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BAY60-6583 acts as a partial agonist at adenosine A_{2B} receptors

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Abbreviations: ADA, adenosine deaminase; AR, adenosine receptor(s); BAY60-6583, 2-((6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-yl)sulfanyl)acetamide; BSA, bovine serum albumin; CCPA, 2-chloro-N⁶-cyclopentyladenosine; CGS-21680, (2-*p*-[2-carboxyethyl]phenethylamino)-5'-N-ethylcarboxamidoadenosine; CHO, Chinese hamster ovary; CI-IB-MECA, 2-chloro-N⁶-(3-iodobenzyl)-9-[5-(methylcarbamoyl)-β-D-ribofuranosyl]adenine; COPD, chronic obstructive pulmonary disorder; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; FCS, fetal calf serum; G418, geneticin; HA, human influenza haemagglutinin tag; hA_{2B} receptor, human A_{2B} receptor; HBSS, Hank's Balanced Salt Solution; HEK, human embryonic kidney; KO, knock-out; MSX-2, 3-(3-hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine; NECA, 5'-N-ethylcarboxamidoadenosine; PSB-10, 8-ethyl-1,4,7,8-tetrahydro-4-methyl-2-(2,3,5-trichlorophenyl)-5*H*-imidazo[2,1-*i*]purin-5-one; PSB-36, 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1*H*)-yl)-3,7-dihydro-3-(3-hydroxypropyl)-1*H*-purine-2,6-dione; PSB-601, 8-(4-(4-(4-chlorobenzyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine; PSB-603, 8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine; PSB-1115, 1-propyl-8-(4-sulfofenyl)xanthine; R-PIA, N⁶-((*R*)-2-phenylisopropyl)adenosine; Ro 20-1724, 4-(3-butoxy-4-methoxyphenyl)methyl-2-imidazolidone; RPMI, Roswell Park Memorial Institute; rt, room temperature; wt, wildtype.

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ABSTRACT

BAY60-6583 (2-((6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-yl)-sulfanyl)acetamide) is the most potent and selective adenosine A_{2B} receptor (A_{2B} AR) agonist known to date. It has therefore been widely used for *in vitro* and *in vivo* experiments. In the present study we investigated the binding and functional properties of BAY60-6583 in various native and recombinant cell lines with different A_{2B} AR expression levels. In cAMP accumulation and calcium mobilization assays, BAY60-6583 was found to be significantly less efficacious than adenosine or the adenosine derivative NECA. When it was tested in HEK293 cells, its efficacy correlated with the A_{2B} expression level of the cells. In Jurkat-T cells BAY60-6583 antagonized the agonistic effect of NECA and adenosine as determined in cAMP accumulation assays. Based on these results we conclude that BAY60-6583 acts as a partial agonist at adenosine A_{2B} receptors. At high levels of the physiological agonist adenosine BAY60-6583 may act as an antagonist and block the effects of adenosine at A_{2B} receptors. This has to be considered when applying the A_{2B} -selective “agonist” BAY60-6583 in pharmacological studies, and previous research results may have to be reinterpreted.

INTRODUCTION

Adenosine receptors (ARs) belong to the superfamily of G protein-coupled receptors and are divided into four subtypes designated A₁, A_{2A}, A_{2B} and A₃ (Fredholm et al., 2011). The A₁ and A₃ receptors signal through G_i (G₀) proteins thereby inhibiting adenylyate cyclase, while A_{2A} and A_{2B} ARs are coupled to G_s (or G_{olf}) proteins, that activate adenylyate cyclase (Fredholm et al., 2001). Coupling to other second messenger systems has also been described. For A_{2B} ARs calcium mobilization through G_q proteins via activation of phospholipase C_β appears to be quite common (Feoktistov et al., 1999; Linden et al., 1999; Panjehpour et al., 2005). In the human leukemia cell line Jurkat-T an increase in intracellular calcium concentrations independent of phospholipase C activation was described (Mirabet et al., 1997), but the mechanism involved is still unknown. A_{2B} ARs show a wide expression in many different cell types, organs and tissues (Aherne et al., 2010).

A_{2B} ARs appear to be involved in various diseases such as coronary artery disorders and atherosclerosis (Eckle et al., 2007; Toldo et al., 2012), asthma (Feoktistov and Biaggioni, 1996; Wilson et al., 2009; Walaschewski et al., 2013), pulmonary hypertension associated with interstitial lung disease (Karmouty-Quintana et al., 2012), liver ischemia and reperfusion injury (Zimmerman et al., 2013), colitis (Frick et al., 2009), type II diabetes (Volpini et al., 2003), and cancer (Ryzhov et al., 2008; Wei et al., 2013). Under pathophysiological conditions such as oxidative stress, inflammation, or in cancer an upregulation of A_{2B} AR expression was observed (Kong et al., 2006, Ma et al., 2010; Cekic et al., 2012). Therefore, A_{2B} ARs are of considerable interest as novel drug targets.

A large number of selective A_{2B} AR antagonists belonging to different chemical classes has been developed, and the xanthine derivative GS-6201 (CVT-6883, see Figure 1) was the first

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antagonist evaluated in phase I clinical trials for the treatment of asthma (Müller and Jacobson, 2011). 1-Propyl-8-*p*-sulfophenylxanthine (Hayallah et al., 2002) has been frequently used for in vivo studies due to its high water-solubility. The most potent and selective antagonist known so far is PSB-603 (Figure 1), which can also be used in tritium-labeled form as an A_{2B}-specific antagonist radioligand (Borrmann et al., 2009).

In contrast, only few A_{2B} AR agonists have been developed so far. They can be divided into adenosine-derived nucleosidic agonists (adenosine, see Figure 1), and non-adenosine-like, non-nucleosidic compounds. NECA was the first adenosine-derived A_{2B} agonist, although it is even more potent at all three other AR subtypes. An improved derivative, 1-deoxy-1-{6-[*N'*-(furan-2-carbonyl)hydrazino]-9*H*-purin-9-yl]-*N*-ethyl- β -D-ribofuranuronamide (Figure 1) was slightly more potent than NECA (**5**) (Baraldi et al., 2007).

The non-nucleosidic, non-adenosine-like compound BAY60-6583, a 2-aminopyridine-3,5-dicarbonitrile derivative (Figure 1), was discovered by Bayer Healthcare (Rosentreter et al., 2001). In recombinant human and mouse cell lines applying a reporter gene assay it displayed EC₅₀ values in the low nanomolar range combined with high selectivity versus the other AR subtypes (Rosentreter et al., 2001; Van der Hoeven et al., 2011). BAY60-6583 was investigated in a rabbit model of ischemia-reperfusion injury and found to exhibit cardioprotective effects (Krahn et al., 2006, Eckle et al., 2007, Kuno et al., 2007). Furthermore, BAY60-6583 was found to be kidney-protective (Grenz et al., 2008) and also protected from intestinal injury, inflammation and permeability dysfunction (Hart et al., 2009).

The activation of the A_{2B} AR has been reported to lead to either proinflammatory or anti-inflammatory effects. Several publications demonstrated anti-inflammatory effects mediated

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by BAY60-6583, e.g. in pulmonary inflammation and lung injury (Eckle et al., 2008b; Schingnitz et al., 2010', Chen et al., 2009). On the other hand the blockade of A_{2B} ARs was also shown to lead to anti-inflammatory effects. Therefore, A_{2B}-selective antagonists were proposed to provide a novel approach for the management and treatment of asthma and chronic obstructive pulmonary disease (Kalla et al., 2006; Elzein et al., 2008; Kalla et al., 2008), intestinal inflammation (Kolachala et al., 2008, El-Tayeb et al., 2011) and inflammatory pain (Bilkei-Gorzo et al., 2008).

The aim of the present study was to perform a detailed characterization of the binding and functional properties of BAY60-6583 (**6**) in human cell lines with various A_{2B} receptor expression profiles and levels. Since BAY60-6583 is the only potent and highly selective A_{2B} AR agonist known so far, which is widely used not only in *in vitro* but also in animal studies, a full and thorough *in vitro* characterization of the compound is indispensable, especially in view of the fact that the role of the A_{2B} AR in inflammation, is highly controversial.

METHODS

Materials. Cell culture media, RPMI-1640, DMEM, DMEM-F12 and penicillin-streptomycin solutions were obtained from Invitrogen (Darmstadt, Germany). FCS and G418 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Oregon Green BAPTA-1/AM was obtained from Invitrogen (Darmstadt, Germany). All other chemicals were purchased from Sigma-Aldrich (Taufkirchen, Germany), or Roth (Karlsruhe, Germany), unless otherwise stated. BAY60-6583 was kindly provided by Dr. Thomas Krahn (Bayer Healthcare, Wuppertal, Germany). Jurkat-T cells were a gift from Prof. Dr. K. Schilling, Anatomic Institute, University of Bonn, Germany. HEK293 and CHO-K1 cells were purchased from the European Tissue Culture Collection (Salisbury, UK). The retroviral transfection vector pLXSN and the packaging cell line GP+envAM12 were provided by Prof. Robert A. Nicholas, Department of Pharmacology, University of North Carolina at Chapel Hill, (USA). Bovine adrenals were obtained from a local abattoir. [³H]cAMP was purchased from Hartmann Analytics (Braunschweig, Germany).

Retroviral transfection. HEK293 and CHO-K1 cells stably transfected with the human A_{2B} AR were generated with a retroviral transfection system. Packaging cells (1.5×10^6 GP+envAM12) were plated into 25 cm² cell culture flasks 24 h before transfection and grown in 5 ml DMEM medium containing 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 1 % ultraglutamine and 0.2 mg/ml hygromycin B. A few hours before transfection, the medium was replaced with 6.25 ml DMEM medium supplemented with 10 % FCS, and 1 % ultraglutamine without antibiotics. Receptor DNA (6.75 µg of pLXSN-hA_{2B}) and 3.75 µg of a vesicular stomatitis virus G protein (VSV-G), which pseudotypes the generated viruses and therefore increases their infection efficiency, were cotransfected. The transfection reagent Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) was used in a ratio of 1 : 2.5 (DNA :

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Lipofectamine). After 12-15 h of incubation, the medium was removed and replaced with 3 ml of DMEM medium containing 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 % ultraglutamine. Additionally, 30 µl of 500 mM sodium butyrate dissolved in water were added to the flasks and the cells were incubated for 48 h at 32°C, 5 % CO₂. Then, the supernatants (3 ml) which contained the virus were filtered and transferred into 25 cm² cell culture flasks of ~ 70 % confluent CHO-K1, or HEK293 cells, respectively. A polybrene solution (6 µl, 4 mg / ml in water) was added. After an infection time of 2.5 h at 32°C, 5 % CO₂, the medium was removed and replaced with 6 ml DMEM/F12 supplemented with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 % ultraglutamine for CHO cells, or replaced with 6 ml DMEM medium containing 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin for HEK293 cells. After 48 h, cells were selected by adding 0.8 mg/ml of G418 to the cell culture medium. After one week the G418 concentration was reduced to 0.2 mg/ml.

Cell culture. GP+envAM12 packaging cells were cultured at 37°C, 5 % CO₂ in HXM medium which consisted of DMEM, 10 % FCS, 100 U/ml penicillin G, 100 µg/ml streptomycin, 1 % ultraglutamine, 0.2 mg/ml hygromycin B, 15 µg/ml hypoxanthine, 250 µg/ml xanthine and 25 µg/ml mycophenolic acid. CHO cells were maintained in DMEM/F12 medium with 10 % FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 1 % ultraglutamine under the same conditions. CHO cells stably transfected with the human A_{2B} AR were maintained at 37°C and 5 % CO₂ in the same medium, however 0.2 mg/ml G418 was added. Jurkat T cells were grown at 37°C and 5 % CO₂ in RPMI-1640 medium supplemented with 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. HEK293 cells were maintained at 37°C and 5 % CO₂ in DMEM medium containing 10 % FCS, 100 U/ml penicillin and 100 µg/ml streptomycin. HEK293 cells stably transfected with the human A_{2B} receptor were cultured in the same medium but in the presence of 0.2 mg/ml G418.

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Membrane preparations. Membranes of CHO cells stably expressing the human A_{2B} receptor were prepared as previously described (Borrmann et al., 2009). The protein concentration was determined by the method of Lowry.

Radioligand binding experiments. Radioligand binding experiments with [³H]PSB-603 were performed as previously described (Borrmann et al., 2009). Sodium shift experiments (Bertarelli et al., 2006) were performed in a final volume of 500 µl containing 25 µl of test compound dissolved in 50 % DMSO / 50 % TRIS-HCl buffer (50 mM, pH 7.4), 150 µl buffer (50 mM TRIS-HCl, pH 7.4), 125 µl buffer (400 mM NaCl dissolved in 50 mM TRIS-HCl, pH 7.4), 100 µl of radioligand solution in 50 mM TRIS-HCl buffer pH 7.4 (final radioligand concentration 0.3 nM) and 100 µl of membrane preparation, 30 µg of protein per tube, in TRIS-buffer containing 2 U/mL ADA (20 min preincubation at rt). Nonspecific binding was determined in the presence of 10 µM (final concentration) 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). After an incubation time of 75 min (at rt), the assay mixture was filtered through GF/B glass fiber filters using a Brandel harvester (Brandel, Gaithersburg, USA). Filters were washed four times (3-4 mL each) with ice-cold 50 mM TRIS-HCl buffer, pH 7.4, containing 0.1 % BSA. Then the filters were transferred into minivials, incubated for 9 h with 2.5 mL of scintillation cocktail (LumaSafe plus, Perkin-Elmer), and counted in a liquid scintillation counter (Tricarb 2700TR, Perkin Elmer, Rodgau, Germany) with a counting efficiency of ~53 %.

Preparation of cAMP binding protein. The preparation of cAMP binding protein was carried out as previously described (Nordstedt and Fredholm, 1990).

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cAMP accumulation in Jurkat-T, HEK293 and HEK-hA_{2B} cells. Cells were removed from two confluent 175 cm² flasks, transferred into a 50 ml falcon tube and centrifuged at 200 g, 4°C for 5 min. After removal of the supernatant, the cell pellet was resuspended in HBSS-buffer, pH 7.4. The cell suspension (200 µl, ~ 300.000 cells per well) containing 2 U/ml of ADA was transferred to 24-well plates. When adenosine was used as an agonist, ADA was omitted. After an incubation time of 1.5 h at 37°C, 5 % CO₂, 25 µl of a solution containing the phosphodiesterase (PDE) inhibitor Ro20-1724 (final concentration 40 µM) dissolved in 100 % HBSS-puffer was added to each well. After 10 min of incubation at 37°C, 12.5 µl of antagonist solution, and after another 10 min of preincubation, 12.5 µl of agonist dissolved in HBSS-buffer containing 10 % DMSO, were added. For assaying agonists 25 µl of test compound was added. For the testing of NECA in the presence of BAY60-6583 both compounds were applied simultaneously. The cells were stimulated with agonist for 15 min at 37°C; the final DMSO concentration did not exceed 1.4 %. cAMP accumulation was stopped by lysing the cells with 250 µL of hot lysis buffer (90°C, 8 mM EDTA, 0.02 % Triton X-100, pH 7.3). The 24-well plates were put on ice and each well was subsequently homogenized by pipetting 6-7 times up and down. For competition binding experiments 50 µl of the cell lysates were transferred into 2.5 ml tubes. Then 30 µl of [³H]cAMP (3 nM final concentration) in lysis buffer (4 mM EDTA, 0.01 % Triton X-100, pH 7.3) and 40 µl of cAMP binding protein in the same buffer (50 µg protein per vial) were added. Total binding was determined with 50 µl of lysis buffer (4 mM EDTA, 0.01 % Triton X-100, pH 7.3), 30 µl of [³H]cAMP solution and 40 µl of cAMP binding protein containing solution. Non-specific filter binding was determined with 90 µl of lysis buffer (4 mM EDTA, 0.01 % Triton X-100, pH 7.3) and 30 µl of [³H]cAMP solution. For a cAMP standard curve 50 µl of known cAMP concentrations were used instead of 50 µl of cell lysate. After an incubation time of 1 h on ice the assay mixture was filtered through GF/B glass fiber filters using a Brandel harvester

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(Brandel, Gaithersburg, USA). Filters were washed three times with ice-cold 50 mM TRIS-HCl buffer, pH 7.4. Then filters were transferred into minivials, incubated for 9 h with 2.5 mL of scintillation cocktail (LumaSafe plus, Perkin-Elmer), and counted in a liquid scintillation counter (Tricarb 2700TR, Perkin Elmer, Rodgau, Germany) with a counting efficiency of ~53 %.

cAMP accumulation in CHO-HA-hA_{2B} cells.

CHO cells were seeded into a 24-well plate at a density of 200,000 cells/well 24 h before performing cAMP assays. Cells were washed with Hank's Balanced Salt Solution (HBSS; 20 mM HEPES, 135 mM NaCl, 5.5 mM glucose, 5.4 mM KCl, 4.2 mM NaHCO₃, 1.25 mM CaCl₂, 1 mM MgCl₂, 0.8 mM MgSO₄, 0.44 mM KH₂PO₄ and 0.34 mM Na₂HPO₄, pH adjusted to 7.3) with 1 U/ml of ADA. Then the cells were incubated in 300 µl HBSS at 37 °C and 5 % CO₂ for 2 h. The PDE inhibitor Ro20-1724 (100 µl, final concentration: 40 µM) was added to the CHO cells, which were subsequently incubated for 15 min at 37 °C and 5 % CO₂. Then 100 µl of various dilutions of agonist, 5'-N-ethylcarboxamidoadenosine and BAY60-6583 respectively, in HBSS containing 5 % DMSO were added and the cells were incubated for 15 min under the same conditions as described above. cAMP formation was stopped by removing the supernatant and subsequently lysing the cells by the addition of 500 µl of hot lysis buffer (90°C; 4 mM EDTA, 0.01 % Triton X-100, pH adjusted to 7.3). After one hour of incubation on ice, cAMP amounts of the lysates were determined by competitive radioligand binding experiments. Competition experiments were performed with aliquots of 50 µl of cell lysates, 30 µl of [³H]cAMP solution in lysis buffer (final concentration 3 nM) and 40 µl of cAMP binding protein diluted in the same buffer (50 µg of protein per vial). For determining cAMP concentrations, instead of cell lysate 50 µl of cAMP solutions containing various cAMP concentrations were added to obtain a standard calibration curve. Total binding

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was determined in the absence of cAMP and non-specific binding was measured in the absence of binding protein. The mixture was incubated for 60 min on ice and then filtered through GF/B glass fiber filters using a cell harvester (Brandel, Gaithersburg, USA). Filters were washed three times with 2-3 ml of ice-cold 50 mM TRIS-HCl buffer, pH 7.4. Radioactivity on the filters was determined in a liquid scintillation counter (TRICARB 2900TR, Packard / Perkin-Elmer) after 9 h of preincubation with 2.5 ml of scintillation cocktail (LumaSafe plus, Perkin-Elmer). Three to five independent experiments were performed, each in duplicates. Amounts of cAMP were calculated by linear regression from a standard curve and normalized to the maximal effect induced by NECA (set at 100 %).

Calcium mobilization in Jurkat-T, HEK293 and HEK-hA_{2B} cells. Ca²⁺ assays were performed using a NOVOstar® plate reader (bmG LabTechnologies, Offenburg, Germany). Cells were removed from two confluent 175 cm² flasks, transferred into a 50 ml falcon tube and incubated for 1 h at 37°C, 5 % CO₂. After incubation, cells were centrifuged (200 g at 4°C for 5 min) and the cell pellet was resuspended in 994 µl of Krebs-HEPES buffer (pH 7.4) containing NaCl 118.6 mM, KCl 4.7 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 4.2 mM, D-glucose 11.7 mM, HEPES (free acid) 10 mM, CaCl₂ 1.3 mM and MgSO₄ 1.2 mM. Then 3 µl of a 1 mM solution of Oregon Green BAPTA-1/AM, was mixed with 3 µl of a 20 % solution of Pluronic F-127 in DMSO and the cell suspension (994 µl) was added. The mixture was incubated for 1 h at rt while continuously rotating the tube under exclusion of light. After incubation, cells were washed three times with 1 ml of Krebs-HEPES buffer each, and diluted in 16 ml of Krebs-HEPES buffer with 2 U/ml ADA (except for experiments with adenosine where no ADA was added). Then 160 µl of the cell suspension (~100,000 cells per well) were transferred into each well of a 96-well plate (Greiner, Frickenhausen, Germany). For antagonist testing, 20 µl of a 10-fold concentrated test compound solution in 10 % DMSO

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were added to 160 μ l of the cell suspension, and for agonist testing 20 μ l of a 90 % Krebs-HEPES buffer / 10 % DMSO mixture were added to 160 μ l of the cell suspension. The plates were incubated for 45 min at rt under exclusion of light. During this time 30 μ l of a 10-fold concentrated agonist solution in 10 % DMSO was pipetted into each well of a 96-well injection plate. When the measurement started, 20 μ l of the agonist solution was injected sequentially into the wells, and fluorescence was measured at 520 nm (bandwidth 25 nm) for 60 intervals (0.4 s each) for HEK293, or HEK-hA_{2B} cells, respectively. Jurkat-T cells were measured for 250 intervals of 0.4 s each because of their delayed calcium-response. The excitation wavelength was 485 nm (bandwidth 20 nm).

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RESULTS

Radioligand binding studies

In order to determine the A_{2B} AR affinity of the agonist BAY60-6583, radioligand binding studies were performed at human ARs recombinantly expressed in CHO cells (CHO-A_{2B}) using the A_{2B} antagonist radioligand [³H]PSB-603 (Table 1) (Borrmann et al., 2009). Data obtained in the same assay for the agonist NECA are provided for comparison (Table 1). In antagonist radioligand binding studies, BAY60-6583 showed high affinity for human (K_i 212 nM) A_{2B} ARs. The non-selective A_{2B} agonist NECA displayed a 5-to 11-fold lower affinity than BAY60-6583 in binding studies versus the antagonist radioligand [³H]PSB-603 (Schiedel et al., 2011).

The curves of agonists of the G_s protein-coupled AR subtypes A_{2A} and A_{2B} determined with an antagonist radioligand are typically shifted to the right in the presence of sodium chloride (100 mM) (Gao and Ijzerman, 2000; Bertarelli et al., 2006; Liu et al., 2012). As expected, the curve for the agonist NECA showed a significant, 8-fold rightward shift in competition binding experiments at CHO-hA_{2B} cell membranes versus 0.3 nM [³H]PSB-603 in the presence of 100 mM sodium chloride (***p* < 0.01, Figure 2A, 2B). When an analogous experiment was performed for the agonist BAY60-6583 only a very slight, insignificant shift could be observed (Figure 2A, 2B). Similarly, the curve for the antagonist PSB-603 did not show any significant shift in the presence of 100 mM sodium chloride (Figure 2A, 2B).

cAMP accumulation assays at recombinant CHO cells

cAMP accumulation assays were performed at CHO cells stably transfected with the hemagglutinin- (HA-) tagged human A_{2B} AR (CHO-HA-hA_{2B} cells). It had previously been shown, that an HA-tag at the N-terminus of the A_{2B} AR neither affected receptor function nor

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binding affinity (Schiedel et al., 2011). Concentration-response curves are shown in Figure 3. While BAY60-6583 ($EC_{50} = 98.7$ nM) was slightly more potent than NECA ($EC_{50} = 318$ nM), NECA was significantly more efficacious than BAY60-6583, the latter showing only about 56 % of the maximal effect of NECA (set at 100%) (Figure 3).

cAMP accumulation in HEK293 cells

Next, we investigated the efficacies of the A_{2B} AR agonists in human embryonic kidney (HEK293) cells, which are natively expressing a low level of A_{2B} ARs (Mundell et al., 1999). We confirmed that HEK293 cells were activated by NECA via A_{2B} , but not via A_{2A} ARs. The non-selective agonist NECA (5 μ M) induced a cAMP signal in nontransfected HEK293 cells which was dose-dependently blocked by the A_{2B} -antagonist PSB-603 (10, 100, 1000 nM), but not by a high concentration (200 nM) of the A_{2A} -antagonist MSX-2 (Supplemental Figure 1). In contrast, the A_{2A} -selective agonist CGS21680 induced only a very small signal at high concentrations of 1 and 10 μ M (Supplemental Figure 1).

Comparing efficacies at native HEK cells, NECA showed the highest efficacy (set at 100 %) followed by adenosine (64 %). Even at high concentrations of up to 100 μ M, BAY60-6583 showed virtually no receptor activation (6 % of the maximal effect of NECA) (Figure 4).

To examine whether the low intrinsic activity of BAY60-6583 depended on the A_{2B} AR expression level, we additionally performed cAMP assays in HEK293 cells that were stably transfected with human A_{2B} receptor and therefore expressed a higher receptor density. In this recombinant HEK-h A_{2B} cell line we determined the A_{2B} AR expression level by homologous competition binding studies using PSB-603 versus the radioligand [3 H]PSB-603. Thereby, a B_{max} value of 131 ± 6 fmol/mg protein was determined, whereas the expression level was too

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low in native, non-transfected HEK cells to be detectable by radioligand binding (see Supplemental Figures 4 and 5). In saturation experiments with the radioligand [³H]MRE2029-F20 a B_{max} value of 450 fmol / mg protein had previously been determined for another A_{2B}-transfected HEK293 cell line (Gessi et al. 2005). In our recombinant HEK293-hA_{2B} cell line, BAY60-6583 was clearly more efficacious than in non-transfected HEK293 cells, but it still showed lower efficacy than NECA and adenosine. The rank order of efficacy was as follows: adenosine (100 %, EC₅₀ 2510 nM) > NECA (100 %, EC₅₀ 200 nM) > BAY60-6583 (49 %, EC₅₀ 505 nM) (Figure 5).

Calcium mobilization in HEK293 cells

In order to investigate a potential bias of BAY60-6583 towards a certain signaling pathway we investigated its potency and efficacy in activating the G_q-coupled signalling pathway by measuring intracellular calcium mobilization. The non-selective agonists NECA (5 μM) and adenosine (10 μM) were able to stimulate Ca²⁺ mobilization in native HEK293 cells, while the A_{2A}-selective agonist CGS21680 (10 μM) failed to induce such a response. Also, the A₃-selective agonist Cl-IB-MECA (200 nM) and the A₁-selective agonist CCPA (0.2 μM) did not generate any effect (Supplemental Figure 2). The selective A_{2B} antagonist PSB-603 significantly blocked the calcium signal induced by 5 μM NECA, which could however, neither be blocked by the selective A_{2A} antagonist MSX-2 (200 nM), nor by the A₁ antagonist PSB-36 (10 nM), nor the A₃ antagonist PSB-10 (200 nM) indicating that it was mediated by the A_{2B} AR (see Supplemental Figure 2).

In contrast to adenosine and NECA, BAY60-6583 only showed a small effect on calcium mobilization in native, non-transfected HEK293 cells. The efficacies were as follows: NECA (set at 100 %) = adenosine (100 %) >> BAY60-6583 (1 %) (Figure 6).

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In recombinant HEK-hA_{2B} cells, which expressed higher levels of A_{2B} ARs compared to nontransfected cells, BAY60-6583 (E_{max}: 57 %) was considerably more efficacious in Ca²⁺ mobilization assays than in non-transfected HEK293 cells, but it was still significantly less efficacious than NECA (set at 100 %) or adenosine (98 %) (Figure 7). NECA (EC₅₀ 438 nM) and BAY60-6583 (EC₅₀ 579 nM) were similarly potent, whereas adenosine (EC₅₀ 990 nM) was slightly weaker.

cAMP accumulation assays in Jurkat-T cells

Jurkat-T cells appear to express a relatively high level of A_{2B} ARs whereas the expression levels of the other AR subtypes seems to be much lower or negligible (Mirabet et al., 1997). We found that the non-selective AR agonist NECA (10 μM) strongly induced cAMP accumulation in Jurkat-T cells, whereas the selective A_{2A} AR agonist CGS21680 had no significant effect at a high concentration of 10 μM (Figure 8). The NECA signal was blocked by the highly selective A_{2B} AR antagonist PSB-603 in a concentration-dependent manner (Figure 8) indicating that it was A_{2B}-mediated. The A_{2B}-selective agonist BAY60-6583 (10 μM), however, had only little effect on cAMP accumulation in Jurkat-T cells (Figure 8). In order to investigate the optimal time frame for cAMP accumulation measurements in Jurkat-T cells, we performed kinetic experiments (incubation times of 2, 5, 10, 30 and 60 min) using two different concentrations of BAY60-6583, 1 and 10 μM. Our results showed that the effect of BAY60-6583 on cAMP accumulation could not be increased by longer incubation times of up to 1 h (Supplemental Figure 3).

A partial agonist will act as an antagonist in the presence of a more efficacious agonist. To examine possible A_{2B} AR-antagonistic effects of BAY60-6583 in Jurkat-T cells versus the full agonists NECA and adenosine, the cells were incubated with a high concentration of

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NECA (10 μ M), or adenosine (250 μ M), respectively, in the presence of increasing concentrations of BAY60-6583. BAY60-6583 showed a concentration-dependent inhibition of the agonists' effects with an IC_{50} value of 7500 ± 1400 nM (versus 10 μ M NECA) and an IC_{50} -value of 25100 ± 5600 nM (versus 250 μ M adenosine) (Figure 9A). BAY60-6583 (100 μ M) inhibited NECA- (10 μ M) induced cAMP accumulation by approximately 82 % and adenosine-induced (250 μ M) cAMP accumulation by 71 %.

To confirm these antagonistic effects, we further determined the K_B -value of BAY60-6583 versus the full agonist NECA. Stimulation of cAMP accumulation by NECA in Jurkat-T cells was measured in the absence and in the presence of 5 μ M BAY60-6583. NECA alone showed an EC_{50} -value of 3810 ± 560 nM whereas the EC_{50} -value of NECA was increased in the presence of 5 μ M BAY60-6583 to 52900 ± 7000 nM (** $p < 0.01$) (Figure 9B). The curve was shifted to the right in a parallel manner. Using the Schild equation a K_B -value of 393 ± 48 nM was calculated. Thus, the determined K_B -value for BAY60-6583 at Jurkat-T cells was in close agreement with the corresponding K_i -value (212 nM) determined in radioligand binding assays using the A_{2B} antagonist radioligand PSB-603 at membrane preparations of CHO cells stably expressing human A_{2B} ARs (Schiedel et al., 2011).

Calcium assays at Jurkat-T cells

Finally, we also performed calcium mobilization assays in Jurkat-T cells. The intracellular calcium release induced by A_{2B} AR activation in Jurkat-T cells had previously been shown not to be mediated by G_q proteins (Mirabet et al., 1997). The calcium release is relatively slow compared to a G_q -mediated calcium signal (Borrmann et al., 2009). We had previously shown, that the calcium signal in Jurkat-T cells induced by 10 μ M NECA could be almost completely inhibited by the A_{2B} antagonist PSB-603, and the residual minor component could

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be blocked by the selective A_{2A} antagonist MSX-2 (Borrmann et al., 2009). Therefore, the calcium mobilization experiments were performed in the presence of MSX-2 (200 nM) to block possible, but minor A_{2A} receptor-mediated effects. While NECA and adenosine showed strong effects in stimulating intracellular calcium release in Jurkat-T cells, BAY60-6583 induced only a small signal (Figure 10). Control experiments in the absence of the A_{2A} antagonist MSX-2 gave the same results (data not shown).

Tables 1 summarizes all determined E_{max} - and EC_{50} -values for NECA, BAY60-6583 and adenosine determined in cAMP and calcium assays at various cell lines.

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Discussion

BAY60-6583, an aminopyridine derivative developed by Bayer (Rosentreter et al., 2001), is the only currently available potent and selective A_{2B} AR agonist. In contrast to all previously described agonists, which are derived from adenosine, it possesses a non-nucleosidic structure. The sugar moiety had previously been believed to be essential for agonism at ARs, while non-nucleosides, e. g. simple adenine derivatives, were found to be antagonists at ARs. We have now undertaken a thorough characterization of this widely applied A_{2B} AR agonist.

In radioligand binding studies using an antagonist radioligand (Auchampach et al., 2009; Schiedel et al., 2011; Seibt et al., 2013), BAY60-6583 showed significantly higher affinity than NECA. This was surprising given the fact that in functional assays BAY60-6583 and NECA had been found to be almost equipotent (Peeters et al., 2010; Schiedel et al., 2011; Seibt et al., 2013; Thimm et al., 2013). To further examine the pharmacological profile of BAY60-6583, we performed sodium shift experiments. Sodium ions shift the equilibrium of receptor conformations to an inactive state to which agonists show reduced affinity (Bertarelli et al., 2006). Our experiments demonstrated, that the curves for NECA showed a significant rightward shift in the presence of sodium chloride (100 mM), while the curve of the antagonist PSB-603 was unaltered. However, to our surprise, the curve for BAY60-6583 was only slightly shifted (Figure 2A, 2B). BAY60-6583 actually behaved more like an antagonist than an agonist.

We subsequently investigated the efficacy of BAY60-6583 in various cell lines with different human A_{2B} AR expression levels, including CHO cells recombinantly expressing the A_{2B} AR, native HEK cells with a low expression level, recombinant HEK cells expressing a higher A_{2B} AR level, and Jurkat-T cells which natively express A_{2B} ARs. In all functional assays in

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which we determined EC₅₀ values BAY60-6583 and NECA were similarly potent, while adenosine was somewhat less potent, in agreement with literature data (Thimm et al., 2013). However, when we looked at the efficacies of the agonists we found that - while NECA and adenosine were both highly efficacious in all assays - BAY-60-6583 displayed huge differences depending on cell line and assay.

In cAMP accumulation assays at CHO-HA-hA_{2B} cells, NECA showed significantly higher efficacy compared to BAY60-6583 (Figure 3). To examine, whether this partial agonistic effect of BAY60-6583 was dependent on receptor expression levels, we compared effects in native HEK293 cells with those in HEK293 cells stably transfected with the human A_{2B} AR using both, cAMP accumulation and calcium mobilization assays. HEK293 cells were described to natively express A_{2B} receptors which signal to G_s and G_{q/11} proteins (Cooper et al., 1997; Gao et al., 1999; Linden et al., 1999). We confirmed that NECA (5 μM) could induce a cAMP signal in HEK293 cells, while the A_{2A}-selective agonist CGS21680 gave only a slight signal in concentrations up to 10 μM. Moreover the highly selective and potent A_{2B} antagonist PSB-603 could significantly block the cAMP response induced by 5 μM NECA while the selective and potent A_{2A} antagonist MSX-2 (200 nM) had no significant antagonistic effect on cAMP production induced by 5 μM NECA, 10 μM adenosine, or 10 μM BAY60-6583, respectively (Supplemental Figure 1). These results confirmed that mainly A_{2B} receptors play a role in activating G_s proteins in HEK293 cells. When we compared the intrinsic efficacies of NECA, adenosine and BAY60-6583, BAY60-6583 only showed a small effect on cAMP production in HEK293 cells (Figure 4). In HEK293 cells that had been additionally transfected with the human A_{2B} receptor to express a higher receptor level, BAY60-6583 was more efficacious than in non-transfected HEK293 cells, but it was still significantly less efficacious than NECA and adenosine (Figure 5). As expected, the partial

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agonistic effect of BAY60-6583 is more observable in cell lines, which are lacking a receptor reserve, than in cells with high receptor expression. As described earlier (Gao et al., 1999), NECA can activate endogenous A_{2B} receptors in HEK293 cells and thereby induce Ca^{2+} mobilization. We confirmed, that NECA (5 μ M) and adenosine (10 μ M) stimulated Ca^{2+} mobilization, whereas the A_{2A} agonist CGS21680 did not. Likewise, the A_3 agonist Cl-IB-MECA (0.2 μ M) and the A_1 agonist CCPA (0.2 μ M) were inactive. Only the selective A_{2B} antagonist PSB-603 could significantly and nearly totally block the calcium signal induced by 5 μ M NECA, which could not be blocked by the selective A_{2A} antagonist MSX-2 (0.2 μ M), the A_1 antagonist PSB36 (0.01 μ M), or the A_3 antagonist PSB-10 (0.2 μ M) (Supplemental Figure 2). Based on these results, we conclude, that only A_{2B} receptors lead to calcium mobilization in HEK293 cells. When we compared the intrinsic efficacies, BAY60-6583 had only little effect on Ca^{2+} mobilization, whereas NECA and adenosine showed large effects (Figure 6). We again tested HEK293 cells which were transfected with human A_{2B} receptors, and in this overexpressing cell line BAY60-6583 was more efficacious than in native HEK293 cells. However, it was still significantly less efficacious than NECA and adenosine, consistent with a partial agonistic effect of BAY60-6583 (Figure 7).

As described earlier (Fredholm et al., 1987), NECA is able to induce a cAMP signal in Jurkat-T cells, while CGS21680 had no effect at concentrations up to 100 μ M (Van der Ploeg et al., 1996). We confirmed these findings and showed that the selective A_{2B} AR antagonist PSB-603 (Borrmann et al., 2009) could nearly totally block the cAMP response induced by 10 μ M NECA (Figure 8). These results indicated that the cAMP signal induced by NECA in the Jurkat-T cell line that we investigated, was mainly due to activation of A_{2B} ARs. Furthermore, only A_{2B} AR expression, but not that of other AR subtypes, had been detected on Jurkat-T cells by radioligand binding in a study by Mirabet et al. (Mirabet et al., 1997). Using a more sensitive detection method, namely RT-PCR, we had detected the presence of mRNA for all

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four AR subtypes in the same Jurkat T cell line (Schiedel et al., 2013), with the lowest expression level found for the A₃ AR. In contrast, Gessi et al. (2001) detected a high density of A₁ and A₃ AR, and a low density of A₁ AR subtypes on Jurkat-T cells by radioligand binding studies (Gessi et al., 2001). We conclude that it is likely that different clones of Jurkat-T cells with different AR expression profiles may exist. Curiously BAY60-6583 (10 μM) had only little effect on cAMP accumulation in Jurkat-T cells (Figure 8). To examine whether the low efficacy of BAY60-6583 was due to the fact, that the incubation time of 10 min may not have been long enough, we measured a time course of cAMP accumulation ranging from 2 to 60 min (Supplemental Figure 3). BAY-60-6583 was virtually inactive at each time point.

For partial agonists it is well known, that they can act as agonists or as antagonists, depending on the level of receptor expression and activation. Our results showed that BAY60-6583 was able to block NECA- (10 μM) as well as adenosine- (250 μM) induced cAMP accumulation in Jurkat-T cells with IC₅₀-values of 7500 ± 1400 nM, and of 25100 ± 5600 nM, respectively (Figure 9A). NECA alone showed an EC₅₀-value of 3810 ± 560 nM, which is consistent with previously published data at Jurkat-T cells (Van der Ploeg et al., 1996). Its EC₅₀ value was shifted to 52900 ± 7000 nM in the presence of 5 μM BAY60-6583 (Figure 9B). The calculated K_B-value of 393 ± 48 nM is in close agreement with the K_i-value (212 nM) determined for BAY60-6583 in binding assays versus the A_{2B} antagonist radioligand [³H]PSB-603. We had found that the calcium signal induced in Jurkat-T by 10 μM NECA cells could be almost completely blocked by the A_{2B} antagonist PSB-603 (Borrmann et al., 2009); a very small residual signal was blocked by the A_{2A} antagonist MSX-2. To compare intrinsic activities of NECA, adenosine and BAY60-6583 at A_{2B} ARs, the A_{2A} antagonist MSX-2 (200 nM) was added to each experiment in order to eliminate A_{2A} AR responses. Our

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results showed, that NECA and adenosine elicited large calcium signals of similar magnitude, while BAY60-6583 displayed only low efficacy consistent with partial agonism (Figure 10).

Partial agonists are believed to stabilize a conformation of the receptor, which only leads to a submaximal cellular response (Maudsley et al., 2005, Simons et al., 2004). Another theory implies that partial agonists may not bind long enough at the receptor to induce a maximum response (Hoeren et al., 2008). Our data indicate that BAY60-6583 may stabilize a receptor conformation that does not fully activate the G protein.

In conclusion, we showed that BAY60-6583 is a partial agonist at human A_{2B} ARs. These results have to be considered when BAY60-6583 is used as a pharmacological tool in preclinical studies. Depending on the receptor expression level, the local adenosine concentrations, and potential constitutive receptor activity, BAY60-6583 may display a broad spectrum of efficacies ranging from full to partial agonistic, and even to antagonistic activity.

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Authorship Contributions.

Participated in research design: Müller, Hinz

Conducted research experiments: Hinz, Lacher, Seibt

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Performed data analysis: Hinz, Müller

Wrote or contributed to the writing of the manuscript: Müller, Hinz

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Figure legends

Figure 1. Structures of selected A_{2B} adenosine receptor ligands

Figure 2.

A. Competition binding experiments of NECA, BAY60-6583 and PSB-603 versus 0.3 nM [3 H]PSB-603 at CHO-h A_{2B} membranes with or without 100 mM NaCl. The calculated IC_{50} -values without NaCl were 1720 nM \pm 559 nM for NECA (n=3 \pm SEM), 343 nM \pm 176 nM for BAY60-6583 (n=4 \pm SEM) and 0.439 nM \pm 0.044 for PSB-603 (n=4 \pm SEM). The IC_{50} -values with 100 mM NaCl were 13900 nM \pm 3430 nM for NECA (n=3 \pm SEM), 559 nM \pm 350 nM for BAY60-6583 (n=4 \pm SEM) and 0.359 nM \pm 0.050 nM for PSB-603 (n=4 \pm SEM).

B. Comparison of the pIC_{50} -values of PSB-603, NECA and BAY60-6583 at human A_{2B} receptors with or without 100 mM NaCl (n = 3-4 \pm SEM, $**p < 0.01$).

Figure 3. Dose-response curves for NECA and BAY60-6583 on cAMP accumulation at CHO-HA-h A_{2B} cells. The calculated EC_{50} -values were 318 nM \pm 55 nM for NECA (n=5) and 98.7 nM \pm 0.6 nM for BAY60-6583 (n=3). The intrinsic efficacies on cAMP accumulation at CHO-HA-h A_{2B} cells were normalized to NECA (set at 100 %) and to buffer (set at 0 %). BAY60-6583 shows an efficacy of 56 \pm 16 %.

Figure 4. Comparison of the intrinsic efficacies of NECA, BAY60-6583 and adenosine on cAMP accumulation at HEK293 cells. Data were normalized to 100 μ M NECA (set at 100 %) and to buffer (set at 0 %). The intrinsic efficacies on cAMP accumulation at native HEK cells were 6 \pm 4 % for BAY60-6583 (100 μ M) and 64 \pm 3 % for adenosine (300 μ M) (n=3 \pm SEM, $**p < 0.01$, $***p < 0.001$).

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Figure 5. Dose-response curves for NECA (EC_{50} -value: 200 ± 52 nM), adenosine (EC_{50} -value: 2510 ± 593 nM) and BAY60-6583 (EC_{50} -value: 505 ± 231 nM) on cAMP accumulation at HEK-hA_{2B} cells. Data were normalized to NECA (set at 100 %) and to buffer (set at 0 %). The intrinsic efficacies on cAMP accumulation at HEK-hA_{2B} cells were 49 ± 14 % for BAY60-6583 and 100 ± 12 % for adenosine ($n=4$, \pm SEM, *** $p < 0.001$).

Figure 6. Comparison of the intrinsic efficacies of NECA, BAY60-6583 and adenosine on Ca²⁺ mobilization in HEK293 cells. The intrinsic efficacies in Ca²⁺ mobilization assays at HEK293 cells were 1 ± 5 % for BAY60-6583 (100 μ M) and 100 ± 14 % for adenosine (300 μ M). Data were normalized to 100 μ M NECA (set at 100 %) and to buffer (set at 0 %) ($n=4$, \pm SEM, *** $p < 0.001$).

Figure 7. Dose-response curves for NECA (EC_{50} -value: 438 ± 177 nM), adenosine (EC_{50} -value: 990 ± 231 nM) and BAY60-6583 (EC_{50} -value: 579 ± 262 nM) on Ca²⁺ mobilization at HEK-hA_{2B} cells. Data were normalized to NECA (set at 100 %) and to buffer (set at 0 %). The intrinsic efficacies on Ca²⁺ mobilization at HEK-hA_{2B} cells were 57 ± 7 % for BAY60-6583 and 98 ± 4 % for adenosine ($n = 4$, \pm SEM, ** $p < 0.01$, *** $p < 0.001$).

Figure 8. Effect of forskolin, NECA, BAY60-6583 and CGS21680 determined in cAMP accumulation assays and antagonism of PSB-603 on cAMP accumulation induced by 10 μ M NECA at Jurkat-T cells (*** $p < 0.001$). Data were normalized to 10 μ M forskolin (100 %) and buffer (0 %) ($n=2$, \pm SEM).

Figure 9.

A. Dose-response curves for BAY60-6583 in the presence of 10 μ M NECA or 250 μ M adenosine in cAMP accumulation assays at Jurkat-T cells. Data were normalized to 100 % relative to the response of 10 μ M NECA or 250 μ M adenosine. The IC_{50} -value for BAY60-

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6583 versus 10 μ M NECA was 7500 ± 1400 nM and versus 250 μ M adenosine 25100 ± 5600 nM (n=3-4, \pm SEM).

B. Stimulation of cAMP accumulation at Jurkat-T cells by NECA was measured in the absence and presence of 5 μ M BAY60-6583. Data were normalized to 100 % relative to the maximal response induced by NECA (n=3, \pm SEM, **p < 0.01). The calculated K_B -value for BAY60-6583 was 393 nM \pm 48 nM.

Figure 10. Comparison of the calcium signal induced by NECA, adenosine and BAY60-6583 in the presence of 200 nM MSX-2 to block adenosine A_{2A} receptors at Jurkat-T cells. The intrinsic efficacies in Ca^{2+} mobilization assays at Jurkat-T cells were 1 ± 8 % for BAY60-6583 (100 μ M) and 102 ± 10 % for adenosine (300 μ M). Data were normalized to 100 μ M NECA (set at 100 %) and to buffer + 200 nM MSX-2 (set at 0 %) (n=4, \pm SEM, *** p < 0.001).

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Table 1. K_i values, EC₅₀ values and efficacies of agonists at human A_{2B} adenosine receptors

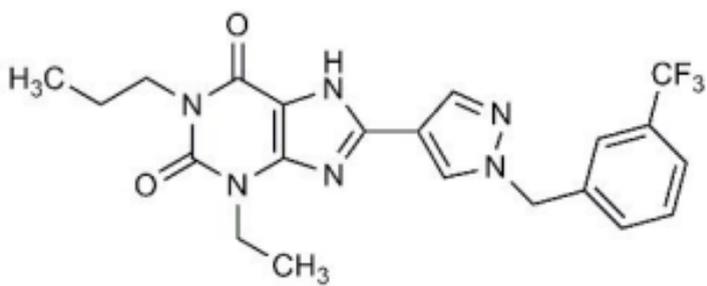
	K_i or EC₅₀ value ± SEM (nM) (maximal effect ± SEM)^a		
	BAY60-6583	NECA	Adenosine
<i>Transfected CHO cells (CHO-hA_{2B})</i>			
Radioligand binding vs. [³ H]PSB-603	212 ± 20	1890 ± 240	n.d. ^b
cAMP	98.7 ± 0.6 (56 ± 16 %)	318 ± 55 (100 %)	n.d. ^b
[Ca ²⁺] _i ^c	No signal (at 0.1-10 μM)	No signal (at 1-100 μM)	No signal (at 0.1-100 μM)
<i>Native HEK293 cells</i>			
cAMP	n.d. ^b (6 ± 4 % at 100 μM)	n.d. ^b (100 % at 100 μM)	n.d. ^b (64 ± 3 % at 300 μM)
[Ca ²⁺] _i	n.d. ^b (1 ± 5 % at 100 μM)	n.d. ^b (100 % at 100 μM)	n.d. ^b (100 ± 14 % at 300 μM)
<i>Transfected HEK293 cells (HEK-hA_{2B})</i>			
cAMP	505 ± 231 (49 ± 14 %)	200 ± 52 (100 %)	2510 ± 593 (100 ± 12 %)
[Ca ²⁺] _i	579 ± 262 (57 ± 7 %)	438 ± 177 (100 %)	990 ± 231 (98 ± 4 %)
<i>Jurkat T cells</i>			
cAMP	7500 ± 1400 (IC₅₀ vs. NECA)	3810 ± 560 (n.d.)	237000 ± 26000 (n.d.)
[Ca ²⁺] _i	n.d. ^b (1 ± 8 % at 100 μM)	663 ± 284 (100 %)	n.d. ^b (102 ± 10 % at 300 μM)

^acompared to the maximal effect of NECA set at 100%

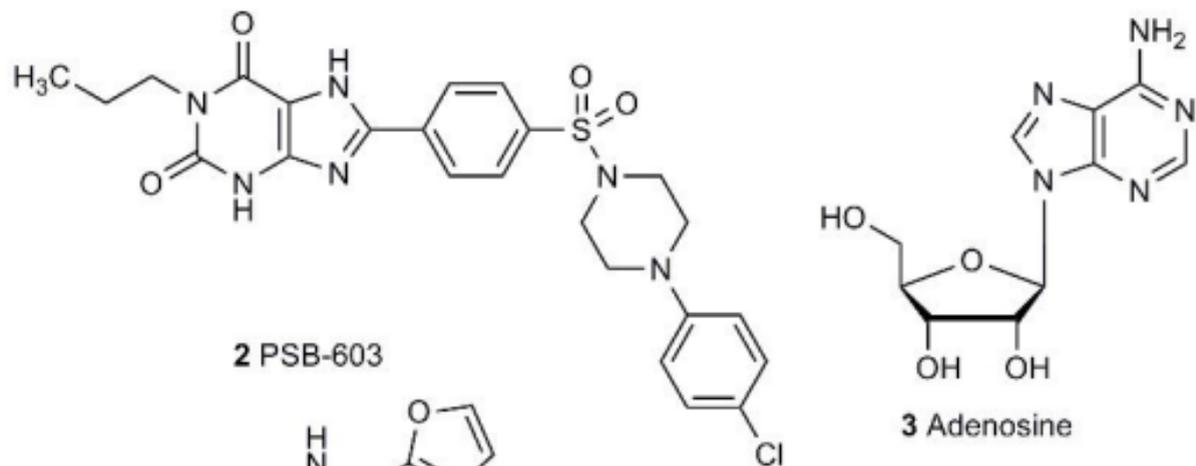
^bn.d., not determined

^cATP and UTP (100 μM each) were used as a positive control. They gave a robust calcium signal (via activation of endogenous G_q-coupled P2Y₂ receptors)

Figure 1

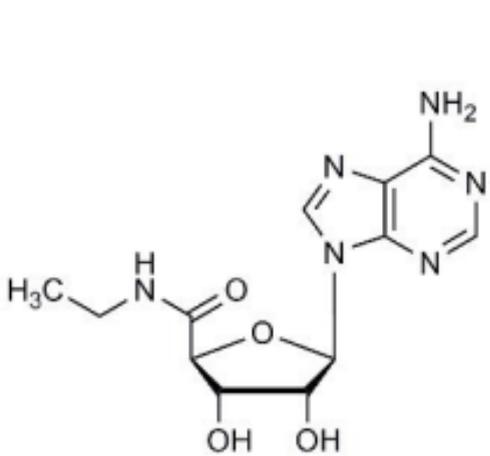


1 GS 6201 (CVT-6883)

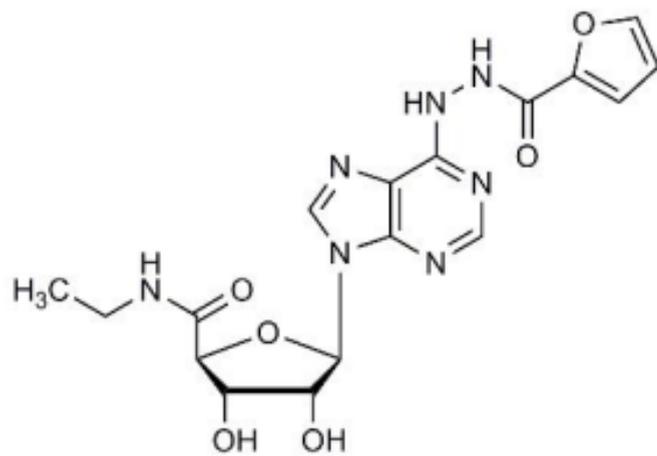


2 PSB-603

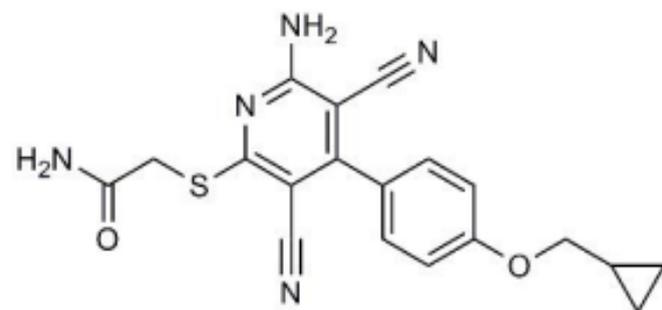
3 Adenosine



4 NECA



5



6 BAY 60-6583

Figure 2A

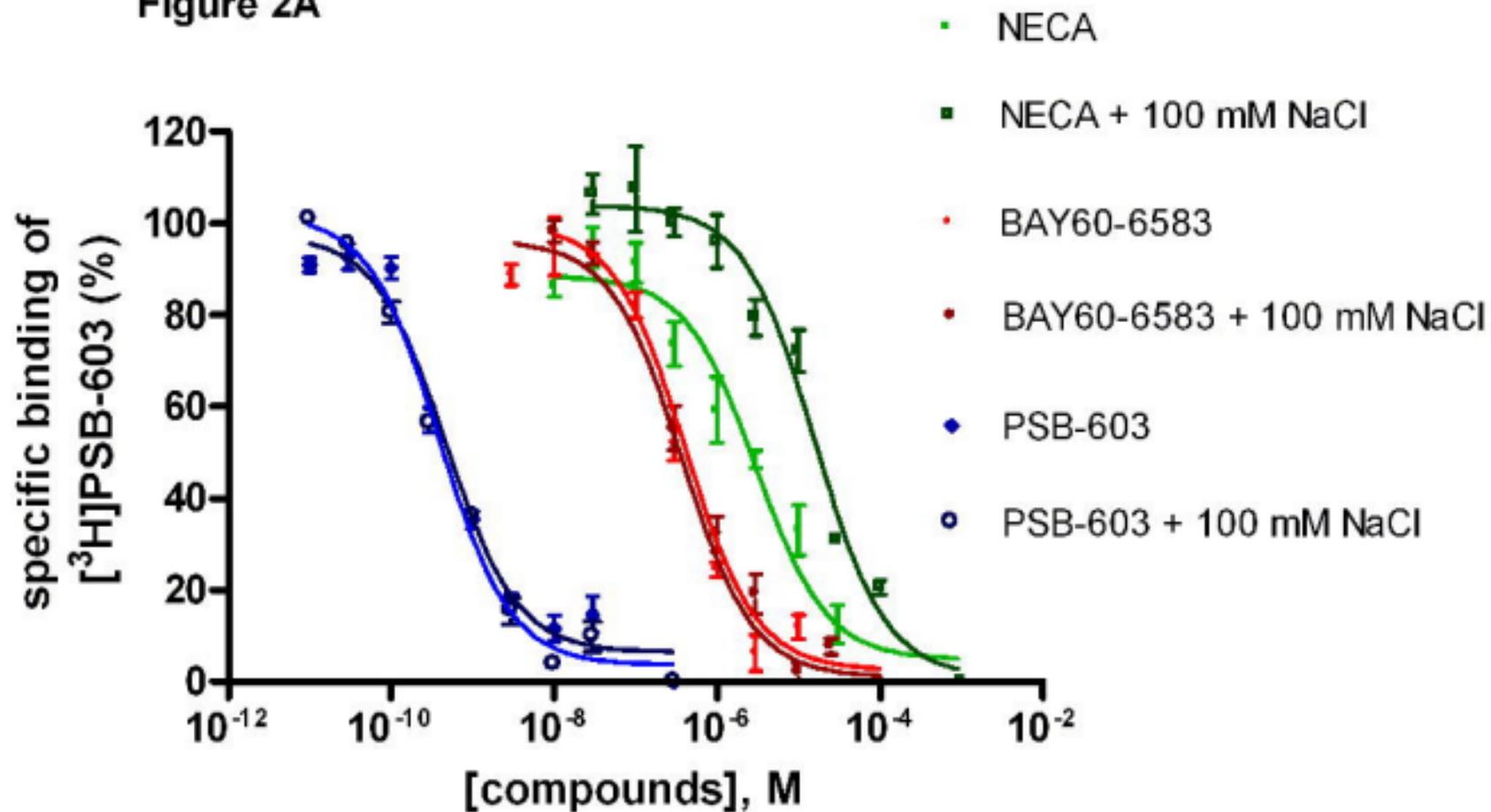


Figure 2B

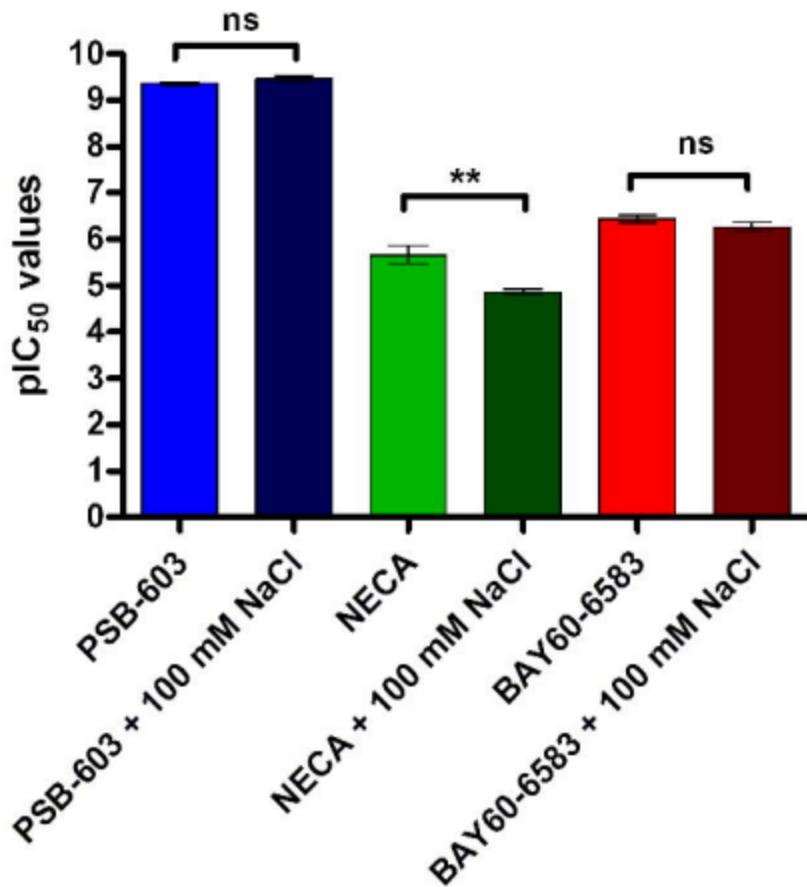


Figure 3

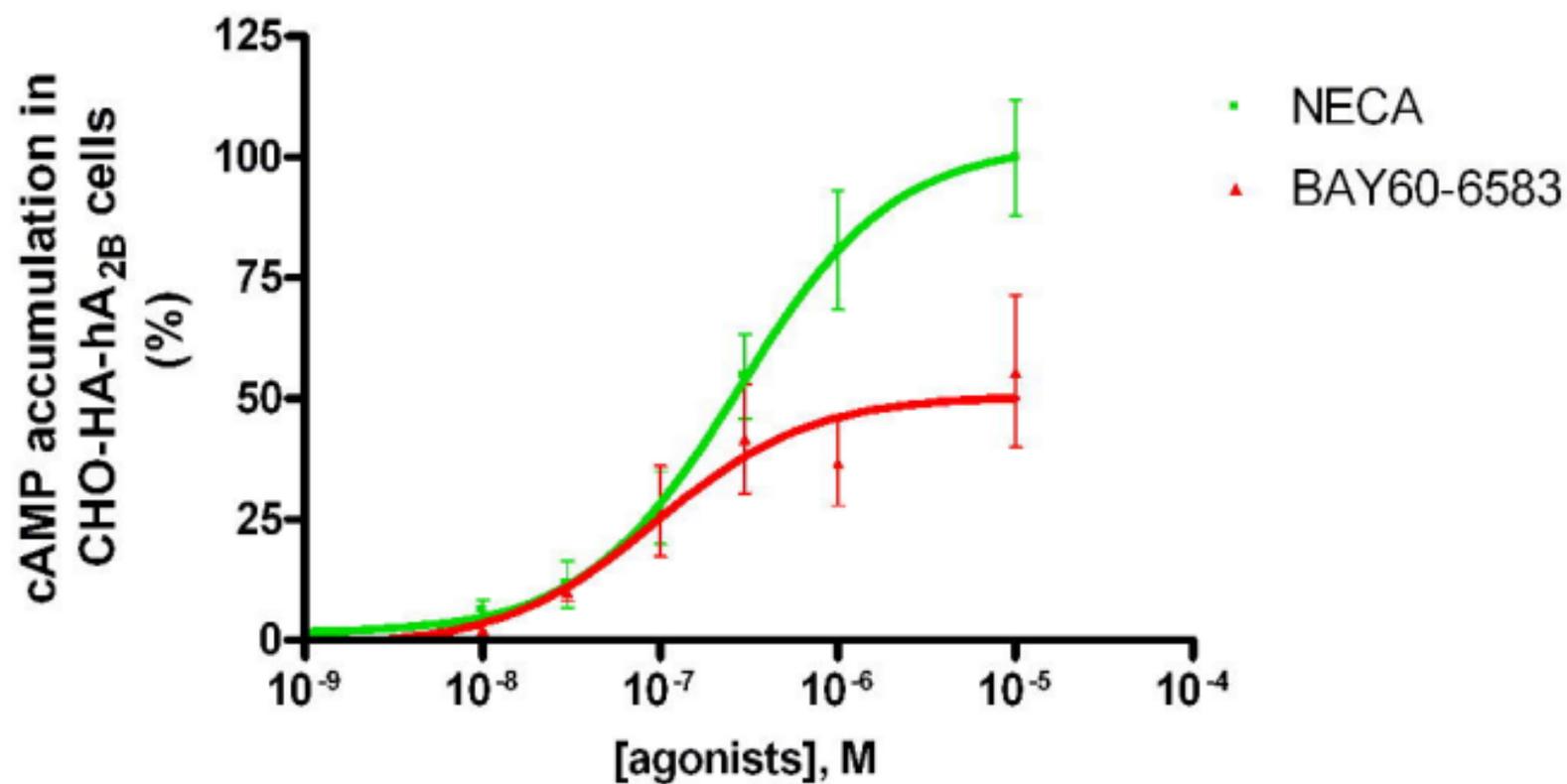


Figure 4

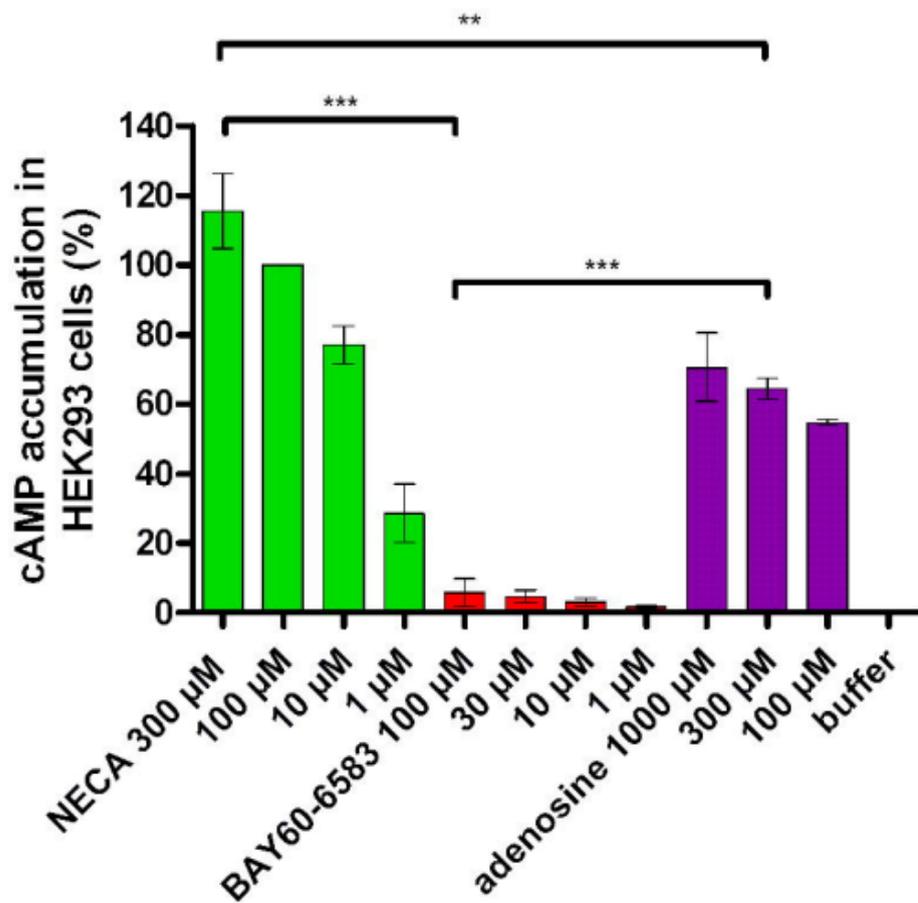


Figure 5

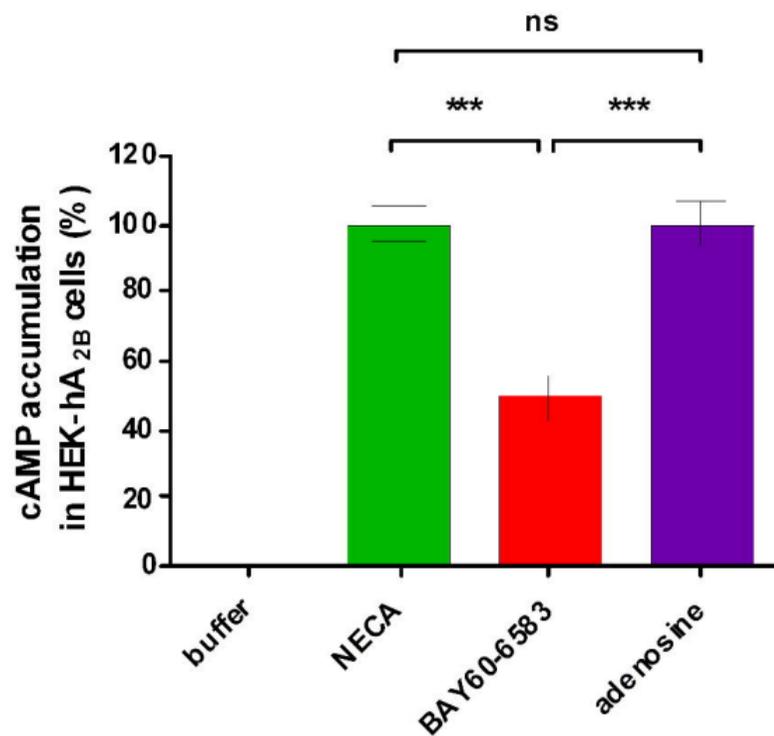
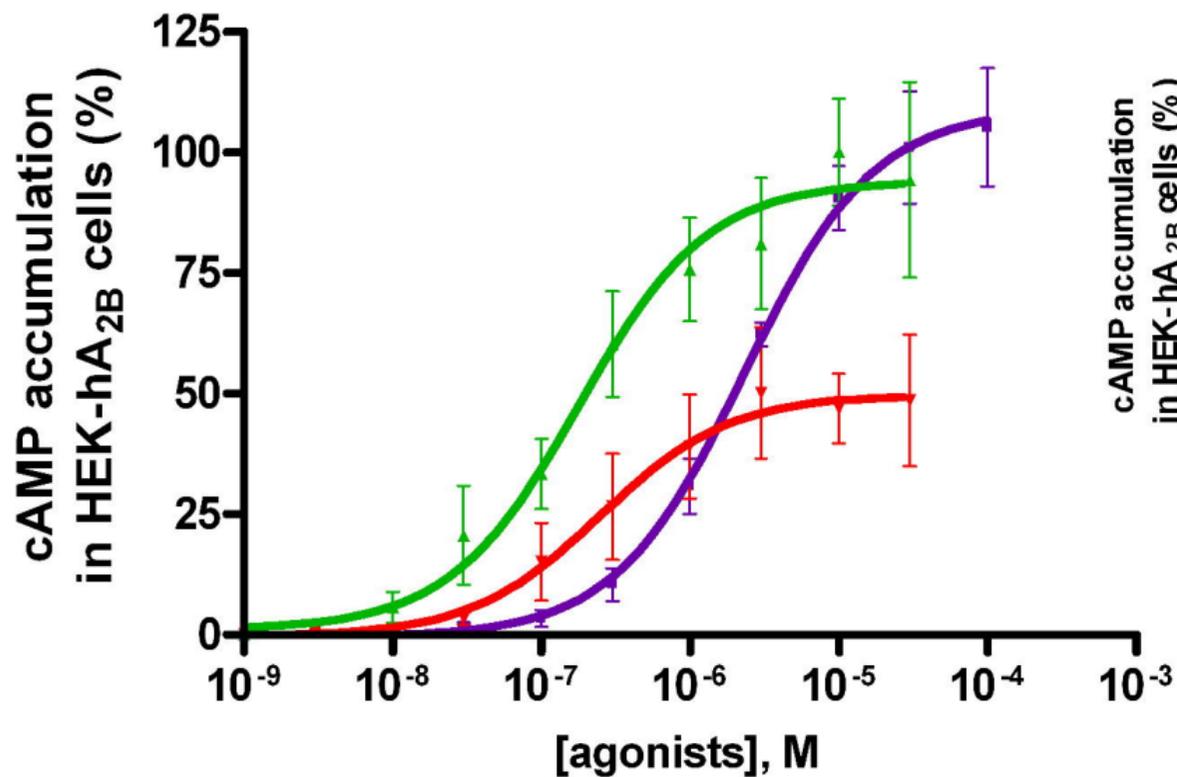


Figure 6

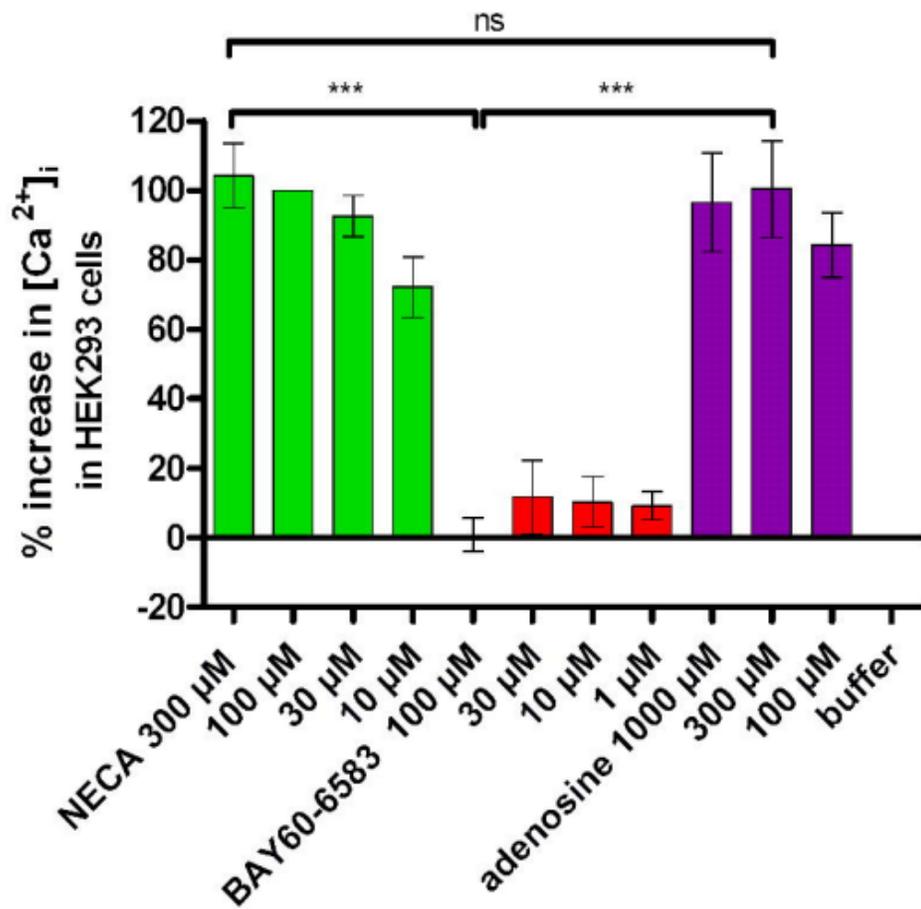


Figure 7

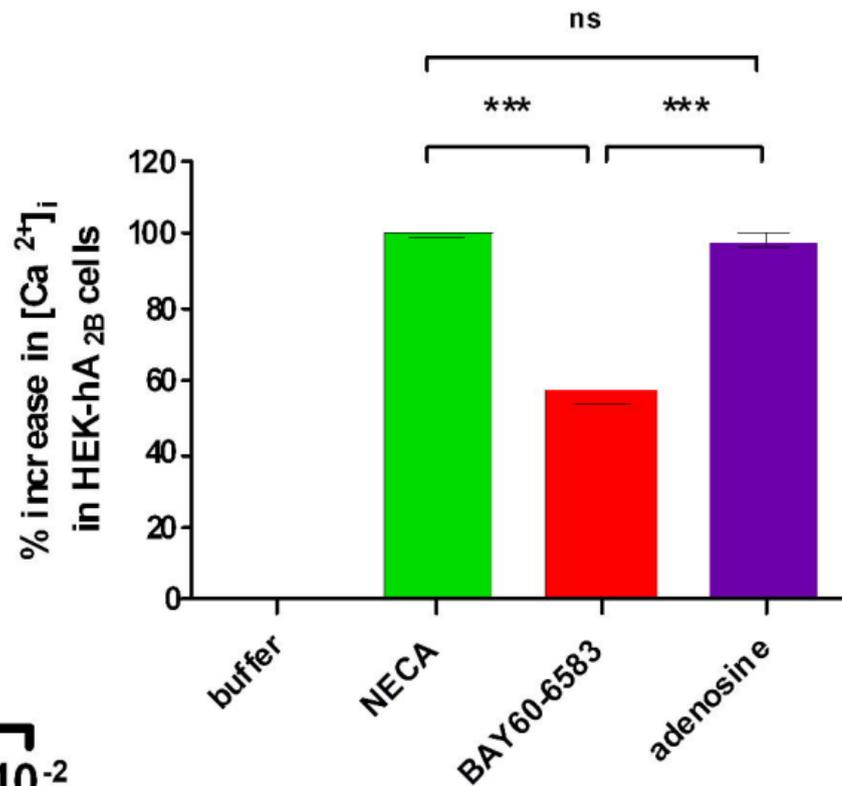
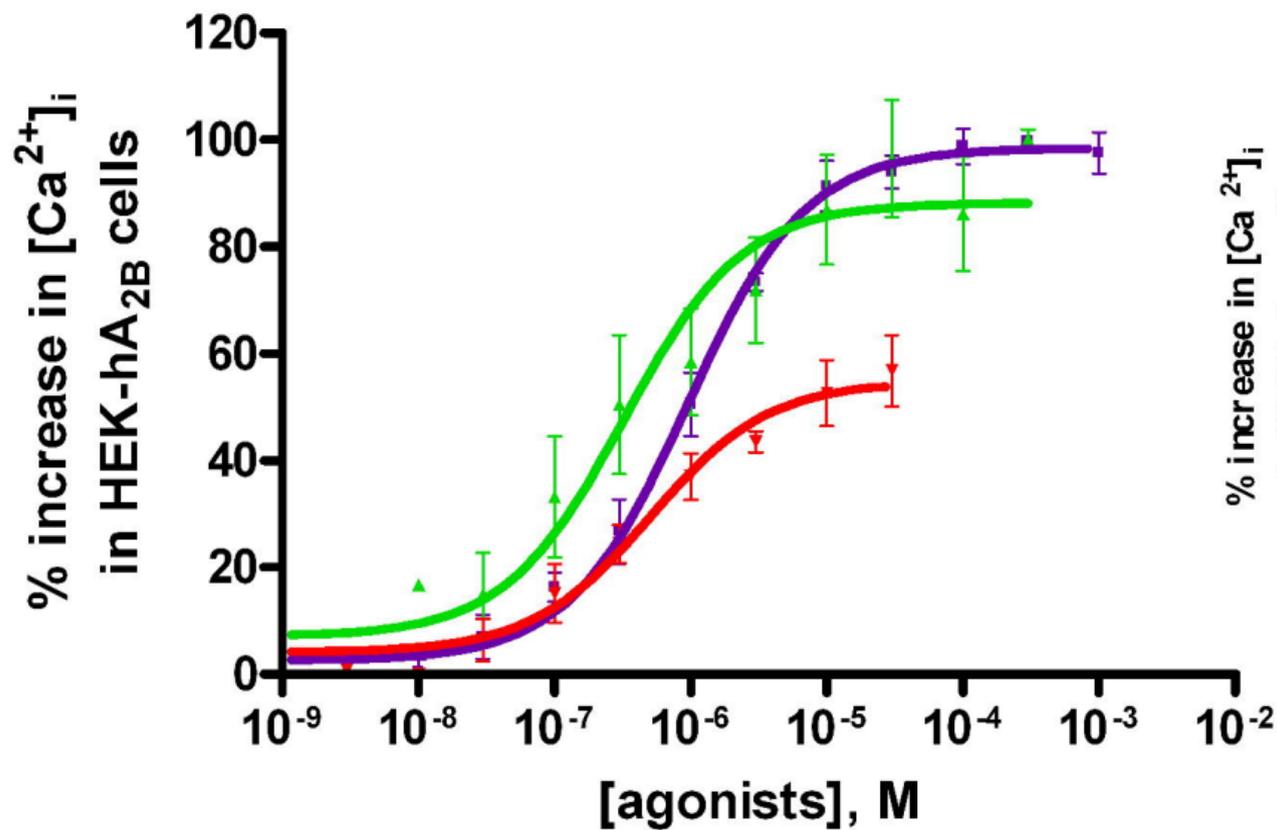


Figure 8

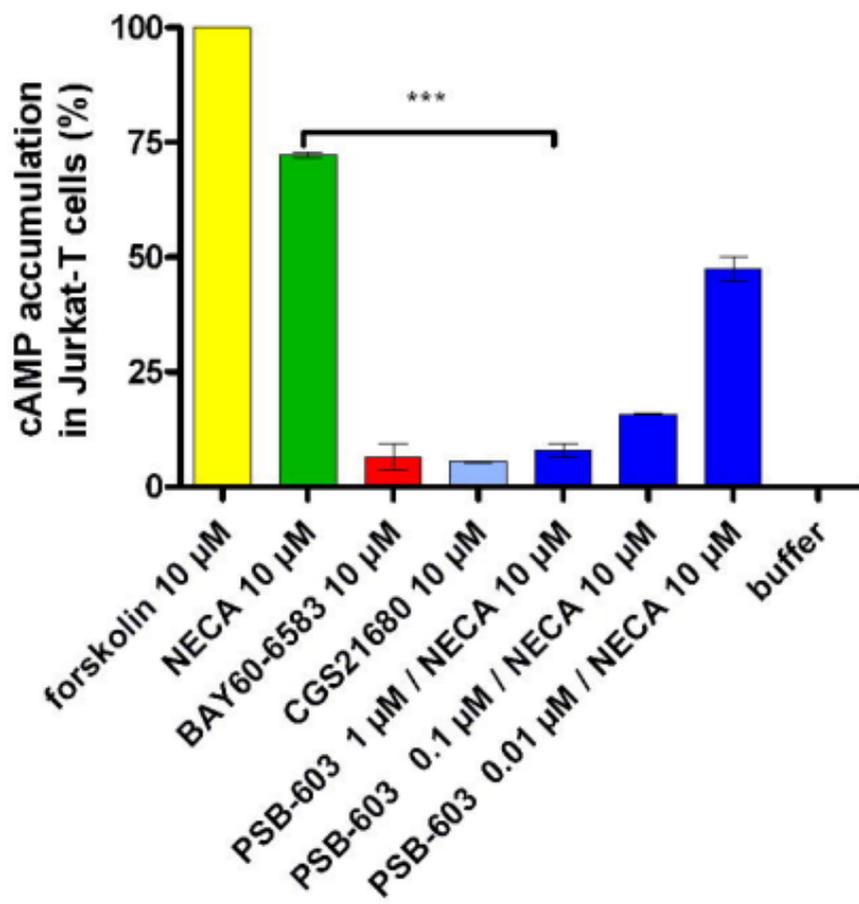


Figure 9A

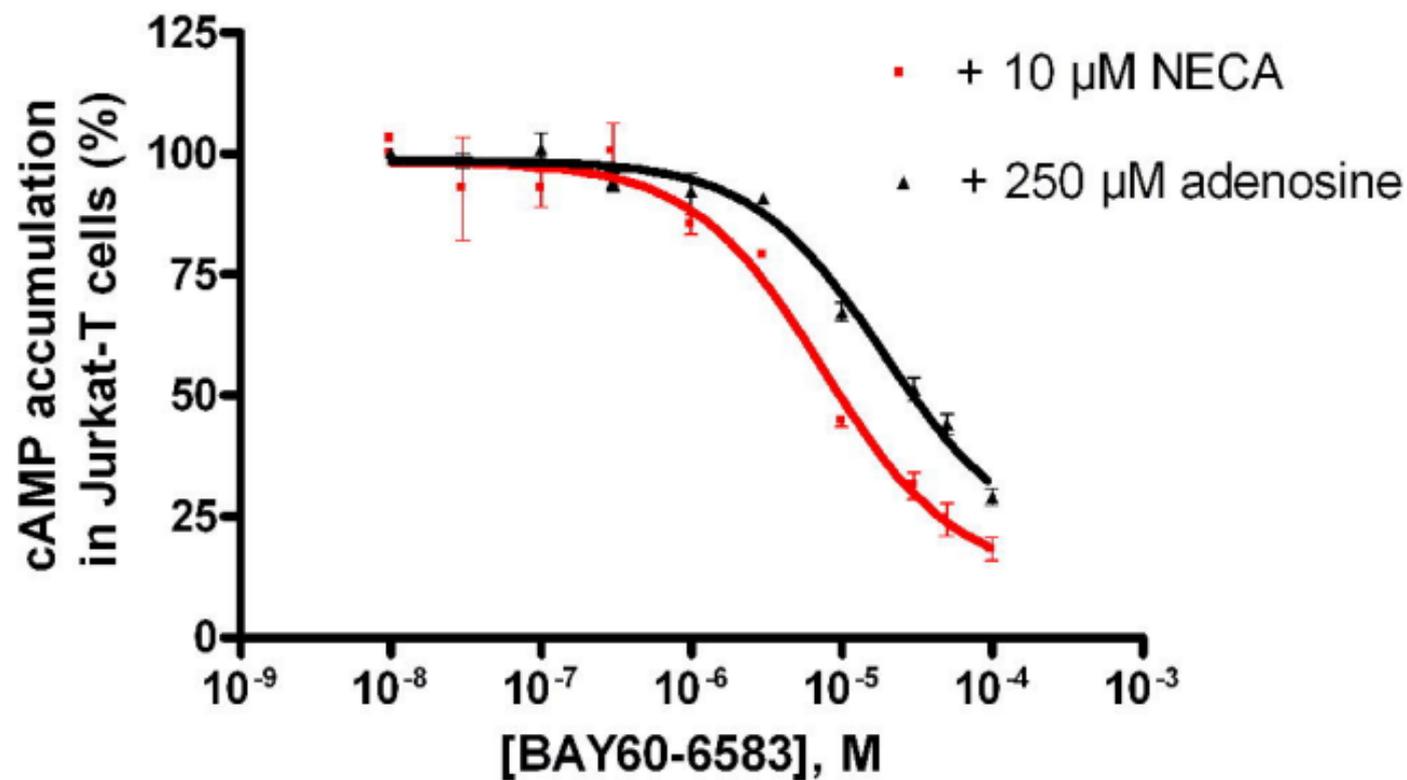


Figure 9B

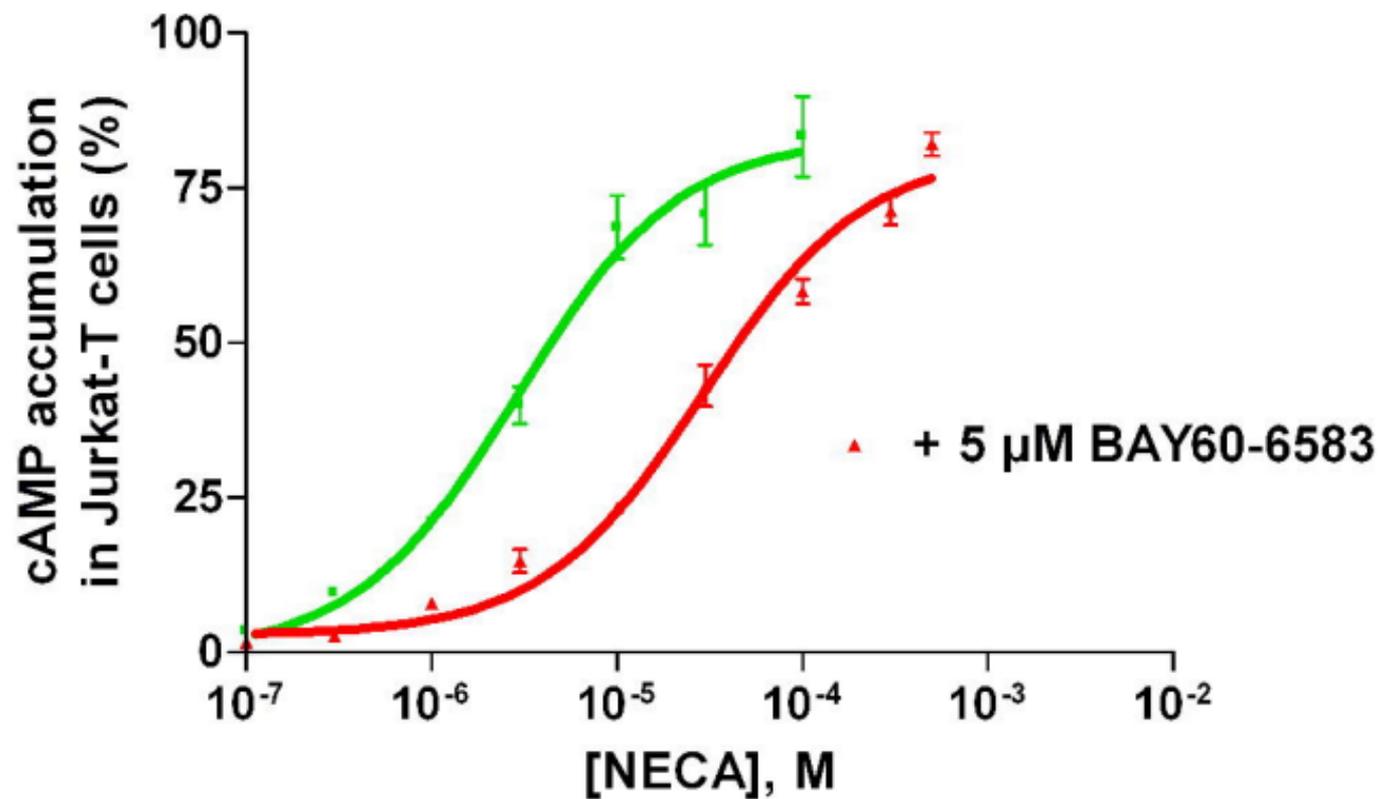


Figure 10

