Fluorosulfonyl- and Bis-(β-chloroethyl)amino-phenylamino Functionalized Pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine Derivatives: Irreversible Antagonists at the Human A_3 Adenosine Receptor and Molecular Modeling Studies

Pier Giovanni Baraldi,*,† Barbara Cacciari,† Stefano Moro,‡ Romeo Romagnoli,† Xiao-duo Ji,§ Kenneth A. Jacobson,§ Stefania Gessi,|| Pier Andrea Borea,|| and Giampiero Spalluto⊥

Dipartimento di Scienze Farmaceutiche, 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, Molecular Modeling Section, Dipartimento di Scienze Farmaceutiche, Università di Padova, Via Marzolo 5, I-35131 Padova, Italy, Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892, 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

Received January 11, 2001

A series of pyrazolotriazolopyrimidines was previously reported to be highly potent and selective human A_3 adenosine receptor antagonists (Baraldi et al. *J. Med. Chem.* **2000**, *43*, 4768−4780). A derivative having a methyl group at the N^8 pyrazole combined with a 4-methoxyphenylcarbamoyl moiety at N^5 position, displayed a K_i value at the hA_3 receptor of 0.2 nM. We now describe chemically reactive derivatives which act as irreversible inhibitors of this receptor. Electrophilic groups, specifically sulfonyl fluoride and nitrogen mustard (bis-(β-chloroethyl)-amino) moieties, have been incorporated at the 4-position of the aryl urea group. Membranes containing the recombinant hA_3 receptor were preincubated with the compounds and washed exhaustively. The loss of ability to bind radioligand following this treatment indicated irreversible binding. The most potent compound in irreversibly binding to the receptor was **14**, which contained a sulfonyl fluoride moiety and a propyl group at the N^8 pyrazole nitrogen. The bis-(β-chloroethyl)amino derivatives displayed a much smaller degree of irreversible binding than the sulfonyl fluoride derivatives. A computer-generated model of the human A_3 receptor was built and analyzed to help interpret these results. The model of the A_3 transmembrane region was derived using primary sequence comparison, secondary structure predictions, and three-dimensional homology building, using the recently published crystal structure of rhodopsin as a template. According to our model, sulfonyl fluoride derivatives could dock within the hypothetical TM binding domain, adopting two different energetically favorable conformations. We have identified two amino acids, Ser247 and Cys251, both in TM6, as potential nucleophilic partners of the irreversible binding to the receptor.

Introduction

In the last 20 years, much effort has been made toward the goal of identifying ligands that interact selectively with the four adenosine receptor subtypes, A_1, A_{2A}, A_{2B}, and A_3.[1,2] In particular, while A_1 and A_{2A} subtypes have been pharmacologically characterized through the use of chemical probes, A_{2B} and A_3 subtypes are still in need of additional probes.[3]

The A_3 subtype, recently cloned from various species (human, rat, and rabbit),[4−9] seems to be involved in a variety of physiological functions. The principal second messengers activated by adenosine A_3 receptors are inhibition of adenylate cyclase[3] and stimulation of phospholipases C[10] and D.[11] In addition, activation of the rat A_3 adenosine receptors causes the release of histamine from mast cells,[12] which leads to processes such as inflammation and hypotension. It has been suggested that the blockade of the A_3 adenosine receptor may be considered as a potential lead toward the treatment of asthma and inflammation.[13,14] For these reasons, and with the aim of obtaining efficient probes for studying this receptor subtype, there has been an active search for potent and selective antagonists. In the past few years, many classes of heterocyclic compounds have been synthesized as A_3 adenosine antagonists, with some showing significant selectivity for the A_3 adenosine receptor subtype.[15] In particular, our group recently reported a class of 5-[[(4-substituted-phenyl)amino]carbonyl]amino-8-alkyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as potent and selective human A_3 adenosine receptor antagonists. These were ineffective on a rat model, due to the well-known differences in sequence homologies (72%) (Table 1).[16,17]

* Address correspondence to Prof. Pier Giovanni Baraldi, Dipartimento di Scienze Farmaceutiche, Via Fossato di Mortara 17-19, I-44100 Ferrara, Italy. Phone: +39-(0)532−291293. Fax: +39-(0)532-291296. E-mail: pgb@dns.unife.it.

† Dipartimento di Scienze Farmaceutiche, Università degli Studi di Ferrara.

‡ Università di Padova.

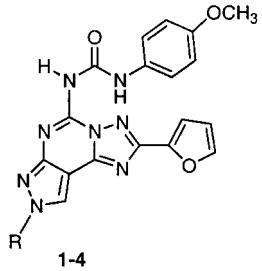
§ National Institute of Diabetes and Digestive and Kidney Diseases, NIH.

|| Dipartimento di Medicina Clinica e Sperimentale-Sezione di Farmacologia, Università degli Studi di Ferrara.

⊥ Università degli Studi di Trieste.

10.1021/jm010818a CCC: $20.00
Published on Web 07/14/2001

Table 1. Structures and Binding Affinity at hA_1, hA_{2A}, hA_{2B}, and hA_3 Adenosine Receptors of Selected Compounds Previously Reported



1-4

compd	R	hA_1 (K_i, nM)[a]	hA_{2A} (K_i, nM)[b]	hA_{2B} (K_i, nM)[c]	hA_3 (K_i, nM)[d]	hA_1/hA_3	hA_{2A}/hA_3	hA_{2B}/hA_3
1	CH_3	1097 (928−1297)	1390 (1220−1590)	261 (226−301)	0.20 (0.17−0.24)	5485	6950	1305
2	C_2H_5	1026 (785−1341)	1040 (830−1310)	245 (188−320)	0.60 (0.51−0.70)	1710	1733	408
3	n-C_3H_7	1197 (1027−1396)	140 (120−155)	2056 (1637−2582)	0.80 (0.63−0.1.00)	1496	175	2570
4	n-C_4H_9	296 (269−326)	80 (65−92)	303 (260−352)	0.32 (0.27−0.34)	925	250	946

[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]-SCH58261 binding at human A_{2A} receptors expressed in CHO cells (n = 3−6). [c] Displacement of specific [^{3}H]-DPCPX binding at human A_{2B} receptors expressed in HEK-293 cells (n = 3−6). [d] Displacement of specific [^{3}H]MRE-3008F20 binding at human A_3 receptors expressed in CHO cells (n = 3−6). Data are expressed as geometric means, with 95% confidence limits.

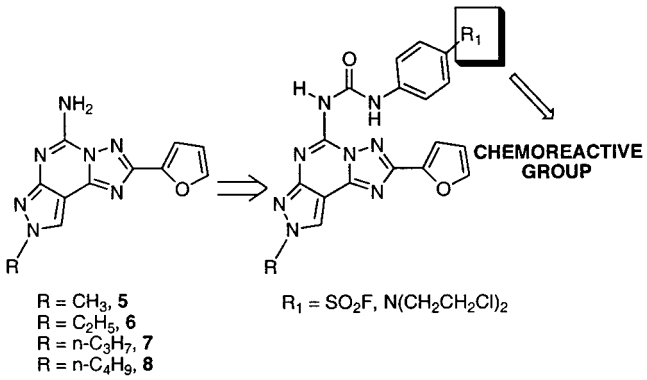


Figure 1. Rational design for the synthesis of irreversible human A_3 adenosine antagonists.

It is evident from the binding data summarized in Table 1 that affinity increased when the 4-methoxyphenyl carbamoyl moiety at the 5 position and small alkyl chains (methyl (**1**), ethyl (**2**), propyl (**3**), butyl (**4**)) at the N^8 pyrazole nitrogen were combined simultaneously. These derivatives are the most potent and selective human A_3 adenosine receptor antagonists ever reported, with affinity at the human A_3 adenosine receptor in the subnanomolar range (0.2−0.8 nM) and selectivities versus other receptor subtypes ranging from 200 to 7000.[17]

Beginning with these experimental observations, we proposed the design of new compounds in this series by replacing the 4-methoxy substituent on the phenyl ring with alkylating moieties such as fluorosulfonyl and bis-(β-chloroethyl)amino groups (Figure 1), as potential irreversibly binding human A_3 adenosine antagonists (**9**−**16**).

The use of irreversible antagonists represents a useful approach to the study of receptor structure and function, such as ligand binding site mapping,[18] physiological receptors functioning,[19] and determining the relationship between receptor occupancy and a tissue or cellular response.[20] A similar approach has been successful with A_1[21−24] and A_{2A}[25] adenosine receptor subtypes, as clearly reported in the literature.

Also for the A_3 receptor, this approach has been used both for agonists (adenosine derivatives)[26] and antagonists (dihydropyridine derivatives).[27] While the agonists showed a good binding profile in a rat model,[26] at the human A_3 adenosine receptors only racemic dihydropyridine derivatives have been tested, and the percentage of irreversibility reached only 56% at 100 nM concentration.[27] Thus, there is a need for improved irreversibly binding A_3 antagonists.

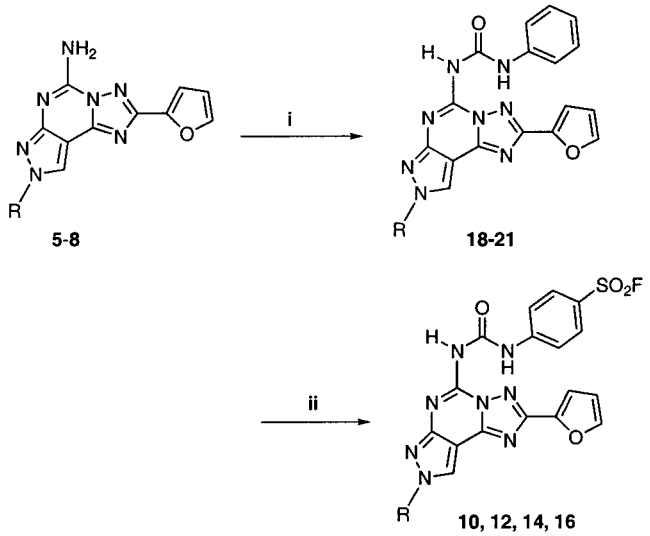
In addition, the introduction of two different alkylating moieties could help to identify the nature of nucleophiles on the receptor which may interact with these derivatives, and consequently this may help identify the nature of some amino acid residues present within the binding site. In fact, while a fluorosulfonyl group is highly reactive[28] and could interact indifferently with any kind of nucleophile (e.g., $-NH_2$, OH, SH), it is well-known that nitrogen mustard, which is less reactive than fluorosulfonyl moiety,[29] reacts only with nitrogen nucleophiles (e.g., $-NH_2$, $-NH-C(NH)=NH_2$).

Chemistry

Potentially irreversible human A_3 receptor antagonists (**9**−**16**) were prepared following, as previously reported,[17] the general synthetic pathway for the preparation of urea derivatives, as summarized in Schemes 1 and 2.

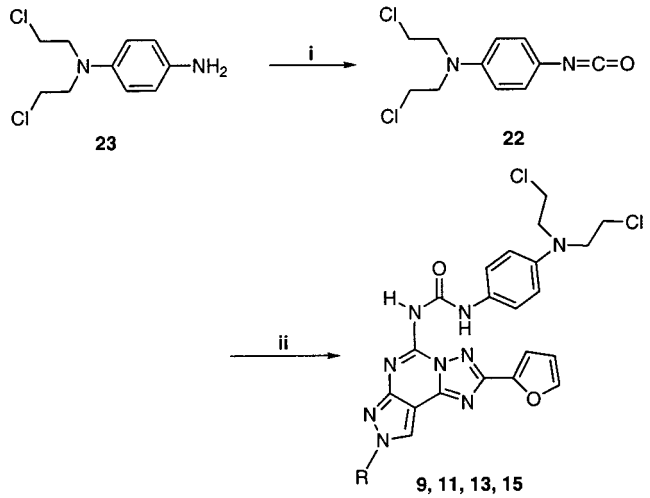
Fluorosulfonyl derivatives (**10**, **12**, **14**, **16**) were prepared starting from the previously reported amino compounds, **5**−**8**, which reacted with the commercially available phenyl isocyanate **17**, to afford the phenylcarbamoyl derivatives, **18**−**21**. After purification by flash chromatography, the resulting compounds, **18**−**21**, reacted at −10 °C with fluorosulfonic acid leading to the final derivatives, **10**, **12**, **14**, and **16**.[25e] The final products were purified by crystallization from EtOAc−light petroleum (Scheme 1).

Scheme 1[a]



[a] Reagents: (i) PhNCO (**17**), THF, reflux, 18 h; (ii) FSO_3H, −10 °C, 2 h.

Scheme 2[a]



[a] Reagents: (i) $ClCOOCCl_3$, Et_3N, dioxane, reflux, 5 h; (ii) **5**−**8**, THF, reflux, 18 h.

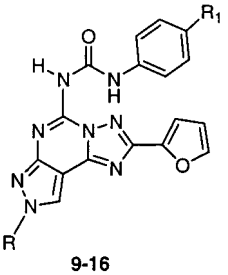
The mustard derivatives (**9**, **11**, **13**, **15**) were prepared in a similar manner by reaction of the amino compounds **5**−**8** with freshly prepared 4-bis-(β-chloroethyl)aminophenyl isocyanate, **22**. The latter compound was prepared starting from the corresponding aniline derivative,[30] **23**, by reaction with diphosgene (1.2 equiv) in the presence of triethylamine (1.2 equiv) in dry dioxane.[31] After 5 h the mixture was concentrated at reduced pressure, and the presence of isocyanate was confirmed by infrared spectroscopy, which showed a characteristic peak at 2250 cm^{-1}. The crude residue was utilized for coupling reactions without further purification (Scheme 2).

Results and Discussion

The reactive pyrazolo-triazolo-pyrimidine derivatives (**9**−**16**) were tested for irreversible binding at the human A_3 adenosine receptors in CHO cells using the radioligand [^{125}I]AB-MECA (Table 2).[32,33]

Prior to radioligand binding, membranes containing hA_3 receptors were preincubated with the reactive antagonist (**9**−**16**), followed by a thorough washing, to remove free antagonist.

Table 2. Irreversible Inhibition of Radioligand ([^{125}I]AB-MECA) Binding to Human A_3 Adenosine Receptors in CHO Cell Membranes, Following a 1 h Incubation with Compounds (**9**−**16**) at Concentrations of 1, 10, and 100 nM



	ligand		inhibition (% of control)[a]		
	R	R_1	1 nM	10 nM	100 nM
9	CH_3	$N(CH_2CH_2Cl)_2$	0	11 ± 6	22 ± 5
10	CH_3	SO_2F	ND[b]	ND[b]	ND[b]
11	C_2H_5	$N(CH_2CH_2Cl)_2$	0	0	18 ± 17
12	C_2H_5	SO_2F	4.1 ± 5.1	30 ± 5	65 ± 4
13	*n*-C_3H_7	$N(CH_2CH_2Cl)_2$	7.8 ± 10	11 ± 9	16 ±14
14	*n*-C_3H_7	SO_2F	47 ± 7	70 ± 3	79 ± 8
15	*n*-C_4H_9	$N(CH_2CH_2Cl)_2$	0	7.0 ± 4.9	9.0 ± 7.2
16	*n*-C_4H_9	SO_2F	31 ± 6	40 ± 11	73 ± 7

[a] $n = 3-7$. [b] Not determined due to the insolubility of the sample at 1 mM in DMSO.

The data summarized in Table 2 showed that compounds containing a fluorosulfonyl moiety (**10**, **12**, **14**, **16**) proved to be irreversible antagonists with different degrees of potency at the human A_3 adenosine receptor, while the corresponding nitrogen mustard derivatives (**9**, **11**, **13**, **15**) were unable to bind covalently at this receptor subtype.

In the fluorosulfonyl series, derivative **10** could not be evaluated for irreversible receptor binding due to its low solubility in DMSO, which was less than 1 mM. Other fluorosulfonyl derivatives were sufficiently soluble in DMSO to prepare stock solutions for the binding studies. (The final concentration of DMSO in buffer must be maximum 1%.) The three fluorosulfonyl compounds (**12**, **14**, **16**) showed good irreversible properties at concentrations of 1−100 nM. In particular, high efficiency was observed at a concentration of 100 nM, with the percentage of inhibition being 65−79%. This effect was quite evident also at a concentration of 10 nM, with irreversibility in the range of 30−70%. The maximal irreversible inhibition was observed with the derivative having a propyl chain at the N^8 position (**14**, 100 nM, 79%).

In addition, the presence of a small alkyl chain on the N^8 position (Table 1) conferred at the compound an optimized structural feature required for the interaction with human A_3 adenosine receptor, as previously demonstrated.[16,17]

In contrast, derivatives with a nitrogen mustard group on the phenyl ring (**9**, **11**, **13**, **15**) showed much less significant irreversible activity at the human A_3 adenosine receptor. In fact, only at a concentration of 100 nM was slight irreversibility observed (9−22%).

With the aim to evaluate the selectivity of these derivatives vs other adenosine receptor subtypes, the most effective irreversible compound (**14**) was also tested in competitive binding assays at human A_1 and

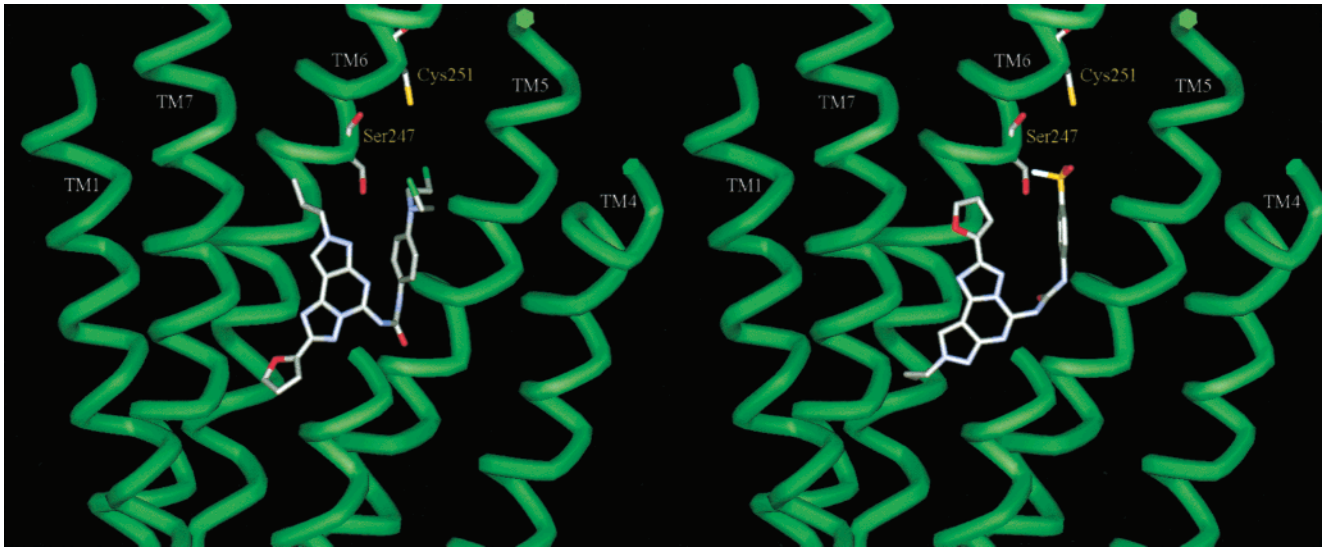


Figure 2. Side view of the A_3-**14** (right side) and A_3-**13** (left side) complex models. The side chains of the two important residues in proximity to the docked ligand molecules are highlighted and labeled.

A_{2A} receptors expressed in CHO cells. At the human A_1 receptor, no significant displacement of binding (<5%) was observed for compound **14** at 100 nM, a concentration which effectively irreversibly inhibited most of the hA_3 receptors. However, at the human A_{2A} receptor, a K_i for binding of **14** was determined to be 50 ± 5 nM. The irreversibility of binding of **14** at the human A_{2A} receptor was not evaluated. This result potentially compromises use of these antagonists as selective hA_3 receptor probes; however, the lower affinity at the A_{2A} receptor would suggest that there likely exists a concentration range of selectivity for irreversible inhibition of the hA_3 adenosine receptor subtype.

This large difference in receptor interaction between the fluorosulfonyl and mustard series can be explained with two different hypotheses. The inactivity of the mustard derivatives can be attributed to the absence in the binding site of a nitrogen nucleophile (e.g., NH_2), which is the only one able to interact with the aziridinium salt generated by the bis-(β-dichloroethyl)amino function, or to the spatial orientation of this group within the binding site, which does not permit the nucleophiles to properly bind.

A molecular modeling study was performed to elucidate the hypothetical binding mode of the new A_3 irreversible antagonists and to determine which amino acid residues in the TM region might bond covalently with the ligand. Very little structural information was available concerning the details of ligand/GPCR interactions. However, a 2.8 Å resolution crystal structure of bovine rhodopsin was very recently published.[34] Using a homology approach and this structure as a template, we built an improved model of the transmembrane region of the human A_3 receptor. This model may be considered a further refinement of the hypothetical binding site model of the A_3 receptor antagonists previously proposed.[35] Details of the model building are given in the Experimental Section.

We selected derivative **14**, which contained a sulfonyl fluoride moiety and a propyl group at the N^8 pyrazole nitrogen, and the corresponding nitrogen mustard analogue **13** as reference ligands in our docking studies. The most stable ligand conformations of both irreversible antagonists are shown in Figure 2.

Interestingly, the receptor−ligand complex containing derivative **14** appeared more energetically stable by approximately 15 kcal/mol. In derivative **13**, the presence of the (bis-(β-chloroethyl)amino) moiety (derivative **13**), characterized by a high degree of conformational flexibility, increased the steric hindrance inside the binding cavity, decreasing the relative stability of the complex. This may be the reason for the low efficacy of the nitrogen mustard derivatives as irreversible antagonists.

As shown in Figure 2, we identified a hypothetical binding site of both **14** and **13** surrounded by TMs 3, 5, 6, and 7 following the Monte Carlo/annealing sampling. Both the sulfonyl fluoride and nitrogen mustard moieties were close to TM6, pointing toward the extracellular environment. Two residues, Ser247 and Cys251, were tentatively implicated in irreversible binding. The side chain of Ser247 (TM6) was 3.6 Å from the S atom of the sulfonyl fluoride and 4.9 Å from one of the two C_α of the β-chloroethyl moiety. Moreover, the side chain of Cys251 (TM6) was 4.2 Å from the S atom of the sulfonyl fluoride, and 5.0 Å from one of the two C_α of the β-chloroethyl moiety. As already anticipated, Cys251 (TM6) would be a more likely candidate for the irreversible binding, based solely on its specific chemical reactivity. However, it is useful to emphasize the proximity of the Ser247 to the putative ligand binding site. A major structural difference among the hypothetical binding sites of adenosine receptor subtypes is that the A_3 receptor does not contain the histidine residue in TM6 common to all A_1 (His251 in h_A_1) and A_2 (His250 in h_A_{2A}) receptors. This histidine has been shown to participate in both agonist and antagonist binding to the A_{2A} receptors. In the A_3 receptor, this histidine in TM6 is replaced with a serine residue, i.e., Ser247, a candidate for the site of irreversible binding, due to its proximity to the electrophilic group.

Nevertheless, it is important to note that these new irreversible antagonists share a common question with all other irreversibly binding compounds: is it necessary

to find a nucleophile residue in proximity to the docked conformation or can they bind provisionally elsewhere on the receptor, on the way down to the TM site, and remain long enough to form a covalent bond? In this study we do not answer this question, but we can speculate that in the proximity of the TM binding domain there are at least two residues tentatively implicated in the irreversible binding.

Conclusions

In summary, a new series of pyrazolo-triazolo-pyrimidines, bearing chemoreactive moieties (4-fluorsulfonyl- and 4-bis-(*β*-chloroethyl)amino-) as irreversible antagonists for the human A_3 adenosine receptor, has been reported. The results from an irreversible binding assay demonstrate that one of the reported compounds, 5-[[(4-fluorosulfonyl-phenyl)amino]carbonyl]amino-8-*n*-propyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (**14**), irreversibly inhibited the binding of [^{125}I]AB-MECA by 79% at a 100 nM concentration. In contrast, all the derivatives bearing the 4-bis-(*β*-chloroethyl)amino function failed to irreversibly bind the hA_3 adenosine receptors. These observations were analyzed by molecular modeling studies, demonstrating that Cys251 or Ser247 are the most likely sites for covalent binding. These hypotheses may now be tested using a site directed mutagenesis approach.

In conclusion, these results provide new pharmacological probes for improving our understanding of the pathophysiological role of the human A_3 adenosine receptor, yielding new information about the structural requirements necessary for the receptor recognition.

Experimental Section

Chemistry. General. Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F_{254} Merck plates). Products were visualized with iodine or potassium permanganate solution. Infrared spectra (IR) were measured on a Perkin-Elmer 257 spectrophotometer. ^{1}H NMR were determined in $CDCl_3$ or DMSO-d_6 solutions with a Bruker AC 200 spectrometer. Peaks positions were given in parts per million (δ), downfield from tetramethylsilane as internal standard, and J values were given in hertz. Light petroleum ether referred to the fractions boiling at 40−60 °C. Melting points were determined on a Buchi-Tottoli instrument and were uncorrected. Chromatography was performed using Merck 60−200 mesh silica gel. All products reported showed IR and ^{1}H NMR spectra in agreement with the assigned structures. Organic solutions were dried over anhydrous magnesium sulfate. Elemental analyses were performed by the microanalytical laboratory of Dipartimento di Chimica, University of Ferrara, and were within ±0.4% of the theoretical values for C, H, and N.

General Procedures for the Preparation of 5-[[(Substituted-phenyl)amino]carbonyl]amino-8-alkyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (9, 11, 13, 15, 18−21). The amino precursor (**5**−**8**) (10 mmol) was dissolved in freshly distilled THF (15 mL), and the appropriate isocyanate (**17**, **22**) (13 mmol) was added. The mixture was refluxed under argon for 18 h. Then the solvent was removed under reduced pressure, and the residue was purified by flash chromatography (EtOAc−light petroleum 4−6) to afford the desired compounds **9**, **11**, **13**, **15**, **18**−**21**.

5-[[(Phenyl)amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (18): yield 90%, yellow solid; mp 165−170 °C (EtOAc−light petroleum); IR (KBr) 3255−2990, 1680, 1620, 1510, 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.20 (s, 3H, N^8-C*H*$_3$); 6.61 (dd, 2H, J = 2, J = 4, 4-*H* Furane); 7.11−7.37 (m, 6H, C_6H_5 and 3-*H* Furane); 7.76 (d, 1H, J = 2, 5-*H* Furane); 8.15 (s, 1H, N^8-C*H*); 8.67 (bs, 1H, CO-N*H*-Ph); 11.16 (bs, 1H, 5-N*H*-CO). Anal. ($C_{18}H_{14}N_8O_2$) C, H, N.

5-[[(Phenyl)amino]carbonyl]amino-8-ethyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (19): yield 94%, yellow solid; mp 180 °C (EtOAc−light petroleum); IR (KBr) 3250−2985, 1675, 1610, 1510, 1460 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.67 (t, 3H, J = 7, C*H*$_3$-CH$_2$-N^8); 4.47 (q, 2H, J = 7, CH$_3$-C*H*$_2$-N^8); 6.62 (dd, 2H, J = 2, J = 4, 4-*H* Furane); 7.16−7.42 (m, 6H, C_6H_5 and 3-*H* Furane); 7.72 (d, 1H, J = 2, 5-*H* Furane); 8.26 (s, 1H, N^8-C*H*); 8.61 (bs, 1H, CO-N*H*-Ph); 11.16 (bs, 1H, 5-N*H*-CO). Anal. ($C_{19}H_{16}N_8O_2$) C, H, N.

5-[[(Phenyl)amino]carbonyl]amino-8-*n*-propyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (20): yield 98%, yellow solid; mp 155 °C (EtOAc−light petroleum); IR (KBr) 3240−2985, 1674, 1615, 1510, 1470 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.00 (t, 3H, J = 7, C*H*$_3$-CH$_2$-CH$_2$-N^8); 2.02−2.13 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$-N^8); 4.36 (t, 2H, J = 7, CH$_3$-CH$_2$-C*H*$_2$-N^8); 6.62 (dd, 2H, J = 2, J = 4, 4-*H* Furane); 7.16−7.49 (m, 6H, C_6H_5 and 3-*H* Furane); 7.69 (d, 1H, J = 2, 5-*H* Furane); 8.24 (s, 1H, N^8-C*H*); 8.61 (bs, 1H, CO-N*H*-Ph); 11.16 (bs, 1H, 5-N*H*-CO). Anal. ($C_{20}H_{18}N_8O_2$) C, H, N.

5-[[(Phenyl)amino]carbonyl]amino-8-*n*-butyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (21): yield 92%, white solid; mp 145 °C (EtOAc−light petroleum); IR (KBr) 3240−2980, 1675, 1610, 1500, 1470 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.96 (t, 3H, J = 7, C*H*$_3$-CH$_2$-CH$_2$-CH$_2$-N^8); 1.30−1.41 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$-CH$_2$-N^8); 1.98−2.06 (m, 2H, CH$_3$-CH$_2$-C*H*$_2$-CH$_2$-N^8); 4.39 (t, 2H, J = 7, CH$_3$-CH$_2$-CH$_2$-C*H*$_2$-N^8); 6.62 (dd, 1H, J = 2, J = 4, 4-*H* Furane); 7.16−7.42 (m, 6H, C_6H_5 and 3-*H* Furane); 7.67 (d, 1H, J = 2, 5-*H* Furane); 8.23 (s, 1H, N^8-C*H*); 8.62 (bs, 1H, CO−N*H*-Ph); 11.16 (bs, 1H, 5-N*H*-CO). Anal. ($C_{21}H_{20}N_8O_2$) C, H, N.

5-[[(4-Bis-*β*-cloroethylamino-phenyl)amino]carbonyl]-amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo-[1,5-*c*]pyrimidine (9): yield 82%, yellow solid; mp 165 °C (EtOAc−light petroleum); IR (KBr) 3230−2990, 1670, 1615, 1510, 1460 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 3.60−3.73 (m, 8H, N(C*H*$_2$C*H*$_2$Cl)$_2$); 4.21 (s, 3H, C*H*$_3$-N^8); 6.62 (dd, 1H, J = 2, J = 4, 4-*H* Furane); 6.66 (d, 2H, J = 9, CO-NH-*Ph*); 7.24 (d, 1H, J = 4, 3-*H* Furane); 7.51 (d, 2H, J = 9, 4-*Ph*- N(CH$_2$CH$_2$Cl)$_2$); 7.66 (d, 1H, J = 2, 5-*H* Furane); 8.22 (s, 1H, N^8-C*H*); 8.60 (bs, 1H, CO-N*H*-Ph); 10.86 (bs, 1H, 5-N*H*-CO). Anal. ($C_{22}H_{21}N_9O_2$-Cl$_2$) C, H, N.

5-[[(4-Bis-*β*-cloroethylamino-phenyl)amino]carbonyl]-amino-8-ethyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo-[1,5-*c*]pyrimidine (11): yield 85%, white solid; mp 185 °C (EtOAc−light petroleum); IR (KBr) 3235−2985, 1675, 1620, 1511, 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 1.67 (t, 3H, J = 6.8, C*H*$_3$-CH$_2$-N^8); 3.62−3.74 (m, 8H, N(C*H*$_2$C*H*$_2$Cl)$_2$); 4.46 (q, 2H, J = 6.8, CH$_3$-C*H*$_2$-N^8); 6.62 (dd, 1H, J = 2, J = 4, 4-*H* Furane); 6.71 (d, 2H, J = 9, CO-NH-*Ph*); 7.24 (d, 1H, J = 4, 3-*H* Furane); 7.51 (d, 2H, J = 9, 4-*Ph*-N(CH$_2$CH$_2$Cl)$_2$); 7.66 (d, 1H, J = 2, 5-*H* Furane); 8.25 (s, 1H, N^8-C*H*); 8.59 (bs, 1H, CO-N*H*-Ph); 11.04 (bs, 1H, 5-N*H*-CO). Anal. ($C_{23}H_{23}N_9O_2Cl_2$) C, H, N.

5-[[(4-Bis-*β*-cloroethylamino-phenyl)amino]carbonyl]-amino-8-*n*-propyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo-[1,5-*c*]pyrimidine (13): yield 78%, white solid; mp 195 °C (EtOAc−light petroleum); IR (KBr) 3230−2985, 1674, 1625, 1520, 1445 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.01 (t, 3H, J = 6.8, C*H*$_3$-CH$_2$-CH$_2$-N^8); 1.97−2.08 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$-N^8); 3.62−3.76 (m, 8H, N(C*H*$_2$C*H*$_2$Cl)$_2$); 4.35 (t, 2H, J = 6.8, CH$_3$-CH$_2$-C*H*$_2$-N^8); 6.62 (dd, 1H, J = 2, J = 4, 4-*H* Furane); 6.71 (d, 2H, J = 9, CO−NH-*Ph*); 7.24 (d, 1H, J = 4, 3-*H* Furane); 7.51 (d, 2H, J = 9, 4-*Ph*- N(CH$_2$CH$_2$Cl)$_2$); 7.66 (d, 1H, J = 2, 5-*H* Furane); 8.23 (s, 1H, N^8-C*H*); 8.59 (bs, 1H, CO−N*H*-Ph); 10.85 (bs, 1H, 5-N*H*-CO). Anal. ($C_{24}H_{25}N_9O_2Cl_2$) C, H, N.

5-[[(4-Bis-*β*-cloroethylamino-phenyl)amino]carbonyl]-amino-8-*n*-butyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo-[1,5-*c*]pyrimidine (15): yield 80%, pale yellow solid; mp 190 °C (EtOAc−light petroleum); IR (KBr) 3235−2980, 1672, 1620, 1510, 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.99 (t, 3H, J = 6.8, C*H*$_3$-CH$_2$-CH$_2$-CH$_2$-N^8); 1.23−1.47 (m, 2H, CH$_3$-C*H*$_2$-CH$_2$-CH$_2$-N^8); 1.89−2.04 (m, 2H, CH$_3$-CH$_2$-C*H*$_2$-CH$_2$-N^8); 3.64−3.74 (m,

8H, N(CH_2CH_2Cl)$_2$); 4.39 (t, 2H, J = 6.8, CH_3-CH_2-CH_2-CH_2-N^8); 6.61 (dd, 2H, J = 2, J = 4, 4-*H* Furane); 6.71 (d, 2H, J = 9, CO-NH-*Ph*); 7.24 (d, 1H, J = 4, 3-*H* Furane); 7.51 (d, 2H, J = 9, 4-*Ph*-N(CH_2CH_2Cl)$_2$); 7.66 (d, 1H, J = 2, 5-*H* Furane); 8.23 (s, 1H, N^8-C*H*); 8.59 (bs, 1H, CO-N*H*-Ph); 10.84 (bs, 1H, 5-N*H*-CO). Anal. ($C_{25}H_{27}N_9O_2Cl_2$) C, H, N.

General Procedures for the Preparation of 5-[[(4-Fluorosulfonyl-phenyl)amino]carbonyl]amino-8-alkyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (10, 12, 14, 16). The appropriate phenyl-carbamoyl derivative (**18**−**21**) (0.086 mmol) were reacted with fluorosulfonic acid (0.1 mL) at −10 °C for 2 h. Then the mixture was quenched with ice water (1 mL), and the precipitate was filtered off to afford the desired compounds (**10**, **12**, **14**, **16**) as a solid in a good yield.

5-[[(4-Fluorosulfonyl-phenyl)amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]-pyrimidine (10): yield 78%, yellow solid; mp 170 °C (EtOAc−light petroleum); IR (KBr) 3230−2995, 1670, 1615, 1500, 1320, 1470, 1160 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 4.14 (s, 3H, CH_3-N^8); 6.76 (dd, 1H, J = 2, J = 4, 4-*H* Furane); 7.29 (d, 1H, J = 4, 3-*H* Furane); 7.89 (d, 2H, J = 9, CO-NH-*Ph*); 7.99 (d, 1H, J = 2, 5-*H* Furane); 8.14 (d, 2H, J = 9, 4-*Ph*-SO_2F); 8.80 (s, 1H, N^8-C*H*); 10.15 (bs, 1H, CO-N*H*-Ph); 11.06 (bs, 1H, 5-N*H*-CO). Anal. ($C_{18}H_{13}N_8SO_4F$) C, H, N.

5-[[(4-Fluorosulfonyl-phenyl)amino]carbonyl]amino-8-ethyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine (12): yield 72%, pale yellow solid; mp 165 °C (EtOAc−light petroleum); IR (KBr) 3235−2990, 1675, 1620, 1510, 1330, 1470, 1160 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 1.5 (t, 3H, J = 6.8, CH_3-CH_2-N^8); 4.42 (q, 2H, J = 6.8, CH_3-CH_2-N^8); 6.76 (dd, 2H, J = 2, J = 4, 4-*H* Furane); 7.29 (d, 1H, J = 4, 3-*H* Furane); 7.96 (d, 2H, J = 9, CO-NH-*Ph*); 7.99 (d, 1H, J = 2, 5-*H* Furane); 8.14 (d, 2H, J = 9, 4-*Ph*-SO_2F); 8.86 (s, 1H, N^8-C*H*); 10.19 (bs, 1H, CO-N*H*-Ph); 11.04 (bs, 1H, 5-N*H*-CO). Anal. ($C_{19}H_{15}N_8SO_4F$) C, H, N.

5-[[(4-Fluorosulfonyl-phenyl)amino]carbonyl]amino-8-*n*-propyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]-pyrimidine (14): yield 75%, white solid; mp 155 °C (EtOAc−light petroleum); IR (KBr) 3235−2990, 1673, 1615, 1515, 1325, 1465, 1150 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.87 (t, 3H, J = 6.8, CH_3-CH_2-CH_2-N^8); 1.90−1.95 (m, 2H, CH_3-CH_2-CH_2-N^8); 4.35 (t, 2H, J = 6.8, CH_3-CH_2-CH_2-N^8); 6.76 (dd, 2H, J = 2, J = 4, 4-H Furane); 7.29 (d, 1H, J = 4, 3-H Furane); 7.89 (d, 2H, J = 9, CO-NH-Ph); 8.01 (d, 1H, J = 2, 5-H Furane); 8.13 (d, 2H, J = 9, 4-Ph-SO_2F); 8.85 (s, 1H, N_8-CH); 10.12 (bs, 1H, CO-NH-Ph); 11.04 (bs, 1H, 5-NH-CO). Anal. ($C_{20}H_{17}N_8SO_4F$) C, H, N.

5-[[(4-Fluorosulfonyl-phenyl)amino]carbonyl]amino-8-*n*-butyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]-pyrimidine (16): yield 77%, white solid; mp 145 °C (EtOAc−light petroleum); IR (KBr) 3225−2980, 1670, 1610, 1525, 1335, 1465, 1155 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 0.97 (t, 3H, J = 7, CH_3-CH_2-CH_2-CH_2-N^8); 1.29−1.34 (m, 2H, CH_3-CH_2-CH_2-CH_2-N^8); 1.98−2.06 (m, 2H, CH_3-CH_2-CH_2-CH_2-N^8); 4.43 (t, 2H, J = 7, CH_3-CH_2-CH_2-CH_2-N^8); 6.65 (dd, 2H, J = 2, J = 4, 4-H Furane); 7.29 (d, 1H, J = 4, 3-H Furane); 7.85 (d, 2H, J = 9, CO−NH−Ph); 8.04 (d, 1H, J = 2, 5-*H* Furane); 8.10 (d, 2H, J = 9, 4-*Ph*-SO_2F); 8.78 (s, 1H, N^8-C*H*); 10.11 (bs, 1H, CO-N*H*-Ph); 11.88 (bs, 1H, 5-N*H*-CO). Anal. ($C_{21}H_{19}N_8SO_4F$) C, H, N.

Biology. hA_1 and hA_{2A} Binding Assays. Binding of [^{3}H]-DPCPX to CHO cells transfected with the human recombinant A_1 adenosine receptor was performed as previously described.[36]

Displacement experiments were performed for 120 min at 25 °C in 0.20 mL of buffer containing 1 nM [^{3}H]-DPCPX, 20 μL of diluted membranes (50 μg of protein/assay), and at least six to eight different concentrations of examined compounds. Nonspecific binding was determined in the presence of 10 μM of CHA, and this is always ≤10% of the total binding.

Binding of [^{3}H]-SCH58261 to CHO cells transfected with the human recombinant A_{2A} adenosine receptors (50 μg of protein/assay) was performed according to Varani et al.[37] In competition studies, at least six to eight different concentrations of compounds were used, and nonspecific binding was determined in the presence of 50 μM NECA for an incubation time of 60 min at 25 °C. Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass-fiber filters using a Micro-Mate 196 cell harvester (Packard Instrument Company). The filter bound radioactivity was counted on Top Count (efficiency 57%) with Micro-Scint 20. The protein concentration was determined according to the Bio-Rad method[38] with bovine albumin as reference standard.

hA_3 Irreversible Binding Assay. Binding of [^{125}I]AB-MECA to membranes prepared from CHO cells stably expressing the human A_3 receptor was performed as described.[32,33] The assay medium consisted of a buffer containing 10 mM Mg^{2+}, 50 mM Tris, and 1 mM EDTA, at pH 7.4. The glass incubation tubes were filled with 100 μL of the membrane suspension (1.0 mg of protein/mL, stored at −80 °C in the same buffer), 20 μL of [^{125}I]AB-MECA (final concentration 0.6 nM), and 50 μL of a solution of the proposed antagonist. Nonspecific binding was determined in the presence of 100 μM NECA. Adenosine deaminase (3 units/mL) was present during the preparation of the membranes, in a preincubation of 30 min at 30 °C and during the incubation with the radioligands.

All nonradioactive compounds (**9**−**16**) were initially dissolved in DMSO and diluted with buffer to the desired final concentartion. The amount of DMSO never exceeded 1%.

Incubations were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester (Brandell, Gaithersburg, MD). The tubes were rinsed three times, each with 3 mL of buffer.

For studies of irreversible binding, membranes were incubated with the indicated (Table 1) concentration of ligand (**9**−**16**) for 1 h at room temperature. Following the incubation, the membrane suspension was centrifuged at 28 000 rpm for 30 min, and the pellet was resuspended in fresh buffer and kept at 4 °C for 30 min.

Membranes were washed a total of 9 times by sequential resuspension and centrifugation with buffer (50 mM Tris, 10 mM Mg^{2+}, and 1 mM EDTA) at pH 7.4 containing 0.02% CHAPS. Adenosine deaminase (3 units/mL) was present during all steps. The final pellet was suspended in a fixed volume of the above buffer solution for the A_3 receptor binding assay as described.[32,33]

Computational Approach. All calculations were performed on a Silicon Graphics O2 R10000 workstation.

The human A_3 receptor model was built and optimized using MOE (2000.02) modeling package[39] based on the approach described by Moro et al.[40] Briefly, transmembrane domains were identified with the aid of Kyte-Doolittle hydrophobicity and E_{min} surface probability parameters.[41] Transmembrane helices were built from the sequences and minimized individually. The minimized helices were then grouped together to form a helical bundle that matched the overall characteristics of the recently published crystal structure of bovine rhodopsin (PDB ID: 1F88). The helical bundle was minimized using the Amber94 all-atoms force field,[42] until the rms value of the conjugate gradient (CG) was <0.01 kcal/mol/Å. A fixed dielectric constant of 4.0 was used throughout these calculations.

All pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine derivatives were fully optimized without geometric constraints using RHF/AM1 semiempirical calculations.[43] Vibrational frequency analyses were used to characterize the minima stationary points (zero imaginary frequencies). The software package Gaussian98 was utilized for all quantum mechanical calculations.[44]

The ligands were docked into the hypothetical TMs binding site using the DOCK docking program, part of the MOE suite.[39] This program incorporates the use of manual and automatic docking procedures in combination with molecular mechanics within the Simulations module of MOE. The docking method employed enables nonbonded van der Waals and electrostatic interactions to be simultaneously monitored during the docking, and several possible conformations for the ligand were evaluated interactively. "Flexible" ligand docking was then used to define the lowest energy position of each ligand using a Monte Carlo/annealing based automated docking protocol. This uses a random iterative algorithm to sample changes in torsion angles and atomic positions while simultaneously recalculating internal and interaction energies. The automated

docking procedure then selected the best structures and applied the totality of the binding site. During the docking, all torsion angles of the side chains on the ligand were allowed to vary. This docking procedure was followed by another sequence of CG energy minimization to a gradient threshold of < 0.1 kcal/mol/Å. Energy minimization of the complexes was performed using AMBER94 all-atom force field.

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

Abbreviations: DMSO, dimethyl sulfoxide; DPCPX, 1,3-dipropyl-8-cytclopentylxanthine; THF, tetrahydrofurane; CHO cells, Chinese hamster ovary cells; EDTA, ethylenediaminotetraacetate; NECA, 5′-(*N*-ethylcarbamoyl)adenosine; TLC, thin-layer chromatography; mp, melting point; EtOAc, ethyl acetate; IR, infrared spectra; NMR, nuclear magnetic resonance; $CDCl_3$, deuterated chloroform; CHAPS, 3-[(3-chloroamidopropyl)-dimethylammino]-1-propanesulfonate; [^{125}I]AB-MECA, [^{125}I]1-[6-[[(4-amino-3-iodophenyl)methyl]amino]-9*H*-purin-9-yl]-1-deoxy-*N*-methyl-*β*-D-ribofuranuronamide; MRE3008-F20, 5-[[(4-methoxyphenyl)amino] carbonyl]amino-8-propyl-2-(2-furyl)-pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine; SCH58261, 5-amino-2-(2-furyl)-7-(2-phenylethyl)pyrazolo[4,3-*e*][1,2,4]-triazolo[1,5-*c*]pyrimidine.

Acknowledgment. This work was supported by MURST (cofin. 2000, ex 40%, prot. n. MM03237197_004) and MEDCO Res., Triangle Park, NC.

References

(1) Fredholm, B. B.; Abbracchio, M. P.; Burnstock, G.; Daly, J. W.; Harden, T. K.; Jacobson, K. A.; Leff, P.; Williams, M. Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* **1994**, *46*, 143−156.

(2) Olah, M. E.; Stiles, G. L. Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu. Rev. Pharmacol. Toxicol.* **1995**, *35*, 581−606.

(3) Jacobson, K. A.; Suzuki, F. Recent developments in selective agonists and antagonists acting at purine and pyrimidine receptors. *Drug. Dev. Res.* **1996**, *39*, 289−300.

(4) Meyerhof, W.; Muller-Brechlin, R.; Richter, D. Molecular cloning of a novel putative G-protein coupled receptor expressed during rat spermiogenesis. *FEBS Lett.* **1991**, *284*, 155−160.

(5) Sajjadi, F. G.; Firestein, G. S. cDNA cloning and sequence analysis of the human A_3 adenosine receptor. *Biochim. Biophys. Acta* **1993**, *1179*, 105−107.

(6) Salvatore, C. A.; Jacobson, M. A.; Taylor, H. E.; Linden, J.; Johnson, R. G. Molecular cloning and characterization of the human A_3 adenosine receptor. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10365−10369.

(7) Linden, J. Cloned adenosine A_3 receptors: Pharmacological properties, species differences and receptor functions. *Trends Pharmacol. Sci.* **1994**, *15*, 298−306.

(8) Zhao, Z. H.; Ravid, S.; Ravid, K. Chromosomal mapping of the mouse A_3 adenosine receptor gene, adora3. *Genomics* **1995**, *30*, 118−119.

(9) Hill, R. J.; Oleynek, J. J.; Hoth, C. F.; Kiron, M. A.; Weng, W. F.; Wester, R. T.; Tracey, W. R.; Knight, D. R.; Buchholz, R.; Kennedy, S. P. Cloning, expression and pharmacological characterization of rabbit A_1 and A_3 receptors. *J. Pharmacol. Exp. Ther.* **1997**, *280*, 122−128.

(10) Abbracchio, M. P.; Brambilla, R.; Kim, H. O.; von Lubitz, D. K. J. E.; Jacobson, K. A.; Cattabeni, F. G-protein-dependent activation of phospholipase-C by adenosine A_3 receptor in rat brain. *Mol. Pharmacol.* **1995**, *48*, 1038−1045.

(11) Ali, H.; Choi, O. H.; Fraundorfer, P. F.; Yamada, K.; Gonzaga, H. M. S.; Beaven, M. A. Sustained activation of phospholipase-D via adenosine A_3 receptors is associated with enhancement of antigen-ionophore-induced and Ca^{2+}-ionophore-induced secretion in a rat mast-cell line. *J. Pharmacol. Exp. Ther.* **1996**, *276*, 837−845.

(12) van Schaick, E. A.; Jacobson, K. A.; Kim, H. O.; IJzerman, A. P.; Danhof, M. Haemodynamic effects and histamine release elicited by the selective adenosine A_3 receptor agonists 2-Cl-IB-MECA in conscious rats. *Eur. J. Pharmacol.* **1996**, *308*, 311−314.

(13) Forsythe, P.; Ennis, M. Adenosine, mast cells and asthma. *Inflammation Res.* **1999**, *48*, 301−307.

(14) Ramkumar, V.; Stiles, G. L.; Beaven, M. A.; Ali, H. The A_3 adenosine receptor is the unique adenosine receptor which facilitates release of allergic mediators in mast cells. *J. Biol. Chem.* **1993**, *268*, 16887−16890.

(15) Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Merighi, S.; Varani, K.; Borea, P. A.; Spalluto G. A_3 Adenosine receptor ligands; history and perspectives. *Med. Res. Rev.* **2000**, *20*, 103−128

(16) Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Spalluto, G.; Klotz, K.-N.; Leung, E.; Varani, K.; Gessi, S.; Merighi, S.; Borea, P. A.. Pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as highly potent and selective human A_3 adenosine receptor antagonists. *J. Med. Chem.* **1999**, *42*, 4473−4478.

(17) Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Spalluto,G.; Moro, S.; Klotz, K. N.; Leung, E.; Varani, K.; Gessi, S.; Merighi, S.; Borea, P. A. Pyrazolo[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine derivatives as highly potent and selective human A_3 adenosine receptor antagonists: Influence of the chain at N8 pyrazole nitrogen. *J. Med. Chem.* **2000**, *43*, 4768−4780.

(18) Dickinson, K. E. J.; Heald, S. L.; Jeffs, P. W.; Lefkowitz, R. J.; Caron, M. G. Covalent labeling of the *β*-adrenergic ligand-binding site with the par-(bromoacetamidyl)benzylcarazolol. A highly potent *β*-adrenergic affinity label. *Mol. Pharmacol.* **1985**, *27*, 499−506.

(19) Minneman, K. P. α_1-Adrenergic receptor subtypes, inositol phosphates, and sources of cell Ca^{2+}. *Pharmacol. Rev.* **1988**, *40*, 87−119.

(20) Nelson, C. A.; Muther, T. F.; Pitha, J.; Baker, S. P. Differential recovery of beta adrenoreceptor antagonist and agonist high affinity binding sites in the guine pig lung after irreversible blockade. *J. Pharmacol. Exp. Ther.* **1986**, *237*, 830−836.

(21) (a) Scammels, P. J.; Bakes, S. P.; Belardinelli, L.; Olsson, R. A. Substituted 1,3-dipropylxanthines as irreversible antagonists of A_1 adenosine receptors. *J. Med. Chem.* **1994**, *37*, 2704−2712. (b) Jacobson, K. A.; Barone, S.; Kammula, U.; Stiles, G. L. Electrophilic derivatives of purines as irreversible inhibitors of A_1-adenosine receptors. *J. Med. Chem.* **1989**, *32*, 1043−1051. (c) Boring, D. L.; Ji, X.-D.; Zimmet, J.; Taylor, K. E.; Stiles, G. L.; Jacobson, K. A. Trifunctional agents as a design strategy for tailoring ligand properties: Irreversible inhibitors of A_1 adenosine receptors. *Bioconjugate Chem.* **1991**, *2*, 77−88.

(22) (a) Srinivas, M.; Shryock, J. C.; Scammels, P. J.; Baker, S. P.; Belardinelli, L. A novel irreversible antagonist of the A_1-adenosine receptor. *Mol. Pharmacol.* **1996**, *50*, 196−205. (b) Stiles, G. L.; Jacobson, K. A. High affinity acylating antagonists for the A_1 adenosine receptor: identification of binding subunit. *Mol. Pharmacol.* **1988**, *34*, 724−728. (c) Jacobson, K. A.; Olah, M. E.; and Stiles, G. L. Trifunctional ligands: A radioiodinated high affinity acylating antagonist for the A_1 adenosine receptor. *Pharmacol. Commun.* **1992**, *1*, 145−154.

(23) (a) Srinivas, M.; Shryock, J. C.; Dennis, D. M.; Baker, S. P.; Belardinelli, L. Differential A_1 adenosine receptor reserve for two action of adenosine on guinea pig myocytes. *Mol. Pharmacol.* **1997**, *52*, 683−691. (b) Dennis, D.; Jacobson, K. A.; Belardinelli, L. Evidence of spare A_1-adenosine in guinea pig atrioventricular node. *Am. J. Physiol.* **1992**, *262*, H661−671. (c) Zhang, J.; Belardinelli, L.; Jacobson, K. A.; Otero, D. H.; Baker, S. P. Persistent activation of A_1 adenosine receptors by an acylating adenosine derivative and its receptor reserve in DDT1 MF-2 cells and heart. *Mol. Pharmacol.* **1997**, *52*, 491−498.

(24) (a) Jacobson, K. A.; Fischer, B.; Ji, X.-D. A "cleavable trifunctional" approach to receptor affinity labeling: regeneration of binding to A_1-adenosine receptors, *Bioconjugate Chem.* **1995**, *6*, 255−263. (b) Beauglehole, A. R.; Baker, S. P.; Scammels, P. J. Fluorosulfonyl-substituted xanthines as selective irreversible antagonists for the A_1-adenosine receptor. *J. Med. Chem.* **2000**, *43*, 4973−4980.

(25) (a) Jacobson, K. A.; Barrington, W. W.; Pannell, L. K.; Jarvis, M. F.; Ji, X.-D.; Williams, M.; Hutchison, A. J.; Stiles, G. L. Agonist-derived molecular probes for A_2-adenosine receptors. *J. Mol. Recognit.* **1989**, *2*, 170−178. (b) Jacobson, K. A.; Stiles, G. L.; Ji, X.-D. Chemical modification and irreversible inhibition of striatal A_{2a}-adenosine receptors. *Mol. Pharmacol.* **1992**, *42*, 123−133. (c) Ji, X.-D.; Gallo-Rodriguez, C.; Jacobson, K. A. 8-(3-Isothiocyanatostyryl)caffeine is a selective irreversible inhibitor of striatal A_2-adenosine receptors. *Drug Dev. Res.* **1993**, *29*, 292−298. (d) Niiya, K.; Jacobson, K. A.; Silvia, S. K.; Olsson, R. A. Covalent binding of a selective agonist irreversibly activates guinea pig coronary artery A_2 adenosine receptors. *Naunyn-Schmeideberg's Arch. Pharmacol.* **1993**, *347*, 521−526. (e) Shryock, J. C.; Snowdy, S.; Baraldi, P. G.; Cacciari, B.; Spalluto, G.; Monopoli, A.; Ongini, E.; Baker, S. P.; Belardinelli, L. A_{2A}-adenosine receptor reserve for coronary. *Circulation* **1998**, *98*, 711−718.

(26) Ji, X.-D.; Gallo-Rodriguez, C.; Jacobson, K. A. A selective agonist affinity label for A_3 adenosine receptors. *Biochem. Biophys. Res. Commun.* **1994**, *203*, 570−576.

(27) Li, A.-H.; Chang, L.; Ji, X.-D.; Melman, N.; Jacobson, K. A. Functionalized congeners of 1,4-dihydropyridines as antagonist molecular probes for A_3 adenosine receptors. *Bioconjugate Chem.* **1999**, *10*, 667−677.

(28) (a) Bruncel, E. Mechanistic aspects of the chemistry of fluorosulfates. *Mech. React. Sulfur Compounds* **1970**, *5*, 71−74. (b) Fukuda, H, Frank, F. S.; Truce, W. E. Synthesis of β-disulfones from sulfonyl fluorides and organometallic compounds. *J. Org. Chem.* **1963**, *28*, 1420.

(29) Panthananickal, A.; Hansch, C.; Leo, A.; Quinn, F. R. Structure−activity relationships in antitumor aniline mustards. *J. Med. Chem.* **1978**, *21*, 16−26.

(30) Bardos, T. J.; Datta-Gupta, N.; Hebborn, P.; Triggle, D. J. A study of comparative chemical and biological activities of alkylating agents. *J. Chem. Soc.* **1965**, *8*, 167−174.

(31) Kurita, K.; Iwakura, Y. Trichloromethyl-Chloroformate as a phosgene equivalent: 3-isocyanatopropanoyl chloride. *Org. Synth. Coll. Vol. VI* **1988**, 715−718.

(32) Karton, Y.; Jiang, J.-L.; Ji, X.-D.; Melman, N.; Olah, M. E.; Stiles, G. L.; Jacobson, K. A. Synthesis and biological activities of flavonoid derivatives as A_3 adenosine receptor antagonists. *J. Med. Chem.* **1996**, *39*, 2293−2301.

(33) Olah, M. E.; Gallo-Rodriguez, C.; Jacobson, K. A.; Stiles, G. L. [^{125}I]AB-MECA, a high affinity radioligand for the rat A_3 adenosine receptor. *Mol. Pharmacol.* **1994**, *45*, 978−982.

(34) Palczewski, K.; Kumasaka, T.; Hori, T.; Behnke, C. A.; Motoshima, H.; Fox, B. A.; Trong, I. L.; Teller, D. C.; Okada, T.; Stenkamp, R. E.; Yamamoto, M.; Miyano, M. Crystal structure of rhodopsin: a G protein-coupled receptor. *Science* **2000**, *289*, 739−745.

(35) Moro, S.; Li, A. H.; Jacobson, K. A. Molecular modeling studies of human A_3 adenosine antagonists: structural homology and receptor docking. *J. Chem. Inf. Comput. Sci.* **1998**, *38*, 1239−1248.

(36) Varani, K.; Merighi, S.; Gessi, S.; Klotz, K. N.; Leung, E.; Baraldi, P. G.; Cacciari, B.; Romagnoli, R.; Spalluto, G.; Borea, P. A. [^{3}H]-MRE3008-F20: a novel antagonist radioligand for the pharmacological and biochemical characterization of human A_3 adenosine receptors. *Mol. Pharmacol.* **2000**, *57*, 968−975.

(37) Varani, K.; Gessi, S.; Dionisotti, S.; Ongini, E.; Borea, P. A. [^{3}H]-SCH 58261 Labelling of functional A_{2A} adenosine receptors in human neutrophil membranes. *Br. J. Pharmacol.* **1998**, *123*, 1723−1731.

(38) Bradford, M. M. A rapid sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(39) *Molecular Operating Environment* (MOE 2000.02), Chemical Computing Group, Inc.: 1255 University St., Suite 1600, Montreal, Quebec, Canada, H3B 3X3.

(40) Moro, S.; Guo, D.; Camaioni, E,; Boyer, J. L.; Harden K. T.; Jacobson, K. A. Human $P2Y_1$ receptor: molecular modeling and site-directed mutagenesis as tools to identify agonist and antagonist recognition sites. *J. Med. Chem.* **1998**, *41*, 1456−1466.

(41) Kyte, J.; Doolittle, R. F. A simple method for displaying the hydrophobic character of a protein. *J. Mol. Biol.* **1982**, *157*, 105−132.

(42) Weiner, S. J.; Kollman, P. A.; Nguyen, D. T.; Case, D. A. An all-atom force field for simulation of protein and nucleic acids. *J. Comput. Chem.* **1986**, *7*, 230−252.

(43) Dewar, M. J. S. E.; Zoebisch, G.; Healy, E. F. AM1: A New General Purpose Quantum Mechanical Molecular Model. *J. Am. Chem. Soc.* **1985**, *107*, 3902−3909.

(44) Frisch, M.; Trucks, G. W.; Schlegel, H.; Scuseria, G.; Robb, M.; Cheeseman, J.; Zakrzewski, V.; Montgomery, J.; Stratmann, R. E.; Burant, J. C.; Dapprich, S.; Millam, J.; Daniels, A.; Kudin, K.; Strain, M.; Farkas, O.; Tomasi, J.; Barone, V.; Cossi, M.; Cammi, R.; Mennucci, B.; Pomelli, C.; Adamo, C.; Clifford, S.; Ochterski, J.; Petersson, G. A.; Ayala, P. Y.; Cui, Q.; Morokuma, K.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Cioslowski, J.; Ortiz, J. V.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Gomperts, R.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Gonzalez, C.; Challacombe, M.; Gill, P. M. W.; Johnson, B. G.; Chen, W.; Wong, M. W.; Andres, J. L.; Head-Gordon, M.; Replogle, E. S.; Pople, J. A., *Gaussian 98* (revision A.6); Gaussian, Inc.: Pittsburgh, PA, 1998.

JM010818A