CHEMISTRY

General: Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated F_{254} Merck plates) and products visualized with iodine or aqueous potassium permanganate. Infrared spectra (IR) were measured on a Perkin Elmer 257 instrument. ^{1}H NMR were determined in D_2O or DMSO-d_6 solutions with a Bruker AC 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. Electrospray mass spectra were recorded on a Perlin-Elmer PE SCIEX API 1 spectrometer, and compounds were dissolved in methanol. Chromatography was performed with 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.

5-[[(4-pyridyl)amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine (3) Amino compound (**8**) (0.5 g, 1.95 mmmol) was dissolved in freshly distilled THF (15 mL) and the freshly prepared 4-pyridyl-isocyanate[1-3] (**5**) (1.17 g, 9.75 mmol, 5 eq) was added. The mixture was refluxed under argon for 18 hours. Then the solvent was removed under reduced pressure and the residue was purified by flash chromatography (EtOAc-Methanol 9:1 gradient EtOAc-Methanol 7:3) on silica gel pretreated with ammonia gas, to afford the desired compounds **3** as a pale yellow solid 365 mg, 50%). mp 269-272 °C (dec) (Methanol-diethyl ether); IR (KBr): 3240-2950, 1668, 1625, 1610, 1510 , 1450 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ: 4.09 (s, 3H); 6.72 (dd, 1H, J = 2, J = 4); 7.22 (d, 1H, J = 4); 7.50 (d, 2H, J = 9); 7.93 (d, 1H, J =2); 8.43 (d, 2H, J =9); 8.70 (s, 1H); 10.18 (bs, 1H); 10.91 (bs, 1H). ES-MS: (MH^+) 376.5 Anal. ($C_{17}H_{13}N_9O_2$) C, H, N.

5-[[(4-pyridyl)amino]carbonyl]amino-8-methyl-2-(2-furyl)-pyrazolo[4,3-e]1,2,4-triazolo[1,5-c]pyrimidine hydrochloride (4) Compound **3** (50 mg, 0.13 mmol) was dissolved in methanol (1 mL) and a saturated solution of methanol with HCl gas (2 mL) was added at 0 °C. The reaction was stirred at the same temperature for 30

min, then the solvent was removed under reduced pressure to afford the correspondent salt as a white solid in quantitative yield. mp 223-225 °C (dec) (Methanol-diethyl ether); IR (KBr): 3420-2910, 1667, 1620, 1610, 1510, 1450 cm^{-1}; ^1H NMR (D_2O) δ: 3.64 (s, 3H); 6.20 (dd, 1H, J = 2, J = 4); 6.62 (d, 1H, J = 4); 7.25 (d, 2H, J = 9); 7.64 (d, 1H, J =2); 7.93 (d, 2H, J =9); 8.27 (s, 1H). Anal. ($C_{17}H_{14}N_9O_2Cl$) C, H, N.

Determination of Rm values by C_{18} RP-HPTLC

The HPTLC determinations were carried out on Whatman KC18F plates as previously described.[4] Solvent mixtures of methanol-water buffer at pH 7 were used as mobile phase. The methanol concentration ranged from 60% to 90%. The solutes were detected under UV 254 nm light.

BIOLOGY

CHO Membranes preparation

The expression of the human A_1, A_{2A} and A_3 receptors in CHO cells has been previously described.[5] The cells were grown adherently, and maintained in Dulbecco's modified Eagle's medium with nutrient mixture F12 without nucleosides, containing 10% fetal calf serum, penicillin (100 U/mL), streptomycin (100 μg/mL), L-glutamine 2 mM and geneticin (0.2 mg/mL) at 37 °C in 5% CO_2/95% air. Cells were split two or three times weekly and then the culture medium was removed for membrane preparations. The cells were washed with phosphate-buffered saline and scraped off flasks in ice cold hypotonic buffer (5 mM Tris HCl, 2 mM EDTA, pH 7.4). The cell suspension was homogenized with Polytron and the homogenate was centrifuged for 30 min at 48,000 g. The membrane pellet was resuspended in 50 mM Tris HCl buffer at pH 7.4 for A_1 adenosine receptors, in 50 mM Tris HCl, 10 mM $MgCl_2$ at pH 7.4 for A_{2A} adenosine receptors, in 50 mM Tris HCl, 10 mM $MgCl_2$, 1 mM EDTA at pH 7.4 for A_3 adenosine receptors and were utilized for binding and adenylate cyclase assays.

Human cloned A_1, A_{2A}, A_{2B} and A_3 adenosine receptor binding assay

Binding of [^{3}H]-DPCPX to CHO cells transfected with the human recombinant A_1 adenosine receptor was performed as previously described.[6]

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 6-8 different concentrations of examined compounds. Non-specific binding was determined in the presence of 10 μM of CHA and this was always ≤10% of the total binding.

Binding of [^{3}H]-ZM241385 to CHO cells transfected with the human recombinant A_{2A} adenosine receptors (50 μg of protein/assay) was performed according to Ongini et al.[7] In competition studies, at least 6-8 different concentrations of compounds were used and non-specific binding was determined in the presence of 50 μM NECA for an incubation time of 60 min at 25 °C.

Binding of [^{3}H]-DPCPX to HEK-293 cells (Receptor Biology Inc., Beltsville, MD, USA) transfected with the human recombinant A_{2B} adenosine receptors, was performed as already described.[8] In particular, assays were carried out for 60 min at 25 °C in 0.1 mL of 50 mM Tris HCl Buffer, 10 mM $MgCl_2$, 1 mM EDTA, 0.1 mM benzamidine pH 7.4, 2 IU/ml adenosine deaminase containing 40 nM [^{3}H]-DPCPX, diluted membranes (20 μg of protein/assay) and at least 6-8 different concentration of tested compounds. Non specific binding was determined in the presence of 100 μM of NECA and was always ≤30 % of the total binding.

Binding of [^{3}H]MRE3008-F20 to CHO cells transfected with the human recombinant A_3 adenosine receptors was performed according to Varani et al.[8] Competition experiments were carried out in duplicate in a finale volume of 250 μL in test tubes containing 1 nM [^{3}H] MRE3008-F20, 50 mM Tris HCl buffer, 10 mM $MgCl_2$, pH 7.4 and 100 μL of diluted membranes (50 μg protein/assay) and at least 6-8 different concentrations of examined ligands. Incubation time was 120 min at 4 °C, according to the results of previos time-course experiments.[8] Non-specific binding was defined as binding in the presence of 1 μM of MRE3008-F20 and was about 25% of total binding. 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[9] with bovine albumin as reference standard.

Rat A_1, A_{2A}, and A_3 adenosine receptor binding assay

Fetal bovine serum (FBS) and penicillin/streptomycin were from Gibco BRL (Gaithersburg, MD). [^{125}I]I-AB-MECA and [^{3}H]R-PIA were from Amersham (Arlington Heights, IL), and [^{3}H]CGS 21680 was from Perkin-Elmer (Boston, MA). Adenosine deaminase (ADA) was from Roche Molecular Biochemicals (Indianapolis, IN). Adenosine derivatives were purchased from Sigma-RBI (St. Louis, MO). All other materials were from standard local sources and of the highest grade commercially available.

RBL-2H3 cells were grown in Dulbecco's modified Eagle's medium containing 10 % fetal bovine serum and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively) at 37 °C in a 5 % CO_2 atmosphere, and membrane homogenates were prepared as reported.[10,11] Composition of the lysis buffer was: 10 mM Tris/ 5mM EDTA, pH 7.4 at 5°C. Binding of [^{125}I]N^6-(4-amino-3-iodobenzyl)adenosine-5'-(N-methyluronamide) ([^{125}I]AB-MECA) to the RBL-2H3 cell membranes was performed as described.[10,12] Assays were performed in a buffer containing 50 mM Tris, 10 mM $MgCl_2$, and 1 mM EDTA, pH 8.26 at 5 °C. The glass tubes contained 100 μL of the membrane suspension, 50 μL of [^{125}I]AB-MECA (final concentration 0.3 nM), and 50 μL of a solution of the proposed antagonist. The antagonist was initially dissolved in DMSO at a concentration of 5 mM and diluted with buffer to the final concentration, where the amount of DMSO never exceeded 1%. Duplicate incubations were carried out for 1 h at 37 °C, and 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 buffer each. The radioactivity on the filters was determined with a Beckman 5500B γ-counter. Nonspecific binding was determined in the presence of 200 μM N-ethylcarboxamidoadenosine (NECA).

Binding of [^{3}H]*R*-N^6-phenylisopropyladenosine ([^{3}H]*R*-PIA) to A_1 receptors from rat cerebral cortex membranes and of [^{3}H]CGS 21680 to A_{2a} receptors from rat striatal membranes was performed essentially as described previously.[13,14] Adenosine deaminase (3 U/mL) was present during the preparation of the brain membranes but not added again during the incubation with the radioligands. Duplicate incubations were carried out for 90 min and were terminated by rapid filtration over Whatman GF/B filters, using a Brandell cell harvester as described above. The binding to A_1 receptors was carried out at 37 °C, and the binding to A_{2A} receptors was carried out at 25 °C. Nonspecific binding was determined in the presence of 2-chloroadenosine (100 μM for A_1 and 400 μM for A_{2A}).

In all binding assays at least six different concentrations of antagonist, spanning 3 orders of magnitude as adjusted appropriately for the IC_{50} of each compound, were used. IC_{50} values, computer-generated using the nonlinear regression method implemented in the InPlot program (Graph-PAD, San Diego, CA), were converted to apparent K_i values using K_d values of 1.0 nM, 14 nM, and 3.6 nM for [^{3}H]*R*-PIA, [^{3}H]CGS 21680, and [^{125}I]AB-MECA binding, respectively, applying the Cheng-Prusoff equation.[15]

Adenylate cyclase assay

Membranes were suspended in 0.5 mL of incubation mixture (50 mM tris HCl, $MgCl_2$ 10 mM, EDTA 1 mM, pH 7.4) containing GTP 5 μM, 0.5 mM 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (Ro 20-1724) as phosphodiesterase inhibitor, 2.0 IU/mL adenosine deaminase and preincubated for 10 min in a shaking bath at 37 °C. Then Cl-IB-MECA or antagonists examined plus ATP (1 mM) and forskolin 10 μM were added to the mixture and the incubation continued for an additional 10 min. The potencies of antagonists were determined by antagonism of the inhibition of cyclic-AMP production induced by Cl-IB-MECA (100 nM). The reaction was terminated by transfer to a boiling water bath. After 2 min. at boiling temperature the tubes were cooled to

4 °C and centrifuged at 2,000 g for 10 min. Supernatants (100 μL) were used in a protein competition assay for c-AMP carried out essentially according to Varani et al.[8]

Samples of cyclic AMP standards (0-10 pmol) were added to each test tube containing the incubation buffer (Trizma base 0.1 M; aminophylline 8.0 mM; 2-mercaptoethanol 6.0 mM, pH 7.4) and [^{3}H]-cyclic AMP in a total volume of 0.5 mL. The binding protein, previously prepared from beef adrenals, was added to the samples previously incubated at 4 °C for 150 min and, after the addition of charcoal were centrifugated at 2,000 g for 10 min. The clear supernatant (0.2 mL) was mixed with 4 mL of scintillation fluid and counted in a LS-1800 Beckman scintillation counter.

Cyclic AMP Accumulation Assay (K_B determination)

Cyclic AMP levels were measured with a competitive protein binding method.[16,17] CHO cell that expressed recombinant human A_3 adenosine receptors were harvested by trypsinization. After centrifugation and resuspension in medium, cells were deposited in 24-well plates in volumes of 1 mL. After 24 hr, the medium was removed and cells were washed three times with 1 mL Dulbecco's modified Eagle's medium, containing 50 mM HEPES, pH 7.4. Cells were then treated in the presence of rolipram (10 μM) and adenosine deaminase (3 Units/mL) with a range of concentrations of the agonist Cl-IB-MECA alone or in combination with fixed concentrations of the antagonist. After 45 min forskolin (10 μM) was added to the medium, and the incubation was continued an additional 15 min. The reaction was terminated by removal of the supernatant, and cells were lysed upon the addition of 200 μL of ice-cold 0.1 M HCl. The cell lysate was resuspended and stored at -20°C. For determination of cyclic AMP production, protein kinase A (PKA) was incubated with [^{3}H]cyclic AMP (2 nM) in K_2HPO_4/EDTA buffer (K_2HPO_4, 150 mM; EDTA, 10 mM), 20 μL of the cell lysate, and 30 μL 0.1 M HCl or 50 μL of cyclic AMP solution (0-16 pmol/200 μL for standard curve). Bound radioactivity was separated by rapid filtration through Whatman GF/C filters and washed once with cold buffer. Bound radioactivity was measured by liquid scintillation counter.

For each Cl-IB-MECA concentration-response curve an EC_{50} value for cyclic AMP accumulation were determined using GraphPAD Prism software (GraphPAD, San Diego, CA). A Schild analysis[18] was carried out on the antagonism, and a K_B value of 0.20 ± 0.03 nM (n=3) for compound **4** was calculated.

COMPUTATIONAL APPROACH.

All calculations were performed on a Silicon Graphics Octane R12000 workstation.

The human A_3 receptor model was built and optimized using MOE (2001.01) modeling package[19] based on the approach described by Moro et al.[20] Briefly, transmembrane domains were identified with the aid of Kyte-Doolittle hydrophobicity, and E_{min} surface probability parameters.[21] Transmembrane helices were built from the sequences and minimized individually. The minimized helices were then grouped together to form a helical bundle matching 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,[22] until the *rms* value of the conjugate gradient (CG) was <0.01 kcal/mol/Å. A fixed dielectric constant = 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 geometry constraints using RHF/AM1 semiempirical calculations.[23] Vibrational frequency analyses were used to characterize the minimal stationary points (zero imaginary frequencies). The software package Gaussian98 was utilized for all quantum mechanical calculations.[22]

The ligands were docked into the hypothetical TMs binding site by using the DOCK docking program, part of the MOE suite. This program incorporates the use of manual and automatic docking procedures in combination with molecular mechanics within the Simulations module of MOE.[24] 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 subjected 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.

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