Synthesis, Biological Properties, and Molecular Modeling Investigation of the First Potent, Selective, and Water-Soluble Human A_3 Adenosine Receptor Antagonist

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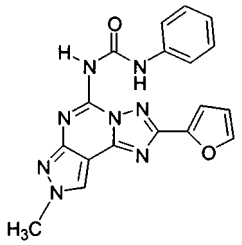
Abstract: A new, highly potent, selective, and water-soluble antagonist of the hA_3 adenosine receptor was synthesized and tested in binding and functional assays. Compound **4** (5-[[(4-pyridyl)amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo-[4,3-*e*]1,2,4-triazolo[1,5-*c*]pyrimidine hydrochloride) displayed high water solubility (15 mM) and the highest affinity (K_i = 0.01 nM) and selectivity for the hA_3 versus A_1, A_{2A}, and A_{2B} receptors (>10000-fold) ever reported. A Schild analysis of the antagonism by **4** of agonist-induced inhibition of cAMP production in CHO cells expressing the hA_3 receptor indicated a K_B value of 0.20 nM.

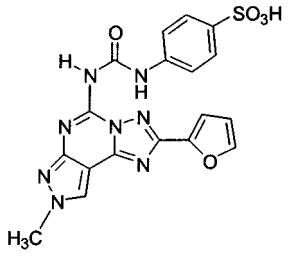
Introduction. It is well-known that adenosine regulates many physiological processes through the interaction with four known receptor subtypes classified as A_1, A_{2A}, A_{2B}, and A_3.[1,2] In particular, the A_3 adenosine receptor subtype, which is distributed in different organs (lung, liver, kidney, heart, and, with a lower density, the brain),[3] exerts its action through the modulation of two second messenger systems: inhibition of adenylate cyclase[4] and stimulation of phospholipases C[5] and D.[6] The potential therapeutic applications of activating or antagonizing this receptor subtype have been investigated in recent years. In particular, antagonists for the A_3 receptor promise to be useful for the treatment of inflammation[7] and in the regulation of cell growth.[8,9] Consequently, much effort has been directed toward searching for potent and selective human A_3 adenosine antagonists.[10] Recently, Baraldi and co-workers reported a large series of pyrazolotriazolopyrimidines, bearing substituted phenylcarbamoyl residues at the amino group at the 5-position, as highly potent and selective antagonists of the human A_3 adenosine receptor.[11−13] In particular, **1** (5-[[(phenyl)-amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-*e*] 1,2,4-triazolo[1,5-*c*]pyrimidine (Chart 1) showed the most favorable binding affinity and selectivity for the human A_3 adenosine receptor ever reported.[13]

Chart 1. Structures, Biological Characterization, and Water Affinities of Reference Compounds



1

hA_1 K_i = 594 nM; hA_{2A} K_i = 381 nM
hA_{2B} K_i = 222 nM; hA_3 K_i = 0.16 nM
hA_1/hA_3 = 3,713; hA_{2A}/hA_3 = 2,381
hA_{2B}/hA_3 = 1,388
Rm = 4.06 ± 0.05



2

hA_1 K_i = >10,000 nM; hA_{2A} K_i = 594 nM
hA_{2B} K_i = >10,000 nM; hA_3 K_i = 25 nM
hA_1/hA_3 = > 400; hA_{2A}/hA_3 = 24
hA_{2B}/hA_3 = > 400
Rm = 1.66 ± 0.18

Unfortunately, a major problem within this class of compounds is the typical low water solubility, which has limited their use as pharmacological and diagnostic tools. The hydrophobicity of compound **1** is indicated by its high R_m value of 4.06. Previously an attempt for obtaining a water-soluble derivative has been made through the introduction of a sulfonic acid group at the para position of the phenyl ring (Chart 1) affording compound **2** (4-[3-(2-furan-2-yl-8-methyl-8*H*-pyrazolo-[4,3-*e*][1,2,4]triazolo[1,5-*c*]pyrimidin-5-yl)ureido]benzenesulfonic acid). As expected, a completely water-soluble derivative (R_m = 1.66, water solubility greater than 20 mM) was obtained; however, a significant loss of affinity (156-fold) and selectivity was observed.[13] A hypothesis for the dramatic loss of affinity was provided through molecular modeling studies, which indicated that steric control seemed to be taking place around the para position of the phenyl ring in the putative A_3 receptor binding site.[13] Taking into account these observations and with the aim of obtaining derivatives with high affinity, selectivity, and water solubility, we synthesized

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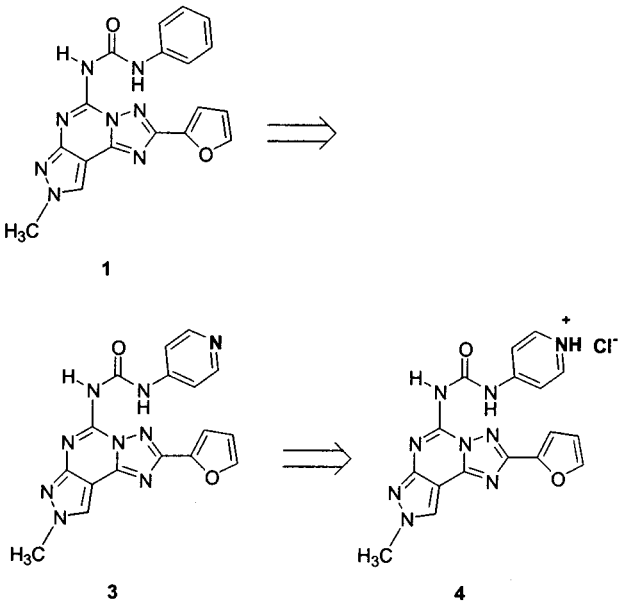
Chart 2. Rational Design of Water-Soluble hA_3 Adenosine Receptor Antagonist



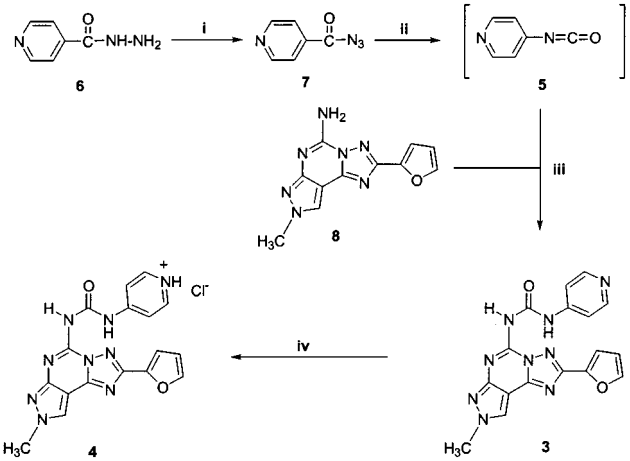
a new derivative (Chart 2) by bioisosteric replacement of the phenyl ring with the 4-pyridyl moiety (**3**), thus providing higher water solubility while avoiding the steric hindrance of a substituent at the para position, which seemed to be responsible for the reduction of hA_3 adenosine receptor affinity.

In fact, the introduction of a basic nitrogen was intended to further improve water solubility when protonated, i.e., in the form of the corresponding HCl salt (**4**).

Results and Discussion. The synthesis of the desired compound has been performed following the general strategy depicted in Scheme 1. The major problem in the synthesis of derivative **3** involved the preparation of a 4-pyridyl isocyanate (**5**), which could not be synthesized by the usual method, i.e., reaction of the corresponding amine with phosgene or a phosgene equivalent, because of the reactivity and instability of pyridyl isocyanates.[14]

For this reason, **5** was prepared as reported in the literature starting from the commercially available isonicotinoyl hydrazide (**6**), which after reaction with sodium nitrite under acid conditions afforded the corresponding acyl azide **7**.[15] The latter was in turn converted into **5** upon Curtius rearrangement induced by heating **7** at reflux in dry benzene for 2 h.[16] The crude isocyanate was refluxed overnight in dry THF with the precursor containing the tricyclic system bearing a methyl group at the N8 position (**8**). The desired product **3** was purified using flash chromatography and eluted

Scheme 1[a]



[a] (i) $NaNO_2$, aqueous HCl, 0 °C, 1 h; (ii) benzene, reflux, 2 h; (iii) THF reflux overnight; (iv) HCl/MeOH, 0 °C, 30 min.

with a MeOH/EtOAc gradient of 0−30%. The corresponding hydrochloride **4** was obtained by treatment of **3** for 30 min at 0 °C with methanol saturated with HCl gas. The hydrophobicity of the newly synthesized substances was measured in reverse-phase TLC experiments and reported as R_m values[17] ($R_m = \log(1/R_f - 1)$) as shown in Table 1. As expected, both derivatives showed increased hydrophilicity with respect to reference **1**, but most importantly, the hydrochloride salt **4** freely dissolved in water to a maximum concentration of 15 mM. Table 1 also compares the receptor binding affinities of **3** and **4** determined at human A_1,[18] A_{2A},[19] A_{2B},[20] and A_3[20] receptors expressed in CHO (A_1, A_{2A}, A_3) and HEK-293 (A_{2B}) cells.

Surprisingly, both substances showed very high affinity at the human A_3 adenosine receptor subtype, with K_i values in the picomolar range (10−40 pM) and with high levels of selectivity. In particular, the hydrochloride salt **4** showed increased affinity and selectivity with respect to the reference compound bearing the phenylcarbamoyl moiety **1**. The K_i value of **4** at the hA_3 receptor was 0.01 nM, thus indicating high selectivity versus other subtypes: $hA_1/hA_3 = 35\,000$, $hA_{2A}/hA_3 = 10\,000$, $hA_{2B}/hA_3 = 25\,000$. These values were more favorable than the selectivity ratios for compound **1**, for which the K_i value at the hA_3 was reported to be 0.16 nM: $hA_1/hA_3 = 3700$, $hA_{2A}/hA_3 = 2400$, $hA_{2B}/hA_3 = 1400$. Moreover, this class of compounds, as previously demonstrated, proved to be inactive in a rat model with K_i values at the rA_3 typically greater than 1 μM.[11] **4** at 1 μM displaced only 35% of the specific binding of [^{125}I]I-AB-MECA at A_3 receptors in membranes of rat basophilic RBL-2H3 cells.[21b] Concerning the affinity differences observed between the salt and neutral species, we

Table 1. Binding Affinity at hA_1, hA_{2A}, hA_{2B}, and hA_3 Adenosine Receptors and Water Solubility of Synthesized Compounds

compd	$R_m(0)$[a]	hA_1 K_i (nM)[b]	hA_{2A} K_i (nM)[c]	hA_{2B} K_i (nM)[d]	hA_3 K_i (nM)[e]	hA_1/hA_3	hA_{2A}/hA_3	hA_{2B}/hA_3
3	3.06 ± 0.06	250 ± 23	60 ± 10	200 ± 16	0.04 ± 0.009	6250	1500	5000
4	2.29 ± 0.05	350 ± 22	100 ± 12	250 ± 24	0.01 ± 0.005	35000	10000	25000

[a] The R_m values of **3** and **4** were measured with a mobile phase of different concentrations of MeOH/H_2O. R_m values are reported as theoretical at 0% organic solvent in the mobile phase ($R_m(0)$). [b] Displacement of specific [^{3}H]DPCPX[26] binding at human A_1 receptors expressed in CHO cells (n = 3−6). [c] Displacement of specific [^{3}H]ZM 241385[26] binding at human A_{2A} receptors expressed in HEK-293 cells. [d] Displacement of specific [^{3}H]DPCPX binding at human A_{2B} receptors expressed in HEK-293[26] cells (n = 3−6). [e] Displacement of specific [^{3}H]MRE3008-F20[26] binding at human A_3 receptors expressed in HEK-293 cells. Data are expressed as K_i ± SEM (n = 3−6). The affinity of **4** at the rat adenosine receptor in RBL-2H3 cells was also determined using previously reported methods.[21] K_i values (n = 3) were 226 ± 50 nM (A_1), 97.6 ± 26.2 nM (A_{2A}), and >1 μM (A_3).

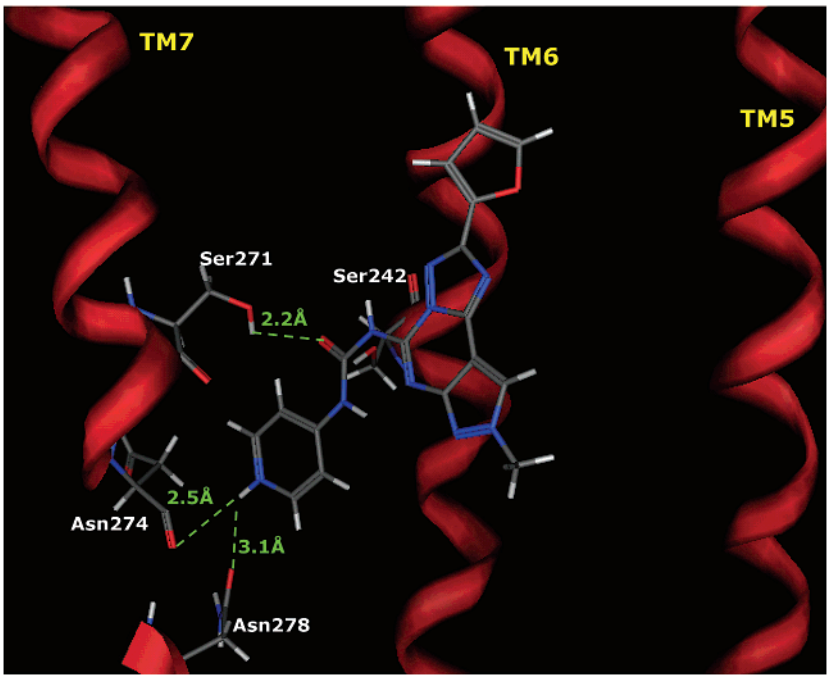


Figure 1. Side view of the human A_3−**4** complex model. The side chains of the important residues in proximity to the docked **4** molecule are highlighted and labeled.

speculate that a difference in their relative dissociation/association rates during the biological assay could be a reasonable explanation. These results not only represent the first example of a highly potent, selective, and water-soluble human A_3 adenosine antagonist but strongly suggest an involvement of the pyridine nitrogen in the receptor recognition. Using a homology modeling approach based on the crystal structure of the rhodopsin[22] as a template, we have built an improved model of the transmembrane helical domains (TMs) of the human A_3 receptor,[23] which can be considered a further refinement of the hypothetical binding site for A_3 receptor antagonists already proposed.[13,22,24] As shown in Figure 1, after the Monte Carlo/annealing sampling, we propose that the hypothetical binding site of **4** is surrounded by TMs 3, 5, 6, and 7, with the furan ring pointing toward the extracellular environment. Similar to conformational results already described for other pyrazolotriazolopyrimidines,[12,13,22] the lowest energy conformation of **4** featured the carbamoyl moiety in the 5-position surrounded by four polar amino acids: Ser242 (TM6), Ser271 (TM7), His274, and Ser275 (TM7).

Accordingly, this region seemed to be critical for the recognition of this class of antagonists. Moreover, additional strong electrostatic interactions appeared to occur between the positively charged pyridinium moiety of **4** and the carbonyl oxygen atoms of Asn274 (N^+H···OC distance = 2.5 Å) and Asn278 (N^+H···OC distance = 3.1 Å), both located on TM7. These electrostatic interactions might be responsible for the increase of the affinity in the protonated form, i.e., the hydrochloride derivative **4**. Interestingly, these two asparagine residues (Asn274 and Asn278) are largely conserved among a number of GPCRs. It should be noted, however, that this human A_3 receptor model, based on the TM region of the receptor, is not able to clearly explain the corresponding increase of selectivity for this subtype even taking into account which of the important TM residues are different in the other receptor subtpyes.

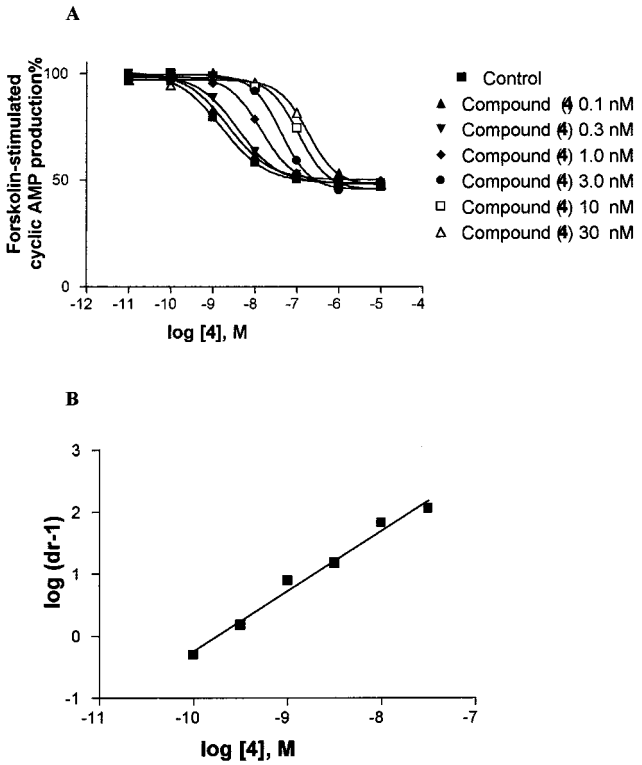


Figure 2. Effects of **4** on the inhibition of cyclic AMP production induced by the agonist Cl-IB-MECA in human A_3 adenosine receptor-expressing CHO cells (A) and Schild analysis (B) of the data. The procedures used are described in Supporting Information. The data shown in panel A were derived from one experiment performed in duplicate and are typical of three independent experiments giving similar results. The K_B value for antagonism by **4** was calculated from three independent experiments.

However, as already reported by Jacobson and coworkers, multiple regions of the adenosine receptors, including a segment of the second extracellular loop, are involved in ligand recognition.[25] Other investigations

are in progress in our lab to better describe the role of the extracellular domain on the ligand recognition process. For confirmation of the high potency of this compound in a functional assay, the inhibition of cAMP[26] generation by Cl-IB-MECA in membranes of CHO cells stably transfected with the human A_3 receptor was evaluated.

Consistent with its binding affinity, **4** showed an IC_{50} value of 0.7 ± 0.06 nM, compared to an IC_{50} of 2.10 ± 0.21 nM for **1**. A Schild analysis of the antagonism of the effects of **4** on Cl-IB-MECA[26]-induced inhibition of forskolin-stimulated cAMP was carried out (Figure 2).[19] A K_B value of 0.20 ± 0.03 nM was calculated, thus demonstrating **4** to be the most potent antagonist of the human A_3 receptor ever reported.

Conclusions. The present study revealed a novel, potent, selective, and most importantly, water-soluble hA_3 adenosine receptor antagonist. This derivative featured a basic 4-pyridylcarbamoyl moiety at the N5 position of the pyrazolotriazolopyrimidine nucleus, and the corresponding hydrochloride salt **4** displayed a K_i value of 0.01 nM at the hA_3 and selectivities versus the other adenosine receptor subtypes ranging from 10 000 to 35 000. This increase of affinity compared to neutral arylcarbamate derivatives could be attributed in receptor modeling to strong electrostatic interactions between the pyridinium moiety of **4** and the side chain carbonyl oxygen atoms of Asn274 and Asn278, both located on TM7. In view of the potency, selectivity, and water solubility, **4** could be an ideal candidate for pharmacological and clinical investigation of the hA_3 adenosine receptor subtype.

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

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(26) Abbreviations: DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; [^{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; THF, tetrahydrofuran; CHO, Chinese hamster ovary; HEK, human embryonic kidney; 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; ZM 241385, 5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine; Cl-IB-MECA, 2-chloro-3-iodobenzyl-5′-(*N*-methylcarbamoyl)adenosine; cAMP, cyclic adenosine-5′-monophosphate′.

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