H, N.

Isopropyl 4,5-Dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate (34). A suspension of **32**[8] (0.96 mmol) in thionyl chloride (12 mL) was heated at reflux for 4 h. The excess of thionyl chloride was removed under reduced pressure, and the residue was treated with cyclohexane (30 mL). Evaporation of the solvent under reduced pressure gave a solid, which was rapidly reacted with 2-propanol (0.261 mmol). The reaction mixture was heated at reflux for 1 h and then was stirred at room temperature for 15 h. Evaporation of the solvent under reduced pressure gave a solid, which was treated with a little diethyl ether and collected by filtration. Yield 70%; mp 294−296 °C (acetone); ^{1}H NMR (DMSO-d_6) δ 1.36 (t, 6H, $2CH_3$, J = 6.00 Hz), 5.20−5.27 (m, 1H, CH), 7.34−7.54 (m, 3H, ar), 8.11 (d, 1H, ar, J = 8.05 Hz), 12.45 (s, 1H, NH); IR 3300, 1750, 1680 cm^{-1}. Anal. ($C_{13}H_{12}N_4O_3$) C, H, N.

Benzyl 4,5-Dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-carboxylate (35). A suspension of **32**[38] (0.96 mmol) in thionyl chloride (12 mL) was heated at reflux for 4 h. The excess of thionyl chloride was removed under reduced pressure, and the residue was treated with cyclohexane (30 mL). Evaporation of the solvent under reduced pressure gave a solid, which was suspended in anhydrous tetrahydrofuran (60 mL). Then, benzyl alcohol (13 mL) was added to the resulting suspension, and the reaction mixture was heated at reflux for 8 h. Evaporation of the solvent at small volume under reduced pressure gave a solid, which was collected by filtration and washed with diethyl ether. Yield 50%; mp 253−255 °C (ethanol); ^{1}H NMR (DMSO-d_6) δ 5.46 (s, 2H, CH_2), 7.39−7.55 (m, 8H, ar), 8.10 (d, 1H, ar, J = 8.42 Hz), 12.47 (s, 1H, NH); IR 1750, 1680 cm^{-1}. Anal. ($C_{17}H_{12}N_4O_3$) C, H, N.

4,5-Dihydro-4-oxo-1,2,4-triazolo[1,5-*a*]quinoxaline-2-benzylcarboxamide (36). Equimolar amounts of *N*-(3-dimethylamino)propyl-*N*′-ethylcarbodiimide hydrochloride and 1-hydroxybenzotriazole (1.50 mmol), followed by an excess of benzylamine (1.94 mmol), were added to a solution of **32**[38] (1.3 mmol) in anhydrous dimethylformamide (25 mL). The reaction mixture was heated at reflux for 24 h. The resulting solid was eliminated by filtration, and the clear solution was evaporated under reduced pressure. When the residue was treated with a little ethyl acetate/methanol 1:1, a solid precipitated, which was collected by filtration and washed with diethyl ether. Yield 32%; mp 291−293 °C (ethanol); ^{1}H NMR (DMSO-d_6) δ 4.47 (d, 2H, CH_2, J = 6.20 Hz), 7.26−7.53 (m, 8H, ar), 8.10 (d, 1H, ar, J = 8.00 Hz), 9.58 (t, 1H, NH, J = 5.86 Hz), 12.4 (br s, 1H, NH); IR 3400, 1690 cm^{-1}. Anal. ($C_{17}H_{13}N_5O_2$) C, H, N.

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 48.

Human A_1, A_{2A}, and A_3 Receptor Binding. Binding experiments at hA_1 and hA_3 ARs, stably expressed in CHO cells, were performed as previously described,[28] using [^{3}H]CHA and [^{125}I]AB-MECA, respectively, as radioligands. Displacement of [^{3}H]NECA from hA_{2A} ARs, stably expressed in CHO cells, was performed as reported in ref 49.

The concentration of the tested compounds that produced 50% inhibition of specific [^{3}H]CHA, [^{3}H]CGS 21680, [^{125}I]AB-MECA, or [^{3}H]NECA 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.[50] The dissociation constants (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, [^{3}H]NECA, and [^{125}I]AB-MECA in hA_1, hA_{2A}, and hA_3 ARs in CHO cell membranes were 1.9, 30, and 1.4 nM, respectively.

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.[51]

Homology modeling, energy calculation, and docking studies were performed using the Molecular Operating Environment (MOE, version 2004.03) suite.[52]

The ground-state geometries of all tautomeric docked structures were fully optimized without geometry constraints using RHF/AM1 semiempirical calculations. Vibrational frequency analysis was used to characterize the minima stationary points (zero imaginary frequencies). Enthalpies of formation in water were calculated using the SM5.4/A solvation model implemented in Spartan O2 software.[53] The software package Spartan O2 was utilized for all quantum mechanical calculations.

Homology Model of the hA_3 AR. Based on the assumption that GPCRs share similar TM boundaries and overall topology,[54] 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 proline 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 has to be given to the second extracellular (E2) loop, which has been described in bovine rhodopsin as folding back over transmembrane helices[54] 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.[55] 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^{-1} $Å^{-1}$.

Molecular Docking of the hA_3 AR antagonists. All antagonist structures were docked into the hypothetical TM binding site by using the DOCK docking program, part of the MOE suite. Searching is conducted within a user-specified 3D docking box, using the Tabù Search protocol[56] and the MMFF94 force field.[57−63] 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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Supporting Information Available: Elemental analyses of the newly synthesized compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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