5-Amino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one: A Versatile Scaffold To Obtain Potent and Selective A_3 Adenosine Receptor Antagonists†

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Binding assays on human A_1, A_{2A}, and A_3 adenosine receptors (ARs) and functional studies on A_{2B} ARs revealed that various 2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1,5(6*H*)-diones **VIII**, previously reported as ligands at the central benzodiazepine receptor (BzR), possess nanomolar affinity at the A_3 AR. Replacement of the amide of **VIII** with an amidine moiety gave the 5-amino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-ones **IX**, which maintain a nanomolar potency at the A_3 AR with selectivity over the BzR. Insertion of a *p*-methoxybenzoyl at the 5-amino moiety enhanced A_3 AR affinity and selectivity over the A_1, A_{2A}, and A_{2B} ARs. The best result of our lead optimization efforts is 9-chloro-5-(4-methoxybenzoyl)amino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one (**23**), which displayed a K_i of 1.6 nM at the A_3 AR and no significant affinity at the other ARs or the BzR. Docking simulations on selected ligands into a model of the A_3 AR allowed us to rationalize the structure−activity relationships of phenyltriazolobenzotriazindiones **VIII** and aminophenyltriazolobenzotriazinones **IX** at the molecular level.

Introduction

Adenosine is a ubiquitous homeostatic substance released from most cells and acts as an agonist at the A_1, A_{2A}, A_{2B}, and A_3 adenosine receptors (ARs), all of which belong to the superfamily of the G-protein-coupled receptors.[1−3] ARs from different species show 82−92% amino acid sequence identity, the only exception being the A_3AR, which exhibits a 74% identity between rats and humans.[4−6]

Responses to activation of ARs are mediated by different second messenger systems, such as calcium or potassium channels (A_1 AR), phospholipase C (A_1 AR, A_{2B} AR, A_3 AR), and phospholipase D (A_3 AR).[7] Furthermore, all four AR subtypes are differently coupled with adenylate cyclase, which is inhibited by activation of A_1 and A_3 ARs, thus decreasing cyclic AMP (cAMP) production, and is stimulated by activation of A_{2A} and A_{2B} ARs, thus increasing cAMP levels.[7] Each AR subtype is currently considered as an attractive target for pharmacological intervention in many pathological conditions such as renal failure, cardiac and cerebral ischemeia, central nervous system (CNS) disorders, neurodegenerative diseases, and inflammatory pathologies, including asthma.[8,9] Accordingly, A_1 AR antagonists have a therapeutic potential as potent diuretics for the prevention of renal failure,[10] arousal stimulators, or cognitive enhancers in Alzheimer's disease and memory disorders;[11] A_{2A} AR antagonists may be promising new drugs to treat neurodegenerative disorders, such as Parkinson's disease;[11,12] A_3 AR antagonists may be employed in the acute treatment of stroke and glaucoma and as antiasthmatic, anti-allergenic and cerebroprotective agents.[13] Furthermore, the significant overexpression of A_3 AR in several types of tumor cells[14,15] has recently led Baraldi and co-workers to propose that A_3 AR antagonists, in view of the prosurvival and antiapoptotic effect of this subtype, might sensitize tumor cells to chemotherapeutic drugs.[16,17]

The best known classes of A_3 AR antagonists are triazoloquinazolines **I**,[18,19] imidazopurinones **II**,[20,21] pyrazolotriazolopyrimidines **III**,[17,22,23] triazoloquinoxalines **IV** and **V**,[24−26] isoquinolines **VI**,[27−29] and pyridines **VII**[30−32] (Chart 1). All these compounds share a nitrogen-containing polyheterocycle analogous to the one featured by phenyltriazolobenzotriazindiones **VIII** (PTBTs, Chart 1), recently described by us as ligands at the central benzodiazepine receptor (BzR).[33] This structural similarity, as well as our experience in switching a series of 3-aryltriazinobenzimidazoles (ATBIs) from Bz[34] to A_1AR binding,[35,36] provided the rationale for the screening of available phenyltriazolobenzotriazindiones as AR antagonists. The nanomolar affinity for the A_3AR displayed by most of the compounds tested prompted the design of further analogues that hopefully may possess an improved potency at the A_3AR and selectivity over the A_1 and A_{2A} ARs, as well as the BzR. Specifically, the first subset of phenyltriazolobenzotriazindiones evolved toward the 5-amino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]-benzotriazin-1-ones **IX** (APTBTs, Chart 1) bearing the same amidine moiety featured by the AR antagonists **I** and **III**−**V**. The structures of the investigated phenyltriazolobenzotriazindiones **1**−**8** and aminophenyltriazolobenzotriazinones **9**−**29** are indicated in Table 2.

Our lead optimization strategy was based on the following modifications: (i) insertion of small groups (H, F, Cl, Me, OMe) in the 9-position of the tricyclic scaffold (R_1) and in the 4′-position of the pendent phenyl ring (R_2) (**10**−**16**); (ii) functionalization of the amidine 5-NH_2 group of compounds **9**, **11**, **12**, **14**, and **16** to yield N^5-substituted benzoyl (**17**−**25**) and N^5-substituted carbamoyl derivatives (**27**−**30**). The R_3 substituents

† This paper is dedicated to Yvonne C. Martin on the occasion of her retirement from Abbott Laboratories.

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Chart 1. Structures of Known (**I**−**VII**) and New (**VIII** and **XI**) A_3 AR Antagonists

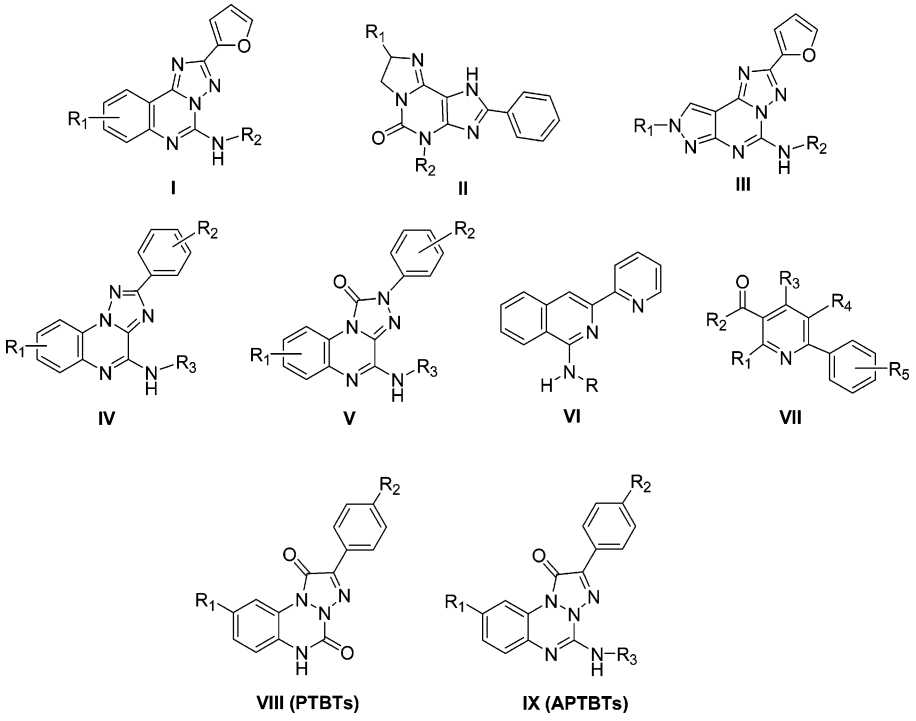
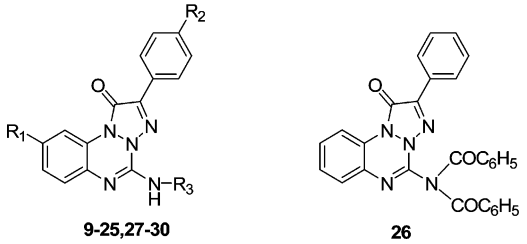


Table 1. Physical Properties of 5-Amino-[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one Derivatives **9**−**30**



compd	R_1	R_2	R_3	yield (%)	mp (°C)[a]	Anal. (formula) C, H, N[b]
9	H	H	H	67	236−238	$C_{15}H_{11}N_5O$
10	H	F	H	60	242−244	$C_{15}H_{10}FN_5O$
11	H	Cl	H	61	273−275	$C_{15}H_{10}ClN_5O$
12	H	CH_3	H	67	254−255	$C_{16}H_{13}N_5O$
13	H	OCH_3	H	60	253−255	$C_{16}H_{13}N_5O_2$
14	Cl	H	H	65	246−248	$C_{15}H_{10}ClN_5O$
15	Cl	Cl	H	62	255−257	$C_{15}H_9Cl_2N_5O$
16	Cl	CH_3	H	69	203−205	$C_{16}H_{12}ClN_5O$
17	H	H	COC_6H_5	42	235−236	$C_{22}H_{15}N_5O_2$
18	H	H	COC_6H_4−4-OCH_3	44	200−202	$C_{23}H_{17}N_5O_3$
19	H	H	COC_6H_4−3-Cl	59	240−242	$C_{22}H_{14}ClN_5O_2$
20	H	Cl	COC_6H_4−4-OCH_3	44	168−170	$C_{23}H_{16}ClN_5O_3$
21	H	CH_3	COC_6H_4−4-OCH_3	52	237−239	$C_{24}H_{19}N_5O_3$
22	H	CH_3	COC_6H_4−3-Cl	44	220−222	$C_{23}H_{16}ClN_5O_2$
23	Cl	H	COC_6H_4−4-OCH_3	47	158−160	$C_{23}H_{16}ClN_5O_3$
24	Cl	H	COC_6H_4−3-Cl	43	234−236	$C_{22}H_{13}Cl_2N_5O_2$
25	Cl	CH_3	COC_6H_4−4-OCH_3	46	208−210	$C_{24}H_{18}ClN_5O_3$
26				51	223−225	$C_{29}H_{19}N_5O_3$
27	H	H	$CONHC_6H_5$	45	190−192	$C_{22}H_{16}N_6O_2$
28	H	H	$CONHC_6H_4$−4-OCH_3	44	209−211	$C_{23}H_{18}N_6O_3$
29	H	H	$CONHC_6H_4$−3-Cl	48	218−220	$C_{22}H_{15}ClN_6O_2$
30	H	H	$CONHCH_2C_6H_5$	43	235−236	$C_{23}H_{18}N_6O_2$

[a] Calculated after recrystallization from ethanol. [b] Elemental analysis results for C, H, and N were within ±0.4% of the calculated values.

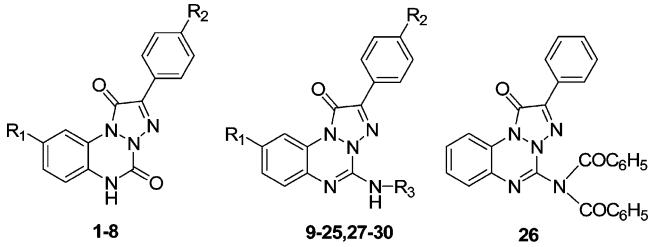
attached to N^5 were selected taking into account literature data about the key role of NH-acyl side chains in ligand recognition by the A_3 AR.[19,23,26,28]

Chemistry

The synthetic pathways yielding the novel aminophenyltriazolobenzotriazinones **9**−**30** are outlined in Scheme 1. The starting material is represented by the 1-(2-amino-5-substituted phenyl)-4-(4-substituted phenyl)-5-oxo-1,2,3-triazoles **31**−**38**, obtained as previously reported.[33] Reaction of **31**−**38** with cyanogen bromide in methanolic solution at room temperature gave the target tricyclic derivatives **9**−**16** (Table 1).

Compounds **9**, **11**, **12**, **14**, and **16** were selected to be suitably functionalized at the 5-NH_2 group by acylation or nucleophilic

Table 2. Binding Activity at Human A_1, A_{2a}, and A_3 ARs of Compounds **1**−**29**



compd	R_1	R_2	R_3	K_i (nM)[a] hA_1[b]	hA_{2A}[c]	hA_3[d]
1	H	H		339.5 ± 54.2	>10000	91.2 ± 8.2
2	H	F		592.8 ± 32.9	>10000	97.0 ± 6.0
3	H	Cl		>10000	>10000	41.9 ± 5.2
4	H	CH_3		4400.5 ± 345.2	>10000	86.5 ± 11.2
5	H	OCH_3		1070.5 ± 79.8	>10000	49.8 ± 8.1
6	Cl	H		960 ± 85	>10000	382.5 ± 22.9
7	Cl	Cl		108.9 ± 15.4	>10000	96.2 ± 7.1
8	Cl	CH_3		890.3 ± 76.5	>10000	39.6 ± 3.3
9	H	H	H	43.9 ± 3.9	134 ± 20.5	34.6 ± 7.2
10	H	F	H	65.2 ± 3.5	20.1 ± 4.6	368.5 ± 55.2
11	H	Cl	H	104.2 ± 14.0	493.3 ± 18.0	33.2 ± 2.1
12	H	CH_3	H	46.0 ± 3.6	50.6 ± 7.7	29.2 ± 4.6
13	H	OCH_3	H	89.5 ± 6.4	9.0 ± 0.6	51.0 ± 3.4
14	Cl	H	H	80.2 ± 6.6	4.7 ± 0.22	295.2 ± 32.1
15	Cl	Cl	H	106.7 ± 7.2	67.5 ± 4.8	557.3 ± 79.3
16	Cl	CH_3	H	19.0 ± 1.5	2.3 ± 0.1	103.0 ± 5.2
17	H	H	COC_6H_5	204.5 ± 44.5	1030.0 ± 166.1	101.2 ± 7.8
18	H	H	COC_6H_4−4-OCH_3	99.0 ± 4.5	>10000	4.3 ± 0.4
19	H	H	COC_6H_4−3-Cl	389.2 ± 26.0	>10000	15.0 ± 1.2
20	H	Cl	COC_6H_4−4-OCH_3	>10000	>10000	55.7 ± 3.3
21	H	CH_3	COC_6H_4−4-OCH_3	80.9 ± 7.1	>10000	1.1 ± 0.01
22	H	CH_3	COC_6H_4−3-Cl	256.0 ± 27.0	>10000	41.5 ± 1.3
23	Cl	H	COC_6H_4−4-OCH_3	2700.0 ± 100.1	>10000	1.6 ± 0.07
24	Cl	H	COC_6H_4−3-Cl	916.0 ± 49.0	>10000	>1000
25	Cl	CH_3	COC_6H_4−4-OCH_3	>10000	>10000	12.3 ± 3.5
26				116.5 ± 11.4	4800.0 ± 348.8	9.2 ± 0.8
27	H	H	$CONHC_6H_5$	91.1 ± 3.2	460.2 ± 66.9	97.0 ± 8.3
28	H	H	$CONHC_6H_4$−4-OCH_3	462.2 ± 55.2	262.1 ± 76.9	49.1 ± 3.3
29	H	H	$CONHC_6H_4$−3-Cl	764.0 ± 13.1	625.0 ± 14.8	49.0 ± 5.1
30	H	H	$CONHCH_2C_6H_5$	>10000	120.1 ± 9.1	43.4 ± 2.5
DPCPX				0.5	337	>1000
NECA				14	16	73
CI-IBMECA				890	401	0.22

[a] The K_i values are means ± SEM derived from an iterative curve-fitting procedure (Prism program, GraphPad, San Diego, CA). [b]Displacement of specific [^{3}H]DPCPX binding in membranes obtained from hA_1 AR stably expressed in CHO cells. [c]Displacement of specific [^{3}H]NECA binding in membranes obtained from hA_{2A} AR stably expressed in CHO cells. [d]Displacement of specific [^{125}I]AB-MECA binding in membranes obtained from hA_3 AR stably expressed in CHO cells.

addition (procedures i−iii, Scheme 1). In particular, the preparation of N^5-benzoyl compounds **18**−**25** was performed by acylation with *p*-anisoylbenzoyl chloride or *m*-chlorobenzoyl chloride in anhydrous dichloromethane at room temperature in the presence of pyridine (Scheme 1, procedure i; Table 1). When **9** was allowed to react with benzoyl chloride under the same conditions, only the dibenzoyl derivative **26** was obtained (Scheme 1, procedure i; Table 1). We modified the reaction procedure and found that the desired compound **17** was formed with good yields by acylation of **9** with benzoic anhydride in freshly distilled dimethylformamide at 70 °C in the presence of triethylamine and a catalytic amount of (dimethylamino)-pyridine (DMAP) (Scheme 1, procedure ii; Table 1). The N^5-carbamoyl derivatives **27**−**30** were obtained starting from **9** by treatment with the appropriate substituted phenyl isocyanate or benzyl isocyanate in tetrahydrofuran solution at room temperature (Scheme 1, procedure iii; Table 1).

The syntheses of compounds **1**−**8** were performed as previously reported.[33]

Biology

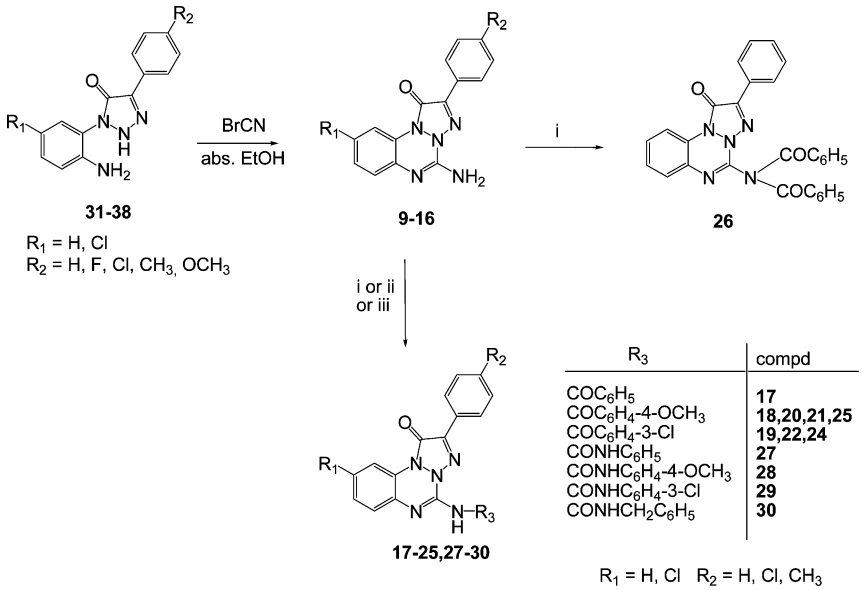
The affinities of the new compounds toward human A_1, A_{2A}, and A_3 ARs were evaluated by competition experiments assessing their respective abilities to displace [^{3}H]DPCPX, [^{3}H]-NECA, or [^{125}I]AB-MECA binding from transfected Chinese hamster ovary (CHO) cells. Experiments were performed as described elsewhere.[36] Compounds **3**, **21**, **23**, and **25**, which showed a high A_3 affinity, were also tested in functional assays at human A_{2B} and A_3 ARs by measuring their effects on NECA-mediated cAMP modulation in transfected CHO cells.[37]

A subset of compounds (**9**, **11**, **12**, **14**, **17**, **23**, **27**, and **30**), selected as the most representative of the new AR ligands, were evaluated for their affinity at the BzR in bovine brain membranes by competition experiments against the radiolabeled antagonist [^{3}H]flumazenil at a fixed concentration of 10 μM.[33]

Results and Discussion

Table 2 lists the binding affinities at the human A_1, A_{2A}, and A_3 ARs of phenyltriazolobenzotriazindiones **1**−**8** and ami-

Scheme 1. Synthesis of Target Compounds **9**−**30**[a]



[a] Reagents. For **9**, **11**, **12**, **14**, **16**: (i) acyl chloride, pyridine in dichloromethane. For **9**: (ii) benzoic anhydride, DMAP, triethylamine in DMF; (iii) substituted phenyl isocyanate or benzyl isocyanate in THF.

nophenyltriazolobenzotriazinones **9**−**30** expressed as K_i values or inhibition percentages.

Compounds **1**−**8** are moderately potent at the A_1 AR and completely inactive at the A_{2A} AR, with nanomolar affinity at the A_3 AR. Compounds **3**, **5**, and **8** are also selective over the A_1 and A_{2A} ARs. A few combinations of R_1 and R_2 different from a hydrogen slightly increase the potency at the A_1 AR (compare **7** vs **1**) or at the A_3 AR (compare **8** vs **1**).

A pairwise comparison of the binding data of compounds **1**−**8** with those of compounds **9**−**16** reveals that the replacement of the amide with an amidine moiety enhances the binding to the A_1 AR and, to a much greater extent, to the A_{2A} AR, whereas it does not significantly improve or worsen the affinity for the A_3 AR.

As mentioned, the choice of functionalizing the 5-NH_2 of compound **9** with acyl groups relied on the well-established role of specific $NHCOR_3$ side chains in conferring high potency and selectivity on A_3 AR antagonists.[19,23,26,28]

Insertion of a benzoyl chain into the structure of **9**, to give **17**, slightly lowers the affinity at the ARs. This fact might be interpreted by hypothesizing that the attractive interactions created by the benzoyl group at each AR are slightly outweighed by the disadvantageous ones (e.g., solvation, steric bulk). The introduction of a methoxy in the para position of the benzoyl moiety of **17**, to yield **18**, increases the A_3 AR affinity by a 23-fold and also improves selectivity over the A_1 and A_{2A} ARs. Compared with the *p*-methoxy group, an *m*-chlorine on the same benzoyl is less effective but still beneficial for the affinity and selectivity at the A_3 AR (compare **19** vs **17**). The above gains in A_3 AR affinity might reflect a hydrogen bond and hydrophobic interactions established by the receptor with the *p*-methoxy of **18** and the *m*-chlorine of **19**, respectively.

Taking compound **18** as a reference structure, further slight improvements in the potency at the A_3 AR can be achieved by modulating the nature of R_1 and R_2 (compare the 4′-methyl derivative **21** and the 9-chlorine derivative **23** vs the unsubstituted counterpart **18**). The simultaneous presence of a 4′-methyl and a 9-chlorine in the same compound does not lead to additive favorable effects on A_3 AR affinity but only to a significant drop in A_1 AR affinity (compare **25** vs **18**).

Table 3. Potency of Compounds **3**, **21**, **23**, and **25** versus hA_{2B} and hA_3 Adenosine Receptor Subtypes

	IC_{50} [a] (nM) of cAMP assays	
compd	for hA_{2B} CHO cells	for hA_3 CHO cells
3	>1000 (26%)	12 ± 1.2
21	1527 ± 150	0.58 ± 0.05
23	>1000 (15%)	3.14 ± 0.3
25	>1000 (26%)	11.61 ± 1.02

[a] The data are expressed as the mean ± SEM of four independent experiments performed in triplicate. In parentheses are indicated the percentage of inhibition, at 10 μM compound, of the cAMP levels, stimulated by 100 nM NECA for A_{2B} adenosine receptors.

Compound **23** stands out for its remarkable potency and selectivity at the A_3 AR (K_i values at the A_1, A_{2A}, and A_3 ARs are 2700, >10000 nM, and 1.6 nM, respectively), thus representing the best result of our lead optimization efforts.

Analogous attempts to enhance the potency of **19** at the A_3 AR by changing R_1 and/or R_2 were unsuccessful (compare **22** and **24** vs **19**).

Finally, two benzoyl groups attached to a hexocyclic nitrogen negatively affect A_1 and A_{2A} AR affinities while they enhance A_3 AR binding by 4-fold (compare **26** vs **9**).

Replacement of the benzoyl of **17** with the phenylcarbamoyl to give **27** does not greatly influence affinities at the ARs. The insertion of a *p*-methoxy or an *m*-chlorine on the phenylcarbamoyl of compound **27** to yield **28** or **29**, respectively, increases affinity at the A_3 AR only 2-fold. Homologation of **27** to give **30** does not give better results for the A_3 AR affinity.

Compounds **3**, **21**, **23**, and **25** were also tested at the A_{2B} AR by evaluating their inhibitory effects on NECA-mediated cAMP accumulation in CHO cells stably expressing this receptor subtype (Table 3). These derivatives proved to be inactive in this assay, thus indicating a complete lack of affinity toward the A_{2B} AR.

Given the recent discoveries of non-nucleoside AR agonists,[38,39] the efficacy profile of compounds **3**, **21**, **23**, and **25** were evaluated in cAMP functional assays at the A_3 AR (Table 3). Expectedly, all these compounds displayed full antagonism, with potencies similar to their binding affinity. Furthermore, when tested in the absence of NECA, they did not show any

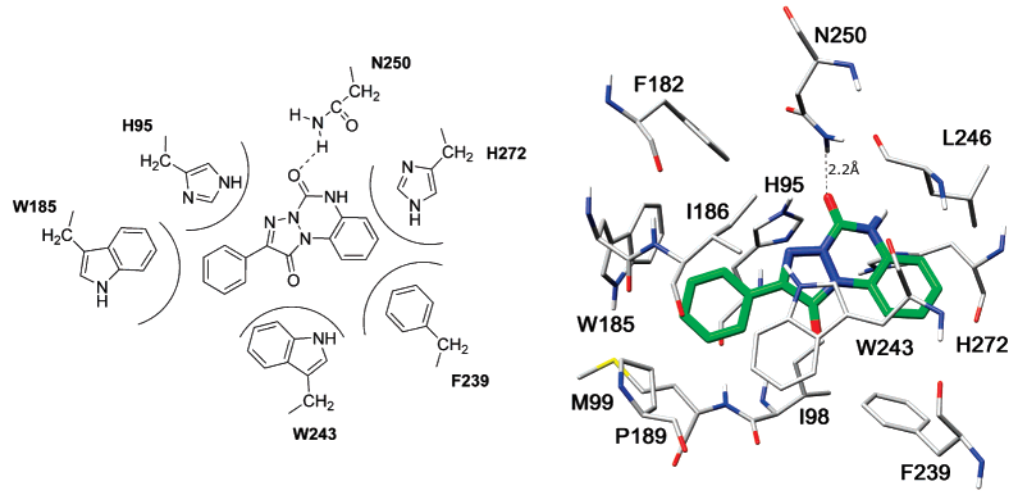


Figure 1. Compound **1** docked into the A_3 AR model.[40]

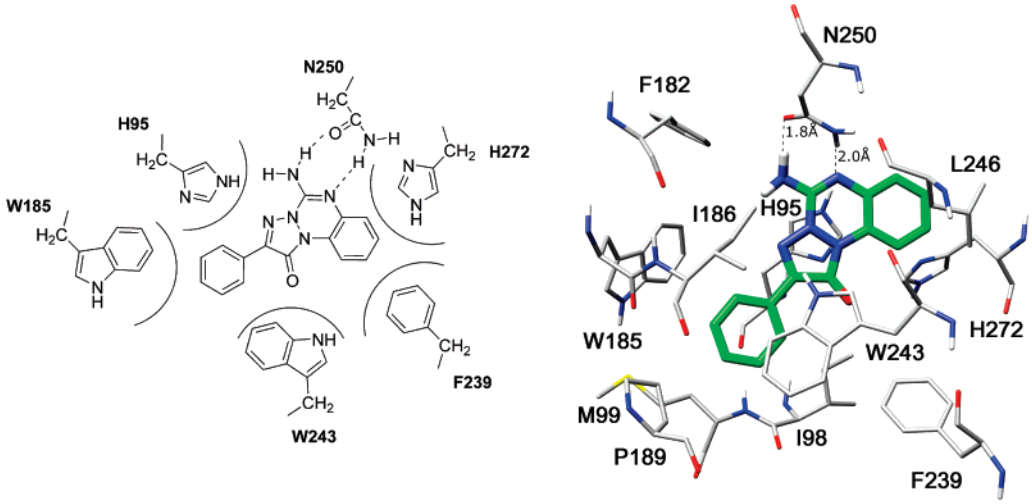


Figure 2. Compound **9** docked into the A_3 AR model.[40]

significant effect on the cAMP level even after stimulation by forskolin, indicating neutral antagonism (data not shown).

A subset of compounds (**9**, **11**, **12**, **14**, **17**, **23**, **27**, and **30**), selected as the most representative of the entire series of new AR ligands, were evaluated for their affinity to the BzR. None of the compounds tested inhibited [^{3}H]flumazenil binding by more than 50% at the fixed concentration of 10 μM (data not shown).

The following chemical rules summarize the affinity/selectivity data of the new AR antagonists: (i) two carbonyl groups in positions 1 and 5 of the triazolobenzotriazine scaffold (compounds **1**−**8**) produce good A_3 AR antagonists with a fair or high selectivity toward the A_1 and A_{2A} ARs, respectively; (ii) replacement of the C═O(5) with an amine fragment to give the amidine derivatives **9**−**16** enhances affinity at the A_1 AR and, to a much greater extent, at the A_{2A} AR, with no significant effect at the A_3 AR, leading to a number of potent and moderately A_{2A}-selective ligands (compounds **13**, **14**, and **16**); (iii) functionalization of the 5-NH_2 group with acyl or carbamoyl substituents generally affords ligands with a nanomolar potency at the A_3 AR, with a different extent of selectivity over the A_1 and A_{2A} ARs (compounds **17**−**30**); in particular, if the acyl group is a *p*-methoxybenzoyl, it is possible to achieve a high affinity and selectivity at the A_3 AR (compounds **23** and **25**).

A number of our ligands (**1**, **9**, **17**−**19**, **23**, **26**, and **28**) were docked into our recently published human A_3 AR model[40] using the AUTODOCK 3.0 program[41] and were then submitted to 1.2 ns of molecular dynamics simulation (see Experimental Section for details). Their overall binding disposition appeared to be very similar to the one recently hypothesized for the triazoloquinazolines **I** and pyrazolo[4,3-*e*][1,2,4]triazolo[1,5-*c*]-pyrimidines **III**.[40]

As shown in Figure 1, the main scaffold of compound **1** interacts with the TM3 and TM5−7 domains of the receptor, made up mostly of lipophilic amino acids (H95 (3.37), I98 (3.40), F182 (5.43), F239 (6.44), W243 (6.48), L246 (6.51), H272 (7.43)); the pendent phenyl is likewise surrounded by lipophilic residues (M99 (3.41), L102 (3.44), W185 (5.46), I186 (5.47), P189 (5.50)); the 5-carbonyl oxygen receives an H-bond from the N250 (6.55) side chain.

Replacement of the 5-oxo moiety of compound **1** with a hexocyclic amino group produced **9** and a corresponding 3-fold gain in potency. The docking simulations performed on compound **9** revealed that this ligand maintains all the hydrophobic interactions of compound **1**, with its amidine moiety making two H-bonds with the N250 (6.55) side chain (Figure 2).

As outlined previously, the introduction of a *p*-methoxy group on the benzoyl moiety of **17** to yield **18** increased the A_3 AR affinity 23-fold. This fact seems to be consistent with a putative H-bond accepted by the *p*-methoxy of compound **18** from the K152 (EL2) ammonium moiety (see Figure 3). Such an H-bond

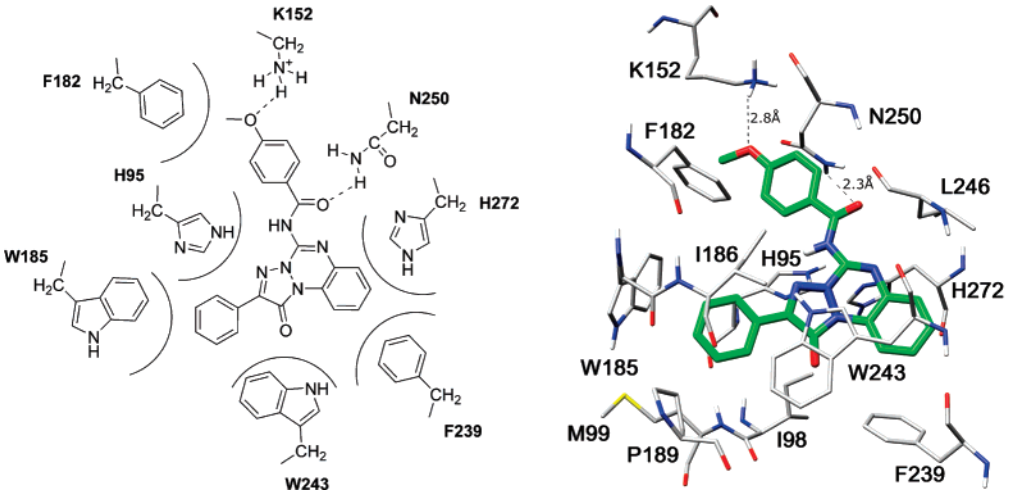


Figure 3. Compound **18** docked into the A_3 AR model.[40]

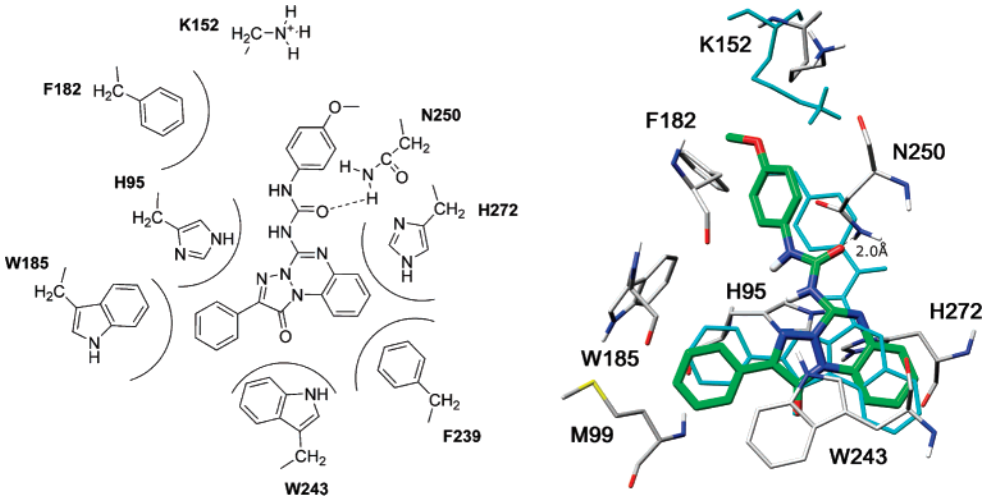


Figure 4. Compound **28** docked into the A_3 AR model[40] superimposed on the A_3 AR−**18** complex (colored sky blue).

might explain mutagenesis data,[42] which assign an important role to K152 (EL2) in the binding of VUF850 (**V**, R = COC_6H_4-4-OCH_3), a highly potent A_3 AR antagonist sharing with compound **18** the same *p*-methoxybenzoylamino side chain.

Favorable hydrophobic interactions between the *m*-chlorine of compound **19** and the F182 (5.43) phenyl ring seem to account for the 6.7-fold higher A_3 AR affinity of this ligand, compared with its unsubstituted counterpart **17**.

Compound **28**, featuring a *p*-methoxyphenylcarbamoylamino side chain, was 10-fold less potent than **18**. Docking studies revealed that, unlike compound **18**, the *p*-methoxy group of **28** is unable to engage an H-bond with K152 (EL2) (Figure 4 shows an overlay of the two corresponding docking models). It is noted that both of the carbonyl oxygens of the two ligands receive an H-bond from N250 (6.55) while their *p*-methoxyphenyl moieties present different dispositions in the receptor binding cleft.

Compound **23** shows an A_3 affinity similar to that of compound **18**, but it possesses a much higher selectivity over the A_1 AR probably because of a steric interference of the 9-chlorine atom with the A_1 AR protein. As shown in Figure 5, **23** is orientated similarly to **18** within the A_3 AR binding site. Although the 9-chlorine of **23** does not seem to be involved in strong interactions, it determines a slight translation of the ligand toward the extracellular side of the receptor.

Finally, our binding hypotheses received further support from the docking of the highly potent compound **26**. In spite of the presence of two sterically demanding benzoyl groups, this ligand adopts an overall orientation within the A_3 AR binding site quite similar to those displayed by the N^5-benzoyl APTBT derivatives. According to our model, while one benzoyl group of compound **26** forms an H-bond with N250 (6.55), the other one is stabilized by hydrophobic interactions with F182 (5.43), W185 (5.46), and I186 (5.47).

In conclusion, the aminophenyltriazolobenzotriazinone system proved to be a versatile scaffold to obtain potent and selective A_3 AR antagonists, provided that suitable groups are attached to the 5-amino moiety and in the 4′-position and/or 9-position. The best result of our lead optimization efforts was represented by compound **23** (R_1 = Cl, R_2 = H, and R_3 = *p*-methoxybenzoyl), which displayed a K_i of 1.6 nM at the A_3 AR and no significant affinity at the other ARs or the BzR. Docking simulations on selected ligands into a model of the A_3 AR allowed us to develop structure−activity relationships at the molecular level, which will be useful in the design of novel A_3 AR antagonists.

Experimental Section

Chemistry. Melting points were determined using a Reichert Köfler hot-stage apparatus and are uncorrected. Infrared spectra were recorded with a Nicolet/Avatar FT-IR spectrometer in Nujol mulls. Routine nuclear magnetic resonance spectra were recorded in DMSO-d_6 solution on a Varian Gemini 200 spectrometer operating at 200 MHz. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm

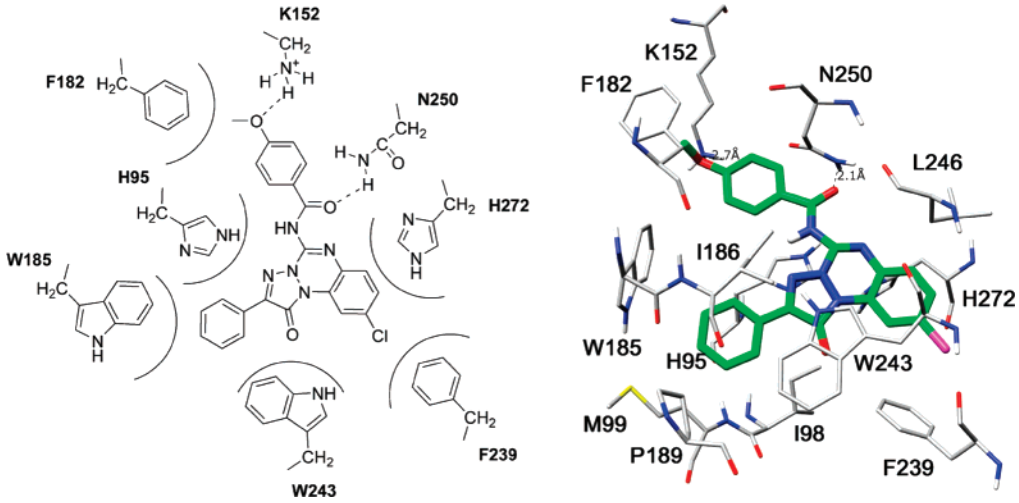


Figure 5. Compound **23** docked into the A_3 AR model.[40]

precoated silica gel aluminum sheets (60 F-254). Elemental analyses were performed by our Analytical Laboratory and agreed with theoretical values to within ±0.4%.

The 9-substituted-2-(4-substituted phenyl)[1,2,3]triazolo[1,2-*a*]-[1,2,4]benzotriazin-1,5(6*H*)-diones **1**−**8** and the 1-(2-amino-5-substituted phenyl)-4-(4-substituted phenyl)-5-oxo-1,2,3-triazole derivatives **31**−**38** were prepared essentially as previously described in ref 33.

General Procedure for the Synthesis of 5-Amino-9-substituted-2-(4-substituted phenyl)[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one Derivatives 9−**16.** Cyanogen bromide (0.090 g, 0.85 mmol) was added in a single portion to a stirred solution of the appropriate 1-(2-amino-5-substituted phenyl)-4-(4-substituted phenyl)-5-oxo-1,2,3-triazole derivative **31**−**38**[33] (0.85 mmol) in 7.0 mL of absolute methanol. The mixture was allowed to react at room temperature under a nitrogen atmosphere for 12−36 h, and the reaction was monitored by TLC analysis. The precipitated crude products **9**−**16** that formed were collected and purified by recrystallization from ethanol (Table 1 and Supporting Information).

5-Benzoylamino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]-benzotriazin-1-one 17. A solution of triethylamine (1.1 mL, 0.76 mmol) in 1.0 mL of freshly distilled dimethylformamide was added dropwise to an ice-cooled, stirred suspension of 5-amino-2-phenyl-[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one **9** (0.140 g, 0.51 mmol), benzoic anhydride (0.340 g, 1.52 mmol), and (dimethylamino)pyridine (0.007 g, 0.006 mmol) in 2.0 mL of the same solvent. The mixture was heated at 70 °C under a nitrogen atmosphere for 72 h. After the mixture was cooled, the precipitated crude product **17** was collected, washed with 5% $NaHCO_3$ aqueous solution, and purified by recrystallization from ethanol (Table 1 and Supporting Information).

General Procedure for the Synthesis of 9-Substituted-2-(4-substituted phenyl)-5-(substituted benzoylamino)[1,2,3]triazolo-[1,2-*a*][1,2,4]benzotriazin-1-one Derivatives 18−**25.** A solution of the appropriate benzoyl chloride (4-methoxybenzoyl chloride, 3-chlorobenzoyl chloride) (0.7 mmol) in 1.0 mL of anhydrous dichloromethane was added dropwise at 0 °C and under a nitrogen atmosphere to a stirred suspension of the appropriate amino derivatives **9**, **11**, **12**, **14**, and **16** (0.43 mmol) and anhydrous pyridine (0.16 mL) in 3.0 mL of the same solvent. The mixture was stirred at room temperature for 24−48 h, the reaction being monitored by TLC analysis. The precipitate obtained was collected, washed with diethyl ether, and purified by recrystallization from ethanol (Table 1 and Supporting Information).

5,5-Dibenzoylamino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]-benzotriazin-1-one 26. A solution of benzoyl chloride (0.11 mL, 0.7 mmol) in 1.0 mL of anhydrous dichloromethane was added dropwise at 0 °C and under a nitrogen atmosphere to a stirred suspension of 5-amino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]-benzotriazin-1-one **9** (0.120 g, 0.43 mmol) and anhydrous pyridine (0.16 mL) in 3.0 mL of the same solvent. The mixture was stirred at room temperature for 24 h, and the precipitate obtained was collected and washed with diethyl ether. The crude product **26** was then purified by recrystallization from ethanol (Table 1 and Supporting Information).

General Procedure for the Synthesis of 9-Substituted-2-(4-substituted phenyl)-5-(substituted phenylaminocarbamoyl)-[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one Derivatives 27−**29 and 2-Phenyl-5-(benzylaminocarbamoyl)[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one 30.** The appropriate substituted isocyanate (phenyl, 4-methoxyphenyl, 3-chlorophenyl, and benzyl isocyanates) (0.8 mmol) was added dropwise to a stirred solution of 5-amino-2-phenyl[1,2,3]triazolo[1,2-*a*][1,2,4]benzotriazin-1-one **9** (0.137 g, 0.50 mmol) in 25.0 mL of anhydrous tetrahydrofuran. The mixture was heated at 50 °C for 24−48 h (TLC analysis). After the mixture was cooled, the precipitate that formed was collected and washed with tetrahydrofuran. The organic solution was concentrated, and the separated precipitate was collected to yield an additional amount of crude product. The quantities of ureido derivatives obtained from the initial insoluble precipitate and from the organic solution were variable, depending on the solubility of the various compounds. Finally, the crude products **27**−**30** were purified by recrystallization from ethanol (Table 1 and Supporting Information).

Biological Methods. Materials. [^{3}H]DPCPX, [^{3}H]NECA, and [^{125}I]AB-MECA were obtained from DuPont-NEN (Boston, MA). Adenosine deaminase (ADA) was from Sigma Chemical Co. (St. Louis, MO). All other reagents were from standard commercial sources and of the highest commercially available grade. CHO cells, stably expressing human A_1, A_{2A}, A_{2B}, and A_3 receptors were kindly supplied by Prof. K. N. Klotz, Wurzburg University, Germany.[43]

Adenosine Receptor Binding Assay. Human A_1 Adenosine Receptors. Aliquots of membranes (50 μg proteins) obtained from A_1 CHO cells were incubated at 25 °C for 180 min in 500 μL of T_1 buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 2 units/mL ADA, pH 7.4) containing [^{3}H]DPCPX (3 nM) and six different concentrations of the newly synthesized compounds. Nonspecific binding was determined in the presence of 50 μM R-PIA.[36] The dissociation constant (K_d) of [^{3}H]DPCPX A_1 CHO cell membranes was 3 nM.

Human A_{2A} Adenosine Receptors. Aliquots of cell membranes (80 μg) were incubated at 25 °C for 90 min in 500 μL of T_2 buffer (50 mM Tris-HCl, 2 mM $MgCl_2$, 2 units/mL ADA, pH 7.4) in the presence of 30 nM of [^{3}H]NECA and six different concentrations of the newly synthesized compounds. Nonspecific binding was determined in the presence of 100 μM NECA.[36] The dissociation constant (K_d) of [^{3}H]NECA in A_{2A} CHO cell membranes was 30 nM.

Human A_3 Adenosine Receptors. Aliquots of cell membranes (40 μg) were incubated at 25 °C for 90 min in 100 μL of T_3 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 units/mL ADA, pH 7.4) in the presence of 1.4 nM [^{125}I]AB-MECA and six different

concentrations of the newly synthesized compounds. Nonspecific binding was determined in the presence of 50 μM R-PIA.[36] The dissociation constant (K_d) of [^{125}I]AB-MECA in A_3 CHO cell membranes was 1.4 nM.

Measurement of Cyclic AMP Levels on hA_{2B} and hA_3 CHO Cells. Intracellular cAMP levels were measured using a competitive protein-binding method.[37] CHO cells, expressing recombinant hA_{2B} or hA_3 ARs, were harvested by trypsinisation. After centrifugation and resuspension in medium, cells (about 60 000) were plated in 24-well plates in 0.5 mL of medium. After 48 h, the medium was removed and the cells were incubated at 37 °C for 15 min with 0.5 mL of DMEM in the presence of Ro20-1724 (20 μM), as a phosphodiesterase inhibitor, and ADA (1 U/mL). The antagonistic profile of the new compounds toward A_{2B} AR was evaluated by assessing their ability to inhibit 100 nM NECA-mediated accumulation of cAMP. The antagonistic profile of the new compounds toward A_3 AR was evaluated by assessing their ability to counteract 100 nM NECA-mediated inhibition of cAMP stimulated by 1 μM forskolin. Cells were incubated in the reaction medium (15 min at 37 °C) with different compound concentrations (1 nM to 10 μM) and then treated with NECA. In parallel, aliquots of cells were treated with the compound alone (10 μM) in the absence or in the presence of forskolin. The reaction was terminated by the removal of the medium and the addition of 0.4 N HCl. After 30 min, lysates were neutralized with 4 N KOH and the suspension was centrifuged at 800*g* for 5 min. For determination of cAMP production, cAMP binding protein (isolated from calf adrenal glands) was incubated with [^{3}H]cAMP (2 nM) and 50 μL of cell lysate or cAMP standard (0−16 pmol) at 4 °C for 150 min in a total volume of 300 μL. Bound radioactivity was separated by rapid filtration through GF/C glass fiber filters and washed twice with 4 mL of 50 mM Tris/HCl, pH 7.4. The radioactivity was measured by liquid scintillation spectrometry.

All compounds were routinely dissolved in DMSO and diluted with the assay buffer to the final concentration, where the amount of DMSO never exceeded 2%. Percentage inhibition values of specific radiolabeled ligand binding at 1−10 μM are the mean ± SEM of at least three determinations. For IC_{50} compound determination, at least six different ligand concentrations were used. IC_{50} values, computer-generated using a nonlinear regression formula on a computer program (Graph-Pad, San Diego, CA), were converted to K_i values, knowing the K_d values of radioligands in the different tissues, and using the Cheng and Prusoff equation.[44] K_i values are the mean ± SEM of at least three determinations.

Computational Methods. The ligands were submitted to a conformational search of 1000 steps. The algorithm used was the Montecarlo method with MMFFs as the force field and a distance-dependent dielectric constant of 1.0. The ligands were then minimized using the conjugated gradient method until a convergence value of 0.05 kcal Å^{-1} mol^{-1}, using the same force field and dielectric constant used for the conformational search.[45] Then the ligands were docked into the recently published A_3 receptor[40] using the AUTODOCK 3.0 program.[41] The regions of interest used by AUTODOCK were defined by considering N250 (6.55) as the central residue of a grid of 44, 54, and 50 points in the *x*, *y*, and *z* directions. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the energy map calculations.

By use of the Lamarckian genetic algorithm, the compounds were subjected to 100 runs of the AUTODOCK search with 500 000 steps of evaluation energy; the default values of the other parameters were used. Cluster analysis was performed on the docked results using an rms tolerance of 1.0 Å.

The best docked structures were then subjected to 1.2 ns of molecular dynamics (MD) calculation. All simulations were performed using AMBER 8.0.[46] MD simulations were carried out at 300 K using the parm94 force field. Chlorine ions were added as counterions to neutralize the system. Prior to MD simulations, a minimization stage was carried out by applying a constraint of 30 kcal mol^{-1} Å^{-1} on the α carbon of the TMs. The minimization consisted of 5000 steps with a combined algorithm, namely, the sequential use of steepest descent and conjugate gradient methods for the first 1000 and the last 4000 steps, respectively.

Molecular dynamics trajectories were run using the minimized structure as a starting input. The time step of the simulations was 2.0 fs with a cutoff of 12 Å for the nonbonded interaction, and SHAKE was employed to keep all bonds involving hydrogen atoms rigid. The 1.2 ns of MD simulation was carried out by applying a decreasing constraint on the α carbon of the TMs from 30 to 5 kcal mol^{-1} Å^{-1}. The final structure of the complexes was obtained as the average of the last 600 ps of MD simulation minimized with the conjugate gradient method until a convergence of 0.05 kcal Å^{-1} mol^{-1} was obtained.

In reference to specific amino acids, both the sequence number and the numbering system proposed by Ballesteros and Weinstein[47] (in parentheses) were employed. For the latter, the most highly conserved residue in each transmembrane helix (TMH) was assigned a value of 0.50 and this number was preceded by the TMH number. The other residues in the helix were given a locant value relative to this.

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Supporting Information Available: Spectral and analytical data of compounds **9**−**30**. This material is available free of charge via the Internet at http://pubs.acs.org.

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