

Original article

Synthesis, biological and modeling studies of 1,3-di-*n*-propyl-2,4-dioxo-6-methyl-8-(substituted) 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purines as adenosine receptor antagonists

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Received 28 February 2005; received in revised form 27 April 2005; accepted 28 April 2005

Available online 14 June 2005

Abstract

A new series of potential adenosine receptor antagonists with a [1,2,4]-triazolo-[3,4-*f*]-purine structure bearing at the 1 and 3 position *n*-propyl groups have been synthesized, and their affinities at the four human adenosine receptor subtypes (A_1 , A_{2A} , A_{2B} and A_3) have been evaluated. In this case the presence of *n*-propyl groups seems to induce potency at the A_{2A} and A_3 adenosine receptor subtypes as opposed to our previously reported series bearing methyl substituents at the 1 and 3 positions. In particular the non-acylated derivative **17** showed affinity at these two receptor subtypes in the micromolar range. Indeed, preliminary molecular modeling investigations according to the experimental binding data indicate a modest steric and electrostatic antagonist-receptor complementarity.

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Keywords: Adenosine receptors; Antagonists; Xanthine; G-protein-coupled receptors; Cycloaddition

1. Introduction

Adenosine is a widely distributed modulator, which regulates many physiological functions [1,2]. The action of adenosine occurs through the stimulation of P1 purinergic receptors, which are subdivided into A_1 , A_{2A} , A_{2B} and A_3 adenosine receptors subtypes [1,2]. All these receptors are coupled to G-proteins and while A_1 and A_3 stimulation induces adenylyl cyclase inhibition and consequently reduced cAMP levels, the activation of A_{2A} and A_{2B} produces the opposite effect [3–6]. All these receptors are widely distributed and they are strongly involved in several patho-physiological functions and/or disorders as extensively reported [1].

For this reason great efforts have been dedicated to the search for potent and selective agonists and antagonists over

the last decades. Such compounds could be used as pharmacological tools for receptor characterization as well as potential drug candidates [7,8].

In the field of antagonists, naturally occurring xanthines like caffeine or theophylline are adenosine receptor ligands with micromolar affinity. These compounds represent an ideal starting point for the development of potent and selective adenosine receptor antagonists.

Several substitutions at the 1-, 3-, and 8-positions led to the discovery of potent and selective antagonists for the different adenosine receptor subtypes. (Fig. 1) In particular, substitution at the 8-position with a cyclopentyl group led to DPCPX **1** [9] which is one of the most potent and selective A_1 adenosine receptor antagonists. The introduction at the same position of styryl (**2**) [10] or bulky aryl substituents (**3**) [11,12] permitted to obtain A_{2A} and A_{2B} adenosine receptor antagonists (**2**, **3**). Recently it has been demonstrated that analogs constrained between 7- and 8- (**4**) [13] or 3- and 4- positions (**5**) [14] possess A_3 affinity and selectivity (Fig. 1).

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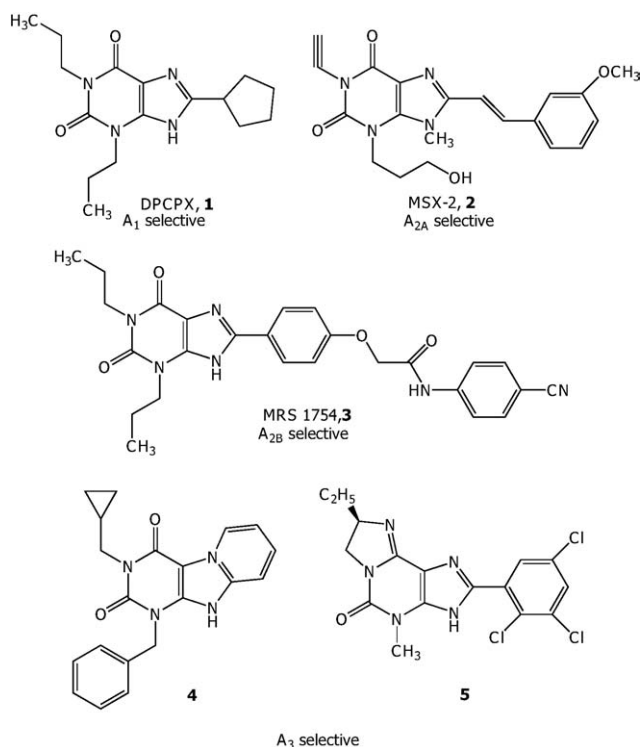


Fig. 1. Structures of xanthine derivatives as potent and selective adenosine receptor antagonists.

Taking into account these experimental observations we have recently reported a new series of 1,3-dimethyl-2,4-dioxo-6-methyl-8-(substituted) 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purines of general formula **6** as potential adenosine receptor antagonists. (Fig. 2) Unfortunately, all the synthesized compounds resulted to be totally inactive at all four adenosine receptor subtypes [15].

A molecular modeling study clearly indicated that a poor steric and electrostatic complementarity for the hA₃ binding site. In particular, this inactivity could be attributed to the

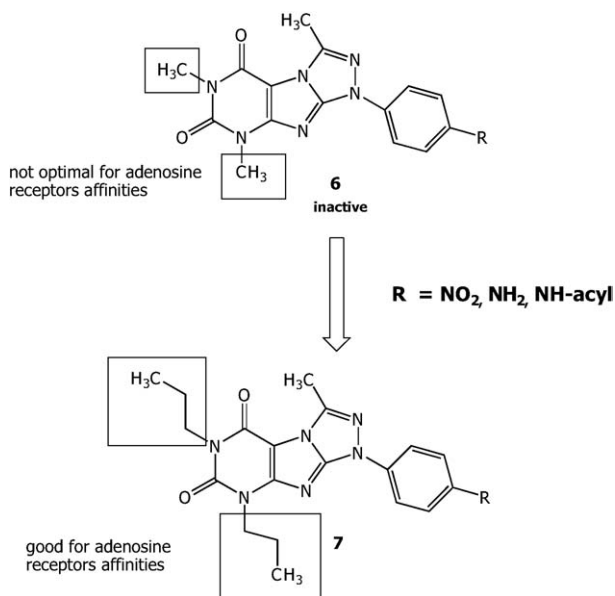


Fig. 2. Rational design of newly synthesized derivatives.

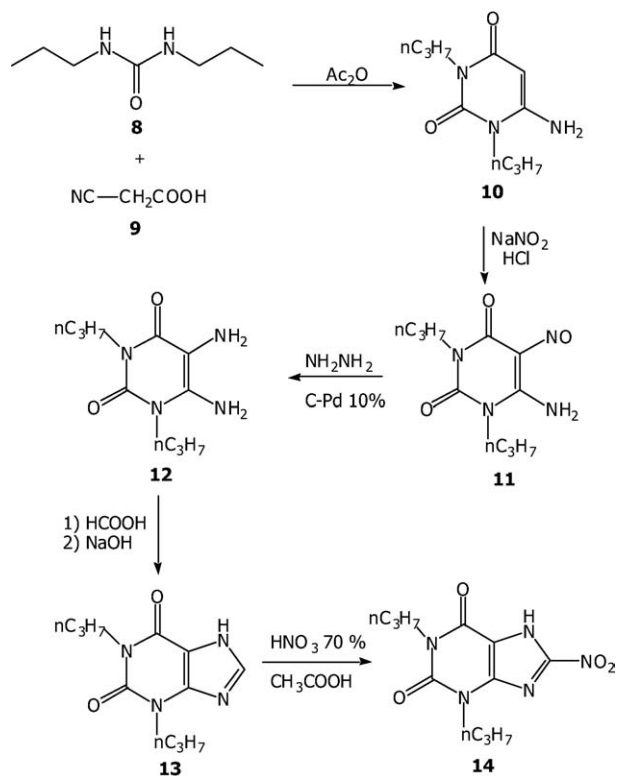
absence of *n*-propyl or bulkier groups at the 1 and 3 positions (see reference compounds **1–5**), which on these bases could be considered an ideal structural requirement for having binding affinity at the adenosine receptor subtypes.

For this reasons we designed a new series of derivatives in which the methyl groups at the 1 and 3 positions were replaced by *n*-propyl groups that are characteristics of adenosine receptor antagonists with the xanthine nucleus (Fig. 2).

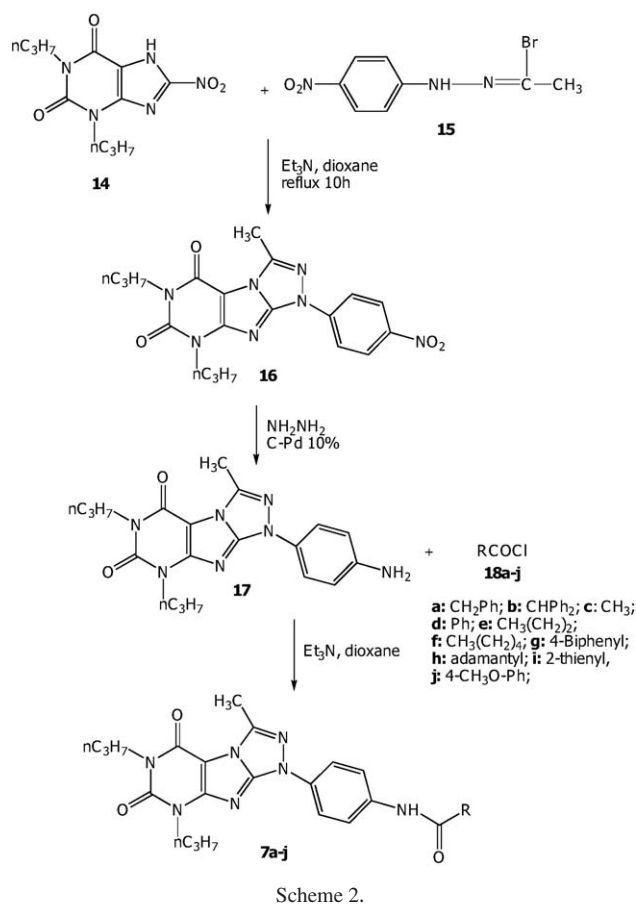
2. Chemistry

The designed compounds (**7a–j**) have been prepared by one-pot cycloaddition reaction between the 8-substituted xanthine with an appropriate hydrazonoyl halide [16,17] as previously reported. The appropriate 8-substituted dipropyl xanthine was prepared following a standard procedure and briefly reported in Scheme 1.

Reacting *N,N'*-dipropyl urea **8** with cyanacetic acid **9** in presence of acetic anhydride afforded the 5-aminouracil **10**, which by treatment with sodium nitrite in acidic conditions gave the nitroso derivative **11**. The latter was then reduced with hydrazine and C–Pd 10% to afford the diaminouracil **12** [18] which was then cyclized to dipropylxanthine **13** by treatment with formic acid and then with NaOH [19,20]. Reaction of **13** with HNO₃ 70% yield to the desired 8-nitrodipropyl xanthine **14** [21–23]. Finally, reacting at reflux (10 h) in presence of triethylamine, compound **14** with the *N*-(2-bromo-4-nitrophenyl) acetohydrazonoyl bromide (**15**) [24] the corresponding cycloadduct **16** was obtained in a good yield



Scheme 1.



[25]. The latter was then reduced with hydrazine in the presence of *C*/Pd 10% [18] to afford the corresponding amino derivative **17**, which was reacted in dioxane at room temperature in the presence of an equivalent amount of triethylamine with an appropriate acyl chloride (**18a–j**) to yield the final compounds **7a–j** (Scheme 2).

3. Results and discussion

In Tables 1 and 2 the receptor binding affinities of the synthesized compounds (**16**, **17**, **7a–j**) are reported. They were determined at the rat A_1 , (from rat cerebral cortex membranes) with [^3H]- N^6 -phenylisopropyladenosine ([^3H]R-PIA), [26] rat A_{2A} (from rat striatal membranes), with [^3H]-2-[4-[(2-carboxyethyl)-phenyl]ethylamino]-5'- N -ethylcarbamoyladenosine, ([^3H]-CGS21680) (A_{2A}) [27].

Binding affinities at human A_1 , A_{2A} , and A_3 receptors expressed in CHO cells were determined using [^3H]-1,3-dipropyl-8-cyclopentyl xanthine ([^3H]DPCPX), (A_1) [28,58], [^{125}I]- (4-(2-[7-amino-2-(2-furyl) [1,2,4]triazolo[2,3-a] [1,3,5]triazin-5-yl-amino]ethyl)-4-iodo-phenol, ([^{125}I]-ZM241385) (A_{2A}) [29] and [^{125}I]- N^6 -(4-amino-3-iodobenzyl)-5'- N -methylcarbamoyladenosine ([^{125}I]-AB-MECA) (A_3) [30] as radioligands.

Instead of evaluating binding affinity at human A_{2B} receptors due to the lack of a suitable radioligand, the potency of

Table 1
Binding affinities at rat A_1 and rat A_{2A} adenosine receptors of synthesized compounds

Compound R	rA_1^a % displ (10 μM) (%)	rA_{2A}^b % displ (10 μM) (%)
16 NO ₂	< 10	< 10
17 NH ₂	< 10	47 \pm 4
7a NHCOCH ₂ Ph	< 10	< 10
7b NHCOCHPh ₂	< 10	< 10
7c NHCOCH ₃	< 10	24 \pm 4
7d NHCOPh	< 10	< 10
7e NHCO(CH ₂) ₂ CH ₃	< 10	< 10
7f NHCO(CH ₂) ₄ CH ₃	< 10	< 10
7g NHCOPh-4-Ph	< 10	< 10
7h NHCO-Adamantyl	< 10	< 10
7i NHCO-Thienyl	< 10	< 10
7j NHCO-4-OCH ₃ -Ph	< 10	< 10

^a Displacement of specific [^3H]R-PIA binding (A_1) in rat brain membranes.

^b Displacement of specific [^3H]CGS 21680 binding (A_{2A}) in rat striatal membranes.

Table 2
Binding affinities (A_1 , A_{2A} , A_3) and potency (A_{2B}) of selected compounds at four human adenosine receptor subtypes

Compound	hA_1^a % displ (10 μM)	$hA_{2A} K_i$ nM or ^b % displ (10 μM)	hA_{2B}^c EC ₅₀ (μM)	$hA_3 K_i$ nM or ^d % displ (10 μM)
17	28	2,050 \pm 520	> 100	1,330 \pm 470
7a	24	25%	> 30	26%
7c	24	64%	> 30	48%
7f	18	42%	> 30	56%
7h	16	23%	> 30	15%
7j	24	24%	> 100	5%

^a Displacement of specific [^3H]DPCPX binding at human A_1 receptors expressed in HEK-293 cells.

^b Displacement of specific [^{125}I]-ZM241385 binding at human A_{2A} receptors expressed. In HEK-293 cells. Data are expressed as $K_i \pm$ S.E.M. in nM ($n = 3–6$).

^c Measurement of adenylyl cyclase activity in CHO cells stably transfected with human recombinant A_{2B} adenosine receptor, expressed as EC₅₀ (μM).

^d Displacement of specific [^{125}I]-I-AB-MECA binding at human A_3 receptors expressed in CHO cells. Data are expressed as $K_i \pm$ S.E.M. in μM ($n = 3–6$).

antagonists was determined in adenylyl cyclase experiments in Chinese hamster ovary (CHO) cells expressing human A_{2B} adenosine receptors [31,32].

In a preliminary screening at the rA_1 and rA_{2A} adenosine receptors, all the synthesized compounds resulted to be totally inactive (Table 1).

Nevertheless, considering the differences between species for the adenosine receptors [31] we tested some selected compounds (**17**, **7a**, **c**, **f**, **h**, **j**) at the four cloned human adenosine receptor subtypes (Table 2).

As clearly indicated while the derivatives acylated at the amino function (**7a**, **c**, **f**, **h**, **j**) resulted to be inactive at all four adenosine receptor subtypes the unsubstituted derivative **17** displayed affinity in the micromolar range at the human A_{2A} and A_3 receptors. This activity could be most probably attributed at the structural similarity between derivative **17** and ref-

erence compounds **2** and **4**. In order to analyze these results in more detail we performed a molecular modeling investigation, at the human A₃ adenosine receptor.

In fact, we have recently revisited the rhodopsin-based model of the human A₃ receptor in its resting state (antagonist-like state), which can be considered a further refinement in building the hypothetical binding site of the A₃ receptor antagonists already proposed [33–37]. Special care had to be given to the second extracellular (EL2) loop, which has been described in bovine rhodopsin to fold back over transmembrane helices, and, therefore, controls the size of the recognition cavity. Similar to rhodopsin, the A₃ receptor model reveals a seven-helix bundle with a central cavity surrounded by helices 1–7 and 5–6. TM4 is not part of the cavity wall and makes contacts only with TM3. Moreover, hairpin (EL2) between TM4 and TM5 controls access of ligands from the periplasmic environment. This hairpin lies between the helices, roughly parallel to the membrane surface. It has contacts with side chains of most of the helices. The most prominent contact is a disulfide bridge to helix 3 (TM3). The ligand recognition seems to occur in the upper region of the TM helical bundle. TM 3, 5, 6 and 7 seem to be crucial for the recognition of both agonists and antagonists. To test the quality of our theoretical model, we decided to mutate several residues of the hA₃ receptor within TMs 3 and 6 and EL2, which have been predicted by previous molecular modeling to be involved in the ligand recognition, including His95, Trp243, Leu244, Ser247, Asn250, and Lys152. The N250A mutant receptor lost the ability to bind both radiolabeled agonist and antagonist [38–40]. We carried out molecular docking simulations to evaluate the complementarity between the TM cleft and the new series of the hA₃ receptor antagonists. According to the experimental binding data a modest steric and electrostatic complementarity has been observed for all the new synthesized triazolo-purines. One of the best docked conformation of derivative **17** is shown in Fig. 3.

Indeed, the most crucial interactions detected between the receptor cavity and the antagonist structures are missing for

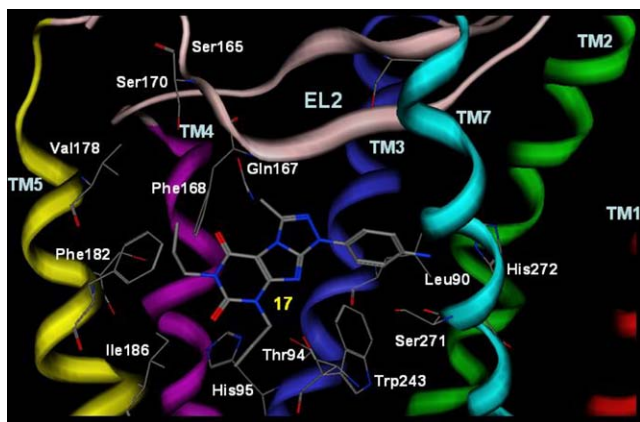


Fig. 3. Triazolo-purine derivatives binding site in the human A₃ receptor. Derivative **17** docked into the ligand binding crevice of the human A₃ receptor viewed from the membrane side facing TM helices 5 and 6. TM helical 6 is not shown to facilitate the inspection of the receptor cavity. Hydrogen atoms are not displayed.

other compounds or their intensities are questionable, in particular with the key residues His95 (TM3), Ser247 (TM6), Asn250 (TM6), and Lys152 (EL2). An intense modeling evaluation is running to improve receptor complementarity using this interesting triazolo-purine scaffold. In fact, identification of microscopic complementarity between residues of the putative receptor binding site and the docked high-affinity ligands, i.e. energetically stabilizing elements in ligand recognition, has an useful tool in the design process of new potent and selective antagonists.

4. Experimental section

4.1. Chemistry

General Remarks: Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F₂₅₄ Merck plates) and products visualized with iodine or aqueous potassium permanganate. Infrared spectra (IR) were measured on a Jasco FT-IR instrument using KBr powder (DRIFT system). ¹H NMR spectra were determined in DMSO-d₆ solutions, unless otherwise noted, at 200 MHz, with a Varian Gemini 200 spectrometer, peaks positions are given in parts per million (δ) downfield, and *J* values are given in Hz. Melting points were determined on a Buchi-Tottoli instrument and are uncorrected. Chromatographies were performed with Merck 60–200 mesh silica gel. All products reported showed IR and ¹H NMR spectra in agreement with the assigned structures. Organic solutions were dried over anhydrous magnesium sulfate. Elemental analyses (C, H, N) were performed by the microanalytical laboratory of Dipartimento di Chimica, University of Trieste, and were within ±0.4% of the theoretical values.

4.1.1. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-(4-nitrophenyl) 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**16**)

To a mixture of hydrazonoyl bromide **15** (0.46 g, 1.8 mmol) and 8-nitrodipropylxanthine **14** (0.5 g, 1.8 mmol) in dry dioxane (10 ml), was added triethylamine (0.26 ml, 1.8 mmol). The mixture was then refluxed for 10 h. The excess of solvent was removed under reduced pressure, the crude solid residue was then filtered, washed with ethanol and crystallized from ethyl acetate–light petroleum to give final product **16** as a brown solid in a good yield (0.68 g, 92%). MW 411.42; m.p.: 246 °C (ethyl acetate–light petroleum); IR-DRIFT (KBr): cm⁻¹ 1684, 1673, 1610, 1545, 1350; ¹H NMR (DMSO): δ: 1.01 (t, 6H, *J* = 6.7), 1.71–1.78 (m, 4H), 2.95 (s, 3H), 3.96 (t, 2H, *J* = 6.7), 4.12 (t, 2H, *J* = 6.7), 8.26 (d, 2H, *J* = 9), 8.33 (d, 2H, *J* = 9); Anal. (C₁₉H₂₁N₇O₄): Calc.: C 55.47, H 5.14, N 23.83; Found: C 55.08; H 5.09, N 23.57.

4.1.2. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-(4-aminophenyl) 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**17**)

To a solution of nitroderivative **16** (1 g, 2.4 mmol) in dioxane (20 ml), hydrazine hydrate (0.52 ml, 16.37 mmol) and

C–Pd 10% (0.08 mg) were added. The mixture was then stirred at reflux for 20 min. The mixture was then filtered through a celite pan and the solvent removed under reduced pressure. The crude solid was crystallized from ethyl acetate-light petroleum to give the amino derivative **17** as a white solid in excellent yield (0.90 g, 99%). MW 381.44; m.p.: 225 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3353, 1685, 1675, 1611; ^1H NMR (DMSO): δ : 1.02 (t, 6H, $J = 6.7$), 1.73–1.81 (m, 4H), 2.90 (s, 3H), 3.85 (bs, 2H), 3.95 (t, 2H, $J = 6.7$), 4.14 (t, 2H, $J = 6.7$), 6.80 (d, 2H, $J = 9$), 7.81 (d, 2H, $J = 9$); Anal. ($\text{C}_{19}\text{H}_{23}\text{N}_7\text{O}_2$): Calc.: C 59.83, H 6.08, N 25.70; Found: C 56.03; H 6.25, N 25.43.

4.1.3. General procedures for the preparation of 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-(substituted) 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine. (**7a–j**)

To a solution of amino compounds **17** (106 mg, 0.28 mmol) in dry dioxane (10 ml) triethylamine (48.5 μl , 0.34 mmol) and appropriate acyl chloride **18a–j** (1.2 eq) were added. Then the mixture was stirred at room temperature for 6 h. The triethylamine hydrochloride was removed by filtration and the solvent concentrated under reduced pressure. The crude residue was crystallized from an appropriate mixture of solvents to afford final compounds **7a–j** in a good yield as solids.

4.1.4. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-((phenylacetyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7a**)

Yield: 80%; white solid; MW 499.57; m.p.: 202 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3345, 1698, 1683, 1675, 1600; ^1H NMR (DMSO): δ : 1.04 (t, 6H, $J = 6.7$), 1.70–1.80 (m, 4H), 2.91 (s, 3H), 3.97 (t, 2H, $J = 6.7$), 4.15 (t, 2H, $J = 6.7$), 5.10 (s, 2H), 7.28–7.45 (m, 5H), 7.62 (d, 2H, $J = 9$), 7.88 (d, 2H, $J = 9$), 9.20 (bs, 1H); Anal. ($\text{C}_{27}\text{H}_{29}\text{N}_7\text{O}_3$): Calc.: C 64.92, H 5.85, N 19.63; Found: C 65.12; H 5.99, N 19.78.

4.1.5. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-((diphenylacetyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7b**)

Yield: 80%; white solid; MW 575.67; m.p.: 230 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3320, 1690, 1670, 1665, 1610; ^1H NMR (DMSO): δ : 1.04 (t, 6H, $J = 1.70$ –1.80 (m, 4H), 2.85 (s, 3H), 3.96 (t, 2H, $J = 6.7$), 4.20 (t, 2H, $J = 6.7$), 4.87 (s, 1H), 7.17–7.35 (m, 10H), 7.75 (d, 2H, $J = 9$), 7.83 (d, 2H, $J = 9$), 10.35 (bs, 1H); Anal. ($\text{C}_{33}\text{H}_{33}\text{N}_7\text{O}_3$): Calc.: C 68.85, H 5.78, N 17.03; Found: C 68.60; H 5.71, N 16.91.

4.1.6. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-(acetylamino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7c**)

Yield: 95%; white solid; MW 423.47; m.p.: 263–265 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3335, 1690, 1675, 1668, 1605; ^1H NMR (DMSO): δ : 1.01 (t, 6H, $J = 6.7$), 1.71–1.76 (m, 4H), 2.05 (s, 3H), 2.77 (s, 3H), 3.92 (t, 2H, $J = 6.7$), 4.23 (t, 2H, $J = 6.7$), 7.65 (d, 2H, $J = 9$), 7.84

(d, 2H, $J = 9$), 10.10 (bs, 1H); Anal. ($\text{C}_{21}\text{H}_{25}\text{N}_7\text{O}_3$): Calc.: C 59.56, H 5.95, N 23.15; Found: C 59.81; H 6.01, N 23.01.

4.1.7. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-(benzoylamino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7d**)

Yield: 77%; white solid; MW 473.53; m.p.: 215–218 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3325, 1690, 1672, 1665, 1605; ^1H NMR (DMSO): δ : 1.04 (t, 6H, $J = 6.7$), 1.73–1.80 (m, 4H), 2.77 (s, 3H), 3.90 (t, 2H, $J = 6.7$), 4.21 (t, 2H, $J = 6.7$), 7.45–7.57 (m, 5H); 7.88 (s, 4H), 10.31 (bs, 1H); Anal. ($\text{C}_{25}\text{H}_{27}\text{N}_7\text{O}_3$): Calc.: C 63.41, H 5.75, N 20.71; Found: C 63.18; H 5.83, N 20.48.

4.1.8. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-((4-methoxyphenylcarbonyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7e**)

Yield: 93%; pale yellow solid; MW 515.57; m.p.: 265 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3323, 1688, 1675, 1667, 1605; ^1H NMR (DMSO): δ : 1.02 (t, 6H, $J = 6.7$), 1.73–1.81 (m, 4H), 2.77 (s, 3H), 3.30 (s, 3H), 3.88 (t, 2H, $J = 6.7$), 4.20 (t, 2H, $J = 6.7$), 7.02 (d, 2H, $J = 9$); 7.89 (s, 4H), 7.98 (d, 2H, $J = 9$), 10.23 (bs, 1H); Anal. ($\text{C}_{27}\text{H}_{29}\text{N}_7\text{O}_4$): Calc.: C 62.90, H 5.67, N 19.02; Found: C 63.08; H 5.70, N 18.89.

4.1.9. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-((butanoylamino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7f**)

Yield: 77%; white solid; MW 451.53; m.p.: 205 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3325, 1694, 1673, 1665, 1610; ^1H NMR (DMSO): δ : 0.97 (t, 3H, $J = 6.7$), 1.02 (t, 6H, $J = 6.7$), 1.22–1.27 (m, 2H), 1.73–1.81 (m, 4H), 2.15 (t, 2H, $J = 6.7$), 2.78 (s, 3H), 3.85 (t, 2H, $J = 6.7$), 4.11 (t, 2H, $J = 6.7$), 7.70 (d, 2H, $J = 9$); 7.91 (d, 2H, $J = 9$), 10.13 (bs, 1H); Anal. ($\text{C}_{23}\text{H}_{29}\text{N}_7\text{O}_3$): Calc.: C 61.18, H 6.47, N 21.71; Found: C 60.93; H 6.53, N 21.52.

4.1.10. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-((esanoyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7g**)

Yield: 76%; white solid; MW 479.58; m.p.: 195 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3323, 1692, 1673, 1663, 1610; ^1H NMR (DMSO): δ : 0.91 (t, 3H, $J = 6.7$), 1.02 (t, 6H, $J = 6.7$), 1.21–1.30 (m, 4H), 1.47–1.59 (m, 2H), 1.47–1.59 (m, 2H), 1.73–1.81 (m, 4H), 2.29 (t, 2H, $J = 6.7$), 2.81 (s, 3H), 3.89 (t, 2H, $J = 6.7$), 4.17 (t, 2H, $J = 6.7$), 7.72 (d, 2H, $J = 9$); 7.97 (d, 2H, $J = 9$), 10.01 (bs, 1H); Anal. ($\text{C}_{25}\text{H}_{33}\text{N}_7\text{O}_3$): Calc.: C 62.61, H 6.94, N 20.44; Found: C 62.33; H 7.00, N 20.63.

4.1.11. 1,3-Di-*n*-propyl-2,4-dioxo-6-methyl-8-[4-((4-biphenyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-*f*]-purine (**7h**)

Yield: 92%; white solid; MW 561.64; m.p.: 229 °C (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3330, 1693,

1677, 1665, 1605; ^1H NMR (DMSO): δ : 1.03 (t, 6H, $J = 6.7$), 1.70–1.83 (m, 4H), 2.75 (s, 3H), 3.90 (t, 2H, $J = 6.7$), 4.22 (t, 2H, $J = 6.7$), 7.20–7.35 (m, 4H), 7.47–7.90 (m, 9H); 10.32 (bs, 1H); Anal. ($\text{C}_{32}\text{H}_{31}\text{N}_7\text{O}_3$): Calc.: C 68.43, H 5.53, N 17.46; Found: C 68.17; H 5.60, N 17.21.

4.1.12. *1,3-Di-n-propyl-2,4-dioxo-6-methyl-8-[4-((adamantyl-carbonyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-f]-purine (7i)*

Yield: 80%; pale yellow solid; MW 544.68; m.p.: 164 °C (ethyl acetate/light petroleum); IR-DRIFT (KBr): cm^{-1} 3335, 1693, 1675, 1662, 1600; ^1H NMR (DMSO): δ : 1.03 (t, 6H $J = 6.7$), 1.53–2.03 (m, 10H), 2.06–2.18 (m, 6H), 2.57–2.88 (m, 3H), 2.82 (s, 3H), 3.04–3.15 (m, 1H), 3.93 (t, 2H, $J = 6.7$), 4.20 (t, 2H, $J = 6.7$), 7.90 (s, 4H), 9.45 (bs, 1H); Anal. ($\text{C}_{30}\text{H}_{38}\text{N}_7\text{O}_3$): Calc.: C 66.15, H 7.03, N 18.00; Found: C 65.84; H 6.97, N 17.82.

4.1.13. *1,3-Di-n-propyl-2,4-dioxo-6-methyl-8-[4-((2-thienyl-carbonyl)amino)phenyl] 1,2,3,4-tetrahydro [1,2,4]-triazolo [3,4-f]-purine (7j)*

Yield: 73%; pale yellow solid; MW 491.57; m.p.: 250–253 °C (dec.) (ethyl acetate-light petroleum); IR-DRIFT (KBr): cm^{-1} 3335, 1693, 1670, 1661, 1620, 1600; ^1H NMR (DMSO): δ : 1.03 (t, 6H, $J = 6.7$), 1.70–1.83 (m, 4H), 2.78 (s, 3H), 3.92 (t, 2H, $J = 6.7$), 4.18 (t, 2H, $J = 6.7$), 7.20–7.25 (m, 1H), 7.73–7.81 (m, 1H), 7.87 (s, 4H), 7.91–8.02 (m, 1H), 10.33 (bs, 1H); Anal. ($\text{C}_{24}\text{H}_{25}\text{N}_7\text{O}_3\text{S}$): Calc.: C 58.64, H 5.13, N 19.95, S 6.52; Found: C 58.38; H 5.04, N 20.04, S 6.47.

4.2. Biology

4.2.1. Binding at rA_1 and rA_{2A} adenosine receptors assays

Procedures for preparation of rat brain membranes were reported previously [41,42]. For binding experiments, membrane membranes were frozen and stored at -20 °C for ≤ 2 months. Adenosine deaminase (ADA) was from Boehringer Mannheim (Indianapolis, IN). [^3H]R-PIA was from Amersham (Arlington Heights, IL), and [^3H]CGS 21680 was from DuPont NEN (Boston, MA).

Binding of [^3H]R-PIA to A_1 receptors from rat cortical membranes and of [^3H]CGS 21680 to A_{2A} receptors for rat striatal membranes was performed as described previously [43]. ADA (2 units ml^{-1}) was present during the preparation of brain membranes. Additional deaminase was not added during incubation with the radioligand.

4.2.2. Radioligand binding to human A_1 , A_{2A} and A_3 adenosine receptors

[^3H]DPCPX, [^{125}I]-ZM241385, and ([^{125}I]-AB-MECA) were utilized in radioligand binding assays to membranes prepared from CHO cells expressing recombinant human A_1 , A_{2A} , and A_3 ARs, respectively, as previously described [28,30]. ADA (3 units ml^{-1}) was present during the prepara-

tion of the membranes, in a preincubation of 30 min at 30 °C, and during the incubation with the radioligands. All nonradioactive compounds were initially dissolved in DMSO and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 2%. 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 with 3 ml of buffer each. At least six different concentrations of competitor, spanning three orders of magnitude adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, calculated with the nonlinear regression method implemented in Graph-Pad (Prism, San Diego, CA), were converted to K_i values as described [44]. Hill coefficients of the tested compounds were in the range of 0.8–1.1.

4.2.3. Adenylyl cyclase activity

Due to the lack of a suitable radioligand the affinity of antagonists and the relative potency of agonists at A_{2B} adenosine receptors was determined in adenylyl cyclase experiments. The procedure was carried out as described previously [31,32] with minor modifications. Membranes were incubated with about 150,000 cpm of [α - ^{32}P]ATP for 20 min in the incubation mixture as described [31,32] without EGTA and NaCl. For agonists the EC_{50} -values for the stimulation of adenylyl cyclase were calculated with the Hill equation. Hill coefficients in all experiments were near unity. IC_{50} -values for concentration-dependent inhibition of NECA-stimulated adenylyl cyclase caused by antagonists was calculated accordingly. Dissociation constants (K_i) for antagonist were then calculated with the Cheng and Prusoff equation [45].

4.2.4. Computational methodologies

All molecular modeling studies were carried out on a six CPU (PIV 2.0–3.0 GHz) linux cluster running under open-Mosix architecture [46].

Homology modeling, energy calculation, and docking studies have been done using Molecular Operating Environment (MOE, version 2003.03) suite [47].

The ground state geometry of all, charged and uncharged, docked structures were fully optimized without geometry constraints using RHF/3-21G(*) ab initio calculations. Vibrational frequency analysis was used to characterize the minima stationary points (zero imaginary frequencies). The software package Spartan O2 was utilized for all quantum mechanical calculations [48].

4.2.4.1. *Homology model of the hA_3 AR.* Based on the assumption that GPCRs share similar TM boundaries and overall topology, a homology model of the hA_3 receptor was constructed. First, the amino acid sequences of TM helices of the A_3 receptor were aligned with those of bovine rhodopsin, guided by the highly conserved amino acid residues, including the DRY motif (D3.49, R3.50, and Y3.51) and three Pro residues (P4.60, P6.50, and P7.50) in the TM segments of GPCRs. The same boundaries were applied for the TM heli-

ces of the A₃ receptor as were identified from the X-ray crystal structure for the corresponding sequences of bovine rhodopsin, the C_a coordinates of which were used to construct the seven TM helices for the human A₃ receptor. The loop domains of the hA₃ receptor were constructed by the loop search method implemented in MOE. In particular, loops are modeled first, in random order. For each loop, a contact energy function analyzes the list of candidates collected in the segment searching stage, taking into account all atoms already modeled and any atoms specified by the user as belonging to the model environment. These energies are then used to make a Boltzmann-weighted choice from the candidates, the coordinates of which are then copied to the model. Any missing sidechain atoms are modeled using the same procedure. Sidechains belonging to residues whose backbone coordinates were copied from a template is modeled first, followed by side chains of modeled loops. Outgaps and their sidechains are modeled last. Special caution had to be given to the second extracellular (E2) loop, which has been described in bovine rhodopsin to fold back over transmembrane helices, and, therefore, limits the size of the active site. Hence, amino acids of this loop could be involved in direct interactions with the ligands. A driving force to this peculiar fold of the E2 loop might be the presence of a disulfide bridge between cysteines in TM3 and E2. Since this covalent link is conserved in all receptors modeled in the current study, the E2 loop was modeled using a rhodopsin-like constrained geometry around the E2-TM3 disulfide bridge. After the heavy atoms were modeled, all hydrogen atoms were added, and the protein coordinates were then minimized with MOE using the AMBER94 force field [49]. The minimizations were carried out by 1000 steps of steepest descent followed by conjugate gradient minimization until the *rms* gradient of the potential energy was less than 0.1 kcal mol⁻¹ Å.

4.2.4.2. Molecular docking of the hA₃ AR antagonists. All antagonist structures were docked into the hypothetical TMs binding site by using the DOCK docking program, part of the MOE suite. Searching is conducted within a user-specified 3D docking box, using Tabù Search protocol [50] and MMFF94 force field [51–57]. MOE-Dock performs a user-specified number of independent docking runs (50 in our specific case) and writes the resulting conformations and their energies to a molecular database file. The resulting docked complexes were subjected to MMFF94 energy minimization until the *rms* of conjugate gradient was <0.1 kcal mol⁻¹ Å⁻¹. Charges for the ligands were imported from the Spartan output files.

The interaction energy values were calculated as follows: $\Delta E_{\text{binding}} = E_{\text{complex}} - (E_{\text{ligand}} + E_{\text{receptor}})$. 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.

Acknowledgments

We wish to thank the Regione Friuli Venezia Giulia (Fondo 2000) and the University of Trieste (fondo 60%) for financial support. The molecular modeling work coordinated by S. Moro has been carried out with financial support from Associazione Italiana per la Ricerca sul Cancro (AIRC), Milan, and the Italian Ministry for University and Research (MIUR), Rome, Italy. S. Moro is also grateful to Chemical Computing Group for their scientific and technical partnership. We wish also to thank Dr. Ken A. Jacobson and Dr. Zhan-Guo Gao (Bethesda) for binding data and helpful discussion.

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