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Communications to the Editor

Pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]-pyrimidine Derivatives as Highly Potent and Selective Human A_3 Adenosine Receptor Antagonists

Pier Giovanni Baraldi,*,§ Barbara Cacciari,§ Romeo Romagnoli,§ Giampiero Spalluto,† Karl-Norbert Klotz,‖ Edward Leung,▽ Katia Varani,‡ Stefania Gessi,‡ Stefania Merighi,‡ and Pier Andrea Borea‡

Dipartimento di Scienze Farmaceutiche and Dipartimento di Medicina Clinica e Sperimentale-Sezione di Farmacologia, Università degli Studi di Ferrara, Via Fossato di Mortara 17-19, I-44100 Ferrara, Italy, Dipartimento di Scienze Farmaceutiche, Università degli Studi di Trieste, Piazzale Europa 1, I-34127 Trieste, Italy, Institut für Pharmakologie, Universität of Würzburg, D-97078 Würzburg, Germany, and Medco Research, Research Triangle Park, North Carolina 27709

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Introduction. Adenosine modulates many physiological functions through activation of four known receptor subtypes, classified as A_1, A_{2A}, A_{2B}, and A_3.[1,2] The adenosine A_1 and A_{2A} receptor subtypes have been pharmacologically characterized through the use of selective ligands.[3]

The adenosine A_3 receptor was initially cloned from a rat testis cDNA library.[4] Subsequently, A_3 receptors have been cloned from human,[5,6] sheep,[7] mouse,[8] and rabbit.[9] Considerable differences in sequence homology (72%) for the A_3 receptors have been observed between species.[10,11] Affinities for antagonist ligands also showed differences between species. For these reasons, the hypothesis of the existence of two different adenosine A_3 receptor subtypes has been proposed. However different A_3 receptor subtypes within a single species have not yet been demonstrated.[12]

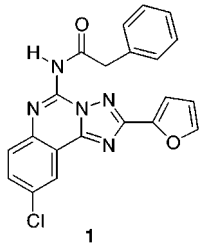
The adenosine A_3 receptor appears to be responsible for many physiological effects, but in some cases the observed responses to chronic agonist and antagonist exposure are the exact opposite to the predicted effects based on short-term exposure to these agents.[12]

Activation of adenosine A_3 receptors has been shown to stimulate phospholipase C[13] and D[14] and to inhibit adenylate cyclase.[3] Activation of A_3 adenosine receptors also causes the release of inflammatory mediators such as histamine from mast cells.[11,15] These mediators are responsible for processes such as inflammation[10] and hypotension.[11] It has also been suggested that the A_3 receptor plays an important role in brain ischemia,[16,17] immunosuppression,[18] and bronchospasm in several animal models.[19]

Taking into account these results, highly selective A_3 adenosine receptor antagonists have been indicated as potential drugs for the treatment of asthma and inflammation.[10,20]

In the past few years, different classes of compounds have been reported to be A_3 adenosine receptor antagonists. Four classes of compounds with non-xanthine structures have been synthesized: dihydropyridine and pyridine analogues,[21−24] flavonoid,[25,26] isoquinoline,[27−28] and triazoloquinazoline derivatives.[29−30]

In the latter class of compounds, Jacobson and coworkers,[29] started from the experimental observation that the 5-amino-9-chloro-2-2−furyl[1,2,4]triazolo[1,5-*c*]quinazoline (CGS15943) possesses affinity for the human A_3 adenosine receptor (K_i hA_3 14 nM). In fact, by its acylation with a phenylacetyl group at the amino function at the 5-position was obtained MRS 1220 (**1**), the most potent but not highly selective A_3 adenosine receptor antagonist reported in the literature.



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In the past few years, we have synthesized a large series of pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine

* Correspondence to: Prof. Pier Giovanni Baraldi. Phone: +39-(0)-532-291293. Fax: +39-(0)532-291296. E-mail: pgb@dns.unife.it.
§ Dipartimento di Scienze Farmaceutiche, Ferrara.
† Università degli Studi di Trieste.
‖ Universität of Wüzburg.
▽ Medco Research.
‡ Dipartimento di Medicina Clinica e Sperimentale, Ferrara.

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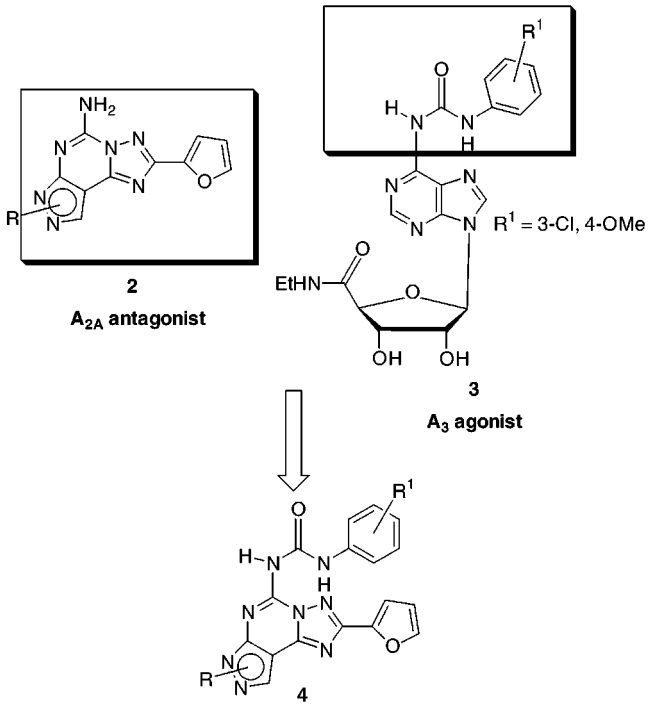
Chart 1. Rational Design of hA_3 Adenosine Receptor Antagonists



derivatives of general formula **2**, structurally related to CGS15943, which turned out to be potent and selective A_{2A} antagonists.[31−34] In addition, several N^6-(substituted phenylcarbamoyl)adenosine-5′-uronamides of general formula **3** have been reported to act as potent agonists for the rat A_3 adenosine receptor subtype (Chart 1).[35,36] Specifically, we observed that the introduction of the 3-chlorophenyl and 4-methoxyphenyl moieties gave the best results in terms of affinity at rat A_3 receptors (K_i 4.4 and 6.6 nM, respectively).

On this basis, we decided to link the amino group at the 5-position, of compounds of general formula **2**, with the two bulky substituents (3-chlorophenylcarbamoyl and 4-methoxyphenylcarbamoyl moieties), which displayed the best results in the field of A_3 agonists,[35,36] in an attempt to modulate the affinity and the selectivity at the human A_3 receptors. (Chart 1). Maintaining the substituents on the phenyl ring (3-chloro or 4-methoxy) at the 5-position, the effects of the lipophilic groups, such as small alkyl and aralkyl moieties, on the pyrazole nitrogen of the synthesized hybrid molecules of general formula **4** have been evaluated.

Chemistry. Compounds **5**−**12** were prepared following the general synthetic strategy summarized in Schemes 1 and 2. Compounds **5**−**12** were synthesized according to a well-known procedure for the synthesis of the pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidines.[32]

Alkylation of 5-amino-4-cyanopyrazole (**13**) with the appropriate alkyl halide in dry dimethylformamide led to an approximately 1:4 mixture of N^1 and N^2 regioisomers (**14**−**17**) as an inseparable mixture, used for the following steps without any further purification (Scheme 1).[32]

Pyrazoles **14**−**17** were transformed into the corresponding imidates **18**−**21** through refluxing in triethyl orthoformate. The imino ethers **18**−**21** were reacted with 2-furoic hydrazide in refluxing 2-methoxyethanol to provide the pyrazolo[3,4-*d*]pyrimidine intermediates. These were converted through a thermally induced cyclization in diphenyl ether to the desired derivatives **22**−**25** in good overall yield (50−63%), after separation of N^7 (minor product) and N^8 (major product) regioisomers by flash chromatography.[32]

Hydrolysis with aqueous 10% HCl afforded the aminotriazoles **26**−**29**, which were in turn converted into the 5-amino-8-(substituted)-2-(2−furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine derivatives **30**−**33**. The final compounds **5**−**12** were obtained by a coupling reaction of derivatives **30**−**33** with the commercially available isocyanates **34** and **35** (Scheme 2).[35]

The coupling reaction with isocyanates was successful using N^8 alkylated compounds, but it failed in the case of the N^7 pattern of substitution (classic A_{2A} antagonists; e.g. 5-amino-7-(2−phenylethyl)-2-(2−furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine, SCH 58261), probably due to the less nucleophilic character of the amino group at the 5-position. In fact, the nitrogen lone pair of the 5-amino group in N^7 derivatives is highly delocalized on the heterocyclic ring. This does not happen in the case of the N^8 derivatives, because the relative mesomeric structures do not allow the same degree of delocalization.

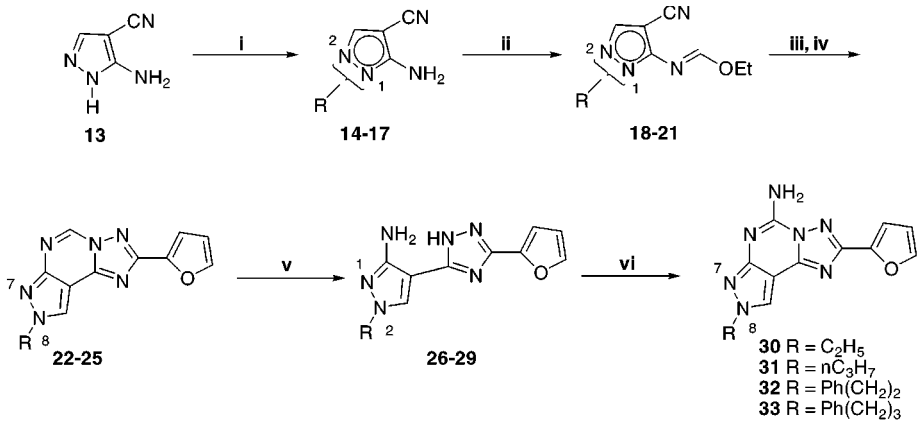
Results and Discussion. Table 1 gives the receptor binding affinities of compounds **5**−**12** and the corresponding N^5-unsubstituted derivatives **30**−**33** determined at rat A_1 and A_{2A} receptors and human A_3 receptors expressed in HEK-293 cells, using [^{3}H]-1,3-dipropyl-8-cyclopentylxanthine ([^{3}H]DPCPX),[37] [^{3}H]-5-amino-7-(2−phenylethyl)-2-(2−furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine ([^{3}H]SCH 58261),[38] and [^{125}I]-N^6-(4−amino−3−iodobenzyl)-5′-(*N*-methylcarbamoyl)-adenosine ([^{125}I]AB-MECA)[39] as radioligands, respectively.

Compounds lacking the phenylcarbamoyl moiety at the N^5 position show high affinity for the A_{2A} receptor with low selectivity versus the A_1 receptor and are inactive at human A_3 adenosine receptor subtypes. As expected, compounds with the greatest affinity at the A_{2A} receptors, **32** and **33**, have the 2-phenylethyl and 3-phenylpropyl chains, respectively, at the pyrazole nitrogen, typical of A_{2A} antagonists. These derivatives are the regioisomers of two of the most potent previously reported A_{2A} antagonists: SCH 58261 and 5-amino-7-(3−phenylpropyl)-2-(2−furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH 63390).[33]

On the contrary, when the substituted phenylcarbamoyl chain is present at the N^5 position, all the synthesized derivatives **5**−**12** show high affinity to human A_3 receptors with a high degree of selectivity versus the other receptor subtypes. In particular the 4-methoxyphenylcarbamoyl moiety (compounds **5**, **7**, **9**, **11**) appears to confer higher affinity to the human A_3 receptor than the 3-chlorophenylcarbamoyl chain (compounds **6**, **8**, **10**, **12**) with a difference of about 2−10 orders of magnitude.

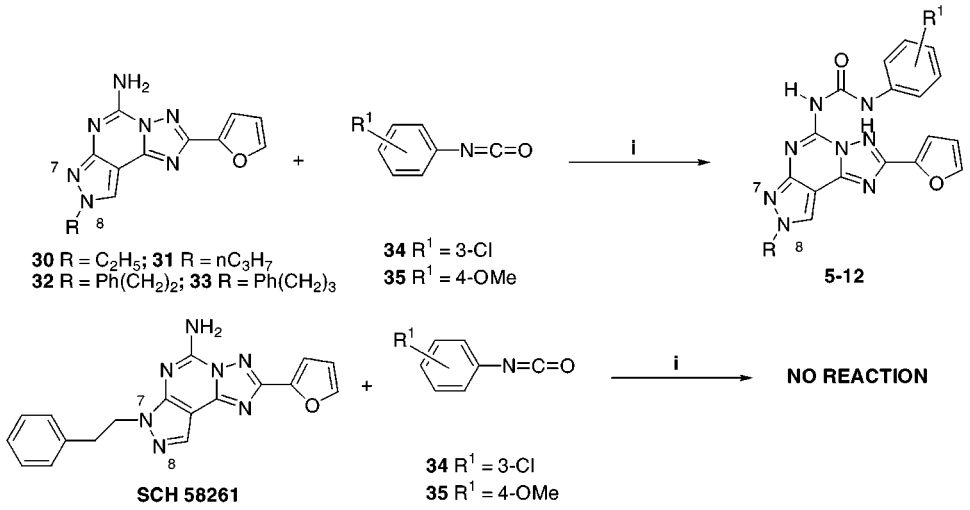
It is evident that small chains such as ethyl and propyl on N^8 of the pyrazole afford compounds with high affinity and selectivity. In particular, compound **5**, with the ethyl group at the N^8 pyrazole combined with the 4-methoxyphenylcarbamoyl moiety at the N^5-position,

Scheme 1[a]



[a] Reagents: (i) NaH, DMF, RX; (ii) $HC(OEt)_3$, reflux; (iii) 2-furoic hydrazide, $MeO(CH_2)_2OH$; (iv) Ph_2O, 260 °C, flash chromatography; (v) HCl, reflux; (vi) NH_2CN, 1-methyl-2-pyrrolidone, pTsOH, 140 °C.

Scheme 2[a]



[a] Reagents: (i) THF, reflux, 12 h.

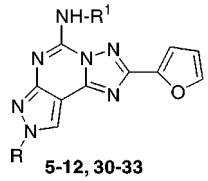
shows the best binding profile with a high human A_3 affinity (K_i 0.28 nM) and selectivity versus A_1 and A_{2A} receptors, higher than 35 000. This derivative could be considered, at present, the most potent and selective hA_3 antagonist ever synthesized. The same degree of affinity, even with a decrease of selectivity, has been observed in compounds with a propyl substituent at N^8 (e.g. compound **7** hA_3 0.29 nM, rA_1/hA_3 >34 000, rA_{2A}/hA_3 6 872). Significant differences have been observed when the phenylethyl and phenylpropyl chains were introduced; in fact, a reduction of affinity of about 10−100-fold is evident (e.g. compound **7** hA_3 0.29 nM vs compound **11** hA_3 19.81 nM).

Earlier experiments using cloned A_1 and A_{2A} human adenosine receptors have shown a significant decrease in selectivity for many ligands whith respect to rat A_1 and A_{2A} receptors.[40] In this study, all the synthesized compounds have been tested on human A_1,[41] human A_{2A},[38] and rat A_3[25a,42] adenosine receptors, to verify the selectivity in the same species (Tables 2, 3).

It is clearly evident, from the results shown in Table 2, that in the human species, these compounds show an increase in affinity of about 10-fold at human A_1 and A_{2A} receptors, with a consequent decrease of selectivity for A_3 receptors. Nevertheless, all the N^5-substituted compounds (**5**−**12**) retain good A_1/A_3 and A_{2A}/A_3 ratios of selectivity. In particular, the most potent compound at human A_3 receptors of this series (**5**) showed again a high level of selectivity versus other receptor subtypes (>3 000). On the contrary, in a rat model (Table 3), all the compounds proved to be almost inactive showing different percentages of inhibition (1−40%) of specific binding at a concentration of 10 μM. These results are in accordance with the low degree of sequence homology (72%) of the A_3 adenosine receptor subtypes.[10,11] All the synthesized compounds were also found to be functional antagonists in a specific functional model where the inhibition of cAMP generation by IB-MECA was measured in membranes of CHO cells stably transfected with the human A_3 receptor (Table 4).[43]

As expected, all the derivatives are antagonists with different degrees of potency. Compounds **30**−**33**, with the free amino group at the 5-position, as observed in binding studies, showed poor activity, inhibiting the effect of 100 nM IB-MECA in a range of 3−40% at 1 μM concentration. On the contrary, the N^5-substituted compounds (**5**−**12**) were more potent in the functional assay than unsubstituted derivatives (**30**−**33**). In particular, the most potent derivatives in binding studies inhibit the effect of IB-MECA at 1 μM concentration from 50% to 88%, displaying IC_{50} values in the nanomolar range (4.5−15.1 nM).

Table 1. Binding Affinity at rA_1, rA_{2A} and hA_3 Adenosine Receptors of Compounds **5**−**12** and **30**−**33**



5-12, 30-33

compd	R	R^1	rA_1 (K_i, nM)[a]	rA_{2A} (K_i, nM)[b]	hA_3 (K_i, nM)[c]	rA_1/hA_3	rA_{2A}/hA_3
1, MRS 1220[d]			52.7 ± 11.8	10.3 ± 3.7	0.65 ± 0.25	81	16
30	C_2H_5	H	95.09 (86.76−104.22)	11.15 (9.84−12.63)	3579 (3376−3793)	0.03	0.003
5	C_2H_5	4-MeO-Ph-NHCO	>10000	>10000	0.28 (0.25−0.32)	>35714	>35714
6	C_2H_5	3-Cl-Ph-NHCO	2699 (2521−2889)	2799 (2621−2989)	2.09 (1.9−2.31)	1291	1339
31	*n*-C_3H_7	H	139 (107−181)	20.23 (16.14−25.36))	613 (582−646	0.22	0.03
7	*n*-C_3H_7	4-MeO-Ph-NHCO	>10000	1993 (1658−2397)	0.29 (0.27−0.32)	>34482	6872
8	*n*-C_3H_7	3-Cl-Ph-NHCO	1582 (1447−1730)	>10000	0.49 (0.47−0.52)	3228	>20408
32	Ph-CH_2-CH_2	H	2.16 (1.9−2.47)	0.70 (0.53−0.91)	2785 (2463−3149)	0.0007	0.0002
9	Ph-CH_2-CH_2	4-MeO-Ph-NHCO	1282 (1148−1432)	1398 (1225−1594)	1.47 (1.22−1.78)	872	951
10	Ph-CH_2-CH_2	3-Cl-Ph-NHCO	1049 (961−1145)	1698 (1524−1892)	13.28 (10.87−16.23)	79	128
33	Ph-CH_2-CH_2-CH_2	H	11.13 (9.34−13.27)	0.59 (0.44−0.81)	2666 (2533−2805)	0.004	0.0002
11	Ph-CH_2-CH_2-CH_2	4-MeO-Ph-NHCO	1514 (1332−1721)	>10000	19.81 (17.61−22.27)	76.4	>504
12	Ph-CH_2-CH_2-CH_2	3-Cl-Ph-NHCO	>10000	3200 (3025−3385)	42.65 (39.92−45.57)	>234	75

[a] Displacement of specific [^{3}H]DPCPX binding (A_1) in rat brain membranes ($n = 3-6$). [b] Displacement of specific [^{3}H]SCH 58261 binding (A_{2A}) in rat striatal membranes. [c] Displacement of specific [^{125}I]AB-MECA binding at human A_3 receptors expressed in HEK-293 cells. Data are expressed as geometric means, with 95% confidence limits. [d] Values taken from ref 30.

Table 2. Binding Affinity at hA_1, hA_{2A}, and hA_3 Adenosine Receptors of Compounds **5**−**12** and **30**−**33**

compd	hA_1 (K_i, nM)[a]	hA_{2A} (K_i, nM)[b]	hA_3 (K_i, nM)[c]	hA_1/hA_3	hA_{2A}/hA_3
30	5.00 (4.05−6.20)	1.90 (1.72−2.09)	3579 (3376−3793)	0.0014	0.0005
5	1026 (785−1341)	1045 (834−1309)	0.28 (0.25−0.32)	3664	3732
6	249 (215−289)	185 (159−214)	2.09 (1.9−2.31)	119	88.5
31	10 (7−14)	2.55 (1.91−3.39)	613 (582−646)	0.016	0.004
7	1197 (1027−1396)	141 (123−161)	0.29 (0.27−0.32)	4127	486
8	30 (23−40)	1221 (882−1689)	0.49 (0.47−0.52)	61	2491
32	1.00 (0.66−1.51)	0.31 (0.20−0.48)	2785 2463−3149)	0.0003	0.0001
9	201 (172−236)	124 (100−155)	1.47 (1.22−1.78)	136	84.3
10	251 (229−276)	110 (93−130)	13.28 (10.87−16.23)	19	8.2
33	3.00 (2.65−3.39)	0.18 (0.07−0.50)	2666 (2533−2805)	0.001	0.00006
11	251 (221−286)	1015 (885−1163)	19.81 (17.61−22.27)	12.6	51.2
12	1500 (1325−1693)	202 (11−216)	42.65 (39.92−45.57)	35.1	4.7

[a] Displacement of specific [^{3}H]DPCPX binding at human A_1 receptors expressed in CHO cells ($n = 3-6$). [b] Displacement of specific [^{3}H]SCH 58261 binding at human A_{2A} receptors expressed in HEK-293 cells. [c] Displacement of specific [^{125}I]AB-MECA binding at human A_3 receptors expressed in HEK-293 cells. Data are expressed as geometric means, with 95% confidence limits.

From these experimental observations, it is possible to hypothesize that the characteristic aralkyl chains of A_{2A} antagonists are not optimal for A_3 receptor interaction, while small chains present the ideal, steric and probably lipophilic, characteristics for the interaction with the human adenosine A_3 receptor subtype.

Conclusions. The present study provides useful information concerning the structural requirements necessary for recognition by the A_3 adenosine receptor. It confirms that a substituted phenylcarbamoyl moiety confers affinity and selectivity for the A_3 adenosine receptor subtype at the pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-

Table 3. Binding Affinity at hA_3 and rA_3 Adenosine Receptors of Compounds **5**−**12** and **30**−**33**

compd	rA_3[a] (% inhibn)	hA_3 (K_i, nM)[b]
30	1	3579 (3376−3793)
5	39	0.28 (0.25−0.32)
6	36	2.09 (1.9−2.31)
31	2	613 (582−646)
7	37	0.29 (0.27−0.32)
8	20	0.49 (0.47−0.52)
32	12	2785 (2463−3149)
9	37	1.47 (1.22−1.78)
10	30	13.28 (10.87−16.23)
33	32	2666 (2533−2805)
11	11	19.81 (17.61−22.27)
12	20	42.65 (39.92−45.57)

[a] Displacement of specific [^{125}I]AB-MECA binding at rat A_3 receptors expressed in CHO cells; data are expressed as percentage of inhibition of specific binding at a concentration of 10 mM. [b] Displacement of specific [^{125}I]AB-MECA binding at human A_3 receptors expressed in HEK-293 cells; data are expressed as geometric means, with 95% confidence limits.

Table 4. Functional Assay: Percentage of Blockade by 1 μM of Each Compound **30**−**33** and **5**−**12** of the Inhibition by 100 nM IB-MECA-Inhibited cAMP Accumulation in CHO Cells Expressing hA_3 Adenosine Receptors[a]

compd	% inhibn	IC_{50} (nM)
30	3 (2−7)	
5	88 (79−97)	6.5 (3.7−11.2)
6	71 (61−81)	15.1 (9.9−23.0)
31	43 (30−61)	40.2 (32.7−49.3)
7	89 (79−100)	4.5 (3.7−5.5)
8	87 (76−99)	5.3 (3.2−8.9)
32	5 (3−7)	
9	81 (75−89)	12.4 (8.0−19.2)
10	62 (43−75)	
33	6 (3−10)	
11	56 (37−7.1)	
12	50 (34−72)	

[a] For compounds **5**−**8** and **31**, IC_{50} values are also shown. Values are the means of at least three experiments, and in parentheses the 95% confidence limits are shown.

c]pyrimidine nucleus, while the N^5-unsubstituted derivatives lacks both affinity and selectivity for human A_3 receptors, showing high affinity for A_1 and/or A_{2A} receptor subtypes. When the pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine nucleus is substituted at the N^8-position with small alkyl groups, higher affinity and selectivity at the human A_3 receptors was observed. When the N^8-ethyl and N^5-4−methoxyphenylcarbamoyl substitutions were combined, the most potent and selective human A_3 adenosine antagonist (**5**) was obtained (K_i = 0.28 nM, rA_1/hA_3 > 35 000, rA_{2A}/hA_3 > 35 000, hA_1/hA_3 > 3 600, hA_{2A}/hA_3 > 3 600).

In addition, the results herein presented and our previous studies[31−33] allow us to propose the pyrazolo-[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine nucleus as a possible template for generating adenosine receptor subtype-selective ligands. In fact, it is quite evident that modifications of the substituents at N^5-, N^7-, and N^8-positions could modulate both affinity and selectivity for the different adenosine receptor subtypes.

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Supporting Information Available: Experimental details. This information is available free of charge via the Internet at http://pubs.acs.org.

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