N^6-Methoxy-2-alkynyladenosine Derivatives as Highly Potent and Selective Ligands at the Human A_3 Adenosine Receptor

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A new series of N^6-methoxy-2-(ar)alkynyladenosine derivatives has been synthesized and tested at the human recombinant adenosine receptors. Binding studies demonstrated that the new compounds possess high affinity and selectivity for the A_3 subtype. Among them, compounds bearing an *N*-methylcarboxamido substituent in the 4′-position showed the highest A_3 affinity and selectivity. In particular, the N^6-methoxy-2-*p*-acetylphenylethynylMECA (**40**; K_i A_3 = 2.5 nM, A_3 selectivity versus A_1 = 21 500 and A_{2A} = 4200) results in one of the most potent and selective agonists at the human A_3 adenosine receptor reported so far. Furthermore, functional assay, performed with selected new compounds, revealed that the presence of an alkylcarboxamido group in the 4′-position seems to be essential to obtain full agonists at the A_3 subtype. Finally, results of molecular docking analysis were in agreement with binding and functional data and could explain the high affinity and potency of the new compounds.

Introduction

Ado[a] is involved in the regulation of many physiological and pathophysiological processes through the activation of four cell membrane receptors, termed A_1, A_{2A}, A_{2B}, and A_3, which belong to the family of G-protein coupled receptors and are ubiquitously expressed in the body.[1−4] In particular, the A_3 receptor is negatively coupled to adenylyl cyclase and positively coupled to phospholipase C, resulting in an increase of intracellular Ca^{++} levels.[5,6] Although the physiological role of the A_3 receptor subtype is not fully understood yet, it has recently attracted considerable interest as a novel drug target.[7] In particular, it has been suggested that A_3 receptor agonists may have potential as cardioprotective[8,9] and cerebroprotective[10] agents.

Furthermore, A_3 agonists can be potential drugs for the treatment of asthma[11,12] and dry eye disorders,[13] in cancer therapy as cytostatics and chemoprotective compounds,[14−16] and as anti-inflammatory and immunosuppressive agents.[17]

A_3 adenosine receptor antagonists might be therapeutically useful as well, for example, for the acute treatment of stroke[7] and glaucoma[18] and also as antiasthmatic and antiallergic drugs,[19] because A_3 receptors can not only mediate anti-inflammatory, but also pro-inflammatory responses.[20]

Hence, the future development of new pharmacological tools, including potent and selective agonists, will facilitate the evaluation of the (patho)physiological role of A_3 receptors and their pharmacological potential.

Selective A_3 receptor agonists have been obtained through modification of the *C*2-, N^6-, and 5′-positions of adenosine;[7] in fact, the Cl-IB-MECA[21] was the first highly selective full agonist for the rat A_3 receptor, however, it showed much lower selectivity at human adenosine receptors.[22]

Some years ago, we have reported the synthesis and binding affinity of a number of Ado derivatives bearing 2-position (ar)-alkynyl chains, which are endowed with good affinity and different degrees of selectivity for the human A_3 Ado receptor subtype.[23−27] Furthermore, the replacement of the hydroxymethyl group in 4′-position of the sugar moiety of these compounds with a methyl or ethylcarboxamido substituent increased A_3 affinity and selectivity.[28] In particular, **1** (PEAdo) showed a K_i A_3 = 16 nM and an A_3 selectivity versus A_1 and versus A_{2A} of 24- and 23-fold, while the corresponding MECA (**2**) and NECA (**3**) derivatives displayed a K_i A_3 of 7.3 and 6.2 nM, respectively, and an A_3 selectivity versus A_1 and A_{2A} of 537- and 241-fold for compound **2** (PEMECA) and 90- and 100-fold for compound **3** (PENECA; Figure 1).[29] These compounds aroused the interest of other researchers, which reported the synthesis of similar 2-alkynylNECA derivatives.[30] Among them is the NECA derivative, with a propynylcyclohexanecarboxymethyl ester in the 2-position, named ATL-146e, which is now in clinical trial for the treatment of acute spinal cord injury.[31]

On the other hand, the introduction of bulky substituents in the N^6 position of 2-alkynylNECA derivatives decreased the A_3 binding affinity,[32,33] whereas the presence of small substituents like a methyl in the same position favored the interaction of the resulting compounds with the human A_3 receptor; consequently, the N^6-methyl-2-phenylethynylAdo (**4**) exhibited a K_i A_3 of 3.4 nM and an A_3 selectivity versus A_1 and A_{2A} of 500- and 2500-fold, respectively.[29] Furthermore, **4a** (HEMADO) although somewhat less selective, showed an even increased affinity for the human A_3 receptor subtype (**4a**; K_i A_3 = 1.1 nM, A_3 selectivity versus A_1 = 300 and versus A_{2A} = 1100, Figure 1).[29] This result prompted us to set up a new synthetic procedure for the synthesis of tritiated HEMADO ([^{3}H]-HEMADO) as a new A_3 radioligand through the introduction in the final step and with high yield of tritiated methylamine.[34] [^{3}H]-HEMADO has

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[a] Abbreviations: Ado, adenosine; Cl-IB-MECA, 2-chloro-N^6-(3-iodobenzyl)-5′-*N*-methylcarboxamidoadenosine; PEAdo, 2-phenylethynyladenosine; MECA, 5′-*N*-methylcarboxamidoadenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; PEMECA, 2-phenylethynyl-5′-*N*-methylcarboxamidoadenosine; PENECA, 2-phenylethynyl-5′-*N*-ethylcarboxamidoadenosine; ATL-146e, 4-{3-[6-amino-9-(5-ethylcarbamoyl-3,4-diidroxytetrahydrofuran-2-yl)-9*H*-purin-2-yl]prop-2-ynyl}cyclohexanecarboxylic acid methyl ester; HEMADO, 2-(hexyn-1-yl)-N^6-methyladenosine; CHO, chinese hamster ovary; CCPA, 2-chloro-N^6-cyclopentyladenosine; NECA, 5′-*N*-ethylcarboxamidoadenosine; *R*-PIA, (*R*)-N^6-phenylisopropyl adenosine; MOE, molecular operating environment; TM, trans-membrane domain; E, extracellular loop.

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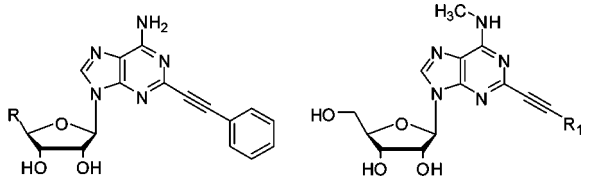


1; R = -CH_2OH

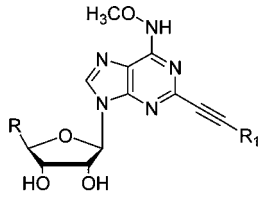
2; R = -CO-NH-Me

3; R = -CO-NH-Et

4; R_1 = -C_6H_5

4a; R_1 = -nC_4H_9

Figure 1. A_3 adenosine receptor agonists.

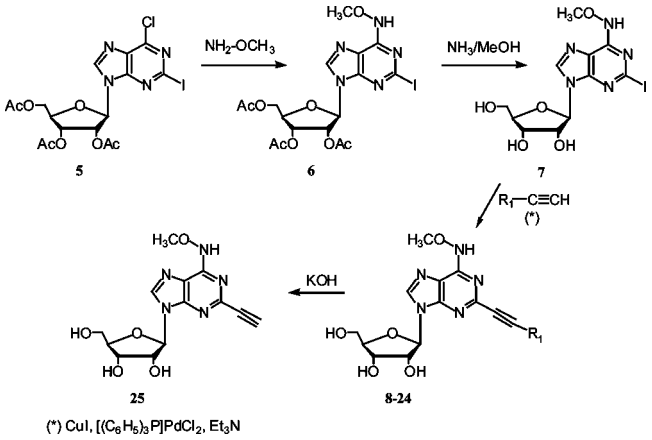


R = -CH_2OH or -CO-NH-Me or -CO-NH-Et

R_1 = H, substituted (ar)alkyl chains

Figure 2. General structure of the synthesized compounds.

Scheme 1



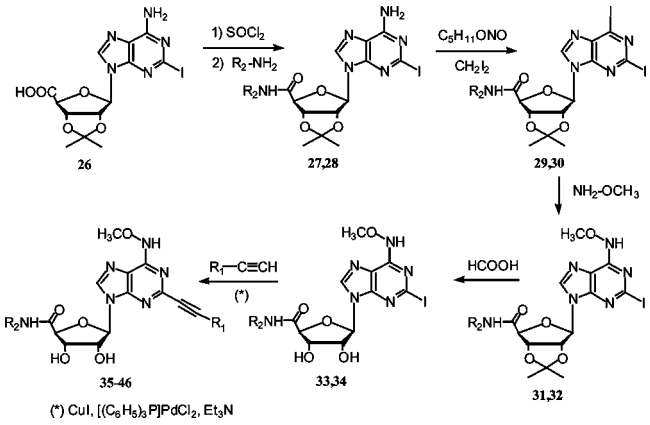
been recently characterized as a novel tool for A_3 receptor binding assays[35] and it is now commercially available.[36]

Based on these results, we attempted to further increase affinity and selectivity of A_3 Ado-receptor agonists. Therefore, the synthesis of 2-alkynylAdo derivatives, bearing a methoxy group in the N^6-position, was undertaken, because a methoxy group has been reported to improve affinity and selectivity of A_3 receptor agonists.[37] Hence, some functionalized alkynyl and cycloalkynyl chains, pyridinylethynyl groups, and substituted phenylethynyls were introduced in the 2-position, and some compounds, which showed the best affinity and selectivity at the A_3 adenosine receptor subtype, were also modified in the 4′-position of the sugar moiety by the introduction of a methyl or an ethyl carboxamido substituent (Figure 2).

Furthermore, selected compounds were submitted to a functional assay to assess their ability to inhibit forskolin-stimulated cAMP production through the human A_3 adenosine receptors, in comparison with the A_3 full agonist Cl-IB-MECA.

Finally, a molecular docking analysis of these compounds was performed utilizing a homology model of the human A_3 receptor based on the bovine rhodopsin crystal structure as template.

Scheme 2



Chemistry

The 2-alkynyl-N^6-methoxyAdo derivatives **8**−**25** were synthesized starting from 6-chloro-2-iodo-9-(2′,3′,5′-tri-*O*-acetyl-*β*-D-ribofuranosyl)-9*H*-purine (**5**), which was obtained from commercially available guanosine in three steps.[23] Reaction of **5** with *O*-methylhydroxylamine hydrochloride gave the corresponding N^6-methoxyamino-2-iodoadenosine derivative **6**, which was treated with methanolic ammonia to obtain the N^6-methoxyamine-2-iodoadenosine **7**. Reaction of this intermediate with the suitable commercially available terminal alkynes, using a modification of the classical palladium-catalyzed cross coupling reaction,[38] gave the 2-alkynyl-N^6-methoxyAdo derivatives **8**−**24** (Scheme 1).

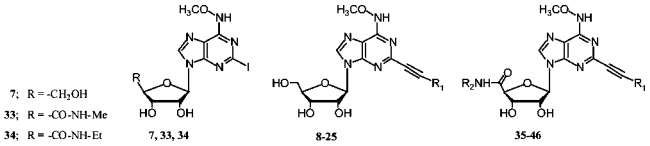
To avoid dimerization of the 2-ethynyl-N^6-methoxyAdo derivative (**25**) during the cross coupling reaction, it was synthesized by deprotection on basic medium of the corresponding trimethylsilylethynyl derivative **10**, obtained by reacting the 2-iodo derivative **7** with trimethylsilylacetylene.

For the synthesis of the derivatives **35**−**46**, substituted also in the 4′-position of the sugar moiety, the 6-(6-amino-2-iodopurin-9-yl)-2,2-dimethyl-tetrahydro-furo[3,4-*d*][1,3]dioxole-4-carboxylic acid (**26**), obtained from guanosine in six steps, was used as starting material (Scheme 2).[23] Treatment of the carboxylic acid **26** with $SOCl_2$ in dry DMF and subsequent reaction with methyl- or ethylamine gave the corresponding alkylcarboxamido derivatives **27** or **28**,[25] which were reacted with isopentylnitrite and diiodomethane to give the 2,6-diiodo derivatives **29** and **30**, respectively. Compounds **29** and **30** were in turn substituted in the N^6-position with *O*-methylhydroxylamine hydrochloride to give **31** and **32**, which were deprotected by treatment with formic acid and then reacted with the suitable alkynes, using the cross coupling reaction condition abovementioned, to obtain the desired trisubstituted Ado derivatives **35**−**46** (Scheme 2).

Results and Discussion

Binding Studies. The new compounds were evaluated at the human recombinant adenosine receptors, stably transfected into CHO cells, utilizing radioligand binding studies (A_1, A_{2A}, A_3) or adenylyl cyclase activity assay (A_{2B}). Receptor binding affinity was determined using [^{3}H]CCPA as radioligand for A_1 receptors, whereas [^{3}H]NECA was used for the A_{2A} and A_3 subtypes. In the case of A_{2B} receptors, K_i values were calculated from IC_{50} values determined by inhibition of NECA-stimulated adenylyl cyclase activity. K_i values are in nM, with 95% confidence intervals in parentheses.[39] The binding affinity of the new compounds is shown in Table 1, with **1**, **2**, and **3** as reference compounds.

Table 1. Affinities of the Ado Derivatives **1** and **7**−**25**, MECA Derivatives **2**, **33**, and **35**−**40**, and NECA Derivatives **3**, **34**, and **41**−**46** in Radioligand Binding Assays at Human A_1, A_{2A}, and A_3 Adenosine Receptors



			K_i nM				
cmpd	R_1	R_2	K_i (A_1)[a]	K_i (A_{2A})[b]	K_i (A_3)[c]	A_1/A_3	A_{2A}/A_3
1			391 (284−556)	363 (285−462)	16 (13−19)	24	23
7			58 (41−82)	12 400 (7860−19 600)	7.4 (6.1−9.0)	8	1700
8	C_6H_5		1210 (893−1630)	4290 (3170−5810)	3.8 (2.6−5.5)	318	1100
25	H		25 (22−29)	3140 (2570−3830)	23 (17−32)	1	136
9	*n*-C_4H_9		97 (75−127)	267 (221−322)	2.8 (1.8−4.3)	35	95
10	$(CH_3)_3Si$		22 (18−27)	5740 (4770−6910)	13 (9.1−17.7)	1.7	441
11	$NC(CH_2)_3$		282 (221−359)	1820 (1310−2520)	4.1 (3.2−5.4)	69	444
12	$CH_3CH(OH)CH_2$		95 (79−114)	758 (542−1060)	5.4 (4.0−7.3)	18	140
13	*c*-$C_6H_{10}(OH)$		34 (26−45)	91 (56−148)	1.7 (0.92−3.2)	20	53
14	2-Py		437 (347−551)	2960 (2660−3290)	2.3 (2.1−2.6)	190	1300
15	3-Py		255 (229−284)	12 500 (9490−16 600)	7.9 (6.8−9.1)	32	1600
16	4-Py		1190 (1030−1360)	6310 (4350−9140)	6.0 (5.2−6.8)	198	1100
17	*p*-CH_3-C_6H_4		1380 (977−1960)	35 100 (1790−6920)	5.9 (4.4−7.8)	234	6000
18	*p*-*n*-C_5H_{11}-C_6H_4		730 (477−1120)	6520 (4770−8920)	39 (21−73)	19	167
19	*p*-F-C_6H_4		2790 (2540−3060)	3960 (3000−5240)	4.4 (3.3−5.8)	634	900
20	*m*-CF_3-C_6H_4		2450 (2310−2600)	8270 (7050−9700)	23 (19−28)	106	360
21	*p*-$NCCH_2$-C_6H_4		766 (611−961)	3080 (2640−3600)	3.7 (2.5−5.5)	207	832
22	*p*-CH_3CO−C_6H_4		1280 (1100−1490)	2680 (2240−3220)	3.0 (1.9−4.7)	427	893
23	*p*-NH_2CO-C_6H_4		901 (708−1150)	3130 (1960−5020)	5.2 (4.0−6.9)	173	602
24	*p*-CH_3O-C_6H_4		1870 (1430−2430)	1780 (1500−2110)	4.3 (2.8−6.5)	435	414
2			3920 (2390−6430)	1760 (1360−2280)	7.3 (4.2−12.7)	537	241
33			153 (107−220)	12 100 (8460−17 300)	1.7 (1.4−2.2)	90	7100
35	C_6H_5	Me	9140 (6680−12 500)	16 300 (12 100−21 900)	1.9 (1.6−2.2)	4800	8600
36	2-Py	Me	3990 (3450−4610)	18 000 (13 600−24 000)	1.1 (0.89−1.3)	3600	16 400
37	*p*-CH_3-C_6H_4	Me	12 000 (9290−15 400)	14 700 (10 600−20 300)	3.3 (2.7−4.1)	3600	4500
38	*p*-*n*-C_5H_{11}-C_6H_4	Me	48 100 (32 300−71 700)	31 700 (18 400−54 900)	55 (40−75)	874	576
39	*p*-F-C_6H_4	Me	3000 (2530−3570)	18 700 (14 800−23 600)	1.9 (1.5−2.4)	1600	9800
40	*p*-CH_3CO-C_6H_4	Me	53 800 (48 100−60 100)	10 400 (8450−12 800)	2.5 (1.8−3.5)	21 500	4200
3			560 (480−650)	620 (300−1300)	6.2 (5.1−7.5)	90	100
34			53 (37−77)	5290 (4270−6540)	2.8 (2.3−3.4)	19	1900
41	C_6H_5	Et	1880 (1330−2660)	6660 (3730−11 900)	3.5 (2.9−4.1)	537	1900
42	2-Py	Et	175 (129−237)	6460 (4250−9820)	1.6 (0.82−3.2)	109	4000
43	*p*-CH_3-C_6H_4	Et	1420 (1240−1620)	4600 (2970−7120)	6.8 (4.1−11)	209	676
44	*p*-*n*-C_5H_{11}-C_6H_4	Et	4090 (2360−7110)	27 100 (19 700−37 300)	68 (52−89)	60	399

Table 1 (Continued)

cmpd	R_1	R_2	K_i nM K_i (A_1)[a]	K_i (A_{2A})[b]	K_i (A_3)[c]	A_1/A_3	A_{2A}/A_3
45	*p*-F-C_6H_4	Et	1880 (1370−2570)	6290 (5280−7490)	3.2 (2.1−4.7)	587	2000
46	*p*-CH_3CO-C_6H_4	Et	1230 (960−1580)	4210 (2710−6520)	4.0 (2.4−6.5)	307	1100

[a] Displacement of specific (^{3}H)CCPA binding in CHO cells, stably transfected with human recombinant A_1 adenosine receptor, expressed as K_i (nM). [b] Displacement of specific (^{3}H)NECA binding in CHO cells, stably transfected with human recombinant A_{2A} adenosine receptor, expressed as K_i (nM). [c] Displacement of specific (^{3}H)NECA binding in CHO cells, stably transfected with human recombinant A_3 adenosine receptor, expressed as K_i (nM).

Most of the tested compounds were inactive at A_{2B} receptors (K_i values >30 μM), with the exception of **13** and **42** (K_i = 3.5 and 7.3 μM, respectively). Therefore, the A_{2B} data are not shown in Table 1. The potency at A_{2B} receptors shown by the N^6-methoxy-2-[2-(1-hydroxycyclohexyl)ethynyl]adenosine (**13**) supports the previously reported observation that the presence of a hydroxyl group in α-position to the triple bond of 2-alkynylAdo derivatives is favorable for A_{2B} potency.[40]

As mentioned above, **1** showed a K_i A_3 = 16 nM and an A_3 selectivity versus A_1 and A_{2A} of 24- and 23-fold, respectively. The introduction of the methoxy group in the N^6-position of **1** led to an increase of A_3 affinity of about 4-fold and a decrease of both A_1 and A_{2A} affinity of 3- and 11-fold, respectively, leading to a significant improvement of A_3 selectivity; in fact, the N^6-methoxy-2-phenylethynylAdo (**8**) showed a K_i A_3 = 3.8 nM and an A_3 selectivity toward A_1 and A_{2A} of 320- and 1100-fold, respectively. Substitution of the phenyl ring in the 2-alkynyl substituent of **8** with hydrogen, alkyls, or functionalized alkyl chains led to compounds **25** and **9**−**13**, endowed with high A_3 affinity, but decreased A_3 selectivity in comparison with **8**. Also, compounds **14**−**16**, bearing pyridinyl substituents in the 2-alkynyl chain, showed high A_3 affinity, but in this case only a minor decrease of A_3 selectivity versus A_1 selectivity was observed, whereas the selectivity versus A_{2A} was maintained or even increased (**14**, K_i A_3 = 2.3 nM, A_3 selectivity vs A_1 = 190 and vs A_{2A} = 1300; **15**, K_i A_3 = 7.9 nM, A_3 selectivity vs A_1 = 32 and vs A_{2A} = 1600; **16**, K_i A_3 = 6.0 nM, A_3 selectivity vs A_1 = 200 and vs A_{2A} = 1100). These results, together with data for **8**, confirm that the presence of an aromatic substituent directly conjugated to the triple bond does not favor the interaction with the A_1 and A_{2A} receptors. The presence of small substituents in the *p*-position of the phenyl ring of **8** was well tolerated by the human A_3 receptor; in fact, compounds **17**, **19**, and **21**−**24** (K_i ranging from 3.0 to 5.9 nM) showed in general comparable A_3 affinity and selectivity with the parent compound **8**. On the contrary, a bulkier substituent in the *p*-position or a small group in the *m*-position decreased the A_3 affinity (**18** and **20**; K_i A_3 = 39 and 23 nM, respectively).

The 5′-*N*-methyl- and 5′-*N*-ethylcarboxamido derivatives of **1** displayed increased A_3 affinity and selectivity in comparison with the parent compound (**2**, K_i A_3 = 7.3 nM, A_3 selectivity vs A_1 = 537 and vs A_{2A} = 241; and **3**, K_i A_3 = 6.2 nM, A_3 selectivity vs A_1 = 90 and vs A_{2A} = 100, compared to **1**, K_i A_3 = 16 nM, A_3 selectivity vs A_1 = 24 and vs A_{2A} = 23). Also, in these cases, the introduction of the methoxy group in N^6-position led to an increase of both A_3 affinity and selectivity; in fact, the N^6-methoxy-2-phenylethynyl-5′-*N*-methylcarboxamidoAdo (**35**) showed a K_i A_3 = 1.9 nM and an A_3 selectivity versus A_1 = 4800 and versus A_{2A} = 8600, and the N^6-methoxy-2-phenylethynyl-5′-*N*-ethylcarboxamidoAdo (**41**) showed a K_i A_3 = 3.5 nM and an A_3 selectivity versus A_1 = 537 and versus A_{2A} = 1900. As in the case of the ribose derivatives, the N^6-methoxy-[2-(2-pyridinyl)ethynyl]MECA and the corresponding NECA derivative (**36** and **42**, respectively) maintained the same high A_3 affinity of the parent compounds **35** and **41**, and, in these cases, the A_3 selectivity versus A_{2A} was greatly improved (**36**, K_i A_3 = 1.1 nM, A_3 selectivity vs A_1 = 3600 and vs A_{2A} = 16 400; and **42**, K_i A_3 = 1.6 nM, A_3 selectivity vs A_1 = 109 and vs A_{2A} = 4000). Once again, a similar trend of A_3 affinity and selectivity was observed in N^6-methoxyMECA and NECA derivatives substituted in the *p*-position of 2-phenylethynyl chain with small groups, in comparison to the corresponding ribose derivatives. It is worthwhile to note that the MECA derivatives resulted to be the most active compounds of the series in terms of binding affinity and selectivity. Hence, compound **40**, bearing a *p*-acetyl group in the 2-phenylethynyl substituent (**40**, K_i A_3 = 2.5 nM, A_3 selectivity vs A_1 = 21 500 and vs A_{2A} = 4200), proved to be one of the most active and selective human A_3 adenosine receptor agonists reported so far.

Table 2. Inhibition of Adenylyl Cyclase Activity via Human A_3 Adenosine Receptors by a Selection of the New Compounds[a]

cmpd	% AC activity after 10 μM forskolin stimulation
Cl-IB-MECA	40 ± 3.9
8	81 ± 2.3
14	94 ± 11.4
22	81 ± 0.6
35	38 ± 3.2
36	34 ± 1.3
40	41 ± 2.8
41	35 ± 4.0

[a] It is shown the percentage of activity remaining after agonist-mediated inhibition of forskolin-stimulated cyclase activity (100%) ± SEM (n = 3).

The three 2-iodo intermediates, **7**, **33**, and **34**, exhibited high A_3 affinity, but low selectivity versus the A_1 receptor subtype (**7**, K_i A_3 = 7.4 nM, A_3 selectivity vs A_1 = 8; **33**, K_i A_3 = 1.7 nM, A_3 selectivity vs A_1 = 90; **34**, K_i A_3 = 2.8 nM, A_3 selectivity vs A_1 = 19).

Adenylyl Cyclase Activity. The ability of selected compounds (**8**, **14**, **22**, **35**, **36**, **40**, and **41**) to inhibit forskolin-stimulated cAMP production via human A_3 adenosine receptors was studied in comparison with the full A_3 agonist Cl-IB-MECA.[39] The functional assay showed that the three N^6-methoxyAdo derivatives are partial agonists (**8**, **22**) or antagonists (**14**) compared with Cl-IB-MECA as a reference. In fact, these compounds are slightly effective or ineffective in inhibiting forskolin-stimulated cAMP production (Table 2). On the contrary, all the N^6-methoxy MECA and NECA derivatives, **35**, **36**, **40**, and **41**, showed an adenylyl cyclase inhibitory activity comparable to that of Cl-IB-MECA, thus behaving as full agonists of the human A_3 receptor subtype.

Molecular Modeling

A molecular docking analysis of these compounds was performed at a homology model of human A_3 receptor built using bovine rhodopsin crystal structure as template, with the aim at getting a possible rationalization of the different binding affinities of the molecules for the human A_3 receptor.

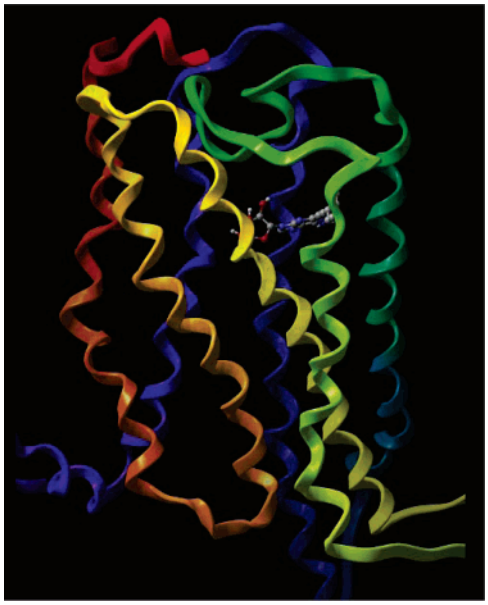


Figure 3. Molecular docking studies of new A_3 receptor agonists. The agonists docking conformations show the adenine scaffold plane almost orthogonal with respect to the receptor transmembrane axis.

The agonists binding region was established with the aid of published site directed mutagenesis data, which indicated in particular that residues H95, S247, N250, and H272 are critical for agonist binding and W243 for receptor activation.[41−44]

It must be underlined that the crystal structure of the resting state of bovine Rhodopsin is not an optimal template for the modeling of G-protein-coupled receptors in the activated form and, consequently, to carry out a rigorous docking analysis of receptor agonists. Anyway, with molecular modeling techniques, it is possible to get information about a ligand−target interaction scheme and to find local modifications of receptor binding site that could possibly lead to receptor activation. That is the case for example of residue W243, whose conformational modification has been associated to possible A_3 receptor activation and, consequently, to agonist activity.[41,42]

The docking conformations present the adenine scaffold plane almost orthogonally with respect to the receptor transmembrane axis, with the 2-phenylethynyl group inserted in a space between TM3 and TM5 (see Figure 3). These conformations are in accordance with the already published docking conformation of **1**.[45]

The analyzed A_3 receptor ligands have the same core structure, with different substituents in the N^6- and the 4′-position. In particular, the different group at the 4′-carbon of the ribose moiety seems to influence both the affinity and the selectivity of the compounds. In Figure 4, the complexes of the human A_3 receptor model are shown with three agonists: **1**, **8**, and N^6-methoxy-2-phenylethynyl-5′-*N*-methylcarboxamidoAdo (**35**). These compounds share a common structure, apart from the functionalization of the ribose group and the N^6-position. The presence of a methoxy group in the N^6-position seems to allow the ligand to interact with the receptor at two points, S247 and N250; this could explain a better affinity of N^6-methoxyAdo derivatives compared to the compounds with an unmodified N^6-position. In addition, the 5′-*N*-methylcarboxamido group of the derivative **35** leads the molecule to interact with the receptor in two points, L90 and S271, compared to the corresponding hydroxymethyl group in molecule **8**, which can have only one H-bond interaction with H272.

The receptor−agonist complexes present the W243 residue oriented toward the external of the receptor, while before the agonist docking/Monte Carlo simulation it is internally oriented. The reorientation of this residue is in accordance with published data,[41,42] which propose this conformational change as one of the possible causes of receptor activation.

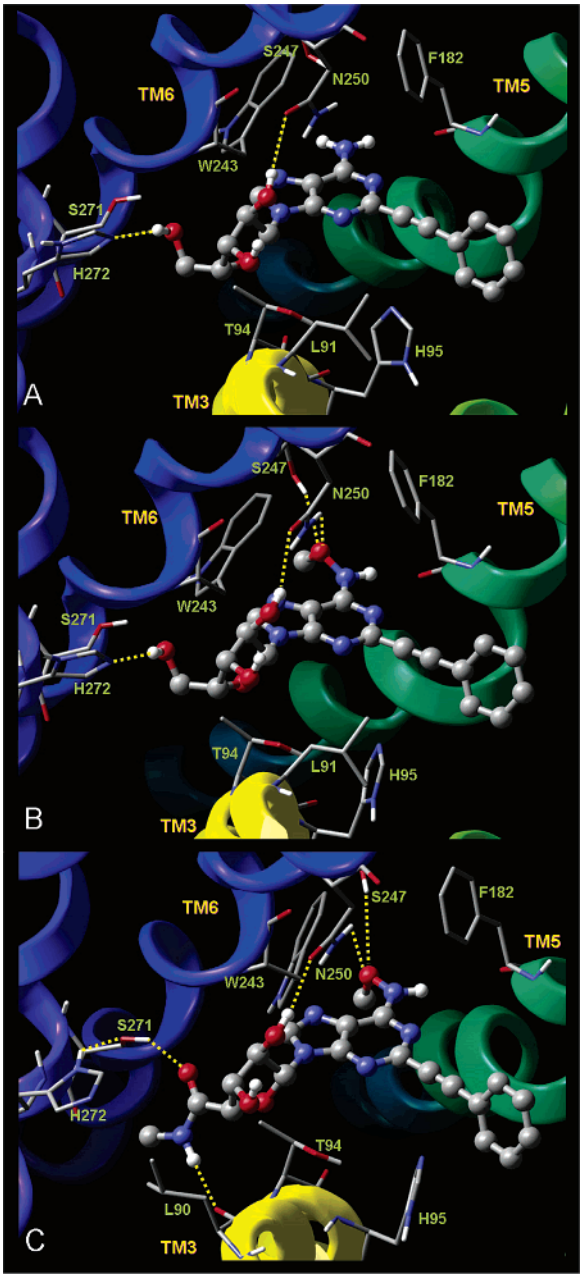


Figure 4. Molecular docking studies of the new A_3 receptor agonists. Here are shown the complexes of the hA_3AR with three agonists, (A) **1**; (B) **8**; (C) **35**. Ligands and protein residues are colored according to atom type. The protein is in *ribbons* representation, and the protein residues interacting with the ligands are shown. H-bond interactions (as indicated by the program) are colored in yellow.

Conclusion

The new 2-aralkynyl-N^6-methoxyAdo derivatives were found to possess high affinity and selectivity for the human A_3 receptor subtype. Among them, compounds bearing a *N*-methylcarboxamido substituent in the 4′-position of the sugar moiety showed the highest A_3 affinity and selectivity. In particular, the N^6-methoxy-2-*p*-acetylphenylethynylMECA (**40**), with a K_i A_3 of 2.5 nM and an A_3 selectivity versus A_1 and A_{2A} of 21 500- and 4200-fold, respectively, and the N^6-methoxy-[2-(2-pyridinyl)-

ethynyl]MECA (**36**), with a K_i A_3 of 1.1 nM and an A_3 selectivity versus A_1 and A_{2A} of 3600- and 16 400-fold, respectively, result to be two of the most potent and selective agonists at the human A_3 adenosine receptor subtype reported so far. Furthermore, adenylyl cyclase activity assay showed that the presence of an alkylcarboxamido group in the 4′-position of the 2-phenylethynylAdo derivatives seems to be essential for full agonistic activity at the A_3 adenosine receptor subtype. Finally, molecular modeling results are in agreement with binding and functional data and help to explain the high affinity and potency of the new trisubstituted adenosine derivatives at the human A_3 adenosine receptor subtype.

Experimental Section

Chemistry. Melting points were determined with a Büchi apparatus and are uncorrected. ^{1}H NMR spectra were obtained with Varian VXR 300 MHz spectrometer; δ in ppm, J in Hz. All exchangeable protons were confirmed by addition of D_2O. TLC were carried out on precoated TLC plates with silica gel 60 F-254 (Merck). For column chromatography, silica gel 60 (Merck) was used. Elemental analyses were determined on Fisons Instruments Model EA 1108 CHNS-O model analyzer and are within ± 0.4% of theoretical values.

2-Iodo-N^6-methoxy-2′,3′,5′-tri-*O*-acetyladenosine (6). To a solution of **5**[23] (3.71 mmol; 2 g) in dry THF (60 mL), *O*-methylhydroxylamine hydrochloride (37.1 mmol; 3.10 g) and Et_3N (44.5 mmol; 6.2 mL) were added. The solvent was removed in vacuo, and the residue was chromatographed on a flash silica gel column, eluting with $CHCl_3-CH_3OH$ (99:1) to give **6**, which was crystallized from $CHCl_3-cC_6H_{12}$ (1:1). Yield 77%; mp 171−173 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.04 (s, 3H, CH_3CO), 2.07 (s, 3H, CH_3CO), 2.14 (s, 3H, CH_3CO), 3.77 (s, 3H, OCH_3), 4.35 (m, 3H, H-4′ and CH_2-5′), 5.61 (m, 1H, H-3′), 5.87 (*p*st, 1H, J = 5.2 Hz, H-2′), 6.18 (d, 1H, J = 5.2 Hz, H-1′), 8.38 (s, 1H, H-8), 11.51 (m, 1H, NH). Anal. ($C_{17}H_{20}IN_5O_8$) C, H, N.

2-Iodo-N^6-methoxyadenosine (7). To compound **6** (3.03 mmol; 1.67 g), methanolic ammonia (30 mL) was added, and the mixture was allowed to stand at rt for 96 h. The solvent was removed in vacuo, and the residue was chromatographed on a flash silica gel column, eluting with $CHCl_3-CH_3OH$ (97:3) to give **7**, which was crystallized from CH_3CN. Yield 84%; mp 93−95 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.62 (m, 2H, CH_2-5′), 3.95 (m, 1H, H-4′), 3.77 (s, 3H, OCH_3), 4.13 (m, 1H, H-3′), 4.55 (m, 1H, H-2′), 5.86 (d, 1H, J = 5.9 Hz, H-1′), 8.42 (s, 1H, H-8), 11.43 (s, 1H, NH). Anal. ($C_{11}H_{14}IN_5O_5$) C, H, N.

General Procedure for the Synthesis of 2-Alkynyl-N^6-methoxyadenosines 8−24. To a solution of **7** (0.35 mmol; 0.15 g) in dry DMF (6 mL), triethylamine (1.4 mL), bis(triphenylphosphine)-palladium dichloride (5 mg; 0.007 mmol), CuI (0.35 mg; 0.002 mmol), and the appropriate terminal alkyne (2.1 mmol) were added. The reaction mixture was stirred under a nitrogen atmosphere at rt, except for compounds **9** and **17** (t = 50 °C). After evaporation in vacuo, the residual oils were purified by silica gel column chromatography, eluting with the suitable mixture of solvents to obtain compounds **8−24** as white solids.

N^6-Methoxy-2-phenylethynyladenosine (8). Reaction of **7** with phenylethyne for 5 h, followed by chromatography, eluting with $CHCl_3-CH_3OH$ (90:10), gave **8**, which was crystallized from CH_3CN. Yield 69%; mp 175−177 °C (dec); ^{1}H NMR (DMSO-d_6) δ 3.65 (m, 2H, CH_2-5′), 3.81 (s, 3H, OCH_3), 3.99 (m, 1H, H-4′), 4.16 (m, 1H, H-3′), 4.57 (m, 1H, H-2′), 5.97 (d, 1H, J = 5.9 Hz, H-1′), 7.50 (m, 3H, H-Ph), 7.68 (m, 2H, H-Ph), 8.58 (s, 1H, H-8), 11.26 (s, 1H, NH). Anal. ($C_{19}H_{19}N_5O_5$) C, H, N.

2-Ethynyl-N^6-methoxyadenosine (25). To compound **10** (0.62 mmol; 0.25 g), dissolved in dry methanol (5 mL), a solution of KOH (1.24 mmol; 0.07 g) in dry methanol (5 mL) was added. The mixture was allowed to stand at rt for 1 h. The solvent was removed in vacuo, and the residue was chromatographed on a flash silica gel column eluting with $CHCl_3-CH_3OH$ (95:5) to give **25**, which was crystallized from $CHCl_3$. Yield 69%; mp 126−128 °C; ^{1}H NMR (DMSO-d_6) δ 3.58 (s, 1H, CH_2-5′), 3.68 (m, 1H, CH_2-5′), 3.78 (s, 3H, OCH_3), 3.96 (m, 1H, H-4′), 4.15 (m, 2H, H-3′ and C≡CH), 4.55 (m, 1H, H-2′), 5.91 (d, 1H, J = 5.7 Hz, H-1′), 8.55 (s, 1H, H-8), 11.24 (s, 1H, NH). Anal. ($C_{13}H_{15}N_5O_5$) C, H, N.

6-Amino-2-iodo-2′,3′-*O*-isopropylidene-5′-*N*-methylcarboxamidoadenosine (27). To compound **26**[23] (0.67 mmol; 0.3 g), $SOCl_2$ (1 mL) and dry DMF (24 μL) were added. The mixture was heated at 50 °C for 2 h under a nitrogen atmosphere. The solvent was removed in vacuo, and the residue was coevaporated three times with dry toluene. To the residue was added dry CH_2Cl_2 (5 mL) and, after cooling at −20 °C, CH_3NH_2 (1 mL) was added. The mixture was stirred 1 h at rt and then partitioned between H_2O and CH_2Cl_2. The organic extracts were collected, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was chromatographed on a flash silica gel column eluting with $CHCl_3-CH_3OH$ (99:1) to give **27** as a white solid. Yield 79%; mp 241−243 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.33 (s, 3H, CH_3), 1.52 (s, 3H, CH_3), 2.37 (m, 3H, NHCH_3), 4.54 (br s, 1H, H-4′), 5.29 (m, 2H, H-2′ and H-3′), 6.24 (s, 1H, H-1′), 7.5 (m, 1H, NH), 7.71 (s, 2H, NH_2), 8.16 (s, 1H, H-8). Anal. ($C_{14}H_{17}IN_6O_4$) C, H, N.

General Procedure for the Synthesis of 2,6-Diiodo Derivatives 29 and 30. To compound **27** or **28** [25] (0.37 mmol), CH_2I_2 (1.78 mL), $C_5H_{11}ONO$ (0.55 mL), and dry DMF (5 mL) were added. The mixture was stirred at 85 °C for 0.5 h. The solvent was removed in vacuo, and the residue was chromatographed eluting with the suitable mixture of solvents to give **29** and **30** as yellow solids.

2,6-Diiodo-2′,3′-*O*-isopropylidene-5′-*N*-methylcarboxamidoadenosine (29). Reaction of **27**, followed by flash chromatography, eluting with $CHCl_3-cC_6H_{12}-CH_3OH$ (80:18:2), gave compound **29**. Yield 56%; mp 125−127 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.37 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 2.27 (d, 3H, J = 4.4 Hz, NHCH_3), 4.60 (s, 1H, H-4′), 5.37 (br s, 2H, H-2′ e H-3′), 6.38 (s, 1H, H-1′), 7.47 (m, 1H, J = 4.4 Hz, NH), 8.66 (s, 1H, H-8). Anal. ($C_{14}H_{15}I_2N_5O_4$) C, H, N.

2,6-Diiodo-2′,3′-*O*-isopropylidene-5′-*N*-ethylcarboxamidoadenosine (30). Reaction of **28**, followed by flash chromatography, eluting with $CHCl_3-cC_6H_{12}-CH_3OH$ (60:39:1), gave compound **30**. Yield 57%; mp 89−91 °C (dec); ^{1}H NMR (DMSO-d_6) δ 0.54 (t, 3H, J = 7.2 Hz, CH_3CH_2), 1.37 (s, 3H, CH_3), 1.54 (s, 3H, CH_3), 2.74 (m, 2H, CH_2NH), 4.60 (s, 1H, H-4′), 5.43 (br s, 2H, H-2′ and H-3′), 6.43 (s, 1H, H-1′), 7.55 (t, 1H, J = 5.7 Hz, NH), 8.67 (s, 1H, H-8). Anal. ($C_{15}H_{17}I_2N_5O_4$) C, H, N.

General Procedure for the Synthesis of 2-Iodo-N^6-methoxy Derivatives 31 and 32. To a mixture of NH_2OCH_3 × HCl (0.58 mmol; 0.49 g), Et_3N (0.97 mL), and dry THF (8.9 mL), stirred under nitrogen atmosphere for 2 h, **29** or **30** (0.58 mmol) was added. The mixture was allowed to stand at 50 °C for 19 h and then partitioned between H_2O and $CHCl_3$. The organic extracts were collected, dried over Na_2SO_4, filtered, and evaporated to dryness. The residue was chromatographed on a silica gel column, eluting with $CHCl_3-CH_3OH$ (99.5:0.5), to obtain **31** or **32**, respectively, as white solids.

2-Iodo-2′,3′-*O*-isopropylidene-N^6-methoxy-5′-*N*-methylcarboxamidoadenosine (31). Yield 53%; mp 115−117 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.40 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 2.35 (m, 3H, NHCH_3), 3.75 (m, 3H, OCH_3), 4.56 (s, 1H, H-4′), 5.32 (br s, 2H, H-2′ and H-3′), 6.29 (s, 1H, H-1′), 7.48 (m, 1H, *NH*CH_3), 8.30 (s, 1H, H-8′), 11.37 (s, 1 H, *NH*OCH_3). Anal. ($C_{15}H_{19}IN_6O_5$) C, H, N.

2-Iodo-2′,3′-*O*-isopropylidene-N^6-methoxy-5′-*N*-ethylcarboxamido Adenosine (32). Yield 78%; mp 116−119 °C; ^{1}H NMR (DMSO-d_6) δ 0.64 (t, 1H, J = 7.2 Hz, $NHCH_2CH_3$), 1.35 (s, 3H, CH_3), 1.53 (s, 3H, CH_3), 2.87 (m, 2H, CH_2NH), 3.74 (s, 1H, OCH_3), 4.55 (m, 1H, H-4′), 5.38 (m, 2H, H-2′ and H-3′), 6.33 (s, 1H, H-1′), 7.47 (t, 1H, J = 5.7 Hz, *NH*CH_2), 8.26 (s, 1H, H-8), 11.36 (s, 1H, NH). Anal. ($C_{16}H_{21}IN_6O_5$) C, H, N.

General Procedure for the Synthesis of 2-Iodo-N^6-methoxy Derivatives 33 and 34. To compound **31** or **32** (0.40 mmol), a 50% solution of HCOOH (14.8 mL) was added, and the mixture was heated at 50 °C for 2 h. The solvent was removed under

vacuum, and the residue was coevaporated three times with H_2O. The residue was chromatographed on a silica gel column, eluting with a gradient of $CHCl_3-CH_3OH$ (98:2) ⇒ (96:4), to give **33** and **34**, respectively, as white solids.

2-Iodo-*N*^{6}-methoxy-5′-*N*-methylcarboxamidoadenosine (33). Yield 77%; mp 225−227 °C (dec); ^{1}H NMR (DMSO-d_6) δ 2.73 (d, 3H, J = 9.6 Hz, NH*CH*$_3$), 3.77 (s, 3H, OCH$_3$), 4.18 (m, 1H, H-3′), 4.32 (m, 1H, H-4′), 4.62 (m, 1H, H-2′), 5.60 (d, 1H, J = 6.2 Hz, OH), 5.72 (d, 1H, J = 8.0 Hz, OH), 5.94 (d, 1H, J = 9.2 Hz, H-1′), 8.03 (m, 1H, *NH*CH$_3$), 8.48 (s, 1H, H-8), 11.48 (br s, 1H, *NH*OCH$_3$). Anal. ($C_{12}H_{15}IN_6O_5$) C, H, N.

2-Iodo-*N*^{6}-methoxy-5′-*N*-ethylcarboxamidoadenosine (34). Yield 67%; mp 160−163 °C; ^{1}H NMR (DMSO-d_6) δ 1.05 (t, 3H, J = 7.4 Hz, CH$_2$*CH*$_3$), 3.23 (m, 2H, *CH*$_2$CH$_3$), 3.76 (s, 3H, OCH$_3$), 4.18 (m, 1H, H-3′), 4.32 (d, 1H, J = 1.8 Hz, H-4′), 4.60 (m, 1H, H-2′), 5.63 (d, 1H, J = 6.2 Hz, OH), 5.73 (d, 1H, J = 4.8 Hz, OH), 5.94 (d, 1H, J = 6.8 Hz, H-1′), 8.14 (t, 1H, J = 5.6 Hz, *NH*CH$_2$), 8.49 (s, 1H, H-8), 11.48 (br s, 1H, *NH*OCH$_3$). Anal. ($C_{13}H_{17}IN_6O_5$) C, H, N.

General Procedure for the Synthesis of 2-Alkynyl-*N*^{6}-methoxy-5′-*N*-methylcarboxamide Derivatives 35−40 and 2-Alkynyl-*N*^{6}-methoxy-5′-*N*-ethylcarboxamide Derivatives 41−46. To a solution of **33** or **34** (0.28 mmol) in dry DMF (10 mL), triethylamine (1.18 mL), bis(triphenylphosphine)palladium dichloride (4.3 mg, 0.006 mmol), CuI (0.22 mg, 0.0012 mmol), and the appropriate terminal alkyne (1.68 mmol) were added. The reaction mixture was stirred under nitrogen atmosphere at rt, except for compounds **37** and **43** (t = 50 °C), and for the time reported in Table 4 in Supporting Information. After evaporation under vacuum, the residue was purified by silica gel column chromatography eluting with the suitable mixture of solvents, to obtain the MECA and NECA derivatives **35**−**40** and **41**−**46**, respectively.

***N*^{6}-Methoxy-2-phenylethynyl-5′-*N*-methylcarboxamidoadenosine (35).** Reaction of **33** with phenylethyne for 24 h, followed by chromatography eluting with $CHCl_3-CH_3OH$ (90:10), gave **35**, which was crystallized from CH_3CN. Yield 60%; mp 164−167 °C; ^{1}H NMR (DMSO-d_6) δ 2.77 (d, 3H, J = 4.8 Hz, NH*CH*$_3$), 3.81 (s, 3H, OCH$_3$), 4.20 (m, 1H, H-3′), 4.34 (s, 1H, H-4′), 4.61 (m, 1H, H-2′), 5.60 (d, 1H, J = 6.2 Hz, OH), 5.76 (d, 1H, J = 4.4 Hz, OH), 6.00 (d, 1H, J = 7.8 Hz, H-1′), 7.49 (m, 3H, H-Ph), 7.64 (m, 2H, H-Ph), 8.48 (m, 1H, *NH*CH$_3$), 8.61 (s, 1H, H-8), 11.32 (br s, 1H, *NH*OCH$_3$). Anal. ($C_{20}H_{20}N_6O_5$) C, H, N.

***N*^{6}-Methoxy-2-phenylethynyl-5′-*N*-ethylcarboxamidoadenosine (41).** Reaction of **34** with phenylethyne for 24 h, followed by chromatography eluting with $CHCl_3-CH_3OH$ (90:10), gave **41**, which was crystallized from CH_3CN. Yield 65%; mp 186−189 °C (dec); ^{1}H NMR (DMSO-d_6) δ 1.03 (t, 3H, J = 7.2 Hz, CH$_2$*CH*$_3$), 3.22 (m, 2H, *CH*$_2$CH$_3$), 3.81 (s, 3H, OCH$_3$), 4.15 (br s, 1H, H-3′), 4.34 (s, 1H, H-4′), 4.65 (m, 1H, H-2′), 5.61 (d, 1H, J = 6.6 Hz, OH), 5.76 (d, 1H, J = 4.4 Hz, OH), 6.10 (d, 1H, J = 7.3 Hz, H-1′), 7.49 (m, 3H, H-Ph), 7.64 (m, 2H, H-Ph), 8.49 (t, 1H, J = 4.0 Hz, *NH*CH$_2$), 8.63 (s, 1H, H-8), 11.32 (br s, 1H, *NH*OCH$_3$). Anal. ($C_{21}H_{22}N_6O_5$) C, H, N.

Biological Evaluation. Binding Studies. Dissociation constants of unlabeled compounds (K_i values) were determined in competition experiments in 96-well microplates as described recently.[38] For A_{2A} and A_3 adenosine receptors the nonselective agonist [^{3}H]NECA (30 nM and 10 nM, respectively) was utilized as radioligand. The A_1-selective agonist [^{3}H]CCPA (1 nM) was utilized for the characterization of A_1 receptor binding. Nonspecific binding was determined in the presence of 100 μM *R*-PIA and 1 mM theophylline, respectively. For details, see Klotz et al.[39] All binding data were calculated by nonlinear curve fitting with the program SCTFIT.[46]

Adenylyl Cyclase Activity. The functional activity of selected new derivatives was determined in adenylyl cyclase experiments. The stimulation of adenylyl cyclase via A_{2B} adenosine receptors and the inhibition of forskolin-stimulated adenylyl cyclase via A_3 receptors were measured as described earlier.[39,47]

Molecular Modeling. Computational Methodologies. All molecular modeling studies were performed on a 2 CPU (PIV 2.0−3.0 GHZ) Linux PC. Homology modeling has been carried out using Molecular Operating Environment (MOE, version 2004.03) suite.[48] Docking studies have been done using Schrodinger Macromodel (version 8.0)[49,50] with Schrodinger Maestro interface.

Homology Model of the Human A_3 Receptor. A homology model of the human A_3 receptor was built using the X-ray crystal structure of the resting state bovine rhodopsin (pdb code: 1L9H;[51] available at the RCSB Protein Data Bank) with a 2.6 Å resolution as template.

The amino acid sequences of TM helices of the human A_3 receptor and bovine rhodopsin were aligned, and in this phase, some GPCRs highly conserved amino acid residues worked as guide, including the DRY motif (D3.49, R3.50, Y3.51, or D107, R108, Y109, respectively) and three Pro residues (P4.60, P6.50, P7.50 or P145, P189, P245, respectively). The boundaries identified from the X-ray crystal structure of bovine rhodopsin were applied for the corresponding sequences of the TM helices of the A_3 receptor. The loop domains of the human A_3 receptor were built by the *loop search method* implemented in MOE. A special care had to be given to the second extracellular (E2) loop, which folds back over TM domains. This loop limits the dimension of the active site, and its amino acids could be involved in direct interactions with the ligands. The presence of a conserved disulfide bridge between cysteines in TM3 and E2 might be the driving force to this particular fold of the E2 domain, so this loop was modeled using a rhodopsin-like constrained geometry around the E2-TM3 disulfide link. Because of the limited sequence similarity between the human A_3 receptors and the template in the C-terminal domain, only a short segment of this region was modeled. In particular, the model ends with the Ser306 residue corresponding to the Asp330 residue of the bovine rhodopsin template. Once the heavy atoms were modeled, all hydrogen atoms were added, and the protein coordinates were then minimized with MOE using the AMBER94[52] force field. The minimizations were performed by 1000 steps of steepest descent, followed by conjugate gradient minimization until the rms gradient of the potential energy was less than 0.05 kJ mol^{-1} Å^{-1}.

Molecular Docking of the Human A_3 Receptor Agonists. All agonist structures were manually docked into the hypothetical TM binding site. This receptor region has been established with the aid of published site directed mutagenesis data, which indicated in particular that residues H95, S247, N250, and H272 are critical for agonist binding and W243 for receptor activation.[41−45] The searching of favorable binding conformations was conducted by a Monte Carlo conformational search protocol implemented in Schrodinger Macromodel. The input structure consisted of the ligand and a shell of receptor amino acids within the specified distance (6 Å) from the ligand. A second external shell of all the residues within a distance of 8 Å from the first shell was kept fixed. During the Monte Carlo conformational searching, the input structure was modified by random changes in user-specified torsion angles (for all input structure residues) and molecular position (for the ligand). Hence, the ligand was left free to be continuously reoriented within the hypothetical binding site, and both ligand and internal shell residues conformations could be explored and reciprocally relaxed. The method consisted of 10 000 conformational search steps with MMFF94s force field.[53−59] Only unique structures within a 50 kJ mol^{-1} energy window above the found global minimum were saved. The final docking complexes were subjected to MMFF94s energy minimization with Schrodinger Macromodel until the rms of the conjugate gradient was less than 0.05 kJ mol^{-1} Å^{-1}.

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Supporting Information Available: Experimental details for the synthesis (Tables 3 and 4), ^{1}H NMR spectral data, and elemental analysis (analytical appendix 1) for compounds **9**−**24**, **36**−**40**, and **42**−**46**. This material is available free of charge via the Internet at http://pubs.acs.org.

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