Electronic Supplementary Information

Studies on enantioselectivity of chiral 4-acetylamino-6-alkyloxy-2-alkylthiopyrimidines acting as antagonists at the human A_3 adenosine receptor

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Materials and methods

Chemistry

Compounds **5** and **7** were obtained as previously reported.[i] 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. The purity of all assayed compounds was determined by HPLC, and it was found to be higher than 95%. HPLC was conducted on a Shimadzu LC-20AD SP liquid chromatograph equipped with a DDA Detector at 210 and 230 nm (column C18 (250 mm x 4.6 mm, 5 μm, Shim pack)). The mobile phase, delivered at isocratic flow, consisted of methanol (90%) and water (10%) and a flow rate of 1.0 mL/min.

Synthesis of N-*[6-(1-Phenylethoxy)-2-(propylsulfanyl)pyrimidin-4-yl]acetamide* ***1***

Acetic anhydride (2 mL, 21.2 mmol), **8** (0.289 g; 1 mmol) and 6 drops of H_2SO_4 were warmed to 50-60 °C for 2 h. The mixture was poured in ice/water (10 mL) and extracted with chloroform (4x10 mL). The organic phase was washed with 0.1 M NaOH solution (10 mL) and with water until pH=7; finally, the organic layer was dried on Na_2SO_4. Removal of the solvent under reduced pressure gave a crude that was purified by flash-chromatography (ethyl acetate/petroleum ether=1:3, v/v as eluant) to give the desired **1** (0.135 g; 41%; mp: 104.9-105.8 °C, white crystals from *n*-hexane). ^{1}H-NMR (DMSO-d_6, δ ppm): 10.70 (bs exch, 1H, NH); 7.39-7.22 (m, 5H, H-Ar); 7.14 (s, 1H, H-5); 6.12 (q, 1H, *J*=6.4 Hz, OCH); 2.96 (t, 2H, *J*=7.3 Hz, SCH_2); 2.07 (s, 3H, CH_3); 1.59-1.53 (m, 5H, CH_2 and CH_3); 0.94 (t, 3H, *J*=7.3 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 170.5; 169.6; 169.1; 158.7; 142.3; 128.4; 127.3; 125.6; 89.8; 73.9; 31.7; 24.0; 22.8; 22.4; 13.2.

Synthesis of N-*{2-[(1-Phenylethyl)sulfanyl]-6-propoxypyrimidin-4-yl}acetamide* ***2***

Operating as above, **9** (0.289 g; 1 mmol) give the desired **2** (0.172 g; 52%) as colorless oil. ^{1}H-NMR (DMSO-d_6, δ ppm): 10.73 (bs, 1H, NH); 7.48-7.44 (m, 2H, H-Ar); 7.36-7.31 (m, 2H, H-Ar); 7.27-7.23 (m, 1H, H-Ar); 7.08 (s, 1H, H-5); 5.00 (q, 1H, *J*=7.1 Hz, SCH); 4.29-4.17 (m, 2H, OCH_2); 2.09 (s, 3H, CH_3); 1.72-1.62 (m, 5H, CH_2 and CH_3); 0.94 (t, 3H, *J*=7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 170.5; 170.0; 169.1; 158.6; 143.0; 128.5; 127.2; 127.1; 89.5; 68.0; 43,5; 24.1; 22.3; 21.6; 10.3.

Synthesis of N-*[2-(Benzylsulfanyl)-6-(butan-2-yloxy)pyrimidin-4-yl]acetamide* ***3***

Operating as above, **10** (0.289 g; 1 mmol) give the desired **3** (0.232 g; 70%; mp: 80.7-82.1 °C, white crystals from *n*-hexane). ^{1}H-NMR (DMSO-d_6, δ ppm): 10.73 (bs, 1H, NH); 7.44-7.41 (m, 2H, H-Ar); 7.34-7.29 (m, 2H, H-Ar); 7.27-7.24 (m, 1H, H-Ar); 7.07 (s, 1H, H-5); 5.15-5.06 (m, 1H, OCH); 4.39 (s, 2H, SCH_2); 2.09 (s, 3H, CH_3); 1.70-1.52 (m, 2H, CH_2); 1.22 (d, 3H, *J*=6.2 Hz, CH_3); 0.86 (t, 3H, *J*=7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 170.5; 169.7; 169.1; 158.6; 137.9; 128.7; 128.4; 127.0; 89.9; 73.8; 33.9; 28.2; 24.1; 19.1; 9.5.

*Synthesis of 6-Amino-2-[(1-phenylethyl)sulfanyl]pyrimidin-4(3*H*)-one* ***6***

(1-Bromoethyl)benzene (1.69 mL; 2.294 g; 12.4 mmol) was added dropwise into a solution of 4-amino-6-hydroxy-2-mercaptopyrimidine monohydrate **4** (1g, 6.2 mmol) in 1M 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, containing a pasty solid, was neutralized with acetic acid obtaining a gummy solid that was solubilized with dichloromethane; the organic layer was separated and dried on Na_2SO_4. Removal of the solvent under reduced pressure gave a crude product that was divided by chromatography, dichloromethane/methanol=10:1, v/v as eluant, to give compound **6** (0.230 g; 15%; colourless oil). ^{1}H-NMR (DMSO-d_6, δ ppm): 11.40 (bs, 1H, NH); 7.46-7.44 (m, 2H, H-Ar); 7.35-7.29 (m, 2H, H-Ar); 7.27-7.23 (m, 1H, H-Ar); 6.49 (bs, 2H, NH_2); 5.02-4.93 (q, 1H, *J*=7.0 Hz, SCH); 4.91 (s, 1H, H-5); 1.67 (d, 3H, *J*=7.0 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 164.3; 163.6; 162.1; 142.7; 128.5; 127.4; 127.3; 81.3; 42.9; 21.8.

*Synthesis of 6-(1-Phenylethoxy)-2-(propylsulfanyl)pyrimidin-4-amine **8***

(1-Bromoethyl)benzene (0.52 mL; 0.888 g; 4.8 mmol) was added dropwise into a suspension of compound **5** (0.450 g; 2.4 mmol) and K_2CO_3 (0.336 g; 2.4 mmol) in anhydrous CH_3CN (6 mL). The reaction mixture was warmed, under stirring, to reflux for 4 h (TLC analysis). After cooling, the K_2CO_3 was removed by filtration and the solvent was evaporated *in vacuo*. The residue was purified by chromatography, ethyl acetate/petroleum ether=1:3, v/v as eluant to give (^{1}H NMR spectrum) compound **8** (0.417 g; 60%; colourless oil). ^{1}H-NMR (DMSO-d_6, δ ppm): 7.35-7.21 (m, 5H, H-Ar); 6.63 (bs exch, 2H, NH_2); 6.01 (q, 1H, *J*=6.4 Hz, OCH); 5.44 (s, 1H, H-5); 2.88 (t, 2H, *J*=7.3 Hz, SCH_2); 1.55 (q, 2H, *J*=7.3 Hz, CH_2); 1.51 (d, 3H, *J*=6.4 Hz, CH_3); 0.92 (t, 3H, *J*=7.3 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 169.2; 167.9; 165.2; 142.3; 128.3; 127.3; 125.5; 82.3; 72.6; 31.5; 23.0; 22.6; 13.3.

*Synthesis of 2-[(1-Phenylethyl)sulfanyl]-6-propoxypyrimidin-4-amine **9** and 6-Amino-2-[(1-phenylethyl)sulfanyl]-3-propylpyrimidin-4(3*H*)-one **9a***

1-Bromopropane (0.54 mL; 6.0 mmol) was added dropwise into a suspension of **6** (0.741 g; 3.0 mmol) in anhydrous CH_3CN (6 mL) and K_2CO_3 (0.414 g; 3.0 mmol). The reaction mixture was kept, under stirring, at 80-85 °C for 4 h. After cooling, the inorganic material was taken off by filtration, and the solvent was removed in vacuo. The solid residue, constituted by a mixture of **9** and **9a** isomers, was resolved by flash-chromatography (dichloromethane/methanol =10:1, v/v as eluant); the faster running band gave pure **9** (0.522 g; 60%; mp 68.7-69.9 °C white crystals from *n*-hexane), the slower one gave compound **9a** (0.138 g; 16%; mp: 157.5-158.6 °C white crystals from *n*-hexane).

*2-[(1-Phenylethyl)sulfanyl]-6-propoxypyrimidin-4-amine **9*** has the following spectroscopic properties: ^{1}H-NMR (DMSO-d_6, δ ppm): 7.45-7.42 (m, 2H, H-Ar); 7.34-7.31 (m, 2H, H-Ar); 7.30-7.23 (m, 1H, H-Ar); 6.66 (bs, 2H, NH_2); 5.39 (s, 1H, H-5); 4.92 (q, 1H, *J*=7.1 Hz, SCH); 4.14-4.10 (m, 2H, OCH_2); 1.67-1.62 (m, 5H, CH_2 and CH_3); 0.91 (t, 3H, *J*=7,4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 168.8; 168.7; 165.1; 143.5; 128.4; 127.2; 126.9; 81.7; 67.0; 43.0; 22.4; 21.9; 10.3.

*6-Amino-2-[(1-phenylethyl)sulfanyl]-3-propylpyrimidin-4(3H)-one **9a*** has the following spectroscopic properties: ^{1}H-NMR (DMSO-d_6, δ ppm): 7.51-7.48 (m, 2H, H-Ar); 7.34-7.31 (m, 2H,

H-Ar); 7.30-7.26 (m, 1H, H-Ar); 6.48 (bs, 2H, NH_2); 5.03 (q, 1H, *J*=7.2 Hz, SCH); 4.89 (s, 1H, H-5); 3.80-3.63 (m, 2H, NCH_2); 1.71 (d, 3H, *J*=7.2 Hz, CH_3); 1.57-1.48 (m, 2H, CH_2); 0.82 (t, 3H, *J*=7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 161.7; 161.3; 159.9; 142.2; 128.6; 127.7; 127.6; 81.1; 44.1; 25.5; 21.7; 21.2; 11.1.

*Synthesis of 2-(Benzylsulfanyl)-6-(butan-2-yloxy)pyrimidin-4-amine **10***

2-Bromobutane (0.52 mL; 0.657 g; 4.8 mmol) was added dropwise into a suspension of compound **7** (0.560 g; 2.4 mmol) and K_2CO_3 (0.336 g; 2.4 mmol) in anhydrous CH_3CN (6 mL). The reaction mixture was warmed, under stirring, to reflux for 4 h (TLC analysis). After cooling, the K_2CO_3 was removed by filtration and the solvent was evaporated *in vacuo*. The residue was purified by chromatography, ethyl acetate/petroleum ether=1:2, v/v as eluant to give compound **10** (0.521 g; 75%; mp: 90.4-91.3 °C, pale yellow crystals from *n*-hexane). ^{1}H-NMR (DMSO-d_6, δ ppm): 7.42-7.38 (m, 2H, H-Ar); 7.32-7.26 (m, 2H, H-Ar); 7.25-7.20 (m, 1H, H-Ar); 6.65 (bs, 2H, NH_2); 5.37 (s, 1H, H-5); 5.00-4.93 (m, 1H, OCH); 4.29 (s, 2H, SCH_2); 1.61-1.50 (m, 2H, CH_2); 1.17 (d, 3H, *J*=6.3 Hz, CH_3); 0.84 (t, 3H, *J*=7.4 Hz, CH_3). ^{13}C-NMR (DMSO-d_6, δ ppm): 168.6; 168.5; 165.1; 138.6; 128.7; 128.3; 126.8; 82.3; 72.4; 33.8; 28.5; 19.4; 9.6.

Enantiomeric separations

The enantiomeric separations were carried out by us as previously reported.[ii]

Biology materials

Each compound was dissolved in DMSO and diluted with assay buffer to the final concentration, where the amount of DMSO never exceeded 2%. [^{3}H]DPCPX and [^{3}H]NECA 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 ARs were kindly supplied by Prof. K.-N. Klotz, Würzburg University, Germany.[iii]

Human A_1 AR binding experiments

Aliquots of cell membranes (30 µg proteins) were incubated at 25 °C for 180 min in 500 µL of binding 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 tested compounds. Non-specific binding was determined in the presence of 50 µM N^6-R-phenylisopropyladenosine (R-PIA).[iv,v] The dissociation constant (K_d) of [^{3}H]DPCPX in human A_1 AR CHO cell membranes was 3 nM as determined by us.

Human A_{2A} AR binding experiments

Aliquots of cell membranes (30 µg) were incubated at 25 °C for 90 min in 500 µL of binding 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 tested compounds. Non-specific binding was determined in the presence of 100 µM (R-PIA).[iv,v] The dissociation constant (K_d) of [^{3}H]NECA in human A_{2A} AR CHO cell membranes was 30 nM as determined by us.

Human A_{2B} AR binding experiments

Aliquots of cell membranes (25 µg) were incubated at room temperature for 180 min in a final volume of 1 ml of T_4 buffer (with the addition of 0.0005% Tween 20, 0.5% albumin from bovine serum, 1 mM DL-dithiothreitol and 2 units/mL ADA) in the presence of 30 nM [^{3}H]NECA and six different concentrations of the tested compounds. Non-specific binding was determined

in the presence of 100 μM NECA.[iv] The dissociation constant (K_d) of [^{3}H]NECA in human A_{2B} AR CHO cell membranes was 30 nM as determined by us.

Human A_3 AR binding experiments

Aliquots of cell membranes (90 μg) were incubated at 25 °C for 180 min in 500 μL of binding buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM EDTA, 2 units/mL ADA, pH 7.4) in the presence of 20 nM [^{3}H]NECA and six different concentrations of the tested compounds. Non-specific binding was determined in the presence of 100 μM R-PIA.[iv,v] The dissociation constant (K_d) of [^{3}H]NECA in human A_3 AR CHO cell membranes was 10 nM as determined by us.

Measurement of cAMP levels in human A_3 AR-transfected CHO cells

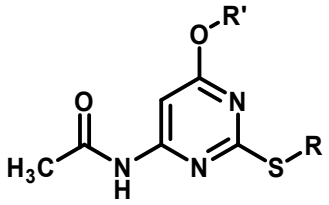
Intracellular cAMP levels were measured using a competitive protein binding method.[vi] A_3 AR CHO cells were harvested by trypsinization. After centrifugation and resuspension in medium, cells (~ 30,000) were plated in 24-well plates in 0.5 mL of medium. After 24 h, the medium was removed, and the cells were incubated at 37 °C for 15 min with 0.5 mL of Dulbecco's Modified Eagle Medium (DMEM) in the presence of ADA (1 U/mL) and the phosphodiesterase inhibitor Ro20-1724 (20 μM). The antagonist efficacy profile of the compounds was evaluated by assessing their ability to counteract Cl-IB-MECA-mediated inhibition of cAMP accumulation in the presence of 1 μM FK. The compounds were incubated 10 min before the addition of the agonist. Following incubation, the reaction was terminated by the removal of the medium and the 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 the determination of cAMP production, bovine adrenal cAMP binding protein was incubated with [^{3}H]cAMP (2 nM) and 50 μL of cell lysate or cAMP standard (0-96 pmol) at 0 °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 50 mM Tris/HCl, pH 7.4. The radioactivity was measured by liquid scintillation spectrometry.

Data analysis

All binding and functional data were analyzed using Graph-Pad Prism 5.0 (GraphPad Software Inc., San Diego, CA). The IC_{50} were obtained using the non-linear regression curve fitting of the concentration-response curves. The K_i values were obtained using the one site fit K_i. All values obtained are mean values of at least three different experiments each performed in duplicate.

Table 1S. Binding affinities of racemates 1-3 for human A_1, A_{2A} and A_{2B} ARs

The binding affinities of racemates **1**-**3** for human A_1, A_{2A} and A_{2B} ARs are expressed as percentage of inhibition of specific radioligand binding determined at 10 μM concentration of the tested racemic mixture. The percentage values are means ± SEM of at least three determinations derived from an iterative curve-fitting procedure.



no	R	R'	hA_1	hA_{2A}	hA_{2B}
(*R/S*)-**1**	$CH_3CH_2CH_2$	$C_6H_5(CH_3)CH$	67% ± 7%	45% ± 4%	7% ± 0.8%
(*R/S*)-**2**	$C_6H_5(CH_3)CH$	$CH_3CH_2CH_2$	59% ± 6%	28% ± 3%	4% ± 0.5%
(*R/S*)-**3**	$C_6H_5CH_2$	$CH_3CH_2(CH_3)CH$	69% ± 7%	56% ± 5%	5% ± 0.5%

Molecular modeling

Models of compounds (*S*)-**1** and (*R*)-**1** were built using the Builder tool and generated with Ligprep module. The Glide program of the Schrödinger package was used to dock these compounds to the human A_3 AR structure.[vi] The receptor grid generation were performed for the box with a center in the putative binding site. The size of the box was determined automatically. The extra precision mode (XP) of Glide was used for the docking. Using this program, a series of compounds were successfully docked into the binding sites of ARs.[vii, viii] The ligand scaling factor was set to 1.0. The binding site was defined as the ligand and all aminoacid residues located within 8 Å from the ligand. All the receptor residues located within 2 Å from

the binding site were used as a shell. The geometry of each ligand-binding site complex was then optimized. The following parameters of energy minimization were used: OPLS2005 force field; water was used as an implicit solvent; a maximum of 5000 iterations of the Polak–Ribier conjugate gradient minimization method was used with a convergence threshold of 0.01 kJ mol^{-1} $Å^{-1}$. The obtained docking poses were analyzed for their consistencies with the available binding affinity data as well as for the corresponding Glide scores.

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