Supporting Information:

Structure-Based Design, Synthesis and *in vivo* Antinociceptive Effects of Selective A_1 Adenosine Receptor Agonists[#]

Riccardo Petrelli,[1] Mirko Scortichini,[1] Carmela Belardo,[2] Serena Boccella,[2] Livio Luongo,[2] Fabio Capone,[3] Sonja Kachler,[4] Patrizia Vita,[1] Fabio Del Bello,[1] Sabatino Maione,[2] Antonio Lavecchia,[3*] Karl-Norbert Klotz,[4] and Loredana Cappellacci[1]*

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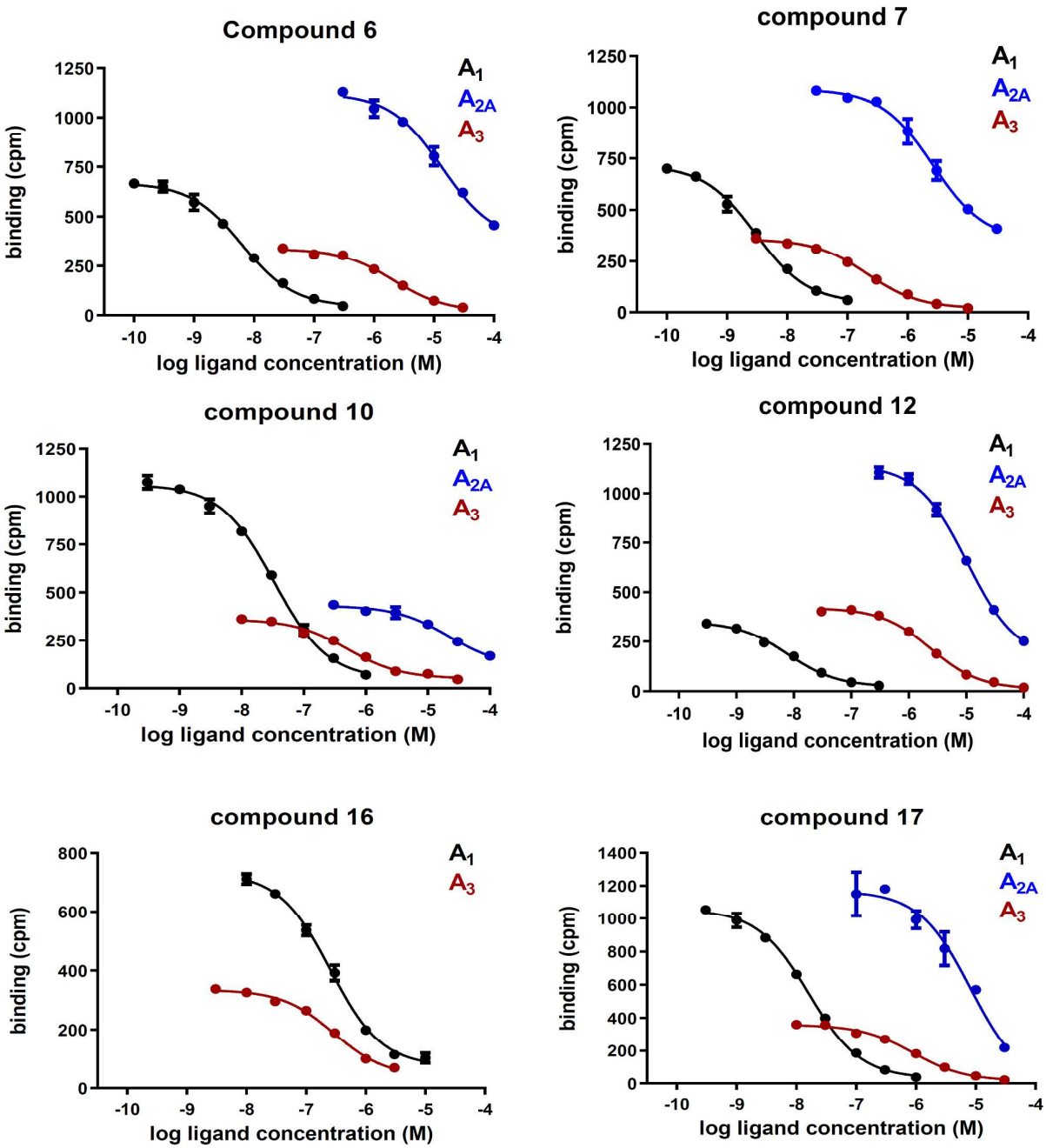

Figure S1. Representative binding curves at hA_1, hA_{2A} and hA_3 ARs for compounds **6**, **7**, **10**, **12**, **16** and **17**.

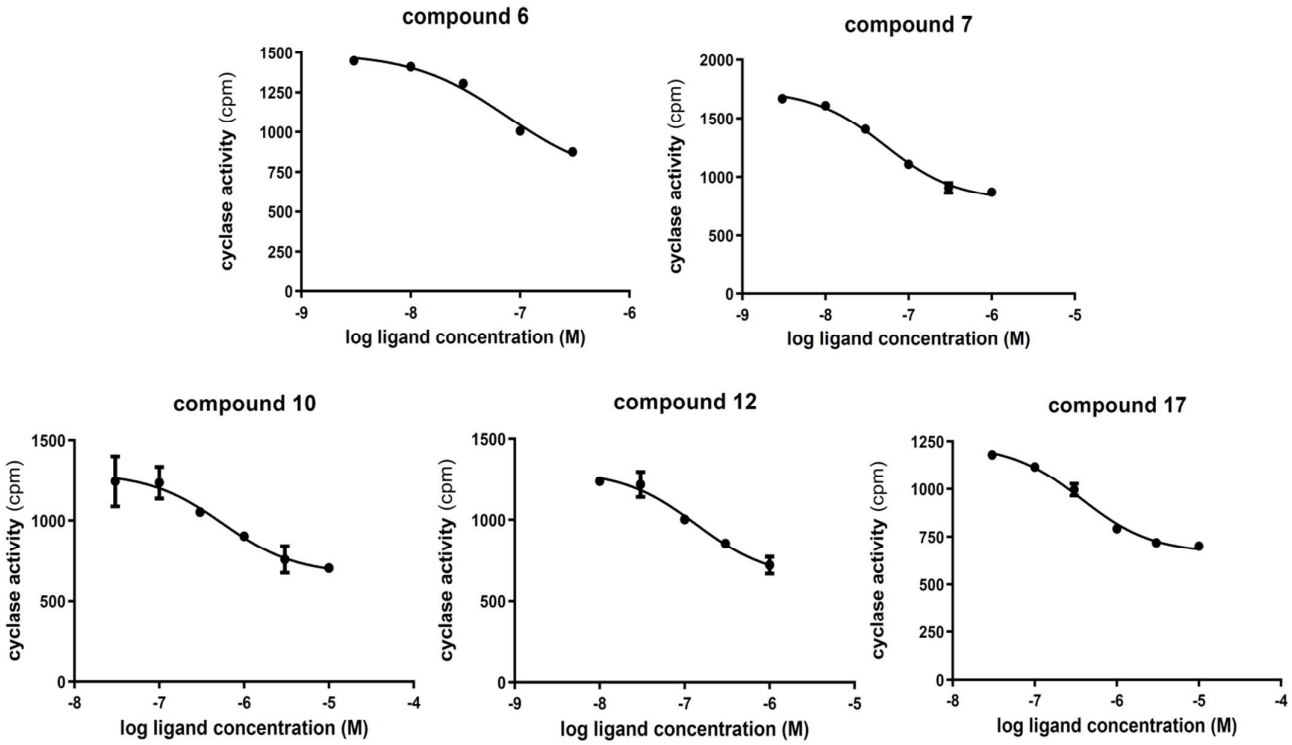

Figure S2. Effect of selected compounds on activity of adenylyl cyclase. **6, 7, 10, 12,** and **17** mediate an inhibition of foskolin-stimulated adenylyl cyclase activity via hA_1ARs. They show the same inhibition as the full agonist CCPA (not shown).

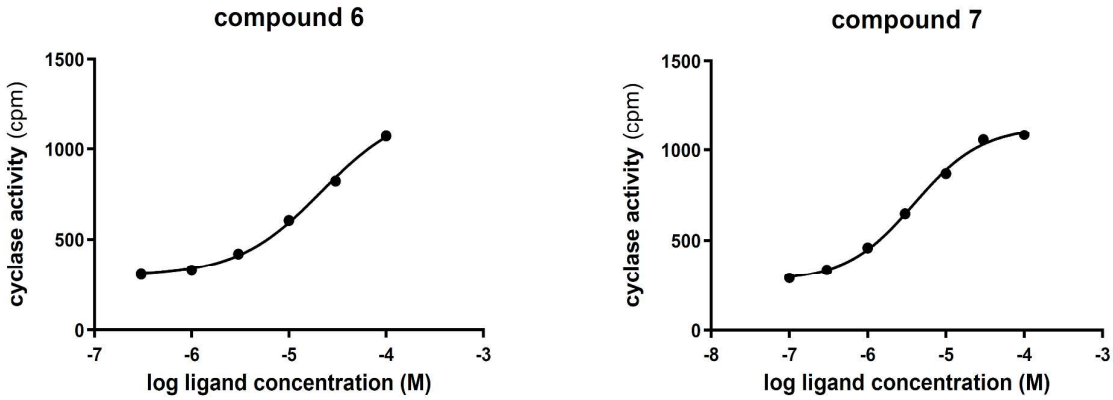

Figure S3. Effect of compounds **6** and **7** on activity of adenylyl cyclase at $hA_{2B}AR$.

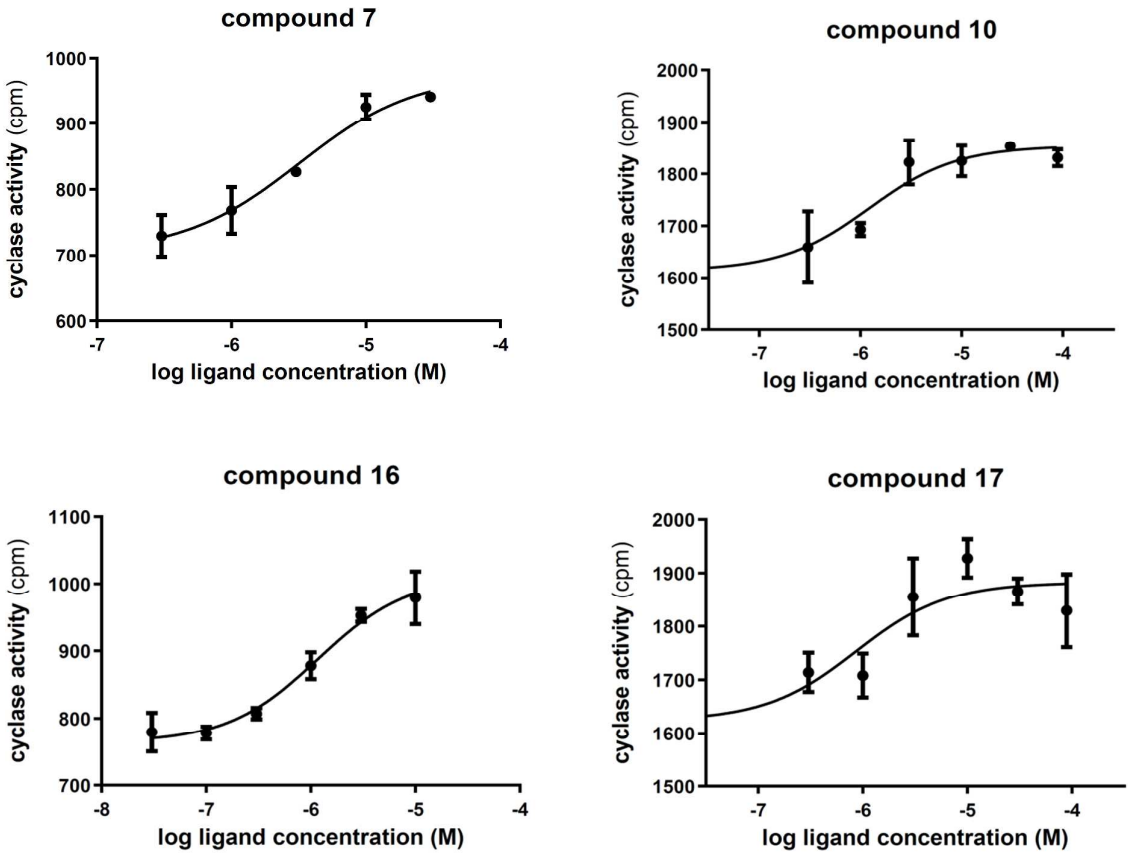

Figure S4. Effect of selected compounds on activity of adenylyl cyclase. **7, 10, 16,** and **17** present as antagonists at the hA_3AR. They fully reverse the NECA-induced inhibition of forskolin-stimulated adenylyl cyclase activity.

Cell culture

Chinese hamster ovary (CHO) cells stable transfected with hARs were grown in Dilbecco's modified Eagle's medium (DMME) with nutrient mixture F12 supplemented with 10% fetal bovine serum (FBS), 100U/ml penicillin, 100 μg/mL streptomycin, 2.5 μg/ml Amphotericin B, 0.1 mg/ml Geneticine and 1 mM sodium pyruvate. They were cultured at 37°C in a humidified atmosphere of 5% CO_2/95% air [1].

Membrane preparation

Membranes for radioligand binding were prepared as described earlier [1]. In brief, after homogenization of CHO cells in ice-cold hypotonic buffer, 5 mM Tris/HCl, 2 mM EDTA, pH 7.4 and stably transfected with the human adenosine receptor subtypes, membranes were prepared in a two-step procedure. A first low-speed centrifugation (1,000 x g) was used to remove cell fragments and nuclei and was followed by a high-speed centrifugation (100,000 x g) of the supernatant in order to sediment a crude membrane fraction. The resulting membrane pellets were resuspended in the specific buffer used for the respective binding experiments (hA_1ARs: 50 mM Tris/HCl buffer pH 7.4; hA_{2A}ARs: 50 mM Tris/HCl, 50 mM $MgCl_2$ pH 7.4; hA_3ARs: 50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM EDTA, pH 8.25), frozen in liquid nitrogen at a protein concentration of 2-4 mg/ml and stored in aliquots at -80°C. Adenylyl cyclase activity was measured in a membrane fraction obtained in a simplified procedure with only one high-speed centrifugation of the homogenate. The resulting crude membrane pellet was resuspended in 50 mM Tris/HCl, pH 7.4 and used immediately for the cyclase assay.

Radioligand Binding and Adenylyl Cyclase Assay.

In competition experiments, a fixed concentration of radioligand (1 nM [^{3}H]CCPA, K_D = 1.1 nM;10 nM [^{3}H]NECA, K_D = 20 nM; 1 nM [^{3}H]HEMADO, K_D = 1.5 nM) was incubated in a 96-well plate with 10 μg of membrane protein and increasing concentrations of the tested compound. Non-specific binding was determined in the presence of 1 mM theophylline for hA_1 AR and 100 μM (*R*)-N^6-phenyliso-propyladenosine (R-PIA) for both hA_{2A} AR and hA_3 AR. Samples were incubated at rt, utilizing the 96-well microplate filtration system Millipore Multiscreen MAFC. After 3 h free radioligand was separated from bound radioligand by filtration through the filter bottom of the microplates. The filters were washed three times with 200 μl of ice-cold binding buffer for the respective receptor subtype and subsequently dried. After the addition of 20 μl of scintillation cocktail, the bound radioactivity was determined using a Wallac Microbeta Trilux scintillation

counter. Dissociation constants (K_i values) were calculated by non-linear curve fitting with Prism 5.0 programme (GraphPAD Software, San Diego, CA, USA). Each concentration was tested in duplicate in atleast threeindependent experiments. K_i values are given as geometric means with 95% confidence intervals [1, 2, 4].

Due to the lack of a useful high-affinity radioligand for A_{2B} ARs, stimulation of adenylyl cyclase activity was measured to determine agonist potency (EC_{50} values) [1]. If only partial agonistic activity was observed, efficacy was compared to 100 μM NECA as a full agonist. All values are given as geometric means with 95% confidence intervals ($n \geq 3$). The functional activity at the hA_1 and hA_3 receptors was determined in adenylyl cyclase experiments. The inhibition of forskolin-stimulated adenylyl cyclase via hA_1 and A_3 receptors was measured as described in detail earlier [1,3]. As reference agonists (efficacy = 100%), CCPA [4] and NECA [3], respectively, were used. Compounds were considered to be A_3 antagonists if they fully reversed (>85%) the NECA-mediated inhibition of adenylyl cyclase activity.

[1] Klotz, K.-N.; Hessling, J.; Hegler, J.; Owman, B.; Kull, B.; Fredholm, B. B.; Lohse, M. J. Comparative pharmacology of human adenosine receptor subtypes-characterization of stably transfected receptors in CHO cells. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1998**, *357*, 1–9.

[2] Klotz, K.-N.; Kachler, S.; Falgner, N.; Volpini, R.; Dal Ben, D.; Lambertucci, C.; Mishra, R. C.; Vittori, S.; Cristalli, G. [^{3}H]HEMADO- a novel highly potent and selective radiolabeled agonist for A_3 adenosine receptors. *Eur. J. Pharmacol.* **2007**, *556*, 14–18.

[3] Klotz, K.-N.; Cristalli, G.; Grifantini, M.; Vittori, S.; Lohse, M. J. Photoaffinity labeling of A_1-adenosine receptors. *J. Biol. Chem.* **1985**, *260*, 14659-14664.

[4] Lohse, M. J.; Klotz, K.-N.; Schwabe, U.; Cristalli, G.; Vittori, S.; Grifantini, M. 2-Chloro-N^6-cyclopentyladenosine: a highly selective agonist at A_1 adenosine receptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **1988**, *337*, 687-689.