# SUPPORTING INFORMATION

## New substituted 9-propyladenine derivatives as $A_{2A}$ adenosine receptor antagonists

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## 1. Experimental section

### 1.1 Chemistry

General: Reactions were routinely monitored by thin-layer chromatography (TLC) on silica gel (precoated $F_{254}$ Merck plates) and products visualized with UV light λ = 254 nm and iodine vapors. Chromatographies were performed using Fluka 230-400 mesh silica gel or Analtech preparative TLC (pTLC). All reported products showed $^1$H NMR spectra in agreement with the assigned structures. Yields were calculated after chromatography. $^1$H NMR spectra were obtained with Varian Mercury 400 MHz spectrometer; $\delta$ in ppm, $J$ in Hz. All exchangeable protons were confirmed by addition of $D_2O$. Melting points were determined on a Büchi instrument and are uncorrected. Elemental analyses were determined on Fisons Instruments Model EA 1108 CHNS-O model analyzer and are within 0.4% of theoretical values. Purity of the compounds was ≥ 95% according to elemental analysis data (Table 1).

#### 1.1.1 General procedure for the synthesis of 2-alkylamino-9-proyladenine derivatives 12-14.

To compound **11**[1] (500 mg; 2.36 mmol) the suitable amine was added and the solution was stirred at 120°C for 24 h. The amine in excess was removed *in vacuo* by oil pump and the residue was flash chromatographed on a silica gel column with the appropriate eluent.

***N*$^2$-benzyl-9-propyl-9*H*-purine-2,6-diamine (12):** Compound **11** was reacted with benzylamine (6.4 mL). The reaction crude was purified eluting with cC$_6$H$_{12}$-EtOAc-MeOH (60:37:3) to give **12** as a white solid (286 mg, 45% yield). M.p.: 130-131°C; $^1$H NMR (DMSO-$d_6$) δ = 0.79 (t, 3H, $J$ = 7.2 Hz, $CH_3$), 1.70 (m, 2H, $CH_2CH_3$), 3.88 (t, 2H, $J$ = 7.2 Hz, $NCH_2$), 4.43 (d, 2H, $NHCH_2$), 6.63 (s, 2H, $NH_2$), 6.79 (t, 1H, $J$ = 6.2 Hz, NH), 7.22 (m, 5H, H-Ph), 7.67 (s, 1H, H-8). Anal calcd for ($C_{15}H_{18}N_6$) C, H, N.

***N*$^2$-phenethyl-9-propyl-9*H*-purine-2,6-diamine (13):** Compound **11** was reacted with 2-phenylethylammine (6.0 mL). The reaction crude was purified eluting with cC$_6$H$_{12}$-EtOAc-MeOH (60:37:3) to give **13** as a white solid (620 mg, 88% yield). M.p. = 78-79 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.83 (t, 3H, $J$ = 7.3 Hz, $CH_3$), 2.73 (m, 2H, $CH_2CH_3$), 2.78 (t, 2H, $J$ = 8.4 Hz, $CH_2$Ph), 3.3 (m, 2H, $NHCH_2$), 3.89 (t, 2H, $NCH_2$), 6.2 (t, 1H, NH), 6.6 (s, 2H, $NH_2$), 7.23 (m, 5H, H-Ph), 7.60 (s, 1H, H-8). Anal calcd for ($C_{16}H_{20}N_6$) C, H, N.

***N*$^2$-(3-phenylpropyl)-9-propyl-9H-purine-2,6-diamine (14):** Compound **11** was reacted with 3-phenylpropylammine (8.79 mL). The reaction crude was purified eluting with EtOAc-cC$_6$H$_{12}$-MeOH (57:38:5) to give **14** as a white solid (549 mg, 75% yield). M.p. 118-119°C; $^1$H NMR (DMSO-$d_6$) δ = 0.80 (t, 3H, $J$ = 5.0 Hz, $CH_3$), 1.75 (m, 4H, $CH_2CH_3$ and $CH_2CH_2$Ph), 2.60 (t, 2H, $J$ = 5.4 Hz, $CH_2$Ph), 3.21 (m, 2H, $NHCH_2$), 3.88 (t, 2H, $J$ = 4.8 Hz, $NCH_2CH_2$), 6.30 (t, 1H, NH), 6.57 (s, 2H, $NH_2$), 7.20 (m, 5H, H-Ph), 7.66 (s, 1H, H-8). Anal calcd for ($C_{17}H_{22}N_6$) C, H, N.

#### 1.1.2 General procedure for the synthesis of the 2-alkylamino-8-bromo-9-propyladenine derivatives 15-17

To 1.0 mmol of the appropriate 2-alkylamino-9-propyladenine **12-14**, dissolved in 18 mL of anhydrous DMF, NBS (272 mg; 1.53 mmol) was added. The reaction is instantaneous, the solvent was removed *in vacuo* and the crude residue was chromatographed by flash silica gel column eluting with the suitable solvent to obtain desired compounds **15-17** as white solids.

***N*$^2$-Benzyl-8-bromo-9-propyl-9*H*-purine-2,6-diamine (15):** Compound **15** was obtained from compound **12**. The crude reaction mixture was purified by eluting with cC$_6$H$_{12}$-EtOAc-MeOH (80:19.5:0.5) to give **15** (82 mg, 50% yield). M.p. 168-169 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.80 (t, 3H, $J$ = 7.4 Hz, $CH_3$), 1.67 (m, 2H, $CH_2CH_3$), 3.88 (t, 2H, $J$ = 6.9 Hz, $NCH_2$), 4.43 (d, 2H, $J$ = 6.4 Hz, $NHCH_2$), 6.84 (bs, 2H, $NH_2$), 7.0 (t, 1H, $J$ = 6.4 Hz, NH), 7.26 (m, 5H, H-Ph). Anal calcd for ($C_{15}H_{17}BrN_6$) C, H, N.

**8-Bromo-*N*$^2$-phenethyl-9-propyl-9*H*-purine-2,6-diamine (16):** Compound **16** was obtained from **13**. The crude reaction mixture was purified by eluting with cC$_6$H$_{12}$-EtOAc-MeOH (80:19.5:0.5) to give **16** (263 mg, 49% yield). M.p. 139-140 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.85 (t, 3H, $J$ = 7.5 Hz, $CH_3$), 1.74 (m, 2H, $CH_2CH_3$), 2.81 (t, 2H, $J$ = 7.5 Hz, $CH_2$Ph), 3.38 (m, 2H, $NHCH_2$), 3.92 (t, 2H, $J$ = 6.9 Hz, $NCH_2$), 6.47 (bt, 1H, NH), 6.83 (bs, 2H, $NH_2$), 7.24 (m, 5H, H-Ph). Anal calcd for ($C_{16}H_{19}BrN_6$) C, H, N.

**8-Bromo-*N*$^2$-(3-phenylpropyl)-9-propyl-9*H*-purine-2,6-diamine (17):** Compound **17** was obtained from **14**. The crude reaction mixture was purified by eluting with cC$_6$H$_{12}$-EtOAc-MeOH (70:29:1) to give **17** (130 mg, 35% yield). M.p. 103-104 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.82 (t, 3H, $J$ = 7.5 Hz, $CH_3$), 1.74 (m, 4H, $CH_2CH_3$ and $NHCH_2CH_2$), 2.60 (t, 2H, $J$ = 7.9 Hz, $CH_2$Ph), 3.21 (m, 2H,

NH*CH*$_2$), 3.89 (t, 2H, *J* = 7.1 Hz, NCH$_2$), 6.46 (bt, 1H, NH), 6.77 (bs, 2H, NH$_2$), 7.21 (m, 5H, H-Ph). Anal calcd for ($C_{17}H_{21}BrN_6$) C, H, N.

**1.1.3 General procedure for the synthesis of 8-(2-furyl)-9-propyladenine derivatives 18-20 and 29-31**

To 0.27 mmol of the appropriate 2-alkylamino-8-bromo-9-propyladenine **15-17** or *N*$^6$-alkyl-8-bromo-9-propyladenine **26-28** solubilized in 1.4 mL of anhydrous THF $(Ph_3P)_2PdCl_2$ (11.4 mg, 0.02 mmol) and (2-tributylstannyl)furane (436 µL, 1.35 mmol) were added. The reaction mixture was left in anhydrous conditions at 60° for 3 h after which another portion of 2-(tributylstannyl)furan (218 µL, 67.5 mmol) was added. After 18 h of total reaction time, the solvent was removed *in vacuo* and the residue was chromatographed on a silica gel column eluting with the suitable solvent, to give the desired compounds **18-20**.

***N*$^2$-benzyl-8-(furan-2-yl)-9-propyl-9*H*-purine-2,6-diamine (18):** Compound **18** was obtained from **15** after a flash column chromatography eluting with EtOAc-c$C_6H_{12}$-$CH_3OH$ (60:38:2) and then by pTLC eluted with EtOAc-c$C_6H_{12}$-$CH_3OH$ (60:37:3) as white solid (52 mg, 55% yield). M.p. = 113-113.4°C; $^1$H NMR (DMSO-$d_6$) δ = 0.79 (t, 3H, *J* = 7.6 Hz, $CH_2$*CH*$_3$), 1.65 (m, 2H, $CH_2$*CH*$_2$CH$_3$), 4.17 (t, 2H, *J* = 7.2 Hz, N*CH*$_2$), 4.46 (d, 2H, *J* = 6.6 Hz, NH*CH*$_2$), 6.68 (m, 1H, H-furyl), 6.82 (bs, 2H, NH$_2$), 6.95 (m, 2H, H-furyl and *NH*CH$_2$), 7.26 (m, 5H, H-Ph), 7.86 (d, 1H, H-furyl). Anal calcd for ($C_{19}H_{20}N_6O$) C, H, N.

**8-(Furan-2-yl)-*N*$^2$-phenethyl-9-propyl-9*H*-purine-2,6-diamine (19):** Compound **19** was obtained from **16** after a flash column chromatography eluting with EtOAc-c$C_6H_{12}$-$CH_3OH$ (60:38:2) as white solid (67 mg, 66% yield). M.p. = 119-120°C; $^1$H NMR (DMSO-*d*$_6$) δ = 0.85 (t, 3H, *J* = 7.5 Hz, $CH_3$), 1.69 (m, 2H, *CH*$_2$CH$_3$), 2.84 (t, 2H, *J* = 7.5 Hz, $CH_2$Ph), 3.33 (m, 2H, NH*CH*$_2$), 4.22 (t, 2H, *J* = 6.9 Hz, NCH$_2$), 6.43 (t, 1H, *J* = 5.6 Hz, *NH*CH$_2$), 6.70 (m, 1H, H-furyl), 6.81 (bs, 2H, NH$_2$), 6.95 (d, 1H, *J* = 3.2 Hz, H-furyl), 7.28 (m, 5H, H-Ph), 7.87 (d, 1H, H-furyl). Anal calcd for ($C_{20}H_{22}N_6O$) C, H, N.

**8-(Furan-2-yl)-*N*$^2$-(3-phenylpropyl)-9-propyl-9*H*-purine-2,6-diamine (20):** Compound **20** was obtained from **17** after a flash column chromatography eluting with EtOAc-c$C_6H_{12}$-$CH_3OH$ (60:38:2) and then by pTLC eluted with EtOAc-c$C_6H_{12}$-$CH_3OH$ (60:35:5) to give **20** as a white solid (71.2 mg, 70% yield). M.p. 107-108°C; $^1$H NMR (DMSO-*d*$_6$) δ = 0.81 (t, 3H, *J* = 7.5 Hz, $CH_3$), 1.75 (m, 4H, *CH*$_2$CH$_3$ and NHCH$_2$*CH*$_2$), 2.62 (t, 2H, *J* = 7.5 Hz, $CH_2$Ph), 3.25 (m, 2H, NH*CH*$_2$), 4.17 (t, 2H, *J* = 7.2 Hz, N*CH*$_2$), 6.46 (t, 1H, NHCH$_2$), 6.68 (m, 1H, H-furyl), 6.77 (bs, 2H, NH$_2$), 6.94 (d, 1H, H-furyl), 7.20 (m, 5H, H-Ph), 7.86 (d, 1H, H-furyl). Anal calcd for ($C_{21}H_{24}N_6O$) C, H, N.

***N*-benzyl-8-(furan-2-yl)-9-propyl-9*H*-purin-6-amine (29):** Compound **29** was obtained from **26**. The crude residue was purified by eluting with c$C_6H_{12}$:EtOAc:$CH_3OH$ (70:29:1), to give **29** (21 mg, 24% yield) as a white solid. M.p. 122 °C; $^1$H NMR (DMSO-*d*$_6$) δ = 0.84 (t, 3H, *J* = 7.2 Hz, $CH_3$), 1.74 (m, 2H, *CH*$_2$CH$_3$), 4.34 (t, *J* =7.4 Hz, 2H, NCH$_2$), 4.69 (bt, 1H, NH*CH*$_2$), 6.75 (m, 1H, H-furyl), 7.23 (m, 6H, H-Ph and H-furyl), 7.98 (s, 1H, H-furyl), 8.21 (s, 1H, H-2) 8,5 (bt, 1H, NH). Anal calcd for ($C_{19}H_{19}N_5O$) C, H, N.

**8-(Furan-2-yl)-*N*-phenethyl-9-propyl-9*H*-purin-6-amine (30):** Compound **30** was obtained from **27**. The crude residue was purified by eluting with c$C_6H_{12}$:EtOAc:$CH_3OH$ (70:29:1), to give **30** (76 mg, 82 % yield) as a vitreous solid. $^1$H NMR (DMSO-$d_6$) δ = 0.84 (t, 3H, *J* = 7.3 Hz, $CH_3$), 1.74 (m, 2H, *CH*$_2$CH$_3$), 2.92 (t, 2H, *J* = 7.7 Hz, *CH*$_2$Ph), 3.70 (m, 2H, NH*CH*$_2$), 4.37 (t, *J* = 7.2 Hz, 2H, NCH$_2$), 6.75 (m, 1H, H-furyl), 7.4 (d, 1H, H-furyl), 7.22 (m, 5H, H-Ph), 7.98 (bs, 2H, NH and H-furyl), 8,25 (s, 1H, H-2). Anal calcd for ($C_{20}H_{21}N_5O$) C, H, N.

**8-(Furan-2-yl)-*N*-(3-phenylpropyl)-9-propyl-9*H*-purin-6-amine (31):** Compound **31** was obtained from **28**. The crude residue was purified by eluting with c$C_6H_{12}$:EtOAc:$CH_3OH$ (75:24:1), to give **31** (59 mg, 61% yield) as a vitreous solid. $^1$H NMR (DMSO-*d*$_6$) δ = 0.84 (t, 3H, *J* = 7.3 Hz, $CH_3$), 1.74 (m, 2H, *CH*$_2$CH$_3$), 1.90 (m, 2H, NHCH$_2$*CH*$_2$), 2.65 (t, 2H, *J* = 7.7 Hz, *CH*$_2$Ph), 3.51 (m, 2H, NH*CH*$_2$), 4.37 (t, 2H, *J* = 7.3 Hz, NCH$_2$), 6.75 (m, 1H, H-furyl), 7.20 (m, 6H, H-Ph and H-furyl), 7.97 (bs, 2H, NH and H-furyl), 8,22 (s, 1H, H-2). Anal calcd for ($C_{21}H_{23}N_5O$) C, H, N.

**6-chloro-9-propyl-9H-purine (22) and 6-chloro-7-propyl-7H-purine (22a):** To commercially available 6-chloropurine (21, 3.0 g, 19.39 mmol), in anhydrous DMF (20 mL), $K_2CO_3$ (2.9 g, 21.12 mmol) and propyl iodide (2.27 mL, 23.28 mmol) were added. The reaction mixture was left in anhydrous conditions at romm temperature for 14 h and then the solvent was removed *in vacuo* and the residue chromatographed on a silica gel flash column eluting with *c*$C_6H_{12}$-EtOAc (80:20 to 70:30), to give 22 (2.45 g, 64% yield), and 22a (1.07 g, 28% yield) as white solids. **22**: $^1$H NMR (DMSO-*d*$_6$) δ = 0.83 (t, 3H, *J* = 7.4 Hz, $CH_3$), 1.85 (m, 2H, *CH*$_2$CH$_3$), 4.24 (t, 2H, *J* = 7.1 Hz, N*CH*$_2$), 8.71 (s, 1H, H-2), 8.76 (s, 1H, H-8). Anal calcd for ($C_8H_9ClN_4$) C, H, N. **22a**: $^1$H NMR (DMSO-*d*$_6$) δ 0.87 (t, 3H, *J* = 7.4 Hz, $CH_3$), 1.85 (m, 2H, *CH*$_2$CH$_3$), 4.43 (t, 2H, *J* = 6.8 Hz, N*CH*$_2$), 8.80 (s, 1H, H-2), 8.83 (s, 1H, H-8). **They were characterized by NMR data comparison of analogue purine derivatives.[1, 2]** Anal calcd for ($C_8H_9ClN_4$) C, H, N.

**1.1.4 General procedure for the synthesis of *N*$^6$-phenylalkyl-9-propyladenine (23-25)**

To a solution of **22** (1.00 g, 5.08 mmol) in anhydrous $CH_3CN$ (92 mL), DMAP (0.75 g, 30 mmol) and the suitable amine (30 mmol) were added. The reaction mixture was stirred, in anhydrous conditions, at 70 °C for 12 h. Then, the solvent was removed *in vacuo* and the residue was chromatographed on a silica gel flash column eluting with c$C_6H_{12}$-$CHCl_3$-$CH_3OH$ (70:29.5:0.5, v/v), to give the desired compounds **23-25** as white solids.

***N*-benzyl-9-propyl-9*H*-purin-6-amine (23):** Compound **23** was obtained by the reaction of **2** with benzylamine as white solid (0.95 g, 78% yield). M.p.: 129-130 °C. $^1$H NMR (DMSO-*d*$_6$) δ = 0.79 (t, 3H, *J* = 7.5 Hz, $CH_3$), 1.80 (m, 2H, *CH*$_2$CH$_3$), 4.09 (t, 2H, *J* = 7.2 Hz, NCH$_2$), 4.69 (m, 2H, NHCH$_2$), 7.25 (m, 5H, H-Ph), 8.15 (s, 1H, H-2), 8.17 (s, 1H, H-8), 8.29 (bs, 1H, NH). Anal calcd for ($C_{15}H_{17}N_5$) C, H, N.

***N*-phenethyl-9-propyl-9*H*-purin-6-amine (24):** Compound **24** was obtained by the reaction of **2** with 2-phenylethylamine as white solid (1.31 g, 92% yield). M.p.: 75-77 °C. $^1$H NMR (DMSO-*d*$_6$) δ = 0.79 (t, 3H, *J* = 10.9 Hz, $CH_3$), 1.77 (m, 2H, *CH*$_2$CH$_3$), 2.87 (t, 2H, *J* = 11.1 Hz, *CH*$_2$Ph), 3.66 (m, 2H, NH*CH*$_2$), 4.06 (t, 2H, *J* = 10.5 Hz, N*CH*$_2$), 7.22 (m, 5H, H-Ph), 7.71 (bs, 1H, NH), 8.18 (s, 1H, H-8), 8.10 (s, 1H, H-2). Anal calcd for ($C_{16}H_{19}N_5$) C, H, N.

***N*-(3-phenylpropyl)-9-propyl-9*H*-purin-6-amine (25):** Compound **25** was obtained by the reaction of **2** with 3-phenylpropylamine (0.79 g, 58% yield) as white solid. M.p.: 67-70 °C; $^1$H NMR (DMSO-*d*$_6$) δ = 0.82 (t, 3H, *J* = 7.5 Hz, $CH_3$), 1.82 (m, 4H, *CH*$_2$CH$_3$ and NHCH$_2$*CH*$_2$), 2.61 (t, 2H, *J* = 7.7 Hz, *CH*$_2$Ph), 3.48 (m, 2H, NHCH$_2$), 4.09 (t, 2H, *J* = 7.0 Hz, NCH$_2$), 7.23 (m, 5H, H-Ph), 7.8 (bs, 1H, NH), 8.12 (s, 1H, H-2), 8.18 (s, 1H, H-8). Anal calcd for ($C_{17}H_{21}N_5$) C, H, N.

**8-Bromo-*N*-(3-phenylpropyl)-9-propyl-9*H*-purin-6-amine (28):** Compound **28** was prepared starting from **33.** The reaction mixture was stirred at 60° for 4 h to give **28** (197 mg, 53%) as a white solid. M.p.: 105-106 °C; $^1$H NMR (DMSO-*d*$_6$) δ = 0.85 (t, 3H,

*J* = 7.4 Hz, $CH_3$), 1.82 (m, 4H, $NHCH_2CH_2$ and $CH_2CH_3$), 2.64 (t, 2H, *J* =7.7 Hz, $CH_2Ph$), 3.46 (m, 2H, $NHCH_2$), 4.09 (t, 2H, *J* =7.8 Hz, $NCH_2$), 7.23 (m, 5H, H-Ph), 8.04 (bs, 1H, NH), 8.20 (s, 1H, H-2). Anal calcd for ($C_{17}H_{20}BrN_5$) C, H, N.

**1.1.5 General procedure for the synthesis of the *N*$^6$-alkyl-8-bromo-9-propyladenine derivatives 26 and 27**

To 1.0 mmol of the appropriate *N*$^6$-alkyl-9-propyladenine **23** and **24**, dissolved in 5.5 mL of anhydrous $CH_3CN$, NBS (267 mg, 1.50 mmol) was added. The reaction was left in anhydrous conditions under stirring at room temperature for 16 h, then, the solvent was removed *in vacuo* and the crude residue was chromatographed by flash silica gel column eluting with the suitable solvent to obtain desired compounds **26** and **27** as white solids.

***N*-benzyl-8-bromo-9-propyl-9*H*-purin-6-amine (26):** Compound **26** was obtained from **23**. The crude reaction mixture was purified by eluting with $cC_6H_{12}$-$CHCl_3$ (80:20 to 50:50), to give **26** (123 mg, 19% yield). M.p. 116-118 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.82 (t, 3H, *J* = 10.9 Hz, $CH_3$), 1.73 (m, 2H, $NCH_2CH_2$), 4.05 (t, 2H, *J* = 10.6 Hz, $NCH_2$), 4.64 (m, 2H, $NHCH_2$), 7.25 (m, 5H, H-Ph), 8.15 (s, 1H, H-2), 8.5 (bt, 1H, NH). Anal calcd for ($C_{15}H_{16}BrN_5$) C, H, N.

**8-Bromo-*N*-phenethyl-9-propyl-9*H*-purin-6-amine (27):** Compound **27** was obtained from **24**. The crude reaction mixture was purified by eluting with $cC_6H_{12}$-$CHCl_3$ (80:20 to 50:50), to give **27** (212 mg, 38% yield). M.p. 85-87 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.84 (t, 3H, *J* = 7.5 Hz, $CH_3$), 1.76 (m, 2H, $CH_2CH_3$), 2.89 (t, 2H, *J* = 7.7 Hz, $CH_2Ph$), 3.69 (m, 2H, $NHCH_2$), 4.08 (t, 2H, *J* = 7.1 Hz, $NCH_2$), 7.22 (m, 5H, H-Ph), 8.04 (bs, 1H, NH), 8.21 (s, 1H, H-2). Anal calcd for ($C_{16}H_{18}BrN_5$) C, H, N.

**8-Bromo-6-iodo-9-propyl-9H-purine (33):** To a solution of **32** (370 mg, 1.44 mmol) in anhydrous $CH_3CN$ (6.3 mL), diiodometane (7.01 mL) and isoamyl nitrite (2.1 mL) were added. The reaction mixture was stirred under anhydrous conditions at 85 °C for 30 min. Then, the solvent was removed *in vacuo* and the residue was flash chromatographed on a silica gel column eluting with $CHCl_3$-$cC_6H_{12}$ (60:40), to give **33** (274 mg, 54% yield). M.p. 138-139 °C; $^1$H NMR (DMSO-$d_6$) δ = 0.87 (t, 3H, *J* = 7.4 Hz, $CH_3$), 1.82 (m, 2H, $CH_2CH_3$), 4.19 (t, 2H, *J* = 7.0 Hz, $NCH_2$), 8.62 (s, 1H, H-2).

**Table 1:** Elemental data analysis of synthesized compounds x-x.

| | | Calculated | | | Found | | |
|---|---|---|---|---|---|---|---|
| **Cpd** | **Mol. formula** | **C** | **H** | **N** | **C** | **H** | **N** |
| **12** | $C_{15}H_{18}N_6$ | 63.81 | 6.43 | 29.77 | 63.98 | 6.64 | 29.35 |
| **13** | $C_{16}H_{20}N_6$ | 64.84 | 6.80 | 28.36 | 65.01 | 6.87 | 28.11 |
| **14** | $C_{17}H_{22}N_6$ | 65.78 | 7.14 | 27.07 | 65.89 | 7.29 | 26.89 |
| **15** | $C_{15}H_{17}BrN_6$ | 49.87 | 4.74 | 23.26 | 49.91 | 4.85 | 23.05 |
| **16** | $C_{16}H_{19}BrN_6$ | 51.21 | 5.10 | 22.39 | 51.37 | 5.23 | 22.21 |
| **17** | $C_{17}H_{21}BrN_6$ | 52.45 | 5.44 | 21.59 | 52.55 | 5.60 | 21.47 |
| **18** | $C_{19}H_{20}N_6O$ | 65.50 | 5.79 | 24.12 | 65.59 | 5.87 | 24.00 |
| **19** | $C_{20}H_{22}N_6O$ | 66.28 | 6.12 | 23.19 | 66.39 | 6.18 | 23.08 |
| **20** | $C_{21}H_{24}N_6O$ | 67.00 | 6.43 | 22.32 | 67.19 | 6.50 | 22.16 |
| **22** | $C_8H_9ClN_4$ | 48.87 | 4.61 | 28.49 | 48.92 | 4.73 | 28.33 |
| **22a** | $C_8H_9ClN_4$ | 48.87 | 4.61 | 28.49 | 48.95 | 4.68 | 28.43 |
| **23** | $C_{15}H_{17}N_5$ | 67.39 | 6.41 | 26.20 | 67.47 | 6.67 | 26.09 |
| **24** | $C_{16}H_{19}N_5$ | 68.30 | 6.81 | 24.89 | 68.41 | 6.81 | 24.79 |
| **25** | $C_{17}H_{21}N_5$ | 69.12 | 7.17 | 23.71 | 69.26 | 7.19 | 23.69 |
| **26** | $C_{15}H_{16}BrN_5$ | 52.04 | 4.66 | 20.23 | 52.14 | 4.69 | 20.18 |
| **27** | $C_{16}H_{18}BrN_5$ | 53.34 | 5.04 | 19.44 | 53.37 | 5.10 | 19.40 |
| **28** | $C_{17}H_{20}BrN_5$ | 54.55 | 5.39 | 18.71 | 54.69 | 5.43 | 18.61 |
| **29** | $C_{19}H_{19}N_5O$ | 68.45 | 5.74 | 21.01 | 68.55 | 5.79 | 20.97 |
| **30** | $C_{20}H_{21}N_5O$ | 69.14 | 6.09 | 20.16 | 69.19 | 6.13 | 20.12 |
| **31** | $C_{21}H_{23}N_5O$ | 69.78 | 6.41 | 19.38 | 69.88 | 6.45 | 19.31 |
| **33** | $C_8H_8BrIN_4$ | 26.18 | 2.20 | 15.27 | 26.28 | 2.22 | 15.25 |

**1.2 Biology**

All pharmacological methods followed the procedures as described earlier.[3] In brief, membranes for radioligand binding were prepared from CHO cells stably transfected with human adenosine receptor subtypes in a two-step procedure. In a first low-speed step (1,000 x g) cell fragments and nuclei were removed. The crude membrane fraction was sedimented from the supernatant at 100,000 x g. The membrane pellet was resuspended in the buffer used for the respective binding experiments, frozen in liquid nitrogen and stored at -80°C. For the measurement of adenylyl cyclase activity only one high speed centrifugation of the homogenate was used. The resulting crude membrane pellet was resuspended in 50 mM Tris/HCl, pH 7.4 and immediately used for the cyclase assay.

For radioligand binding at $A_1$AR, at $A_{2A}$AR, and at $A_3$AR 1 nM [$^3$H]CCPA, 10 nM [$^3$H]NECA, and 1 nM [$^3$H]HEMADO were used, respectively. Non specific binding of [$^3$H]CCPA was determined in the presence of 1 mM theophylline, in the case of [$^3$H]NECA and [$^3$H]HEMADO 100 pM *N*$^6$-(*R*)-phenylisopropyladenosine (R-PIA) was used. *K*i values from competition experiments were calculated with the program SCTFIT.[4] Radioligand binding at $A_{2B}$AR is problematic as no high-affinity ligand is available for this subtype. Therefore, inhibition of NECA-stimulated adenylyl cyclase activity was determined as a measurement of affinity of compounds. $EC_{50}$-values from these experiments were converted to *K*i-values with the Cheng and Prusoff equation.[5]

### 1.3 Molecular Modelling

All molecular modelling studies were performed on a 2 CPU (PIV 2.0-3.0 GHZ) Linux PC. Homology modelling, energy minimization, and docking studies were carried out using MOE (version 2010.10) suite.[6] All ligand structures were optimized using RHF/AM1 semiempirical calculations and the software package MOPAC implemented in MOE was utilized for these calculations.[7]

#### 1.3.1 Refinement of the human $A_{2A}$AR structures

The crystal structure of the $hA_{2A}AR$ in complex with ZM241385 were retrieved from the Protein Data Bank (http://www.rcsb.org; pdb code: 3EML; 2.6-Å resolution[8]).

The structure was re-modelled by firstly removing the T4L external segment and secondly by performing a building of missing receptor regions (i.e. missing sections of EL2 or IL3 domains). The Homology Modelling tool of MOE was employed. In detail, the boundaries identified from the used $hA_{2A}AR$ X-ray crystal structure were applied and the missing loop domains were built by the loop search method implemented in MOE. Once the heavy atoms were modelled, all hydrogen atoms were added, and the protein coordinates were then minimized with MOE using the AMBER99 force field.[9] The minimizations were performed by 1000 steps of steepest descent followed by conjugate gradient minimization until the RMS gradient of the potential energy was less than 0.05 kJ $mol^{-1}$ $Å^{-1}$. Reliability and quality of the model were checked using the Protein Geometry Monitor application within MOE, which provides a variety of stereochemical measurements for inspection of the structural quality in a given protein, like backbone bond lengths, angles and dihedrals, Ramachandran φ-ψ dihedral plots, and sidechain-rotamer and non-bonded contact quality.

#### 1.3.2 Molecular docking analysis

All compound structures were docked into the binding site of the three $hA_{2A}AR$ models using the MOE Dock tool. This method is divided into a number of stages: *Conformational Analysis of ligands*. The algorithm generated conformations from a single 3D conformation by conducting a systematic search. In this way, all combinations of angles were created for each ligand. *Placement*. A collection of poses was generated from the pool of ligand conformations using Triangle Matcher placement method. Poses were generated by superposition of ligand atom triplets and triplet points in the receptor binding site. The receptor site points are alpha sphere centres which represent locations of tight packing. At each iteration a random conformation was selected, a random triplet of ligand atoms and a random triplet of alpha sphere centres were used to determine the pose. *Scoring*. Poses generated by the placement methodology were scored using two available methods implemented in MOE, the *London dG* scoring function which estimates the free energy of binding of the ligand from a given pose, and *Affinity dG* scoring which estimates the enthalpy contribution to the free energy of binding. The top 30 poses for each ligand were output in a MOE database.

#### 1.4.2 Post Docking analysis

The docking poses of each compound were then subjected to AMBER99 force field energy minimization until the RMS gradient of the potential energy was less than 0.05 kJ $mol^{-1}$ $Å^{-1}$. Receptor residues within 6 Å distance from the ligand were left free to move, while the remaining receptor coordinates were kept fixed. AMBER99 partial charges of receptor and MOPAC output partial charges of ligands were utilized. Once the compound-binding site energy minimization was completed, receptor coordinates were fixed and a second energy minimization stage was performed leaving free to move only compound atoms. MMFF94 force field[10-16] was applied. For each compound, the minimized docking poses were then rescored using *London dG* and *Affinity dG* scoring functions and the *dock-p$K_i$* predictor. The latter tool allows estimating the p$K_i$ for each ligand using the "scoring.svl" script retrievable at the SVL exchange service (Chemical Computing Group, Inc. SVL exchange: http://svl.chemcomp.com). The algorithm is based on an empirical scoring function consisting of a directional hydrogen-bonding term, a directional hydrophobic interaction term, and an entropic term (ligand rotatable bonds immobilized in binding). The obtained p$K_i$ values must be considered as docking scores and not as prediction of binding affinity. For each compound, the top-score docking pose according to at least two out of three scoring functions were selected for final ligand-target interaction analysis.

## References


1. C. Lambertucci, G. Cristalli, D. Dal Ben, D. D. Kachare, C. Bolcato, K. N. Klotz, G. Spalluto and R. Volpini, *Purinergic Signal.*, 2007, 3, 339-346.
2. C. Lambertucci, I. Antonini, M. Buccioni, D. Dal Ben, D. D. Kachare, R. Volpini, K. N. Klotz and G. Cristalli, *Bioorg. Med. Chem.*, 2009, 17, 2812-2822.
3. K. N. Klotz, J. Hessling, J. Hegler, C. Owman, B. Kull, B. B. Fredholm and M. J. Lohse, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 1998, 357, 1-9.
4. A. De Lean, A. A. Hancock and R. J. Lefkowitz, *Molecular Pharmacol.*, 1982, 21, 5-16.
5. Y. Cheng and W. H. Prusoff, *Biochem. Pharmacol.*, 1973, 22, 3099-3108.
6. *Molecular Operating Environment; C.C.G., Inc., 1255 University St., Suite 1600, Montreal, Quebec, Canada, H3B 3X3*.
7. J. J. Stewart, *J. Comput. Aided Mol. Des.*, 1990, 4, 1-105.
8. V. P. Jaakola, M. T. Griffith, M. A. Hanson, V. Cherezov, E. Y. Chien, J. R. Lane, A. P. IJzerman and R. C. Stevens, *Science*, 2008, 322, 1211-1217.
9. W. D. Cornell, P. Cieplak, C. I. Bayly, I. R. Gould, K. M. Merz, D. M. Ferguson, D. C. Spellmeyer, T. Fox, J. W. Caldwell and P. A. Kollman, *J. Am. Chem. Soc.*, 1995, 117, 5179-5197.
10. T. A. Halgren, *J. Comput. Chem.*, 1996, 17, 490-519.
11. T. A. Halgren, *J. Comput. Chem.*, 1996, 17, 520-552.
12. T. A. Halgren, *J. Comput. Chem.*, 1996, 17, 553-586.
13. T. A. Halgren, *J. Comput. Chem.*, 1996, 17, 587-615.
14. T. A. Halgren and R. Nachbar, *J. Comput. Chem.*, 1996, 17, 616-641.
15. T. A. Halgren, *J. Comput. Chem.*, 1999, 20, 720-729.
16. T. A. Halgren, *J. Comput. Chem.*, 1999, 20, 730-748.