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ORIGINAL ARTICLE

Tritium-labeled agonists as tools for studying adenosine A_{2B} receptors

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Abstract

A selective agonist radioligand for A_{2B} adenosine receptors (A_{2B}ARs) is currently not available. Such a tool would be useful for labeling the active conformation of the receptors. Therefore, we prepared BAY 60-6583, a potent and functionally selective A_{2B}AR (partial) agonist, in a tritium-labeled form. Despite extensive efforts, however, we have not been able to establish a radioligand binding assay using [^{3}H]BAY 60-6583. This is probably due to its high non-specific binding and its moderate affinity, which had previously been overestimated based on functional data. As an alternative, we evaluated the non-selective A_{2B}AR agonist [^{3}H]NECA for its potential to label A_{2B}ARs. [^{3}H]NECA showed specific, saturable, and reversible binding to membrane preparations of Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells stably expressing human, rat, or mouse A_{2B}ARs. In competition binding experiments, the AR agonists 2-chloroadenosine (CADO) and NECA displayed significantly higher affinity when tested versus [^{3}H]NECA than versus the A_{2B}-antagonist radioligand [^{3}H]PSB-603 while structurally diverse AR antagonists showed the opposite effects. Although BAY 60-6583 is an A_{2B}AR agonist, it displayed higher affinity versus [^{3}H]PSB-603 than versus [^{3}H]NECA. These results indicate that nucleoside and non-nucleoside agonists are binding to very different conformations of the A_{2B}AR. In conclusion, [^{3}H]NECA is currently the only useful radioligand for determining the affinity of ligands for an active A_{2B}AR conformation.

Keywords Adenosine A_{2B} receptor · Agonist radioligand · [^{3}H]BAY 60-6583 · [^{3}H]NECA · Kinetics · Radioligand binding

Abbreviations

[^{125}I]ABOPX	2-[4-[3-[(4-Amino-3-iodobenzyl)]-2,6-dioxo-1-propyl-7H-purin-8-yl]phenoxy]acetic acid
ADA	Adenosine desaminase
AR	Adenosine receptor(s)
BAY 60-6583	2-({6-Amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-yl}sulfanyl)acetamide
BSA	Bovine serum albumin
CADO	2-Chloroadenosine
CCPA	2-Chloro-N^6-cyclopentyladenosine
CGS-15943	9-Chloro-2-(2-furyl)[1,2,4]triazolo-[1,5-*c*]chinazoline-5-amine
CGS-21680	(2-*p*-[2-Carboxyethyl]phenethylamino)-5′-*N*-ethylcarboxamidoadenosine
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
CHO	Chinese hamster ovary
Cl-IB-MECA	2-Chloro-N^6-(3-iodobenzyl)-9-[5-(methyl-carbamoyl)-β-D-ribofuranosyl]adenine
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
FCS	Fetal calf serum
G418	Geneticin
GPCR	G protein-coupled receptor
hA_{2B}	Human A_{2B}
HEK293	Human embryonic kidney
mA_{2B}	Mouse A_{2B}
$MgCl_2$	Magnesium chloride
MRE2029-F20	*N*-Benzo[1,3]dioxol-5-yl-2-[5-(2,6-dioxo-1,3-dipropyl-2,3,6,7-tetrahydropurin-8-yl)-1-methyl-1*H*-pyrazol-3-yloxy]-acetamide
MRS-1754	*N*-(4-Cyanophenyl)2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1*H*-purin-8-yl)-phenoxy]acetamide
MSX-2	3-(3-Hydroxypropyl)-7-methyl-8-(*m*-methoxystyryl)-1-propargylxanthine

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NECA	5′-*N*-Ethylcarboxamidoadenosine
OSIP-3309391	*N*-(2-{2-Phenyl-6-[4-(3-phenylpropyl)piperazine-1-carbonyl]-7*H*-pyrrolo[2,3-*d*]pyrimidin-4-ylaminoethyl)acetamide
PSB-1115	4-(2,3,6,7-Tetrahydro-2,6-dioxo-1-propyl-1*H*-purin-8-yl)-benzenesulfonic acid
PSB-298	(8-{4-[2-(2-Hydroxyethylamino)-2-oxoethoxy]phenyl}-1-propylxanthine
PSB-603	8-(4-(4-Chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine
rA_{2B}	Rat A_{2B}
R-PIA	N^6-(2-Phenylisopropyl)adenosine
ZM 241385	4-(2-[7-Amino-2-(2-furyl)-[1,2,4]-triazolo[2,3-*a*][1,3,5]-triazin-5-ylamino]ethyl)phenol

Introduction

The G protein-coupled adenosine receptors (ARs) are divided into four subtypes: A_1, A_{2A}, A_{2B}, and A_3. While A_1 and A_3ARs are coupled to G_i proteins mediating an inhibition of adenylate cyclase, A_{2A} and A_{2B}ARs are coupled to G_s proteins resulting in a stimulation of the enzyme [1]. The A_{2B}ARs can additionally couple to G_q proteins resulting in phospholipase C activation followed by intracellular calcium release [2]. Depending on the structure of agonists and antagonists, different receptor conformations may be induced and stabilized potentially leading to functional selectivity with regard to different signaling pathways, e.g., G protein activation versus β-arrestin recruitment [3]. GPCRs can also form homo- or heteromeric di- or oligomers [4] which may display an altered pharmacology [5, 6]. ARs are involved in a range of diseases including cardiovascular, nervous system, pulmonary, and kidney disorders, and adenosine mediates various effects including anti-inflammatory and immune-suppressive activity [7]. Therefore, ARs represent therapeutic drug targets, and the development of selective agonists and antagonists for medical applications has been of great interest. The A_{2A} and A_{2B}AR subtypes have recently become major targets in immuno-oncological drug development [8]. The well-investigated A_{2A}AR subtype is typically activated by relatively low nanomolar concentrations of adenosine, whereas the A_{2B}AR subtype requires higher micromolar adenosine concentrations for activation. Only few selective A_{2B}AR agonists have been described, the most prominent one being the non-nucleosidic partial agonist 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide (BAY 60-6583) [9, 10]. Only very few other potent A_{2B}AR agonists, all derived from the nucleoside adenosine, have been reported, mainly by the group of Baraldi's [11–13], but their degree of selectivity is unclear since A_{2B}AR data obtained in cAMP accumulation assays were compared to A_1, A_{2A}, and A_3AR radioligand binding data. However, functional assays are highly dependent on receptor expression levels and receptor reserve [14]. In contrast to the situation with agonists, many potent and selective A_{2B}AR antagonists have been developed [13, 15–21] and evaluated in preclinical and initial clinical trials [22]. PSB-603 is one of the most potent and selective A_{2B} antagonists, which is frequently used as a tool compound to study A_{2B}ARs [23–26]. In Fig. 1, a selection of important non-selective and selective A_{2B}AR agonists and antagonists is depicted including NECA, CADO, BAY 60-6583, caffeine, PSB-603, and CGS-15943.

Radioligand receptor binding is a powerful method for the screening of new potential ligands, and the obtained data provide an ideal basis for structure-activity relationship analyses. Agonist radioligands label an active receptor conformation which displays high affinity for agonists while neutral antagonist radioligands bind to active and inactive receptor conformations with similar affinity. Inverse agonist radioligands label an inactive receptor conformation, for which agonists typically display low affinity [27]. For competition binding assays, agonist or antagonist radioligands can be employed, both of which will provide different information about a test compound. While agonists typically show higher affinity when they are tested against agonist radioligands (which label the so-called high-affinity binding site of an active receptor conformation), they usually display lower affinity for an antagonist/inverse agonist radioligand-labeled, inactive receptor conformation. In some cases, biphasic curves are observed in competition binding assays of agonists versus antagonist radioligands, e.g., for the human A_1AR [28]. However, at A_{2A} and A_{2B}ARs, no biphasic curves have been observed and only inactive, low affinity conformations appeared to be labeled by antagonist radioligands [17, 29]. Consequently, A_{2A}AR agonists were found to display lower affinity versus antagonist radioligands than versus agonist radioligands. Thus, agonist radioligands are important tools to study the affinity of agonists at A_{2A}ARs for their high-affinity binding site in the active receptor conformation. Radioligand binding studies have also been crucial for recent X-ray crystallography [30–32] and NMR studies [33] to prove the correct folding of the receptor proteins after isolation and purification.

Several crystal structures of the human A_{2A}AR in complex with agonists [34–36] and antagonists [37–41] have been published, but the X-ray structure of the A_{2B}AR has not been resolved so far. Recently, crystal structures of the A_1AR have been obtained, which provided insights into selectivity among A_1 and A_{2A}AR subtypes [30, 41].

For the A_{2B}AR, potent and selective antagonist radioligands have been developed [15–18]; however, no selective A_{2B}AR agonist radioligand has been described so far. The non-selective AR agonist [^{3}H]NECA has been reported to

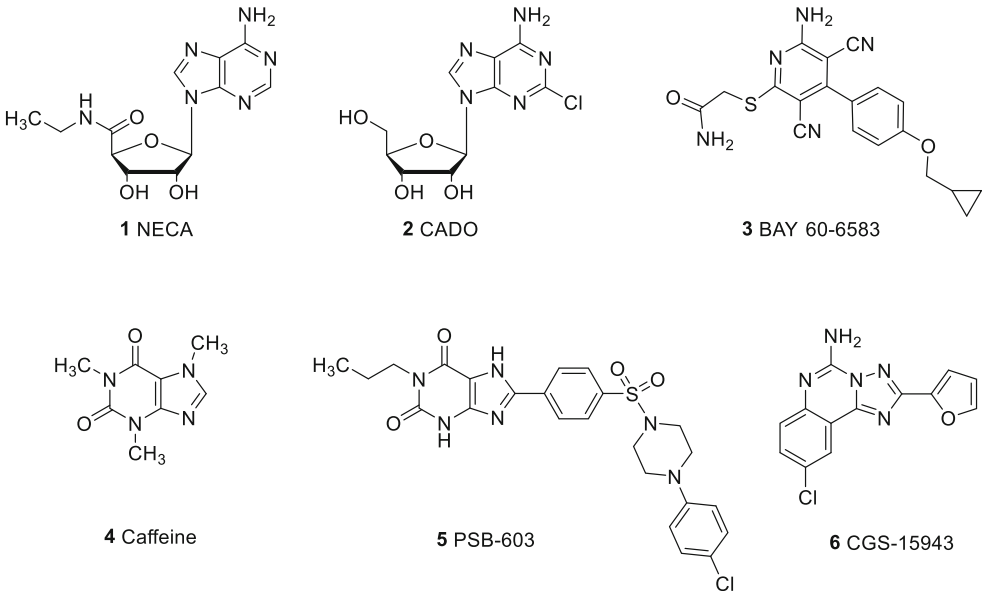

Fig. 1 Structures of the the non-selective AR agonist NECA (1), the non-selective AR agonist CADO (2), the selective (partial) A_{2B}AR agonist BAY 60-6583 (3), the non-selective AR antagonist caffeine (4), the selective A_{2B}AR antagonist PSB-603 (5), and the non-selective AR antagonist CGS-15943 (6)

label A_{2B}ARs [42–45]. But a systematic characterization of [^{3}H]NECA as a radioligand for A_{2B}ARs has not been performed, and the radioligand has not been employed for the determination of affinities of series of ligands. The goal of the present study was to establish an agonist radioligand binding assay at human, rat, and mouse A_{2B}ARs. To this end, our initial strategy was the preparation and utilization of tritium-labeled BAY 60-6583. However, major difficulties in establishing an A_{2B}AR binding assay with [^{3}H]BAY 60-6583 promped us to investigate [^{3}H]NECA as an A_{2B}AR radioligand instead. The radioligand was subsequently used for characterizing the affinities of ligands, in particular agonists, for the high-affinity conformation labeled by the adenosine derivative.

Materials and methods

Materials and (bio)chemicals

[^{3}H]NECA (15.9 Ci/mmol) was supplied by Perkin Elmer Life and Analytical Science (Rodgau-Jügesheim, Germany). All other chemical reagents were purchased from Sigma-Aldrich (Taufkirchen, Germany), Tocris Biosciences (Bristol, UK), or Roth (Karlsruhe, Germany), unless otherwise stated. BAY 60-6583 was synthesized or purchased from Tocris Biosciences (Bristol, UK). All cell culture media and penicillin-streptomycin solutions were obtained from Invitrogen (Darmstadt, Germany). Fetal calf serum (FCS) and G418 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Cell culture materials (flasks and dishes) were obtained from Labomedic (Bonn, Germany) and Sarstedt (Nümbrecht, Germany), respectively.

Retroviral transfection of GP$^+$ env AM12 cells and infection of CHO-K1 cells

CHO cells stably expressing human, rat, or mouse A_{2B}ARs were generated using a retroviral expression system as previously described [46].

Cell culture

CHO cells stably transfected with the human, rat, or mouse A_{2B}AR were maintained in DMEM-F12 medium with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.8 mg/ml G418 at 37 °C and 5% CO_2.

Membrane preparations

Membranes of CHO cells stably expressing human, rat, or mouse A_{2B}ARs were prepared as previously described [16, 46]. Membranes from HEK-hA_{2B} cells which were used for some of the competition binding studies were purchased from Perkin Elmer (Rodgau-Jügesheim, Germany).

Radioligand receptor binding studies

Preparation of [^{3}H]BAY 60-6583

The radiosynthesis was performed starting from an iodine-substituted precursor in a single step by iodine-tritium exchange catalyzed by palladium black in tetrahydrofurane in the presence of triethylamine as a base. The product was purified and analyzed by RP-HPLC using the following conditions: column: Phenomenex AQUA C18, 5 μm, mobile phase: acetonitrile:water (1:1), flow rate of 1 and 5 ml/min, respectively. The analytical HPLC chromatogram with radio

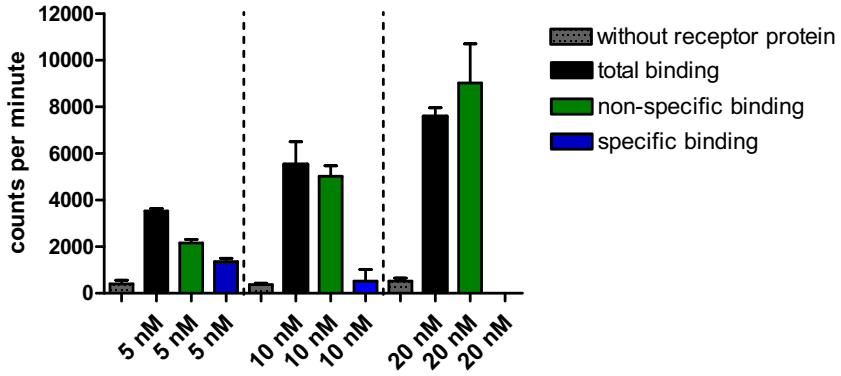

Fig. 2 Binding assays with different concentrations of [^{3}H]BAY 60-6583. Non-specific binding was determined in the presence of 10 μM DPCPX. Assay conditions were as follows: a final volume of 500 μl contained 2.5% DMSO, 50 mM Tris-buffer with 3 mM $MgCl_2$, pH 7.4, as incubation buffer, 5, 10, or 20 nM of [^{3}H]BAY 60-6583, 80 μg of A_{2B}AR membrane preparation per vial, incubation for 140 min at rt, filtration through GF/C filters, and washing with 50 mM ice-cold Tris buffer, pH 7.4. The use of 5 nM of [^{3}H]BAY 60-6583 gave the best results with 30–40% of specific binding. Data points are means ± SEM of three (5 nM) or two independent experiments performed in single values

detection and UV detection (254 nm) showed high purity of the product [^{3}H]BAY 60-6583, which was obtained with a specific radioactivity of 22.7 Ci/mmol (2008.9 MBq/mg) in high radiochemical purity of > 99% (see Supplemental Fig. 1).

Binding experiments with [^{3}H]BAY 60-6583

Initial binding experiments with [^{3}H]BAY 60-6583 were performed in a final assay volume of 0.5 ml containing test compound dissolved in DMSO (25 μl), 275 μl of assay buffer (50 mM Tris-HCl, pH 7.4), 100 μl of radioligand solution (1 nM) in assay buffer, and 100 μl of human A_{2B}AR membrane preparation (30 μg per vial) suspended in assay buffer containing adenosine deaminase (ADA; 2 U/mg protein, 20 min preincubation at rt). Non-specific binding was determined in the presence of 1 mM NECA, 10 μM DPCPX, or 10 μM PSB-1115, respectively. After an incubation time of 30 min at rt, the assay mixture was rapidly filtered through GF/B glass fiber filters using a 24-well Brandel harvester (Brandel, Gaithersburg, MD). Filters were washed three times (3 ml each) with ice-cold 50 mM Tris-HCl buffer, pH 7.4. Then filters were transferred to vials, incubated for 9 h with 2.5 ml of scintillation cocktail (LumaSafe plus, Perkin-Elmer), and counted in a liquid scintillation counter (Tricarb 2700TR) with a counting efficiency of 53%. For additional experiments, a final volume of 0.5 ml, 2.5% DMSO, GF/C filters, higher radioligand concentrations (5, 10, 20 nM), and $MgCl_2$ (3 mM) in the incubation buffer were employed. Additionally, the A_{2B}AR amount was increased to 80 μg of membrane preparation per vial, and the incubation time was increased to 140 min at rt. Further experiments were conducted at 4 °C.

Competition binding experiments with [^{3}H]NECA

Competition binding experiments with [^{3}H]NECA were performed in a final volume of 1 ml containing 25 μl of test compound dissolved in DMSO, 775 μl of assay buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7,4), 100 μl of radioligand solution in assay buffer (final concentration 30 nM), and 100 μl of human, rat, or mouse A_{2B}AR membrane preparation (200–350 μg protein or 30 μg protein (human A_{2B}AR membranes from Perkin Elmer) per vial in assay buffer containing ADA (2 U/mg protein, 20 min incubation at rt). Non-specific binding was determined in the presence of 250 μM NECA. After an incubation time of 4 h at 4 °C, the assay mixture was rapidly filtered through GF/C glass fiber filters using a 24-well Brandel harvester (Brandel, Gaithersburg, MD). Filters were washed four times (3 ml each) with ice-cold 50 mM Tris-HCl buffer, pH 7.4. Then filters were transferred to scintillation vials, incubated for 9 h with 2.5 ml of scintillation cocktail (LumaSafe plus, Perkin-Elmer), and counted in a liquid scintillation counter (Tricarb 2700TR) with a counting efficiency of 53%.

Kinetic experiments with [^{3}H]NECA

Association experiments were performed at 4 °C with eight different time points and over a time period of 240 min. Dissociation experiments were initiated after 240 min of preincubation by the addition of 250 μM NECA. Three separate experiments were performed, each in duplicates. All other conditions were as described above for competition binding experiments.

Saturation binding experiments with [^{3}H]NECA

Saturation experiments at recombinant human and rat A_{2B}ARs were conducted to determine the K_D values for the radiolabeled agonist NECA at the different species. Different amounts of membrane preparation per vial, which were dependent on the receptor expression (200 μg for human and 250 μg for rat), and 7–9 different concentrations of

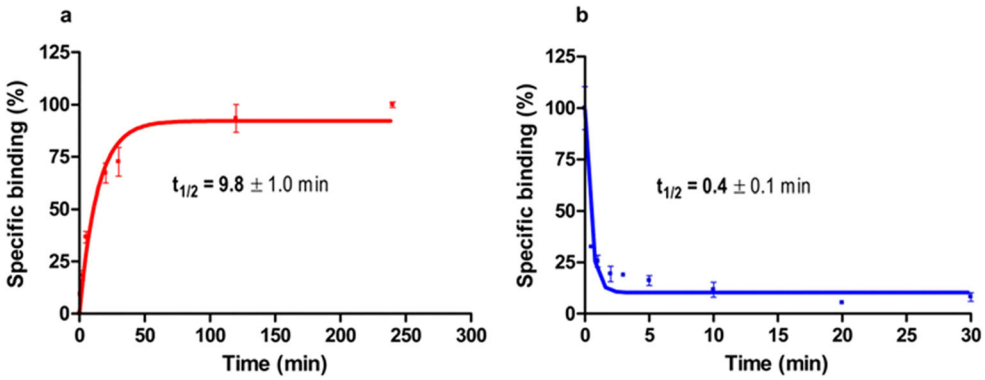

Fig. 3 Binding kinetics of [^{3}H]NECA (30 nM) to membrane preparations of CHO cells recombinantly expressing human A_{2B}ARs. Non-specific binding was determined in the presence of 250 μM NECA. **a** Association experiment. **b** Dissociation experiment. Dissociation was induced after 240 min of pre-incubation by the addition of 250 μM of unlabeled NECA. Data points are means ± SEM of three separate experiments performed at 4 °C

[^{3}H]NECA in a range of 10–2000 nM were employed. Four (human) or two (rat) separate experiments were performed, each in duplicates. All other assay conditions were as described above for competition binding experiments.

Competition binding experiments with [^{3}H]PSB-603

Competition binding experiments with the antagonist radioligand [^{3}H]PSB-603 at human A_{2B}ARs were performed as previously described [16].

Data analysis

All data were analyzed using Graph Pad Prism, version 4.0 (San Diego, CA, USA). K_D values from homologous competition binding experiments were calculated according to the Cheng and Prusoff equation [47]. In homologous competition binding experiments, radioligand and competitor are the same; therefore, K_D is equal to K_i, and the Cheng and Prussoff equation can be simplified to $K_D = IC_{50} - [\text{radioligand}]$.

Results

Attempts to establish an A_{2B}AR binding assay with [^{3}H]BAY 60-6583

The initial goal of the present study was to establish a radioligand binding assay using the A_{2B}AR agonist [^{3}H]BAY 60-6583. The ^{3}H-labeled form of BAY 60-6583 was obtained from its iodo-substituted precursor by catalytic hydrogenation with tritium gas yielding [^{3}H]BAY 60-6583 with a specific radioactivity of 22.7 Ci/mmol and a high radiochemical purity of > 99% (see Supplemental Fig. 1). Initially employed standard radioligand binding assay conditions with 1 nM of [^{3}H]BAY 60-6583, 30 μg of human A_{2B}AR protein per vial, 30 min incubation time at rt, filtration through GF/B filters, and washing with 50 mM Tris pH 7.4 buffer resulted in high non-specific binding of 80–90% for [^{3}H]BAY 60-6583 (see Supplemental Fig. 2). The addition of 0.1% bovine serum albumin (BSA) to the washing buffer did not reduce non-specific binding (see Supplemental Fig. 3). Further experiments performed at 4 °C with an incubation time of 120 min did not show any increase in specific binding of [^{3}H]BAY 60-6583 (see Supplemental Fig. 4). For subsequent experiments, a final volume of 0.5 ml, 2.5% DMSO, GF/C filters, higher radioligand concentrations (5, 10, 20 nM), and the addition of $MgCl_2$ (3 mM) to the incubation buffer were tested. Mg^{2+} may shift the A_{2B}AR to an active conformation to which agonists show high affinity as observed for the A_{2A}AR [48]. Under these conditions and with 5 nM of [^{3}H]BAY 60-6583, an increased degree of specific binding of 30–40% could be achieved (Fig. 2). With higher radioligand concentrations, non-specific binding was much higher as could be expected. The moderate specific binding of [^{3}H]BAY 60-6583 was too low to establish a reliable radioligand binding assay, which would require at least 50%, preferably 70% of specific binding. Therefore, different washing buffers were tested. But non-specific binding could not be further reduced, neither by the addition of 5% DMSO nor by adding 5% ethanol to the washing buffer. Many further modifications were tried, including preincubation of the filters in 1% aqueous polyethylenimine solution for 30 min, preincubation of the filters in 0.1% BSA solution, or preincubation of the filters in 50 μM BAY 60-6583 solution, but none of the numerous experiments resulted in increased levels of specific [^{3}H]BAY 60-6583 binding, even in cell membranes with very high expression levels of ca. 10 pmol/mg protein. Because of major difficulties in establishing an A_{2B}AR binding assay with [^{3}H]BAY 60-6583, we subsequently investigated and characterized [^{3}H]NECA as an A_{2B}AR agonist radioligand instead.

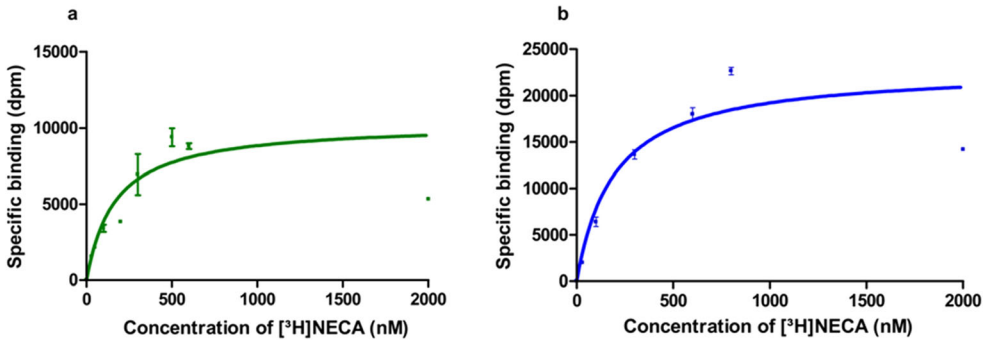

Fig. 4 Saturation binding experiments of [^{3}H]NECA **a** at human and **b** at rat A_{2B}ARs. Non-specific binding was determined in the presence of 250 μM NECA. A K_D value of 441 ± 169 nM and a B_{max} value of 2150 ± 449 fmol/mg protein were determined for human A_{2B}ARs, and a K_D value of 325 ± 144 nM and a B_{max} value of 2680 ± 472 fmol/mg protein were determined for rat A_{2B}ARs. Data points are means ± SEM of four (human) or two (rat) independent experiments each performed in duplicates

Radioligand receptor binding studies with [^{3}H]NECA at recombinant human, mouse, and rat A_{2B}ARs

Radioligand binding assays provide quantitative information about receptor expression (B_{max}) and the affinity of ligands (K_i, K_D) at a defined receptor. Three basic protocols, kinetic, saturation, and competition experiments were performed to determine these parameters.

Kinetic experiments

Due to the expected moderate affinity at the human A_{2B}AR and fast dissociation of NECA from the A_{2B}AR, kinetic experiments were performed at 4 °C using 30 nM of [^{3}H]NECA, which gave sufficiently high specific binding (see below). All experiments were best fitted using a single binding site model (Fig. 3a, b). Association (Fig. 3a) reached equilibrium within less than 30 min with a $t_{1/2}$ of 9.7 ± 1.0 min. Equilibrium binding was stable for at least 240 min. After the addition of a high concentration of unlabeled NECA (250 μM), [^{3}H]NECA binding was rapidly reversed as shown in Fig. 3b. Because the dissociation ($t_{1/2}$ = 0.4 ± 0.1 min) was very fast, much faster than the association, it was not possible to calculate a kinetic K_D value from the k_{on} and k_{off} values.

Next we performed saturation binding experiments to determine K_D and B_{max} values.

Saturation experiments

Saturation binding experiments were carried out using [^{3}H]NECA on membrane preparations recombinantly expressing A_{2B}ARs of different species, human and rat. [^{3}H]NECA binding was saturable, and the obtained data were in all cases best fitted to a monophasic curve. For the human A_{2B}AR, a K_D value of 441 ± 169 nM and a B_{max} value of 2150 ± 449 fmol/mg protein were determined (Fig. 4a). The calculated K_D value was 4-fold lower than the K_i value determined for NECA in competition assays versus the antagonist radioligand [^{3}H]PSB-603 at human A_{2B}ARs (1890 nM) [16]. At rat A_{2B}ARs, a K_D value of 325 ± 144 nM was determined for [^{3}H]NECA, and a B_{max} value of 2680 ± 472 fmol/mg protein was calculated (Fig. 4b). Again, the determined K_D value was about 3-fold lower than the K_i value determined for NECA in competition binding assays versus the antagonist radioligand [^{3}H]PSB-603 at rat A_{2B}ARs (1110 nM) [46].

Competition binding experiments

Next, we performed homologous competition binding experiments measuring concentration-dependent inhibition of unlabeled NECA versus [^{3}H]NECA to determine and compare the K_D and B_{max} values at the different A_{2B}AR species with those obtained in the saturation experiments. At the employed radioligand concentration of 30 nM, specific binding amounted to 50–70% of total binding depending on the harvesting conditions, washing steps, temperature, and membrane preparation. The homologous competition binding experiments resulted in K_D values that were in the same range as those obtained from saturation binding experiments. The calculated K_D and B_{max} values were 663 ± 87 nM and 2220 ± 71 fmol/mg protein for human A_{2B}ARs (Fig. 5a), 532 ± 65 nM and 4400 ± 1500 fmol/mg protein for rat A_{2B}ARs, and 465 ± 104 nM and 1480 ± 451 fmol/mg for mouse A_{2B}ARs (Fig. 5b). The competition binding assay conditions were subsequently used for the determination of the affinities of a structurally diverse set of A_{2B}AR ligands, agonists, and antagonists.

For *agonists* determined versus the *agonist radioligand* [^{3}H]NECA, the following rank order of potency at human A_{2B}ARs was determined: NECA > CADO > BAY 60-6583 (Figs. 5a–d and 6, Supplemental Table 1). For *agonists* determined versus the *antagonist radioligand* [^{3}H]PSB-603, a

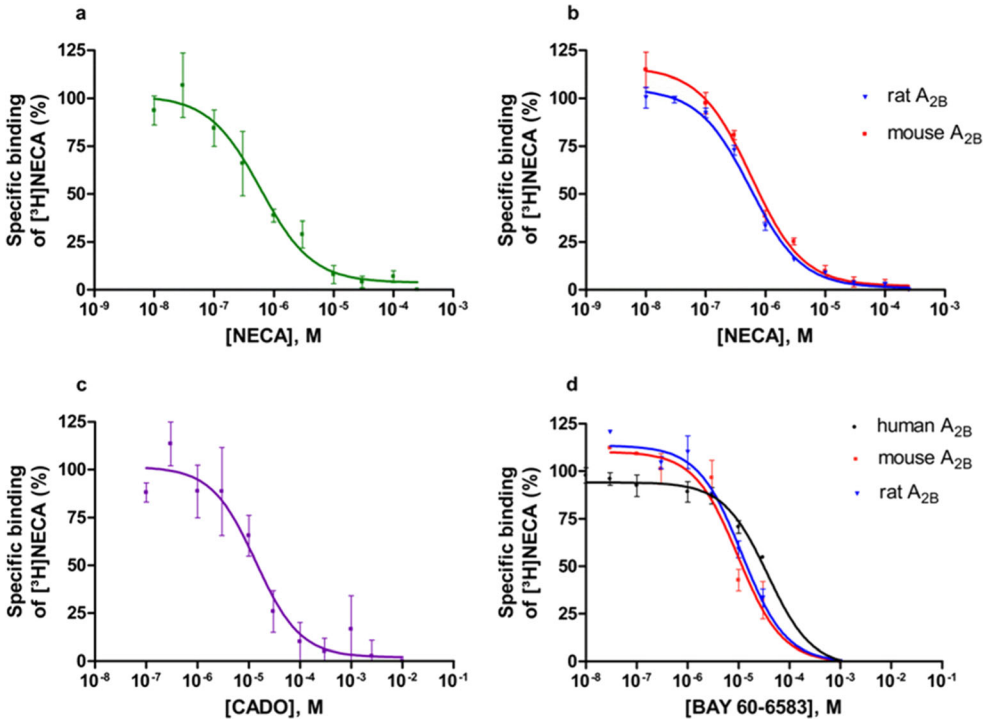

Fig. 5 Homologous competition binding experiments: concentration-dependent inhibition of radioligand binding (30 nM [^{3}H]NECA) by unlabeled NECA at membrane preparations of CHO cells recombinantly expressing **a** human, **b** rat, or mouse A_{2B}ARs. The calculated K_D and B_{max} values were **a** for human A_{2B}ARs: 663 ± 87 nM and 2220 ± 71 fmol/mg protein, **b** for rat A_{2B}ARs: 532 ± 65 nM and 4400 ± 1500 fmol/mg protein, and for mouse A_{2B}ARs: 465 ± 104 nM and 1480 ± 451 fmol/mg protein. Data points are means ± SEM of three to five independent experiments performed in duplicates. **c** Competition binding of CADO versus 30 nM [^{3}H]NECA at human A_{2B}ARs. A monophasic curve was obtained, and a K_i value of of 8570 ± 1110 nM was determined. Data points are means ± SEM of four independent experiments each performed in duplicates. **d** Competition binding of BAY 60-6583 versus 30 nM [^{3}H]NECA at human, mouse, and rat A_{2B}ARs. The curves were best fitted by monophasic isotherms. At human A_{2B}ARs, a K_i value of 31,400 ± 7750 nM, at mouse A_{2B}AR, a K_i value of 10,300 ± 3400 nM, and at rat A_{2B}AR, a K_i value of 10,400 ± 3430 nM were determined. In all experiments, non-specific binding was determined in the presence of 250 μM NECA. Data points are means ± SEM of two (mouse), three (rat), or five (human) independent experiments performed in duplicates (curves were extrapolated because of low solubility of the compound at concentrations > 30 μM)

different rank order of potency at human A_{2B}ARs was observed: BAY 60-6583 > NECA > CADO (Fig. 6, Supplemental Table 1).

For 2-chloroadenosine (CADO), a monophasic curve was obtained (Fig. 5c), and the determined K_i value versus [^{3}H]NECA was about 3-fold lower than that determined versus the antagonist radioligand [^{3}H]PSB-603 (Fig. 6). Likewise, the K_i value determined for NECA at human A_{2B}ARs was significantly (4-fold) lower when determined versus [^{3}H]NECA as compared to the value obtained versus the antagonist radioligand [^{3}H]PSB-603 (Fig. 6). In contrast, displacement of [^{3}H]NECA by BAY 60-6583 at human, mouse, and rat A_{2B}ARs showed a lower apparent affinity of BAY 60-6583 in comparison with the K_i value determined versus [^{3}H]PSB-603 (Figs. 5d and 6, Supplemental Table 1).

As a next step, competition binding experiments for the structurally diverse A_{2B}AR antagonists caffeine, PSB-603, and CGS-15943 versus the antagonist radioligand [^{3}H]PSB-603 and versus the agonist radioligand [^{3}H]NECA were performed (Fig. 7, Supplemental Fig. 5a, b, c, Supplementary Table 1), and the following rank order of potency was determined: PSB-603 > CGS-15943 > caffeine. Data from competition binding experiments of the antagonists versus [^{3}H]NECA were best fitted to a monophasic equation (Supplemental Fig. 5a, b, c). All K_i values determined versus the agonist radioligand [^{3}H]NECA were significantly higher when compared to the K_i values determined versus the antagonist radioligand [^{3}H]PSB-603. For caffeine, the determined K_i value versus [^{3}H]NECA was 3-fold higher than that determined versus the antagonist radioligand [^{3}H]PSB-603 (Fig. 7). Likewise, the K_i value determined for PSB-603 at human A_{2B}ARs was significantly (4-fold) higher when determined versus [^{3}H]NECA as compared to the value obtained versus the antagonist radioligand [^{3}H]PSB-603 (Fig. 7). The largest difference was observed for the adenine-like antagonist CGS-15943 (12-fold difference). All three investigated antagonists were not able to fully block [^{3}H]NECA binding (see Supplemental Fig. 5a, b, c) in contrast to the agonists, which completely displaced [^{3}H]NECA binding.

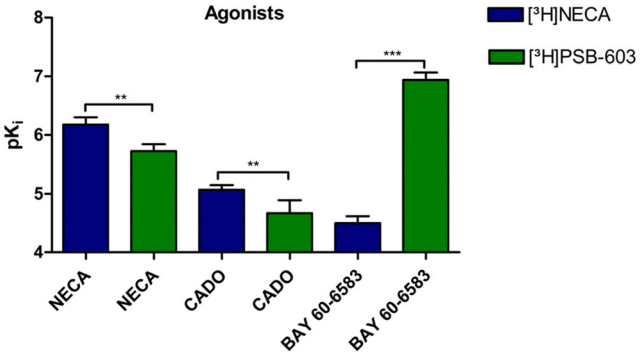

Fig. 6 Affinities of AR agonists determined in competition binding experiments using the agonist radioligand [^{3}H]NECA and the antagonist radioligand [^{3}H]PSB-603 at human A_{2B}ARs. Non-specific binding was determined in the presence of 250 μM NECA ([^{3}H]NECA) or 10 μM DPCPX ([^{3}H]PSB-603). The calculated K_i values versus [^{3}H]NECA were 663 ± 87 nM for NECA, 8570 ± 1110 nM for CADO, and 31,400 ± 7750 nM for BAY 60-6583. The calculated K_i values versus [^{3}H]PSB-603 were 1890 ± 240 nM for NECA [16], 21,400 ± 5700 nM for CADO [16], and 114 ± 36 nM for BAY 60-6583 [50]. Literature data were from our laboratory performed under the same conditions

Discussion

The initial goal of the present study was to establish a radioligand binding assay with the A_{2B}AR agonist [^{3}H]BAY 60-6583 as a tool for labeling an active A_{2B}AR receptor conformation. BAY 60-6583 had previously been reported to display an EC_{50} value in the low nanomolar range (3–10 nM) at the human A_{2B}AR as determined in a gene reporter assay [49] combined with high selectivity versus the other AR subtypes (EC_{50} > 10 μM). However, in radioligand binding assays versus the antagonist radioligand [^{3}H]PSB-603, it had shown a K_i value of only 114 nM [50]. Functional assays are highly dependent on receptor expression levels and receptor reserve, and are therefore often not comparable with a compound's receptor affinity. Because of major difficulties in establishing an A_{2B}AR binding assay with [^{3}H]BAY 60-6583 mainly due to its low affinity and high non-specific binding which could not be further reduced with different optimization steps, the compound was not suitable as an agonist radioligand at A_{2B}ARs (see Fig. 2, Supplemental Figs. 2, 3, 4).

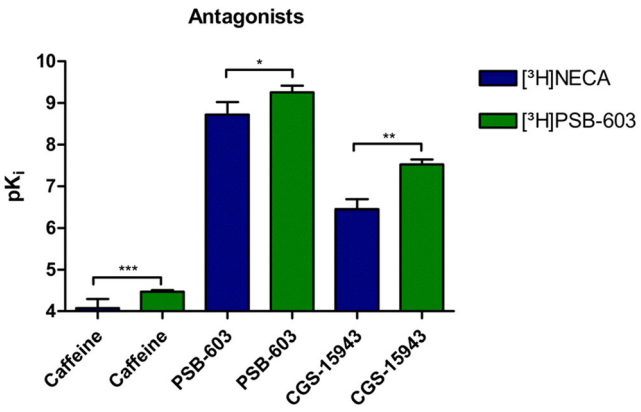

Fig. 7 Competition binding experiments of the antagonists caffeine, PSB-603, and CGS-15943 versus [^{3}H]NECA or [^{3}H]PSB-603 at human A_{2B}ARs. Non-specific binding was determined in the presence of 250 μM NECA ([^{3}H]NECA) or 10 μM DPCPX ([^{3}H]PSB-603). The calculated K_i values versus [^{3}H]NECA were 83,700 ± 6000 nM for caffeine, 1.89 ± 0.78 nM for PSB-603, and 357 ± 107 nM for CGS15943. The calculated K_i values versus [^{3}H]PSB-603 were 33,800 ± 1200 nM for caffeine [16], 0.553 ± 0.103 nM for PSB-603 [16], and 30.0 ± 3.9 nM for CGS-15943 [16]. Literature data were from our laboratory performed under the same conditions

Therefore, we characterized the binding of the tritiated non-specific A_{2B}AR agonist NECA at membrane preparations of CHO or HEK cells stably transfected with human, mouse, or rat A_{2B}ARs. Kinetic experiments at human A_{2B}ARs demonstrated that both association and dissociation are monophasic (Fig. 3a, b). Although the experiments were conducted at 4 °C, the association ($t_{1/2}$ = 9.7 ± 1.0 min) and in particular the dissociation ($t_{1/2}$ = 0.4 ± 0.1 min) were very fast. This relatively fast dissociation is consistent with the moderate affinity of NECA for this receptor subtype. Similarly, fast binding kinetics of [^{3}H]NECA at bovine A_{2B}ARs were reported by Casado et al. [42].

Saturation binding experiments at human and rat A_{2B}ARs were best fitted using a single site binding model (Fig. 4a, b). The determined K_D values of 441 ± 169 nM at human A_{2B}ARs and 325 ± 144 nM at rat A_{2B}AR were in the same range as those determined in homologous competition binding experiments (Fig. 5a, b). For mouse A_{2B}ARs, a K_D value of 465 ± 104 nM was determined in homologous competition binding experiments (Fig. 5b). The B_{max} values determined with [^{3}H]NECA at membrane preparations of recombinant cell lines expressing human, mouse, or rat A_{2B}ARs were around 2000 fmol/mg protein and thus higher than those determined with the antagonist [^{3}H]PSB-603 at human (502 ± 57 fmol/mg protein) and mouse (645 ± 51 fmol/mg protein) A_{2B}ARs [16]. In a native cell system, when no agonist is present, there are generally more receptors in the G protein-uncoupled, inactive conformational state than in the G protein-coupled, active state. It is believed that neutral antagonists bind to all affinity states with similar affinity, whereas inverse agonists preferably bind to an inactive receptor state. Full agonists prefer or even shift the equilibrium to an active receptor conformation, which they stabilize [51]. One reason for the higher B_{max} value determined with [^{3}H]NECA is probably the fact that $MgCl_2$ was included in the agonist assays. Mg^{2+} ions increase the coupling of the A_{2B}ARs to G proteins and therefore promote the active receptor conformation [52]. At A_{2A}ARs, it was found that $MgCl_2$ increased agonist radioligand binding, e.g., for [^{3}H]NECA and [^{3}H]CGS21680, and reduced non-specific binding [53].

In contrast, PSB-603 is an inverse agonist detecting the inactive receptor state. The affinity of the adenosine derivatives NECA and CADO was approximately 3–4-fold higher when tested versus the agonist radioligand than versus

[^{3}H]PSB-603, which would be expected for agonists. In contrast, the determined A_{2B}AR affinities for structurally diverse antagonists were higher when tested versus the antagonist as compared to agonist radioligand (Fig. 7, Supplemental Table 1).

For the A_{2A}AR, similar differences between agonists and antagonists had previously been observed [52]: K_i values of agonists determined versus the antagonist radioligand [^{3}H]MSX-2 were 3–7 times higher than K_i values determined versus the agonist radioligand [^{3}H]CGS-21680 [29]. Also, for the A_1AR, it was shown that there is a significant rightward shift in agonist binding curves, most pronounced for rat brain A_1ARs, when using an antagonist radioligand rather than an agonist radioligand (Supplemental Table 2), and this discrepancy may be used to functionally characterize A_{2A}AR ligands for differentiation between agonists and antagonists. For human recombinant A_1ARs, however, this observation could not be confirmed [54]. It appears that the decrease in affinity may be species or cell context-dependent.

Although BAY 60-6583 is a potent A_{2B}AR agonist [9], it showed a large rightward shift in affinity when tested versus the agonist radioligand [^{3}H]NECA as compared to binding studies versus the antagonist/inverse agonist radioligand [^{3}H]PSB-603 (Figs. 5d and 6, Supplemental Table 1). Thus, BAY 60-6583 behaved like the antagonists, not like the agonists. Because it is structurally very different from adenosine derivatives, which are characterized by a nucleoside structure featuring a ribosyl residue (see Fig. 1), the (partial) agonist BAY 60-6583 might stabilize a very different receptor conformation than the full agonist NECA.

Moreover, it was observed that [^{3}H]NECA binding could not be fully displaced by the antagonists, while [^{3}H]PSB-603 binding was fully blocked by the compounds. This may suggest different binding modes of nucleosides (adenosine-derived agonists) on the one hand and antagonists on the other hand (see Supplemental Fig. 5a, b, c). Another more likely explanation would be the presence of a previously described NECA-binding protein designated adenotin [55, 56]. Adenotin, which was later identified as heat shock protein paralog Grp94 [57], was reported to display submicromolar affinity for NECA and to lack affinity for adenosine receptor antagonists [55, 56]. Saturation analysis with soluble and membrane-derived adenotin had shown a K_D value for NECA of 220 nM (soluble) and 210 nM (membrane-derived) [56]. In a previous study performed in chromaffin plasma cell membranes, the xanthine antagonist DPCPX had shown complete inhibiton of [^{3}H]NECA binding [42], which might be due to the lack of expression of the NECA-binding protein adenotin in the employed cell line. Binding to non-transfected HEK cell membranes showed some specific binding, which was, however, moderate and may be at least partly due to the endogenous expression of A_{2B}ARs [58] (see SI Fig. S6). In order to avoid effects that are due to binding of [^{3}H]NECA to potentially present adenotin/Grp94, it may be advisable to use an A_{2B}AR antagonist for the determination of non-specific binding.

Conclusions

[^{3}H]BAY 60-6583, a potent and functionally selective A_{2B}AR (partial) agonist, was prepared and examined as a tool for labelling an active A_{2B}AR conformation. However, despite excessive efforts, we have not been able to establish a radioligand binding assay with this compound mainly due to its high non-specific binding. Nevertheless, [^{3}H]BAY 60-6583 may still be useful for studying in vivo distribution and metabolism of the drug. For the labeling of human, rat, and mouse A_{2B}ARs, we subsequently investigated binding of the non-selective agonist [^{3}H]NECA as an alternative. This adenosine-like agonist labeled A_{2B}ARs with an affinity in the higher nanomolar range and showed acceptable non-specific binding due to its high polarity. [^{3}H]NECA is currently the method of choice for radioligand binding studies aimed at labeling the "high-affinity" active conformation of A_{2B}ARs. Nevertheless, the development of more potent and selective A_{2B}AR agonists would be highly desirable. It has to be pointed out that different agonists are most likely binding to and stabilizing different receptor conformations. This was observed in the present study for adenosine derivatives in comparison to the non-nucleoside (partial) agonist BAY 60-6583, an aminopyridine derivative. Moreover, large affinity differences may be measured for compounds dependent on the structure of the radioligand that is used for affinity determination, since each radioligand may label (induce, stabilize) a specific receptor conformation. X-ray structures of the A_{2B}AR (active conformation) in complex with nucleoside and non-nucleoside agonists would be of great value to further elucidate the receptor's activation mechanisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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