Derivatives of 4-Amino-6-hydroxy-2-mercaptopyrimidine as Novel, Potent, and Selective A_3 Adenosine Receptor Antagonists

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A number of derivatives of 4-amino-6-hydroxy-2-mercaptopyrimidine (**5**) were synthesized and biologically evaluated as A_3 adenosine receptor (A_3 AR) antagonists. The new compounds were designed as open chain analogues of a triazolopyrimidinone derivative displaying submicromolar affinity for the A_3 AR, which had been previously identified using a 3D database search. Substituents R, R′, and R″ attached to the parent compound **5** were chosen according to factorial design and stepwise lead optimization approaches, taking into account the essentially hydrophobic nature of the A_3 AR binding site. As a result, **5m** (R = *n*-C_3H_7, R′ = 4-$ClC_6H_4CH_2$, R″ = CH_3) was identified among the pyrimidine derivatives as the ligand featuring the best combination of potency and selectivity for the target receptor. This compound binds to the A_3 AR with a K_i of 3.5 nM and is devoid of appreciable affinity for the A_1, A_{2A}, and A_{2B} ARs.

Introduction

Adenosine is a ubiquitous purine nucleoside that plays a pivotal role in a large variety of physiological and pathophysiological processes, which are modulated by its interaction with specific receptors. Four different adenosine receptors (ARs) have been identified, A_1, A_{2A}, A_{2B}, and A_3, all of which belong to the superfamily of G-protein-coupled receptors.[1–3] ARs from different species show 82–92% amino acid sequence homology, the only exception being the A_3 AR, which exhibits 74% homology between rats and humans.[4–6]

AR-mediated signaling occurs through inhibition (A_1 and A_3) or stimulation (A_{2A} and A_{2B}) of adenylate cyclase, resulting in a modification of the intracellular production of cAMP. Adenosine receptor coupling to other second messenger systems has also been described, such as stimulation of phospholipase C (A_1, A_{2B}, and A_3) or activation and inhibition of potassium and calcium channels (A_1).[7]

Each AR is considered an attractive target for pharmacological intervention in many pathological conditions, including renal failure, cardiac and cerebral ischemia, central nervous system disorders, neurodegenerative diseases, and inflammatory pathologies, such as asthma.[8] The development of potent and selective synthetic antagonists of ARs has been the subject of medicinal chemistry research for more than 3 decades.[9] In particular, over the past few years, the interest in blocking the A_3 subtype increased after the discovery of its involvement in cellular growth.[10]

The A_3 AR is widely distributed in mammals, but pronounced differences in expression levels exist between species. In humans, the highest density of this receptor has been found in lung and liver, with lower levels in aorta, brain, and testes.[11] The A_3 AR is involved in a variety of important physiological processes, including modulation of cerebral and cardiac ischemic damage,[11,12] inflammation,[13] modulation of intraocular pressure,[14] regulation of normal and tumor cell growth,[10,15,16] and immunosuppression.[2,17] Accordingly, A_3 AR antagonists may be employed in the acute treatment of stroke and glaucoma and as antiasthmatic, antiallergenic, and cerebroprotective agents.[17] Furthermore, the significant overexpression of A_3 AR in several types of tumor cells and the prosurvival and antiapoptotic effects of A_3 AR stimulation have recently led Baraldi et al. to propose that antagonists for this receptor might sensitize tumor cells to chemotherapeutic drugs.[15,18]

In the past 5 years, considerable progress has been made toward the identification and development of potent and selective ligands for A_3 AR. The best known classes of A_3 AR antagonists (see Chart 1) are imidazopurinones **I**,[19,20] triazoloquinazolines **II**,[21,22] pyrazolotriazolopyrimidines **III**,[15,23,24] triazoloquinoxalines **IV** and **V**,[25–27] isoquinolines **VI**,[28–30] and pyridines **VII**.[31–33]

We have recently described the synthesis and the biological evaluation of a number of A_1 and A_3 AR antagonists designed using database search and lead optimization approaches (structures and binding data are reported in Table 1).[34] Our work proceeded through the following steps (Figure 1). Briefly, (i) the Cambridge Structural Database (CSD)[35] was searched using two substructures as queries extracted by known A_1 and A_3 AR antagonists; (ii) five potential leads (**A**−**E**) were selected to purchase, synthesize, or translate synthetically into less bulky or hydrophilic analogues; (iii) out of the five tested compounds, two displayed micromolar to nanomolar affinity for the A_1 AR (**B** and **C**), four showed nanomolar affinity for the A_3 R (**B**−**E**), while one turned out to be inactive (**A**); (iv) lead optimization efforts gave the best results in the series of 2-(benzimidazol-2-yl)quinoxalines (**C**) yielding compounds **C′** and **C″** as potent and selective antagonists at the A_1 and A_3 ARs, respectively.

Although the triazolopyrimidinone **E** exhibited an interesting selectivity profile at the A_3 AR, simple modifications about the thiazolidine ring yielded compounds equipotent or

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Chart 1. Structures of Known A_3 AR Antagonists

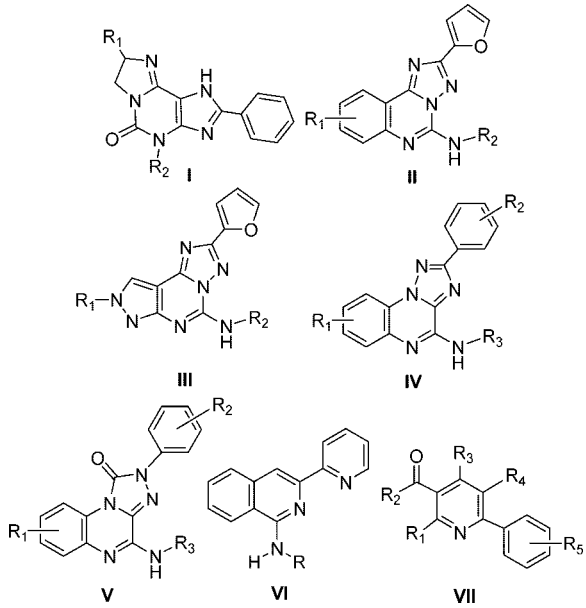
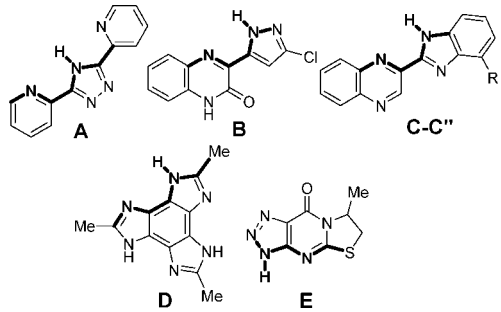


Table 1. Affinities at Human A_1, A_{2A}, and A_3 ARs of Compounds Designed as A_1 AR and/or A_3 AR Antagonists by Means of 3D Database Searching[34]



	K_i (nM)[a]		
compd	hA_1[b]	hA_{2A}[c]	hA_3[d]
A	>10000	>10000	>1000
B	3336 ± 557	>10000	125 ± 20
C (R = H)	50 ± 15	561 ± 17	763 ± 13
C′ (R = SC_2H_5)	0.5 ± 0.01	3440 ± 980	955 ± 215
C″ (R = CH_3)	8000 ± 567	833 ± 67	26 ± 9
D	>10000	>10000	130 ± 3
E[e]	>10000	>10000	440 ± 33
DPCPX[f]	0.5 ± 0.03	337 ± 28	>1000
NECA[g]	14 ± 4	16 ± 3	73 ± 5
Cl-IB-MECA[h]	890 ± 61	401 ± 25	0.22 ± 0.02

[a] The K_i values are the mean ± SEM of at least three determinations derived from an iterative curve-fitting procedure (Prism program, GraphPad, San Diego, CA). [b] Displacement of specific [^{3}H]DPCPX binding in membranes obtained from CHO cells stably expressing hA_1 AR. [c] Displacement of specific [^{3}H]NECA binding in membranes obtained from CHO cells stably expressing hA_{2A} AR. [d] Displacement of specific [^{125}I]AB-MECA binding in membranes obtained from CHO cells stably expressing hA_3 AR. [e] Assayed with a purity degree of 96%. [f] DPCPX: 1,3-dipropyl-8-cyclopentylxanthine. [g] NECA: 5′-*N*-ethylcarboxamidoadenosine. [h] Cl-IB-MECA: 2-chloro-N^6-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide.

inactive in binding experiments. Because the triazolopyrimidinone scaffold was not synthetically versatile, we turned our attention to derivatives of 4-amino-6-hydroxy-2-mercaptopyrimidine (**5**) and 6-amino-2-mercaptopyrimidin-4(3*H*)-one (**6**) as open chain analogues of **E**, which were better suited to lead optimization (Figure 2). Taking into account that the binding site of A_3 AR antagonists is mostly lipophilic, the R, R′, and R″ substituents in the general formulas **5** and **6** were alkyl and phenylalkyl chains.

Preliminary binding assays of the first newly synthesized compounds revealed that derivatives of series **6** were totally devoid of any affinity toward ARs (data not shown) while derivatives of series **5** displayed interesting binding profiles at the A_3 AR. A few precursors of series **5** (**4d**,**e**) and **6** (**3d**,**e**) likewise turned out to be inactive (data not shown). Further research focused on series **5** through the design, synthesis, and testing of additional 4-amino-6-hydroxy-2-mercaptopyrimidines to identify those characterized by the highest affinity and selectivity as A_3 AR antagonists.

Chemistry

The derivatives **5a**−**s** were obtained in three steps starting from the commercially available 4-amino-6-hydroxy-2-mercaptopyrimidine monohydrate (**1**) (Scheme 1). The first was the alkylation of the sulfur atom at the 2-position by reaction with iodomethane or the opportune bromo(phenyl)alkane in 1 M NaOH to give compounds **2a**−**e** (Table 1 of Supporting Information), which were collected by filtration and were sufficiently pure to be used in the next step without further purification. The second step, i.e., the alkylation of the 4-oxygen atom of compounds **2a**−**e** to prepare derivatives **4**, always gave a mixture of the isomeric products **3** and **4** because of the keto–enol equilibrium of the pyrimidinone scaffold. The experimental conditions were extensively studied with regard to solvent (anhydrous DMF, acetonitrile, ...), base quantities, temperature, and time of reaction. The best results in terms of **4** yields were obtained when the reaction was carried out in anhydrous DMF in the presence of an excess of K_2CO_3 as base, which generally gave a 1:3 ratio of isomers **3** and **4**, even though the relative quantities of the two isomers obtained varied depending on the stereoelectronic features of both reagents. Conversely, for example, the use of acetonitrile as solvent increased the formation of the **3** isomer. For only the case of derivatives with R′ = CH_3, the use of methyl iodide always gave an excess of the N-isomer **3** with respect to **4** (see Table 2 of Supporting Information for details). The **4** isomer was then easily isolated by flash chromatography and characterized (Tables 2 and 3 of Supporting Information). The final step was the conversion of the 4-amino group of **4** into the amide group of **5** by reaction with the opportune anhydride and concentrated H_2SO_4 as catalyst (Table 4 of Supporting Information).

Biology

The affinities of the new compounds **5a**−**s** toward human A_1, A_{2A}, and A_3 ARs were evaluated by competition experiments assessing their respective abilities to displace [^{3}H]DPCPX, [^{3}H]NECA, or [^{125}I]AB-MECA binding from transfected Chinese hamster ovary (CHO) cells. Experiments were performed as described elsewhere.[36]

Compounds **5m**, **5o**, **5p**, and **5r**, which all showed high A_3 affinity, were also tested in functional assays at human A_{2B} and A_3 ARs by measuring their effects on NECA-mediated cAMP modulation in transfected CHO cells.[37]

Results and Discussion

Table 2 lists the binding affinities for the human A_1, A_{2A}, and A_3 ARs of the new derivatives of 4-amino-6-hydroxy-2-mercaptopyrimidine (**5a**−**s**). The first eight compounds (**5a**−**h**) were prepared according to a factorial design in which the size/lipophilicity of substituents R, R′, and R″ was explored at two levels. For R and R′ the low and high levels correspond to CH_3

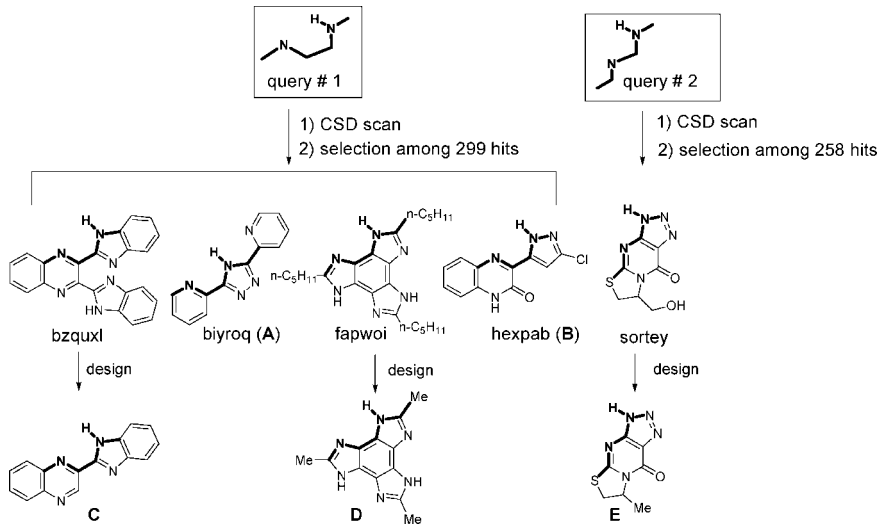


Figure 1. Strategy adopted to identify lead compounds as antagonists at the A_1 and/or A_3 ARs by a 3D search of the CSD.

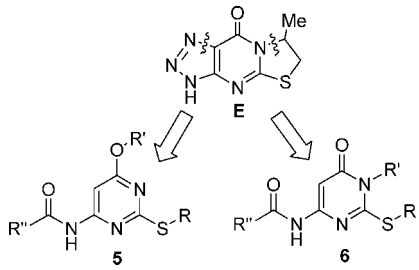
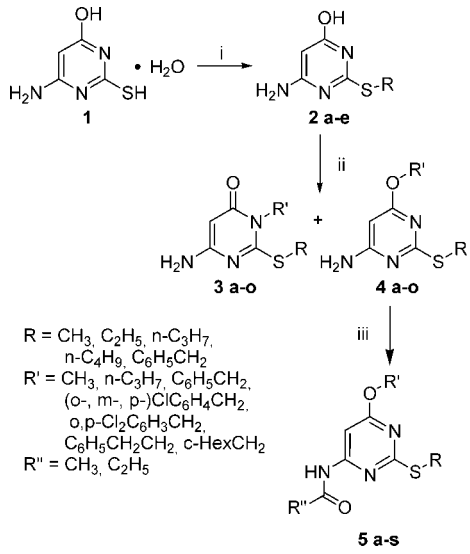


Figure 2. Design of open-chain analogues of triazolopyrimidinone **E** as potential A_3 AR antagonists.

Scheme 1. Synthesis of 4-Amino-6-hydroxy-2-mercaptopyrimidine Derivatives **5a**−**s** (See Table 2 for the Substituents)[a]



[a] Reagents and conditions: (i) methyl iodide or (phenyl)alkyl bromide, 1 M NaOH; (ii) methyl iodide or (substituted phenyl)alkyl bromide, anhydrous DMF, excess K_2CO_3; (iii) acetic or propionic anhydride, catalytic conc H_2SO_4.

and n-C_3H_7, respectively; for R″ the low and high levels correspond to CH_3 and C_2H_5, respectively. The best combination of substituents was found in **5g** (R = R′ = n-C_3H_7, R″ = CH_3), which displayed a K_i value of 26 nM at the A_3 AR, submicromolar affinity at the A_1 AR, and no affinity at the A_{2A} AR.

The binding data of **5a**−**h** suggest that the effects of R, R′, and R″ on A_3 AR affinity are fairly additive and that selectivity for A_3 AR versus A_{2A} AR is conferred by a large R′ and a small R″ (compare **5g** vs **5h**). Moreover, a pairwise comparison of the couples **5c**,**d**, **5e**,**f**, and **5h**,**g** indicates that switching of R″ from CH_3 to C_2H_5 slightly lowers affinity for A_3 AR. Thus, further modifications of R″ were not taken into consideration.

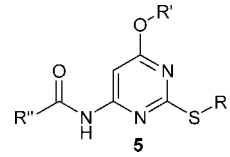
Taking **5g** as the reference structure, further modifications were made to increase potency and selectivity at the A_3 AR. First, we inserted a bulky benzyl group on the S-2 atom, obtaining **5i**, which showed a significant decrease in affinity for the target receptor. Then, by keeping fixed R = n-C_3H_7 and R″ = CH_3, we increased the weight of R′ through substituted benzyl chains (compounds **5j**−**o**). This modification produced a general enhancement in A_3 AR affinity, with **5o** (R′ = 4-$CH_3OC_6H_4CH_2$) being the most active compound (K_i = 1.8 nM). Within this subset, **5m** (R′ = 4-$ClC_6H_4CH_2$) was the best-performing derivative compared with **5g**, showing a K_i of 3.5 nM at the A_3 AR and no affinity for A_1 and A_{2A} ARs. Additional modifications of R′ led to the synthesis of **5p** and **5q**, featuring a $PhCH_2CH_2$, and a c-HexCH_2 chain, respectively. Although these two compounds showed excellent affinity (**5p** was the most active compound of the whole series with a K_i of 1.4 nM) and selectivity at the A_3 AR, they were not significantly better than **5m**.

Finally, to find the optimum size/lipophilicity of R, we took **5m** as the reference structure and replaced the n-C_3H_7 with a C_2H_5 or a n-C_4H_9 to obtain **5r** and **5s**, respectively. Both compounds exhibited nanomolar potency at the A_3 AR (K_i of 2.1 and 8.6 nM, respectively) and a high selectivity over the A_1 and A_{2A} ARs, but they still do not possess the same outstanding affinity/selectivity profile of their homologue, **5m**.

Compounds **5m**, **5o**, **5p**, and **5r** were also tested at the A_{2B} AR by evaluating their inhibitory effects on NECA-mediated cAMP accumulation in CHO cells stably expressing this receptor subtype (Table 3). These derivatives were inactive in this assay, thus indicating a complete lack of affinity for A_{2B} AR.

Given the recent discovery of non-nucleoside AR agonists,[38,39] the antagonistic activities of compounds **5m**, **5o**, **5p**, and **5r** were evaluated in cAMP functional assays at the A_3 AR (Table 3). As expected, all of these compounds displayed full antagonism with potencies comparable to their binding affinities. Furthermore, when tested in the absence of the receptor agonist NECA, they did not show any significant effect on cAMP levels, even following stimulation by forskolin, indicating neutral antagonism (Figure 3).

Table 2. Affinities at Human A_1, A_{2A}, and A_3 ARs of Derivatives of 4-Amino-6-hydroxy-2-mercaptopyrimidines **5a**−**s**

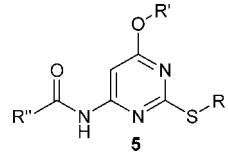


5

				K_i (nM) or % inhibition[a]		
compd	R	R′	R″	hA_1[b]	hA_{2A}[c]	hA_3[d]
5a	CH_3	CH_3	CH_3	30%	29%	49%
5b	CH_3	CH_3	CH_3CH_2	2%	21%	51%
5c	CH_3	$CH_3CH_2CH_2$	CH_3	7%	1121 ± 88	31 ± 2.4
5d	CH_3	$CH_3CH_2CH_2$	CH_3CH_2	19%	398 ± 20	45 ± 0.9
5e	$CH_3CH_2CH_2$	CH_3	CH_3	43%	10%	166 ± 10
5f	$CH_3CH_2CH_2$	CH_3	CH_3CH_2	486 ± 34	4%	276 ± 24
5g	$CH_3CH_2CH_2$	$CH_3CH_2CH_2$	CH_3	5474 ± 275	30%	26 ± 0.8
5h	$CH_3CH_2CH_2$	$CH_3CH_2CH_2$	CH_3CH_2	1462 ± 111	71 ± 3.4	38 ± 0.7
5i	$C_6H_5CH_2$	$CH_3CH_2CH_2$	CH_3	34%	23%	525 ± 21
5j	$CH_3CH_2CH_2$	$C_6H_5CH_2$	CH_3	2445 ± 110	36%	7.5 ± 0.22
5k	$CH_3CH_2CH_2$	2-$ClC_6H_4CH_2$	CH_3	1840 ± 18	4149 ± 400	6.3 ± 0.07
5l	$CH_3CH_2CH_2$	3-$ClC_6H_4CH_2$	CH_3	582 ± 36.3	41%	7.1 ± 0.48
5m	$CH_3CH_2CH_2$	4-$ClC_6H_4CH_2$	CH_3	17%	43%	3.5 ± 0.05
5n	$CH_3CH_2CH_2$	2,4-Cl_2-$C_6H_3CH_2$	CH_3	46%	29%	30 ± 2.7
5o	$CH_3CH_2CH_2$	4-$MeOC_6H_4CH_2$	CH_3	2443 ± 240	47%	1.8 ± 0.15
5p	$CH_3CH_2CH_2$	$C_6H_5CH_2CH_2$	CH_3	2080 ± 205	27%	1.4 ± 0.14
5q	$CH_3CH_2CH_2$	c-Hex-CH_2	CH_3	43%	33%	22 ± 0.4
5r	CH_3CH_2	4-$ClC_6H_4CH_2$	CH_3	4480 ± 440	56%	2.0 ± 0.2
5s	$CH_3(CH_2)_3$	4-$ClC_6H_4CH_2$	CH_3	0%	26%	8.6 ± 0.81

[a] The K_i values are the mean ± SEM of at least three determinations derived from an iterative curve-fitting procedure (Prism program, GraphPad, San Diego, CA). The % inhibition of specific radioligand binding is determined at 10 μM (A_1 and A_{2A} ARs), and 1 μM (A_3 ARs) ligand concentration. [b] Displacement of specific [^{3}H]DPCPX binding in membranes obtained from CHO cells stably expressing hA_1 AR. See K_i values of reference ligands in Table 1. [c] Displacement of specific [^{3}H]NECA binding in membranes obtained from CHO cells stably expressing hA_{2A} AR. See K_i values of reference ligands in Table 1. [d] Displacement of specific [^{125}I]AB-MECA binding in membranes obtained from CHO cells stably expressing hA_3 AR. See K_i values of reference ligands in Table 1.

Table 3. Potency of Compounds **5o**, **5m**, **5p**, and **5r** versus hA_{2B} and hA_3 ARs



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				IC_{50}[a] (nM) of cAMP assays	
compd	R	R′	R″	for hA_{2B} CHO cells	for hA_3 CHO cells
5m	$CH_3CH_2CH_2$	4-$ClC_6H_4CH_2$	CH_3	>10000 (0%)	9.2 ± 0.7
5o	$CH_3CH_2CH_2$	4-$MeOC_6H_4CH_2$	CH_3	>10000 (0%)	2.7 ± 0.2
5p	$CH_3CH_2CH_2$	$C_6H_5CH_2CH_2$	CH_3	>10000 (13%)	1.25 ± 0.09
5r	CH_3CH_2	4-$ClC_6H_4CH_2$	CH_3	>10000 (10%)	1.84 ± 0.1

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

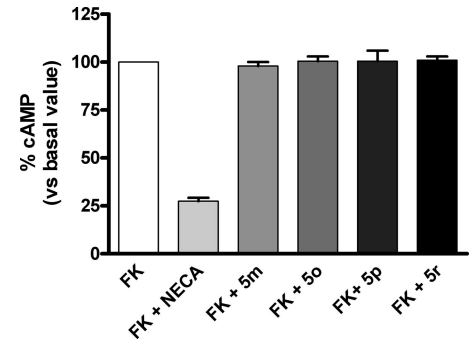

Figure 3. Effect of selected compounds (**5m**, **5o**, **5p**, and **5r**) on forskolin (FK) stimulated cAMP production in A_3 CHO cells. Cells were treated with 1 μM forskolin in the absence or in the presence of 100 nM NECA or 10 μM of each compound. Data are expressed as percentage of cAMP production with respect to FK-stimulated cAMP levels, set to 100%. Each data point represents the mean ± SEM of three different experiments.

The most potent compound at the A_3 AR, **5p**, was docked into our recently published model of this receptor[40] according to a procedure described in the Experimental Section. As shown in Figure 4, **5p** is engaged in several attractive interactions: the ether oxygen receives an H-bond from the N250 (6.55) side chain; the pyrimidine moiety π-stacks with the aromatic rings of H95 (3.37) and W243 (6.48); the phenethyl and the propylthio substituents fill two adjacent hydrophobic pockets delimited by the side chains of F182 (5.43) and W185 (5.46) and of M99 (3.41), L102 (3.44), W185 (5.46), P189 (5.50), and F239 (6.44), respectively. The putative binding mode of **5p** is consistent with the mutagenesis data supporting a key role of H95 (3.37), F182 (5.43), W243 (6.48), and N250 (6.55) in the recognition of several A_3 AR antagonists.[41] This model will probably be exploited to design new A_3 AR antagonists similar to our pyrimidine derivatives. A glance at the Y-like disposition of the three substituents of **5p** within the A_3 AR binding cavity may suggest why compounds of series **6** are devoid of any affinity toward this receptor: their R′ and R substituents are too close, with the result that the former is forced to clash with the binding cleft boundaries.

In summary, we prepared derivatives of 4-amino-6-hydroxy-2-mercaptopyrimidine (**5**) as open chain analogues of the weakly potent A_3 AR antagonist triazolopyrimidinone derivative (**E**). The optimum size/lipophilicity of the R, R′, and R″ substituents born by the new scaffold were sought by a preliminary factorial design followed by a stepwise lead optimization. As a result, we identified **5m** as the ligand displaying the best combination

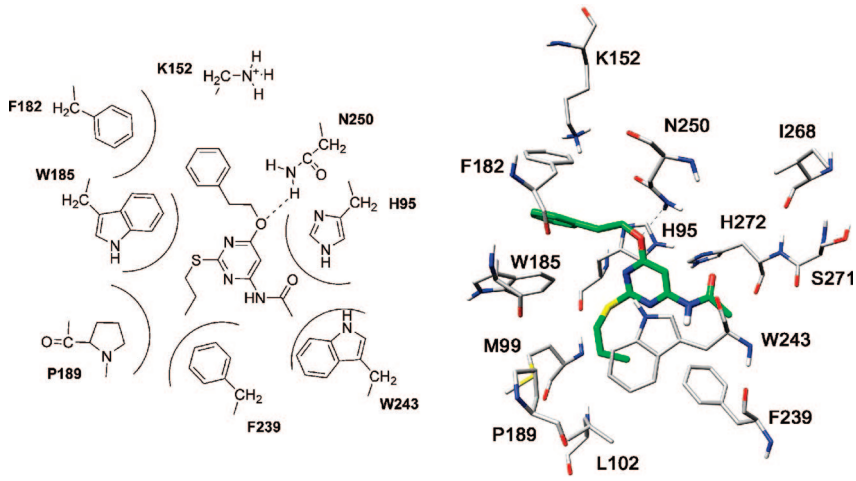

Figure 4. Compound **5p** docked into the A_3 AR model.[40]

of potency at the A_3 AR (K_i = 3.5 nM) and selectivity (K_i > 10 μM at the A_1 and A_{2A} ARs).

Experimental Section

Chemistry. Evaporation was performed in vacuo (rotary evaporator). Analytical TLC was carried out on Merck 0.2 mm precoated silica gel aluminum sheets (60 F-254). Silica gel 60 (230–400 mesh) was used for column flash chromatography. Melting points were determined using a Büchi apparatus B 540 and are uncorrected. Routine nuclear magnetic resonance spectra were recorded on a Varian Mercury 400 spectrometer operating at 400 MHz for the proton and 100 MHz for the carbon in DMSO-d_6 solution. Elemental analyses were performed by our Analytical Laboratory in Pisa, and the results agreed with theoretical values to within ±0.4%.

General Procedure for the Synthesis of 4-Amino-6-hydroxy-2-(phenyl)alkylthiopyrimidines 2a−e. Haloalkane (12.4 mmol) was added dropwise into a solution of 4-amino-6-hydroxy-2-mercaptopyrimidine monohydrate (1g, 6.2 mmol) in 1 M NaOH (12.4 mL). The reaction mixture was kept, under stirring, at 50–55 °C for 4 h and then was left overnight at room temperature. Next, the mixture was neutralized with acetic acid, obtaining a solid that was collected by filtration, washed with petroleum ether, and dried. The solid, identified by ^{1}H and ^{13}C NMR spectra or by comparison with published melting points[42,43] (see Table 1 of Supporting Information for details), was sufficiently pure to be used in the next reaction without further purification.

General Procedure for the Synthesis of 6-Amino-3-(phenyl) alkyl-2-(phenyl)alkylthiopyrimidin-4-ones 3a−p and 4-Amino-6-(phenyl)alkyloxy-2-(phenyl)-alkylthiopyrimidines 4a−p. Haloalkane (10.0 mmol) was added dropwise into a suspension of the opportune compound **2a−e** (10.0 mmol) in anhydrous DMF (30 mL) and K_2CO_3 (30 mmol). The reaction mixture was kept, under stirring, at 80–85 °C for 4 h. After the mixture was cooled, the inorganic material was taken off by filtration and the solvent was removed in vacuo. The solid residue, consisting of a mixture of **3** and **4** isomers, was resolved by flash chromatography. Purification conditions, yields, and physicochemical and spectral data are reported in Tables 2 and 3 of the Supporting Information.

General Procedure for the Synthesis of Compounds 5a−s. A mixture of amine **4a−p** (1.2 mmol), acetic or propionic anhydride (72 mmol), and a catalytic amount of concentrated H_2SO_4 was warmed to 50 °C until disappearance of the substrate (40–120 min), while the progress of the reaction was monitored by TLC. After cooling at room temperature, the mixture was poured into ice/water and extracted with chloroform. The organic phase was washed with a saturated solution of $NaHCO_3$, then with water, and dried on Na_2SO_4. Removal of the solvent under reduced pressure gave the crude products **5a−s**, which were purified by recrystallization from *n*-hexane (Table 4 of Supporting Information).

Biological Methods. Materials. [^{3}H]DPCPX, [^{3}H]NECA, and [^{125}I]AB-MECA were obtained from DuPont-NEN (Boston, MA). Adenosine deaminase (ADA) was from Sigma Chemical Co. (St. Louis, MO). All other reagents were from standard commercial sources and of the highest commercially available grade. CHO cells stably expressing human A_1, A_{2A}, A_{2B}, and A_3 receptors were kindly supplied by Prof. K. N. Klotz, Wurzburg University, Germany.[44]

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

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

Human A_3 Adenosine Receptors. Aliquots of cell membranes (40 μg) were incubated at 25 °C for 90 min in 100 μL of T_3 buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM EDTA, 2 units/mL ADA, pH 7.4) in the presence of 1.4 nM [^{125}I]ABMECA and six different concentrations of the newly synthesized compounds. Nonspecific binding was determined in the presence of 50 μM R-PIA.[36] The dissociation constant (K_d) of [^{125}I]AB-MECA in A_3 CHO cell membranes was 1.4 nM.

Measurement of Cyclic AMP Levels on hA_{2B} and hA_3 CHO Cells. Intracellular cAMP levels were measured using a competitive protein binding method.[37] (Nordstedt) CHO cells expressing recombinant hA_{2B} or hA_3 ARs were harvested by trypsinization. After centrifugation and resuspension in medium, cells (~60 000) were plated in 24-well plates in 0.5 mL of medium. After 48 h, the medium was removed, and the cells were incubated at 37 °C for 15 min with 0.5 mL of DMEM in the presence of Ro20-1724 (20 μM), as phosphodiesterase inhibitor, and ADA (1U/mL). The antagonistic profile of the new compounds toward A_{2B} AR was evaluated assessing their ability to inhibit 100 nM NECA-mediated accumulation of cAMP. The antagonistic profile of the new compounds toward A_3 AR was evaluated by assessing their ability to counteract 100 nM NECA-mediated inhibition of cAMP accumulation stimulated by 1 μM forskolin. Cells were incubated in the reaction medium (15 min at 37 °C) with different compound concentrations (1 nM to 10 μM) and then treated with NECA. In parallel, aliquots of cells were treated with the compound alone (10 μM) in the absence or in the presence of forskolin. The reaction

was terminated by removal of the medium and addition of 0.4 N HCl. After 30 min, lysates were neutralized with 4 N KOH, and the suspension was centrifuged at 800*g* for 5 min. For determination of cAMP production, cAMP binding protein isolated from bovine adrenal glands was incubated with [^{3}H]cAMP (2 nM), 50 μL of cell lysate, or cAMP standard (0–16 pmol) at 4 °C for 150 min in a total volume of 300 μL. Bound radioactivity was separated by rapid filtration through GF/C glass fiber filters and washed twice with 4 mL of 50 mM Tris-HCl, pH 7.4. The radioactivity was measured by liquid scintillation spectrometry.

All compounds were routinely dissolved in DMSO and diluted with assay buffer to the final concentration, where the amount of DMSO never exceeded 2%. Percentage inhibition values of specific radiolabeled ligand binding at 1–10 μM concentration are the mean ± SEM of at least three determinations. For compound IC_{50} determination, at least six different ligand concentrations were used. IC_{50} values, computer-generated using a nonlinear regression formula on a computer program (Graph-Pad, San Diego, CA), were converted to K_i values, based on the K_d values of radioligands in the different tissues and using the Cheng and Prusoff equation.[45] K_i values are the mean ± SEM of at least three determinations.

Computational Methods. The ligands were submitted to 1000 steps of Monte Carlo based conformational search employing the MMFFs force field with a distance-dependent dielectric constant of 1.0.[46] The resulting molecules were then energy-minimized using the conjugated gradient method until a convergence value of 0.05 kcal/(mol·Å) was reached. The obtained structures were docked into a recently published[40] model of the A_3 receptor using the AUTODOCK 3.0 program.[47] The regions of interest used by AUTODOCK were defined by considering N250 as the central residue of a grid of 44, 54, and 50 points in the *x*, *y*, and *z* directions. A grid spacing of 0.375 Å and a distance-dependent function of the dielectric constant were used for the energetic map calculations.

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

The best docking complexes were energy-minimized by applying a constraint of 30 kcal/(mol·Å) on the α carbons of the TMs. Each minimization consisted of 5000 steps with a combined algorithm, namely, the sequential steepest descent and conjugate gradient methods for the first 1000 and the last 4000 steps, respectively.

The resulting geometry-optimized complexes were then subjected to 1.2 ns of molecular dynamics (MD) simulation using AMBER 9.0.[48] MD simulations were carried out at 300 K using the parm94 force field. Chlorine ions were added as counterions to neutralize the system. The time step of the simulations was 2.0 fs, with a cutoff of 12 Å for the nonbonded interaction, and the SHAKE option was employed to keep all bonds involving hydrogen atoms rigid. The MD simulation was carried out by gradually decreasing the constraint on the α carbons of the TMs from 30 to 5 kcal/(mol·Å).

The final structure of each complex was obtained by minimizing the average structure of the last 400 ps of MD using the conjugate gradient method until a convergence of 0.05 kcal/(Å·mol) was reached. During the whole computational simulation, with the exception of the α carbons of the TMs, all the atoms were allowed to move. The root-mean-square deviation of all the heavy atoms between the initial complexes and the final structures was about 2.5 Å.

For specific amino acids, both the sequence number and the numbering system proposed by Ballesteros and Weinstein[49] (in parentheses) were employed. For the latter, the most highly conserved residue in each TM helix (TMH) was assigned a value of 0.50, and this number was preceded by the TMH number. The other residues in the helix were given a locant value relative to this.

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Supporting Information Available: Tables listing yields, chemical−physical properties, and spectral data of compounds **2a**−**e**, **3a**−**o**, **4a**−**o**, and **5a**−**s**, as well as analytical data of **5a**−**s**. This material is available free of charge via the Internet at http://pubs.acs.org.

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