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**Capadenoson, a clinically trialed partial adenosine A₁ receptor agonist, can stimulate
adenosine A_{2B} receptor biased agonism**

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Abstract

The adenosine A_{2B} receptor (A_{2B}AR) has been identified as an important therapeutic target in cardiovascular disease, however *in vitro* and *in vivo* targeting has been limited by the paucity of pharmacological tools, particularly potent agonists. Interestingly, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583), a potent and subtype-selective A_{2B}AR agonist, has the same core structure as 2-amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methylsulfanyl]-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitril (capadenoson). Capadenoson, currently classified as an adenosine A₁ receptor (A₁AR) partial agonist, has undergone two Phase IIa clinical trials, initially in patients with atrial fibrillation and subsequently in patients with stable angina. Capadenoson has also been shown to decrease cardiac remodeling in an animal model of advanced heart failure and a capadenoson derivative, neladenoson bialanate, recently entered clinical development for the treatment of chronic heart failure. The therapeutic effects of capadenoson are currently thought to be mediated through the A₁AR. However, the ability of capadenoson to stimulate additional adenosine receptor subtypes, in particular the A_{2B}AR, has not been rigorously assessed. In this study, we demonstrate that capadenoson does indeed have significant A_{2B}AR activity in physiologically relevant cells, cardiac fibroblasts and cardiomyocytes, which endogenously express the A_{2B}AR. Relative to the non-selective adenosine receptor agonist NECA, capadenoson was a biased A_{2B}AR agonist with a preference for cAMP signal transduction over other downstream mediators in cells with recombinant and endogenous A_{2B}AR expression. These findings suggest the reclassification of capadenoson as a dual A₁AR/A_{2B}AR agonist. Furthermore, a potential A_{2B}AR contribution should be an important consideration for the future clinical development of capadenoson-like therapeutics, as the A_{2B}AR can promote cardioprotection and modulate cardiac fibrosis in heart disease.

Keywords

Capadenoson; adenosine A_{2B} receptor; biased agonist; cardiomyocytes; cardiac fibroblasts

Abbreviations

A₁AR, adenosine A₁ receptor; A_{2A}AR, adenosine A_{2A} receptor; A_{2B}AR, adenosine A_{2B} receptor; A₃AR, adenosine A₃ receptor; ADA, adenosine deaminase; AR, adenosine receptor; ANOVA, analysis of variance; BAY60-6583, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide; BrdU, 5-Bromo-2'-deoxyuridine, BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle medium; [³H]DPCPX, cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2, 3-³H(N)]; ERK1/2, extracellular signal-regulated kinases 1 and 2; FBS, fetal bovine serum; FlpIn-CHO, Chinese hamster ovary cells with FlpIn™ vector; GPCR, G protein-coupled receptor, HIF-1α, hypoxia-inducible factor 1-α; HTRF, homogeneous time resolved fluorescence; IP₁, inositol monophosphate; NECA, 5'-N-ethylcarboxamidoadenosine; NT, Non-transfected; NVCF, neonatal ventricular cardiac fibroblast; NVCM, neonatal ventricular cardiomyocyte; pERK1/2, phosphorylated extracellular signal-regulated kinase 1 and 2; PLC, phospholipase C; PSB-603, 8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl))-1-propylxanthine; RTCA, real-time cell analyzer; SCH 442416, 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine; SEM, standard error of the mean; VCP746, 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-N-(6-(9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-9H-purin-6-ylamino)hexyl)benzamide

Chemical compounds studied in this article

NECA (PubChem CID: 448222); BAY60-6583 (PubChem CID: 11717831); Capadenoson (PubChem CID: 9936489); [3H]DPCPX (PubChem CID: 53319341); PSB-603 (PubChem

CID: 44185871); SLV320 (PubChem CID: 9953065); SCH442416 (PubChem CID:
10668061)

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1. Introduction

The adenosine A_{2B} receptor (A_{2B}AR) belongs to the adenosine family of G protein-coupled receptors (GPCRs), which also includes the adenosine A₁, A_{2A} and A₃ receptors (A₁AR, A_{2A}AR, A₃AR) [1]. Pleiotropically coupled, activating both G_s and G_q proteins upon ligand stimulation [1,2], the A_{2B}AR is up-regulated by hypoxia-inducible factor-1 α (HIF-1 α) [3] and implicated in numerous pathologies, including ischemia-reperfusion injury [4-6], fibrosis [7], diabetes [8] and cancer [9-11]. Despite the A_{2B}AR representing a novel therapeutic target for a variety of disease states, there remains a paucity of potent and selective A_{2B}AR agonists. Currently, agonists for the A_{2B}AR can be classified according to the presence or absence of a nucleoside-like core. Adenosine modifications at the N⁶ position and C² position have generated agonists with increased potency for the A_{2B}AR [12-14]. For example, the N⁶ modified adenosine derivative, 5'-N-ethylcarboxamidoadenosine (NECA; Fig. 1), has commonly been used as one of the highest potency A_{2B}AR agonists, despite being non selective across the different adenosine receptor (AR) subtypes [13,15]. In an effort to enhance subtype selectivity, the focus has shifted to the development of non-nucleoside agonists. Centered around compounds that contain a 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine core, this approach has successfully identified a potent A_{2B}AR agonist, 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583; Fig. 1) [16]. Importantly, and in contrast to adenosine-like compounds, the non-nucleoside partial agonist BAY60-6583 shows greater than 3000-fold selectivity for the A_{2B}AR over other AR subtypes [17,18].

In addition to optimizing ligand affinity, efficacy and subtype selectivity, it is becoming increasingly recognized that refining the signaling repertoire stimulated upon receptor activation will likely enhance clinical translation [19,20]. Biased agonism describes the ability of an agonist to stabilize a unique subset of GPCR conformations and thereby

modulate intracellular signaling in a manner that is distinct to that of the endogenous ligand [21]. Compounds that explore distinct chemical space, such as the non-nucleoside A_{2B}AR ligands, are likely to stabilize a distinct spectrum of GPCR conformations and subsequently engender biased agonism. At the A₁AR, in contrast to prototypical adenosine-like agonists, structurally distinct agonists such as the bitopic agonist 4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-*N*-(6-(9-((2*R*,3*R*,4*S*,5*R*)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydro-furan-2-yl)-9*H*-purin-6-ylamino)hexyl)benzamide (VCP746) and the non-nucleoside agonist 2-amino-6-[[2-(4-chlorophenyl)-1,3-thiazol-4-yl]methylsulfanyl]-4-[4-(2-hydroxyethoxy)phenyl]pyridine-3,5-dicarbonitril (capadenoson; Fig. 1) have been shown to engender biased agonism [22,23]. Furthermore, A_{2B}AR homology modeling, based on a high resolution A_{2A}AR crystal structure, suggested that non-nucleoside and nucleoside-containing agonists exhibit different docking conformations [24], which could contribute to biased signaling. In agreement with this suggestion, BAY60-6583 was recently reported to display biased agonism relative to NECA at the A_{2B}AR [25].

Capadenoson, which previously entered into two Phase IIa clinical trials as an A₁AR partial agonist in patients with atrial fibrillation and stable angina, has been purported to be a selective A₁AR agonist with beneficial cardiovascular effects [26-31]. Strikingly, capadenoson shares the same 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine core structure to that of the non-nucleoside A_{2B}AR agonist BAY60-6583 (Fig. 1). The structural similarity with BAY60-6583 raises the possibility that capadenoson may have previously unappreciated A_{2B}AR activity. Furthermore, similar to that observed at the A₁AR, capadenoson may stimulate A_{2B}AR biased agonism and therefore represent a new pharmacological tool to interrogate A_{2B}AR signal transduction.

Therefore, the aim of the current study was to rigorously characterize the pharmacology of capadenoson at the A_{2B}AR. This was initially performed in FlpIn-CHO cells

1 stably transfected with the human A_{2B}AR and then in HEK293T and primary cardiac cells
2 that endogenously express the A_{2B}AR. In addition, the A_{2B}AR signaling profile for
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4 capadenoson was investigated across multiple pathways and compared to the non-selective
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6 adenosine-like agonist, NECA and the subtype selective non-nucleoside partial agonist,
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9 BAY60-6583. Capadenoson was found to promote A_{2B}AR signal transduction with a unique
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11 bias profile. Furthermore, capadenoson retained the ability to mediate significant A_{2B}AR
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13 activity in primary isolated neonatal rat cardiac myocytes and fibroblasts. Therefore, the
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15 ability of capadenoson to stimulate the A_{2B}AR, in addition to the A₁AR, may contribute to the
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17 desirable cardiovascular effects previously observed *in vivo*.
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2. Materials and Methods

2.1 Materials

Dulbecco's Modified Eagle Medium (DMEM), Fluo-4, trypsin, antibiotic/antimycotic and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). Adenosine deaminase (ADA) and hygromycin-B were purchased from Roche (Basel, Switzerland). Fetal bovine serum (FBS) was purchased from ThermoTrace (Melbourne, Australia). The IP-One homogeneous time resolved fluorescence (HTRF) kit was obtained from Cisbio Bioassays (Codolet, France). Type II collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ). xCELLigence 96-well plates were purchased from ACEA Biosciences (San Diego, CA). AlphaScreen SureFire extracellular signal-regulated kinases 1 and 2 (ERK1/2) kits, AlphaScreen cAMP kits and AlphaScreen LANCE cAMP kits, Ultima Gold scintillation cocktail and cyclopentyl-1, 3-dipropylxanthine, 8-[dipropyl-2, 3-³H(N)] ([³H]DPCPX) were purchased from PerkinElmer (Waltham, MA). Adenosine receptor ligands 2-((6-amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)-2-pyridinyl)thio)acetamide (BAY60-6583), 8-(4-(4-(4-chlorophenyl)piperazine-1-sulfonyl)phenyl)-1-propylxanthine (PSB-603) and 2-(2-furanyl)-7-[3-(4-methoxyphenyl)propyl]-7H-pyrazolo[4,3-e][1,2,4]triazolo[1,5-c]pyrimidin-5-amine (SCH 442416) were from Tocris Bioscience (Bristol, UK) and capadenoson was purchased from MedChemExpress (Shanghai, China). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO) and were of analytical quality.

2.2 Cell culture and membrane preparation

Recombinant FlpIn-CHO cells stably expressing the human A₁AR, A_{2A}AR, A_{2B}AR or A₃AR were generated as previously described [32]. HEK293T cells endogenously expressing the A_{2B}AR were purchased from Invitrogen (Carlsbad, CA). All cell lines were grown in DMEM supplemented with 10% FBS and maintained at 37°C in a humidified incubator

containing 5% CO₂. In FlpIn-CHO cells, stable AR expression was maintained by the addition of the selection antibiotic hygromycin-B (500 µg/mL). For ERK1/2 phosphorylation, calcium mobilization and IP1 accumulation assays, cells were seeded into 96-well culture plates at a density of 4×10^4 cells/well. After 6 h, cells were washed with serum free DMEM and maintained in serum free DMEM for 12 - 18 h at 37°C in 5% CO₂ before assaying. For cAMP accumulation assays, cells were seeded into 96-well culture plates at a density of 2×10^4 cells/well and incubated overnight at 37°C in 5% CO₂ prior to assaying. For all assays, HEK293T cells were seeded onto plates pre-coated with poly-D-lysine and HEK293T cell assays were performed in the presence of SCH 442416 (100 nM) to antagonize endogenously expressed A_{2A}AR [33]. A_{2B}AR-FlpIn-CHO cell membranes were prepared as previously described [34].

2.3 Rat neonatal cardiomyocyte and fibroblast isolation

Animal experiments were conducted in accordance with Monash Institute of Pharmacy and Pharmaceutical Sciences animal ethics committee-approved protocols (MIPS.2015.23) and conformed to the requirements of the National Health and Medical Research Council of Australia *Code of Practice for the Care and Use of Animals for Scientific Purposes*. Isolation of primary neonatal rat ventricular cardiomyocytes (NVCs) and primary neonatal rat ventricular cardiac fibroblasts (NVCFs) was performed as previously described [35]. Briefly, hearts were rapidly excised from 1-2 day old Sprague-Dawley rat pups and ventricles dissected and placed in Hanks balanced salt solution containing 0.1% trypsin overnight at 4°C. Following trypsin deactivation, ventricles were successively digested with type II collagenase (2.5 mg/heart) in Hanks balanced salt solution in a shaking water bath at 37°C for 10 min. After the fourth digestion, supernatants were pooled and centrifuged for 5 min at 485g. The pellet was then resuspended in DMEM containing 10% FBS and pre-plated onto 150-mm culture dishes for 1 h at 37°C in 5% CO₂ to separate neonatal ventricular

cardiomyocytes (NVCMs) from neonatal ventricular cardiac fibroblasts (NVCFs). Non-adherent cells (NVCMs) were removed and transferred to a fresh culture dish for a further 1 h of pre-plating prior to counting with a haemocytometer. NVCMs were subsequently plated into 96-well plates in DMEM containing 10% FBS, 1% antibiotic/antimycotic and 100 μ M 5-bromo-2'-deoxyuridine (BrdU), at a density of 4×10^4 cells/well and incubated at 37°C in 5% CO₂ for 24 h. Media was replaced with DMEM containing 10% FBS and 1% antibiotic/antimycotic and experiments were performed 72 h later.

Culture dishes containing adherent NVCFs were maintained in DMEM containing 10% FBS and 1% penicillin-streptomycin at 37°C in 5% CO₂ humidified incubator and grown to confluence. Cells were passaged after trypsin/versene detachment and subsequently seeded into 96-well plates at a density of 2×10^4 cells/well. For all experiments, NVCFs were used at passage 2 and serum starved for 24 h prior to assaying.

2.4 Radioligand equilibrium competition binding

A_{2B}AR-FlpIn-CHO cell membrane homogenates (100 μ g) were incubated in HEPES-buffered saline solution (10 mM HEPES, 146 mM NaCl, 10 mM D-glucose, 5 mM KCl, 1 mM MgSO₄, 1.3 mM CaCl₂, and 1.5 mM NaHCO₃, pH 7.4) containing 1 U/mL ADA and 10 nM [³H]DPCPX in the absence and presence of increasing concentrations of capadenoson for 1 h at room temperature. Non-specific binding was determined in the presence of 100 μ M NECA. Incubation was terminated by rapid filtration through 0.9% NaCl pre-soaked Whatman GF/B filters using a membrane harvester (Brandel, Gaithersburg, MD). Filters were washed five times with 2 mL of ice-cold 0.9% NaCl and dried, prior to addition of Ultima Gold scintillation cocktail and determination of radioactivity as described previously [36].

2.5 ERK1/2 phosphorylation

ERK1/2 phosphorylation (pERK1/2) assays were performed as described in detail previously [22]. Briefly, pERK1/2 concentration response curves were generated for A_{2B}AR-

1 FlpIn-CHO and HEK293T cells at 37°C upon exposure of cells to A_{2B}AR agonists for the
2 time corresponding to peak pERK1/2 phosphorylation (NECA: 5 min, BAY60-6583: 5 min,
3 Capadenoson: 7 min). Agonist stimulation was terminated by addition of 50 µL SureFire lysis
4 buffer to each well, followed by AlphaScreen detection as previously described [22]. Agonist
5 concentration-response curves were normalized to the response mediated by the positive
6 control, 10% v/v FBS (5 min exposure).
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10 **2.6 Calcium mobilization**

11 Calcium mobilization assays were performed as described in detail previously [22].
12 Briefly, A_{2B}AR-FlpIn-CHO, pre-incubated for 1 h with 1 µM Fluo4, were stimulated with
13 agonist in a FlexStation plate reader (Molecular Devices, Sunnyvale, CA) and fluorescence
14 measurements were obtained. Agonist concentration-response curves constructed for calcium
15 mobilization were normalized to the response mediated by the positive control, adenosine
16 triphosphate (ATP; 100 µM).
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31 **2.7 IP₁ accumulation**

32 Media was removed from 96-well plates and replaced with IP₁ stimulation buffer (10
33 mM HEPES, 1 mM CaCl₂, 0.5 mM MgCl₂, 4.2 mM KCl, 146 mM NaCl, 5.5 mM D-glucose,
34 50 mM LiCl, pH 7.4) and maintained at 37°C for 30 min. Cells were then exposed to IP₁
35 stimulation buffer in the absence or presence of agonist and incubated for an additional 1 h at
36 37°C. Agonist stimulation was terminated by the removal of buffer and the addition of 25
37 µL/well IP-One HTRF lysis buffer. Detection of IP₁ accumulation was performed as
38 described previously [34]. In brief, lysates (14 µL) were transferred to a 384-well proxiplate
39 and a 1:1:40 (v/v/v) dilution of IP₁-d2 conjugate:Anti-IP₁ Lumi4TM-Tb cryptate conjugate:IP-
40 One HTRF lysis buffer (6 µL) was added to each well. Plates were incubated for 1 h at room
41 temperature before fluorescence (calculated as 665 nm / 620 nm ratio) was measured on an
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EnVision plate reader (PerkinElmer; Waltham, MA, USA). Data were normalized to the maximal response elicited by 10 μ M NECA.

2.8 cAMP accumulation

Inhibition of forskolin stimulated cAMP accumulation in A₁AR and A₃AR-FlpIn-CHO cells was performed as described previously [22,37]. Stimulation of cAMP accumulation in A_{2A}AR- and A_{2B}AR-FlpIn-CHO, and HEK293T cells involved the removal of media and addition of cAMP stimulation buffer (140 mM NaCl, 5 mM KCl, 0.8 μ M MgSO₄, 0.2 mM Na₂HPO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 5.6 mM D-glucose, 5 mM HEPES, 0.1% BSA, 1 U/mL ADA and 10 μ M rolipram, pH 7.4) in the absence or presence of PSB-603 (1 μ M), followed by a 30 min incubation at 37°C. Cells were then exposed to agonist for 30 min at 37°C. For NVCFs, media was replaced with stimulation buffer and incubated for 30 min at 37°C, followed by addition of agonist and forskolin (1 μ M) for an additional 20 min at 37°C. For NVCMs, media was replaced with stimulation buffer and the A₁AR antagonist, SLV320 (1 μ M) and incubated for 1 h at 37°C. Agonist and forskolin (1 μ M) were then added for an additional 30 min at 37°C. Stimulation was terminated by removal of cAMP stimulation buffer and addition of 50 μ L/well ice-cold 100% ethanol. Subsequent to evaporation of the ethanol, 50 μ L cAMP lysis buffer (0.1% BSA, 0.3% Tween-20 and 5 mM HEPES, pH 7.4) was added to each well and plates were agitated for 10 min. Detection of cAMP for FlpIn-CHO cells was performed using AlphaScreen methods as described previously [22]. Detection of cAMP for HEK293T, NVCM and NVCF cells was performed using a LANCE cAMP kit. LANCE cAMP detection involved the addition of 1:200:400 (v/v/v) of Alexa Fluor647-anti cAMP antibody:LANCE cAMP detection buffer:lysate in a 384-well optiplate in a total volume of 15 μ L, followed by incubation at room temperature in the dark for 30 min. Subsequently, 10 μ L/well of a pre-equilibrated 1:3.5:5000 (v/v/v) dilution of LANCE Eu-W8044 labeled streptavidin beads:biotinylated

cAMP:LANCE cAMP detection buffer was added. Plates were further incubated for 1 h at room temperature and fluorescence read on an EnVision plate reader (PerkinElmer; Waltham, MA) using standard LANCE settings. All agonist concentration-response curves were normalized to the response mediated by the positive control, forskolin (FSK; 1, 3 or 10 μ M as specified in the results).

2.9 Cellular impedance assay

Agonist-mediated changes in cellular impedance, defined as the cell index, were measured by the xCELLigence real-time cell analyzer (RTCA) single-plate reader (Roche Applied Science and ACEA Biosciences, San Diego, CA). To assess agonist-mediated changes in cellular impedance in NVCMs and NVCFs, media in E-plates was replaced with DMEM containing 10% FBS and placed into the RTCA at 37°C in 5% CO₂ and baseline impedance measured at 10 min intervals. Antagonist or vehicle was then added and cell index values obtained at 15 s intervals for 1 h. Cells were subsequently exposed to agonist or vehicle and cell index values obtained at 15 s intervals for a further 2.5 h. For both NVCMs and NVCFs, data were normalized to the cell index values from the time point immediately prior to agonist or vehicle addition. Data were visualized by plotting the normalized cell index data against time. Agonist-mediated effects on cell index were compared at a single time point, t = 30 min post agonist addition, which corresponded to the approximate time of maximal change in the normalized cell index.

2.10 Data Analysis

Statistical analysis and curve fitting were performed using Prism 6 (GraphPad Software, San Diego, CA). Capadenoson inhibition binding data were fit to a one-site inhibition mass action curve:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(X - Log IC_{50})}} \quad \text{Equation 1}$$

where Top is the specific binding of the radioligand in the absence of unlabeled ligand (X), Bottom is the specific radioligand binding equivalent to non-specific binding and IC₅₀ is the concentration of unlabeled ligand (X) that reduced the radioligand specific binding by half. The resulting IC₅₀ values were converted to dissociation constants, log(K_I) values, using the Cheng & Prusoff equation [38]. Agonist concentration-response data were fit to a three-parameter Hill equation:

$$Response = Basal + \frac{(E_{max} - Basal) \times [A]}{EC_{50} + [A]} \quad \text{Equation 2}$$

where Basal is the magnitude of the response in the absence of agonist (A) and EC₅₀ is the concentration of agonist required to generate a response halfway between Basal and the maximal effect (E_{max}). Signaling bias was quantified by fitting concentration-response curves to a re-parameterization of the operational model of agonism as described previously [22].

All results were expressed as the mean ± standard error of the mean (SEM). Statistical significance was defined as P<0.05 as determined by one-way analysis of variance (ANOVA) with Tukey or Dunnett's multiple comparisons post hoc analysis or t-test as indicated within the results.

3. Results

3.1 Capadenoson has a similar affinity as NECA and BAY60-6583 for the human A_{2B}AR

Given the structural similarity of capadenoson to the prototypical A_{2B}AR agonist BAY60-6583 (Fig. 1), the first objective of the current study was to address whether capadenoson had affinity for the human A_{2B}AR. The affinity of capadenoson, estimated from [³H]DPCPX (10 nM) competition binding on A_{2B}AR-FlpIn-CHO cell membrane homogenates, was in the low micromolar range with a pK_I of 5.49 ± 0.24 (Fig. 2A). The affinity of capadenoson for the human A_{2B}AR was similar to previously published A_{2B}AR affinity values determined under the same conditions for the non-selective adenosine receptor

agonist NECA and the A_{2B}AR-selective agonist BAY60-6583 [39], which are conventionally used as the highest affinity A_{2B}AR agonists [18,40].

3.2 Capadenoson stimulates potent A_{2B}AR-mediated cAMP accumulation

As capadenoson had appreciable affinity for the A_{2B}AR, the ability of capadenoson to stimulate A_{2B}AR-mediated signal transduction was assessed. In A_{2B}AR-FlpInCHO cells, NECA, BAY60-6583 and capadenoson stimulated robust concentration-dependent cAMP accumulation, with all ligands being full agonists and equipotent for this well-coupled pathway (Table 1; Fig. 2B). Capadenoson has previously been shown to mediate robust inhibition of cAMP accumulation in A₁AR-FlpInCHO cells. The AR subtype selectivity of capadenoson was investigated by comparing agonist concentration-response curves at the A_{2A}AR and A₃AR to that at the A₁AR and A_{2B}AR. A_{2A}AR-mediated stimulation and A₃AR-mediated inhibition of cAMP accumulation was assessed in FlpIn-CHO cells stably transfected with the human A_{2A}AR or A₃AR, respectively. In contrast to the robust activity observed at A₁AR and A_{2B}AR, capadenoson was a weak to modest activator of the A_{2A}AR and A₃AR (Table 2). As observed previously, BAY60-6583 had strong subtype selectivity for the A_{2B}AR, mediating minimal stimulation of the A₁AR, A_{2A}AR and A₃AR (Table 2). Importantly, capadenoson-mediated stimulation of cAMP accumulation was not detected in non-transfected FlpIn-CHO cells (data not shown), confirming the effects observed in the transfected FlpIn-CHO cells were mediated by the A_{2B}AR.

3.3 Agonist-mediated signal transduction and biased agonism in A_{2B}AR-FlpIn-CHO cells

The A_{2B}AR is pleiotropically coupled and therefore, in addition to cAMP accumulation, a number of additional pathways stimulated by the A_{2B}AR were also investigated [1,2]. These included inositol monophosphate (IP₁) accumulation, intracellular calcium mobilization and stimulation of ERK1/2 phosphorylation (pERK1/2). As previous,

these experiments were conducted in A_{2B}AR-FlpIn-CHO cells, which allowed for robust detection of signal transduction and quantification of ligand bias.

In contrast to cAMP accumulation, where capadenoson and NECA were equipotent, capadenoson had lower potency than NECA at the other pathways assessed. The rank order of potency for IP₁ accumulation was NECA>BAY60-6583≈capadenoson (Fig. 3A). BAY60-6583 exhibited a partial response for IP₁ accumulation relative to the full agonist NECA, which is in agreement with previous findings that BAY60-6583 behaves as a partial agonist (Table 1) [18,25]. The rank order of potency for both pERK1/2 and calcium mobilization was NECA≈BAY60-6583>capadenoson, with both BAY60-6583 and capadenoson being partial agonists at these pathways (Fig. 3C and D; Table 1).

3.4 Quantification of capadenoson biased agonism in A_{2B}AR-FlpIn-CHO cells

It is evident that the rank order of agonist potency at the A_{2B}AR was not maintained across all pathways measured, a hallmark of biased agonism [41,42]. Quantification of biased agonism used a derivation of the Black-Leff operational model of agonism. This equation allows for the estimation of transduction coefficients, Log(τ/K_A), which encompass both the affinity and efficacy of an agonist for a given pathway. Normalization to a reference ligand, NECA in this study, generates the $\Delta\text{Log}(\tau/K_A)$, which indicates the relative efficiency of the agonist to stimulate a given pathway. Subsequent normalization to a reference pathway, pERK1/2 in this study, generates the $\Delta\Delta\text{Log}(\tau/K_A)$ or Log(Bias Factor), which provides a quantification of the relative bias for each agonist at each pathway assessed (Fig. 4; Table 3).

Relative to pERK1/2, IP₁ accumulation and calcium mobilization, capadenoson had a significant 8-20 fold bias towards cAMP accumulation, when normalized to the reference ligand, NECA (Table 3; P<0.05; One-way ANOVA Tukey post-hoc analysis on the $\Delta\text{Log}(\tau/K_A)$). Interestingly, compared to reference agonist NECA, BAY60-6583 showed a

significant 5-12 fold bias away from IP₁ accumulation relative to the other signaling pathways assessed (Table 3; P<0.05; One-way ANOVA Tukey post-hoc analysis on the $\Delta\text{Log}(\tau/K_A)$).

3.5 Agonist-mediated signal transduction in HEK293T cells endogenously expressing the A_{2B}AR

A contemporary issue in the field of GPCR biased agonism is how biased agonism changes across different cellular backgrounds and whether biased agonism can be detected and quantified in endogenous expression systems [43]. Although the A_{2B}AR-FlpIn-CHO cell line was useful for rapid screening of biased agonism, subsequent studies investigated the translation of bias from a heterologous expression system into cells endogenously expressing the human A_{2B}AR. These studies used HEK293T cells, a human embryonic kidney cell line which endogenously expresses the A_{2B}AR and A_{2A}AR [33]. Exposure of HEK293T cells to NECA, BAY60-6583 or capadenoson stimulated robust cAMP accumulation, with all ligands behaving as full agonists (Fig. 5A; Table 4). The cAMP accumulation was A_{2B}AR-mediated as the response was virtually abolished in the presence of PSB-603 (1 μM), a highly selective A_{2B}AR antagonist (Fig. 5A). Similar to that observed in A_{2B}AR-FlpIn-CHO cells, BAY60-6583 and capadenoson stimulated a partial pERK1/2 response relative to NECA, with capadenoson having relatively low potency (Fig. 5B; Table 4). A_{2B}AR-mediated accumulation of IP₁ and calcium mobilization could not be detected in HEK293T cells (data not shown), which may be due to a lower A_{2B}AR expression in these cells compared to the heterologous A_{2B}AR-FlpIn-CHO cell line.

The bias profiles of BAY60-6583 and capadenoson were quantified relative to the reference ligand, NECA, and the reference pathway, pERK1/2, as described in section 3.4. Capadenoson exhibited an approximate 9-fold bias towards cAMP accumulation over pERK1/2 (Fig. 5C; Table 5; P<0.01, unpaired t-test on $\Delta\text{Log}(\tau/K_A)$), which is comparable to the 14-fold bias towards cAMP accumulation observed in A_{2B}AR-FlpIn-CHO cells.

3.6 Capadenoson stimulates $A_{2B}AR$ signaling in cardiomyocytes and cardiac fibroblasts

The therapeutic effects of capadenoson have been investigated in a number of cardiovascular diseases including angina, atrial fibrillation and heart failure [26,27,29,30]. Within these studies, the disease-modulating capacity of capadenoson was ascribed to signal transduction downstream of the A_1AR . However, given that capadenoson is a biased $A_{2B}AR$ agonist that can stimulate potent cAMP accumulation in $A_{2B}AR$ -FlpIn-CHO and HEK293T cells, the question remains as to whether capadenoson-mediated $A_{2B}AR$ signaling can be observed in cardiac cells that endogenously express the $A_{2B}AR$. Therefore, capadenoson-mediated $A_{2B}AR$ signal transduction was investigated in isolated neonatal ventricular cardiomyocytes (NVCMs) and neonatal ventricular cardiac fibroblasts (NVCFs). Global cellular changes were assessed using a label-free impedance-based technology, the xCELLigence assay, that assesses changes in the electrical impedance of cells adhered to a microelectrode array. Capadenoson (1 μ M) stimulated a significant change in impedance relative to buffer control in both NVCMs and NVCFs (Fig 6A and B). The significant decrease in cellular impedance stimulated by capadenoson was reduced in the presence of the $A_{2B}AR$ antagonist PSB-603 (1 μ M) (Fig. 6C and D; $P < 0.05$; One-way ANOVA Dunnett's post-hoc analysis). In addition to the $A_{2B}AR$ -mediated change in cellular impedance, capadenoson also stimulated robust cAMP accumulation in NVCFs and NVCMs (Fig. 6E and F), which was virtually abolished in the presence of PSB-603 (1 μ M). These results demonstrate that capadenoson can stimulate appreciable $A_{2B}AR$ -mediated signal transduction in physiologically relevant cells.

4. Discussion

Capadenoson, characterized as an A_1AR partial agonist with cardioprotective pharmacology, has progressed into two Phase IIa clinical trials, the first in patients with atrial

1 fibrillation and the second in patients with stable angina [25,29]. Furthermore, neladenoson
2 bialanate, a capadenoson-like compound, is currently under clinical development as a novel
3 selective A₁AR partial agonist for the treatment of heart failure [53]. The current study
4 reveals, for the first time, that capadenoson is a potent A_{2B}AR agonist and furthermore it has
5 biased agonist activity at the A_{2B}AR. We have demonstrated that capadenoson stimulates
6 A_{2B}AR-mediated signal transduction in both recombinant and endogenously expressing cell
7 lines as well as primary isolated cardiac myocytes and fibroblasts. The signaling profile of
8 capadenoson diverged from that of the non-selective adenosine receptor agonist, NECA, and
9 the A_{2B}AR-selective agonist BAY60-6583. Relative to NECA, capadenoson was significantly
10 biased towards stimulation of cAMP accumulation compared to other second messenger
11 pathways.
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26 The A_{2B}AR is described as a low-affinity receptor due to its modest affinity for the
27 cognate ligand adenosine and other prototypical adenosine receptor agonists [44,45]. The
28 current paucity in pharmacological tools has contributed to a limited understanding of the
29 A_{2B}AR relative to the other AR subtypes. The identification of BAY60-6583, the first
30 subtype-selective A_{2B}AR agonist, enabled a clearer understanding of the physiological role of
31 the A_{2B}AR and identified it as a key pharmacological target for a number of conditions
32 including cardiac ischemia-reperfusion injury and fibrosis [4,16,17]. A similar 2-thio-3,5-
33 dicyano-4-phenyl-6-aminopyridine derivative, capadenoson, was subsequently synthesized by
34 Bayer and classified as an A₁AR-selective agonist [26,29,31]. However, our study using
35 [³H]DPCPX binding, has demonstrated that capadenoson has A_{2B}AR affinity in the low
36 micromolar range, similar to the A_{2B}AR affinity of both NECA and BAY60-6583 [39].
37 Interestingly, a recent study characterizing a range of novel capadenoson derivatives failed to
38 observe significant inhibition in the binding of the radiolabelled A_{2B}AR antagonist, [³H]PSB-
39 603, in the presence of 1 μM capadenoson [28]. The negligible A_{2B}AR activity would appear
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1 to contradict our data demonstrating moderate A_{2B}AR affinity, however, the divergence likely
2 reflects the disparity between the affinity of the different radiolabelled antagonists employed.
3 Specifically, at the A_{2B}AR, the affinity of [³H]PSB-603 is approximately 100-fold higher than
4 that of [³H]DPCPX [46,47]. Therefore, the lack of significant [³H]PSB-603 inhibition may be
5 a consequence of the low affinity of capadenoson relative to the high affinity antagonist
6 [³H]PSB-603 and may explain why capadenoson affinity and/or activity at the A_{2B}AR has not
7 been reported previously.
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10 G_s-stimulated cAMP accumulation is an efficiently-coupled pathway downstream of
11 the A_{2B}AR. Capadenoson stimulated robust cAMP accumulation with a similar potency and
12 maximal effect as the potent A_{2B}AR agonists NECA and BAY60-6583. In addition to the
13 effects on cAMP accumulation, capadenoson also stimulated multiple other signaling
14 pathways in A_{2B}AR-FlpIn-CHO cells, including IP₁ accumulation, ERK1/2 phosphorylation
15 and intracellular calcium mobilization. However, in contrast to the response observed for
16 cAMP accumulation, capadenoson had lower potency than NECA for the other pathways
17 assessed and behaved as a partial agonist in pERK1/2 and intracellular Ca²⁺ mobilization.
18 These divergent effects are reflected in the bias analysis of the A_{2B}AR-FlpIn-CHO
19 concentration response curves, demonstrating that relative to the other pathways assessed,
20 capadenoson was significantly biased toward cAMP accumulation, when normalized to the
21 reference agonist NECA. Importantly, the bias of capadenoson towards cAMP accumulation
22 relative to pERK1/2 was recapitulated in HEK293T cells that endogenously express A_{2B}AR.
23 Interestingly, although structurally similar, the bias profile of BAY60-6583 in A_{2B}AR-FlpIn-
24 CHO cells was different to that observed for capadenoson. Specifically, BAY60-6583 was
25 significantly biased away from IP₁ accumulation relative to all other pathways assessed. In
26 HEK293T cells, no significant IP₁ accumulation could be detected upon stimulation of
27 endogenously expressed A_{2B}ARs and therefore the biased agonism of BAY60-6583 away
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1 from IP₁ accumulation could not be further investigated. The absence of significant IP₁
2 accumulation may reflect a lower A_{2B}AR expression in HEK293T cells compared to A_{2B}AR-
3 FlpIn-CHO cells. Capadenoson and BAY60-6583, which have a 2-thio-3,5-dicyano-4-phenyl-
4 6-aminopyridine non-nucleoside core, both stimulate biased agonism relative to the
5 prototypical adenosine-like NECA, supporting the suggestion that structurally distinct
6 compounds have the potential to stimulate physiological responses that differentiate from that
7 of the endogenous agonist. Indeed, previous structure-activity relationship studies of
8 dicarbonitrilepyridines indicated that differential side-chain substitutions could not only affect
9 adenosine receptor selectivity, but also have significant effects on agonist efficacy [13].
10 Accordingly, it would be of interest to further interrogate the structure-activity relationship of
11 dicarbonitrilepyridines with respect to biased agonism.
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26 Biased agonists are thought to enrich a unique spectrum of GPCR conformations
27 relative to that of the endogenous agonist. Biased agonism has considerable therapeutic
28 potential as it provides the opportunity to selectively stimulate desired activity whilst
29 eschewing detrimental signaling [20,48]. However, for effective translation, pathways
30 responsible for the therapeutic and adverse effects must be rigorously assessed in the relevant
31 tissue and/or cell type. The A_{2B}AR has been identified as a novel target for the treatment of
32 ischemia-reperfusion injury in many organs, including the heart [4-6]. In addition, A_{2B}AR
33 signaling has an important role in modulating cardiac fibrosis, however, both pro- and anti-
34 fibrotic effects have been observed [7,49,50]. Intriguingly, the divergent fibrotic effects may
35 arise from differential effector coupling to the pleiotropic A_{2B}AR. In cardiac (myo)fibroblasts,
36 cAMP production stimulated by G_s-coupled GPCRs has been shown to inhibit proliferation
37 and collagen synthesis. In contrast, stimulation of G_{q/11}-coupled GPCRs, such as the
38 angiotensin II type 1 receptor, promote fibrosis [51,52]. As such, an A_{2B}AR agonist with
39 significant bias towards G_s-mediated cAMP accumulation relative to G_q-mediated IP₁
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1 accumulation or Ca^{2+} mobilization may selectively stimulate anti-fibrotic signal transduction.
2 This study has demonstrated that in cardiac cells, $\text{A}_{2\text{B}}\text{AR}$ -mediated global cellular changes
3 and cAMP accumulation can be detected in response to capadenoson. Furthermore, in a
4 heterologous expression system, capadenoson displays an $\text{A}_{2\text{B}}\text{AR}$ bias profile which may be
5 conducive to a potential anti-fibrotic agent. Future studies will investigate the bias profile of
6 capadenoson in cardiac cells. Interestingly, a recent study demonstrated capadenoson exerted
7 anti-fibrotic effects *in vivo* in dogs with heart failure [29], which were attributed to an A_1AR -
8 mediated effect. However, in light of the $\text{A}_{2\text{B}}\text{AR}$ biased agonism observed for capadenoson in
9 the current study, $\text{A}_{2\text{B}}\text{AR}$ -mediated signaling likely contributes to the observed decrease in
10 fibrosis. Notably, a reclassification of adenosine receptor subtype selectivity as a result of
11 atypical cardiovascular pharmacology has occurred previously for clinically trialed
12 compounds. Specifically, the cardioprotection stimulated by AMP579, originally
13 characterized as an $\text{A}_1\text{AR}/\text{A}_{2\text{A}}\text{AR}$ agonist, was found to be mediated by the $\text{A}_{2\text{B}}\text{AR}$, as
14 selective antagonism of $\text{A}_{2\text{B}}\text{AR}$ abolished protective effects in rabbit hearts [53].

33 In conclusion, we have demonstrated that capadenoson stimulates appreciable signal
34 transduction downstream of $\text{A}_{2\text{B}}\text{AR}$ s endogenously expressed in physiologically relevant
35 cells, including cardiomyocytes and cardiac fibroblasts. Furthermore, this study has also
36 demonstrated that capadenoson has a biased agonist profile relative to the adenosine-like
37 agonist, NECA. Our findings highlight that capadenoson should be reclassified as a dual
38 $\text{A}_1\text{AR}/\text{A}_{2\text{B}}\text{AR}$ agonist and future studies should investigate whether some of the desirable
39 cardiovascular effects of capadenoson can be attributed to the hitherto unappreciated $\text{A}_{2\text{B}}\text{AR}$
40 activity. The clinical relevance of this pharmacological reclassification is topical as a
41 capadenoson derivative, neladenoson bialanate, is currently under development as a novel
42 selective A_1AR partial agonist for the treatment of heart failure [54].

Conflict of Interest Statement

Authors have no conflict of interest to declare

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Figure legends

Fig. 1. Structures of the prototypical adenosine-like agonist NECA and the non-nucleoside derivatives capadenoson and BAY60-6583. Capadenoson and BAY60-65-83 share the same 2-thio-3,5-dicyano-4-phenyl-6-aminopyridine core (highlighted in red).

Fig.2. Capadenoson has affinity and efficacy at the human $A_{2B}AR$. (A) Capadenoson has a similar micromolar affinity as NECA or BAY60-6583 for the $A_{2B}AR$ as determined by [^3H]DPCPX radioligand binding on $A_{2B}AR$ -FlpInCHO cell membranes. Dashed line represents the non-linear regression of the specific binding for NECA and BAY60-6583 as taken from previous publication [39]. (B) Capadenoson stimulation of cAMP accumulation was equipotent and equieffective when compared to the prototypical $A_{2B}AR$ agonists NECA and BAY60-6583 in FlpIn-CHO cells stably expressing the human $A_{2B}AR$. Data represent the mean + SEM from 3-5 independent experiments performed in duplicate.

Fig. 3. Capadenoson has relatively low $A_{2B}AR$ potency for (A) IP_1 accumulation, (B) ERK1/2 phosphorylation and (C) intracellular Ca^{2+} mobilization in FlpIn-CHO cells stably expressing the human $A_{2B}AR$. Data represent the mean + SEM from 3-5 independent experiments performed in duplicate or triplicate.

Fig. 4. Capadenoson and BAY60-6583 are $A_{2B}AR$ biased agonists, relative to the reference ligand NECA. The ‘web of bias’ plots bias factors ($\Delta\Delta(\tau/K_A)$) which are calculated by normalizing the transduction ratio (τ/K_A) of each agonist to a reference agonist (NECA) and a reference pathway (pERK1/2). * $P<0.05$; One-way ANOVA Tukey’s post-hoc analysis on the $\Delta\text{Log}(\tau/K_A)$. Data represent the mean + SEM from 3-5 independent experiments performed in duplicate or triplicate.

Fig. 5. Capadenoson acts as a biased agonist at A_{2B}ARs endogenously expressed in HEK293T cells. The robust cAMP accumulation stimulated by the adenosine receptor agonists NECA, BAY60-6583 and capadenoson (solid lines and closed symbols) in HEK293T cells (A) was virtually abolished in the presence of the A_{2B}AR antagonist PSB-603 (1 μM) (dashed lines and open symbols). (B) BAY60-6583 and capadenoson stimulated a partial pERK1/2 response relative to NECA. (C) The bias factors of BAY60-6583 and capadenoson, quantified relative to the reference ligand, NECA, and the reference pathway, pERK1/2, demonstrated that capadenoson exhibited a significant bias towards cAMP accumulation over pERK1/2. **P<0.01, unpaired t-test on $\Delta\text{Log}(\tau/K_A)$. Data represent the mean + SEM from 3-5 independent experiments performed in duplicate.

Fig. 6. Capadenoson can stimulate robust A_{2B}AR-mediated signal transduction in primary isolated neonatal ventricular cardiac fibroblasts (NVCFs) and cardiomyocytes (NVCs). Real-time global cellular changes in response to the AR ligands were assessed in an impedance-based assay (xCELLigence assay). Capadenoson (1 μM) stimulated a significant change in impedance relative to the buffer control in both (A and C) NVCFs and (B and D) NVCs. In (C) NVCFs and (D) NVCs, the maximal effect of capadenoson, which occurred approximately 30 min after agonist addition was significantly reduced in the presence of the A_{2B}AR antagonist PSB-603 (1 μM). Capadenoson stimulated robust cAMP accumulation in (E) NVCFs and (F) NVCs that was abolished in the presence of PSB-603 (1 μM). *P<0.05; ***P<0.001; One-way ANOVA Dunnett's post-hoc analysis. Data represent the mean + SEM from 3-5 independent experiments performed in duplicate or triplicate.

Table 1. The potency (pEC_{50}) and maximal effect (E_{MAX}) of AR agonists for different signaling outputs in A_{2B}AR-FlpIn-CHO cells.

Data represents the mean $\pm$ SEM of 3-5 experiments conducted in duplicate or triplicate.

Ligand/Subtype	cAMP	pERK1/2	IP ₁	iCa ²⁺
	pEC_{50} (E_{MAX})	pEC_{50} (E_{MAX})	pEC_{50} (E_{MAX})	pEC_{50} (E_{MAX})
NECA	8.61 ± 0.47	6.50 ± 0.24	5.76 ± 0.08	7.12 ± 0.05
	(46.27 ± 2.56)	(39.37 ± 4.36)	(113.20 ± 7.54)	(44.48 ± 10.18)
BAY60-6583	8.74 ± 0.42	6.87 ± 0.26	5.05 ± 0.17	7.38 ± 0.11
	(44.43 ± 5.91)	(24.30 ± 1.66)**	(77.73 ± 4.27)*	(24.71 ± 5.28)
Capadenoson	8.94 ± 0.33	6.12 ± 0.46	5.03 ± 0.22	6.20 ± 0.22
	(43.08 ± 3.93)	(8.03 ± 0.89)***	(N.D)	(14.66 ± 4.37)*

* Significantly different, *P<0.05, **P<0.01, ***P<0.001 (one-way ANOVA, Dunnet's post-hoc analysis or unpaired t test), when compared to the E_{MAX} value of NECA at each respective pathway. N.D. Not determined

Table 2. Subtype selectivity of AR agonists (pEC₅₀ and E_{MAX} values) in human AR expressing FlpIn-CHO cells.

Data was quantified from cAMP concentration-response curves and represents the mean $\pm$ SEM of 3-9 experiments conducted in duplicate or triplicate.

Ligand/Subtype	A ₁ AR	A _{2A} AR	A _{2B} AR	A ₃ AR
	pEC ₅₀ (E _{MAX})	pEC ₅₀ (E _{MAX})	pEC ₅₀ (E _{MAX})	pEC ₅₀ (E _{MAX})
NECA	8.84 $\pm$ 0.08*	7.79 $\pm$ 0.10	8.61 $\pm$ 0.47	8.32 $\pm$ 0.15#
	(89.77 $\pm$ 2.9)*	(84.26 $\pm$ 2.71)	(46.27 $\pm$ 2.56)	(80.00 $\pm$ 4.07)#
BAY60-6583	6.95 $\pm$ 0.87	N.D	8.74 $\pm$ 0.42	6.32 $\pm$ 0.76
	(7.00 $\pm$ 7.54)	N.D	(44.43 $\pm$ 5.91)	(15.77 $\pm$ 13.21)
Capadenoson	9.18 $\pm$ 0.07*	5.85 $\pm$ 0.08	8.94 $\pm$ 0.33	6.62 $\pm$ 0.53
	(99.28 $\pm$ 3.17)*	(74.26 $\pm$ 4.02)	(43.08 $\pm$ 3.93)	(43.32 $\pm$ 15.10)

* Denotes data taken from [22]; # Denotes data taken from [37]; N.D. Not determined.

Table 3. Quantification of biased agonism in A_{2B}AR-FlpIn-CHO cells.

An operational model was used to estimate agonist transduction coefficients ($\text{Log}(\tau/K_A)$) for each signaling output. Normalized transduction coefficients ($\Delta\text{Log}(\tau/K_A)$) and $\text{Log}(\text{bias factors})$, as described within the methods, were used to quantify biased agonism. Data represents the mean $\pm$ SEM of 3-5 separate experiments conducted in duplicate or triplicate.

Ligand	Parameter	cAMP	pERK1/2	IP ₁	iCa ²⁺
NECA	$\text{Log}(\tau/K_A)$	8.62 ± 0.43	6.50 ± 0.27	5.74 ± 0.08	7.13 ± 0.04
	$\Delta\text{Log}(\tau/K_A)$	0	0	0	0
	$\text{Log}(\text{bias factor})$	0 (1.0)	0 (1.0)	0 (1.0)	0 (1.0)
BAY60-6583	$\text{Log}(\tau/K_A)$	8.87 ± 0.39	6.49 ± 0.36	4.90 ± 0.27	7.12 ± 0.14
	$\Delta\text{Log}(\tau/K_A)$	0.25 ± 0.08	-0.02 ± 0.09	$-0.84 \pm 0.19^*$	-0.02 ± 0.11
	$\text{Log}(\text{bias factor})$	0.26 ± 0.10	0	-0.82 ± 0.19	-0.07 ± 0.11
		(1.82)	(1.0)	(0.15)	(0.85)
Capadenoson	$\text{Log}(\tau/K_A)$	8.52 ± 0.46	5.24 ± 0.14	4.71 ± 0.12	5.71 ± 0.30
	$\Delta\text{Log}(\tau/K_A)$	$-0.10 \pm 0.11^*$	-1.26 ± 0.14	-1.03 ± 0.05	-1.42 ± 0.27
	$\text{Log}(\text{bias factor})$	1.16 ± 0.11	0 (1.0)	0.23 ± 0.05	-0.16 ± 0.274
		(14.45)		(1.70)	(0.69)

Bias factors are shown in parentheses. * Significantly different, $P < 0.05$ (One-way ANOVA, Tukey post-hoc analysis), when compared to $\Delta\text{Log}(\tau/K_A)$ of corresponding agonist relative to all other signaling pathways.

Table 4. The potency (pEC₅₀) and maximal effect (E_{MAX}) of AR agonists for cAMP accumulation and ERK1/2 phosphorylation in HEK293T cells.

Data represents the mean ± SEM of 3-5 experiments conducted in duplicate.

Ligand/Subtype	cAMP	pERK1/2
	pEC ₅₀ (E _{MAX})	pEC ₅₀ (E _{MAX})
NECA	7.34 ± 0.18	7.52 ± 0.09
	(105.9 ± 1.65)	(68.08 ± 7.96)
BAY60-6583	7.27 ± 0.14	7.64 ± 0.18
	(103.0 ± 1.06)	(27.90 ± 5.38)*
Capadenoson	6.85 ± 0.08	6.60 ± 0.14
	(98.89 ± 1.99)	(22.87 ± 8.41)*

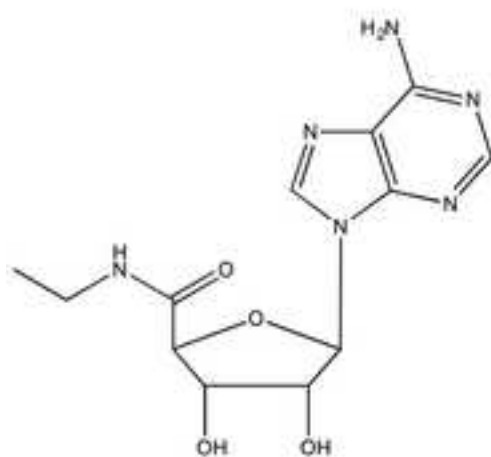
*Significantly different, P<0.05 (one-way ANOVA, Dunnet’s post-hoc analysis), when compared to the E_{MAX} value of NECA at each respective pathway.

Table 5. Quantification of biased agonism in HEK293T cells.

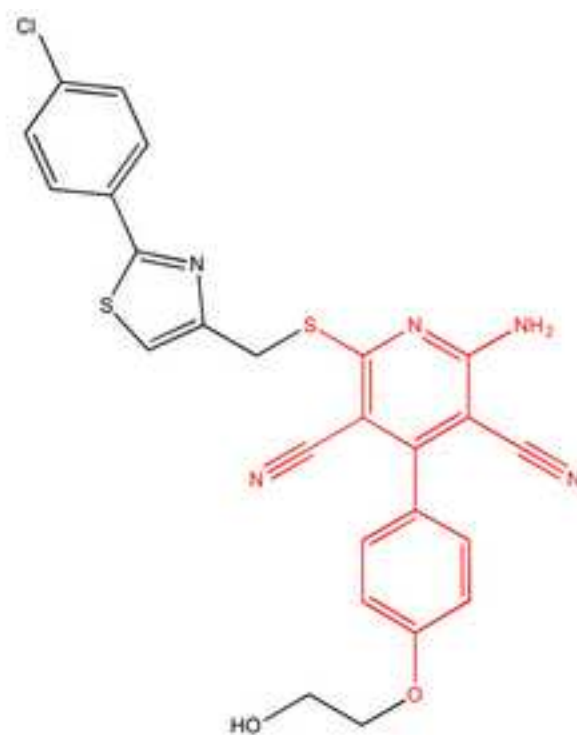
An operational model was used to estimate agonist transduction coefficients ($\text{Log}(\tau/K_A)$) for each signaling output. Normalized transduction coefficients ($\Delta\text{Log}(\tau/K_A)$) and $\text{Log}(\text{bias factors})$, as described within the methods, were used to quantify biased agonism. Data represents the mean $\pm$ SEM of 3-5 separate experiments conducted in duplicate.

Ligand	Parameter	pERK1/2	cAMP
NECA	$\text{Log}(\tau/K_A)$	7.49 ± 0.07	7.38 ± 0.17
	$\Delta\text{Log}(\tau/K_A)$	0	0
	$\text{Log}(\text{bias factor})$	0 (1.0)	0 (1.0)
BAY60-6583	$\text{Log}(\tau/K_A)$	7.06 ± 0.22	7.25 ± 0.14
	$\Delta\text{Log}(\tau/K_A)$	-0.44 ± 0.18	-0.13 ± 0.11
	$\text{Log}(\text{bias factor})$	0 (1.0)	0.31 ± 0.11 (2.04)
Capadenoson	$\text{Log}(\tau/K_A)$	6.00 ± 0.13	6.83 ± 0.09
	$\Delta\text{Log}(\tau/K_A)$	$-1.49 \pm 0.09^*$	-0.55 ± 0.11
	$\text{Log}(\text{bias factor})$	0 (1.0)	0.94 ± 0.11 (8.71)

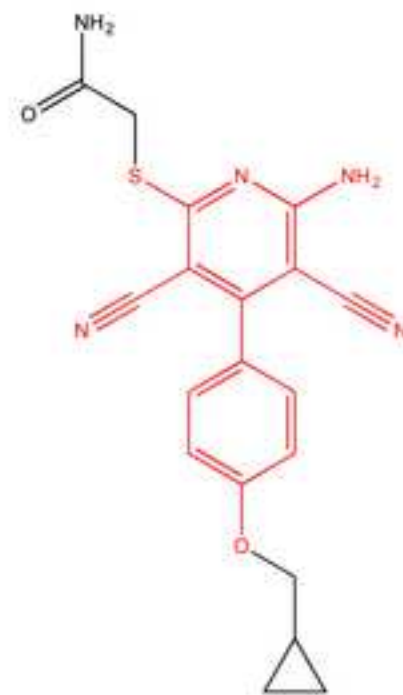
Bias factors are shown in parentheses. *Significantly different, $P < 0.05$ (t -test), when compared to $\Delta\text{Log}(\tau/K_A)$ estimated for corresponding agonist between the two signal transduction pathways



NECA

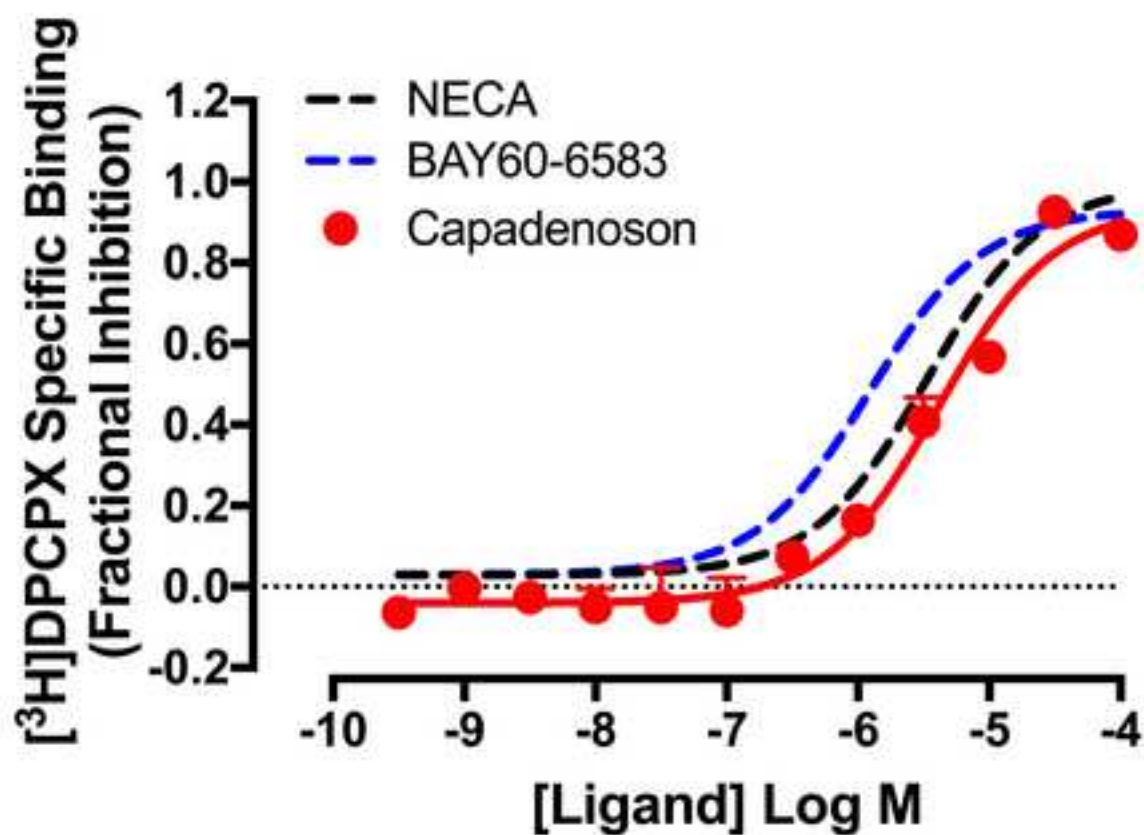


Capadenoson



BAY60-6583

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B

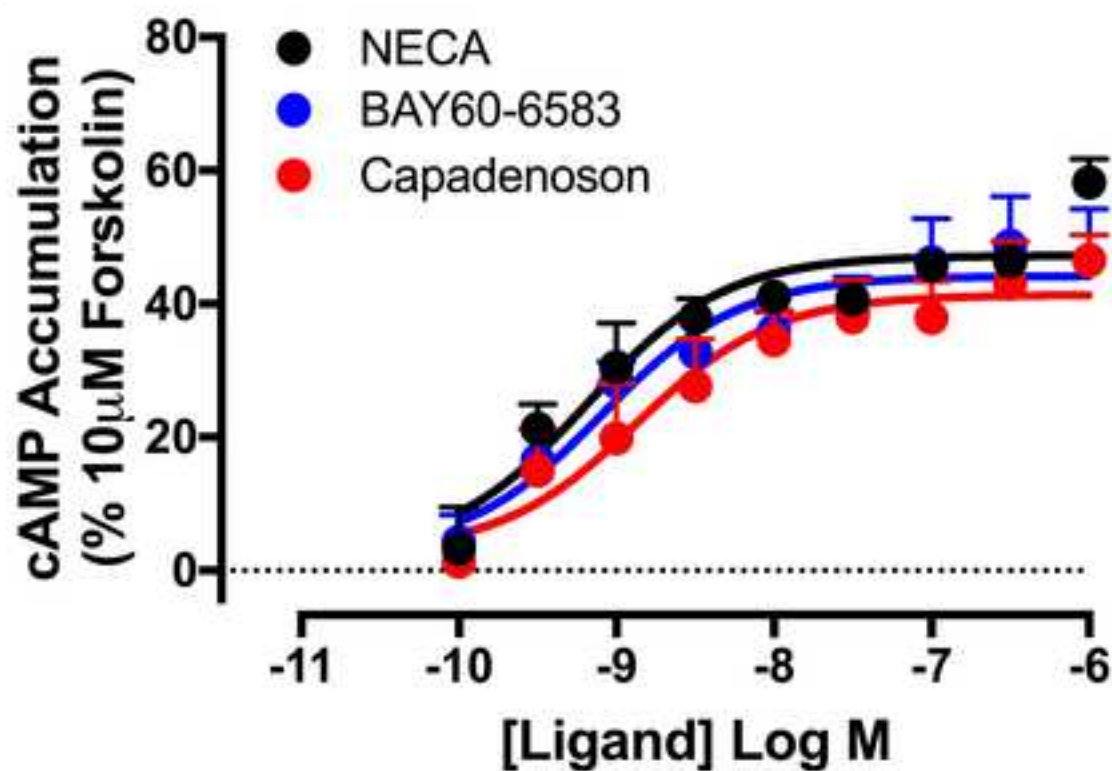


Figure 3_Color
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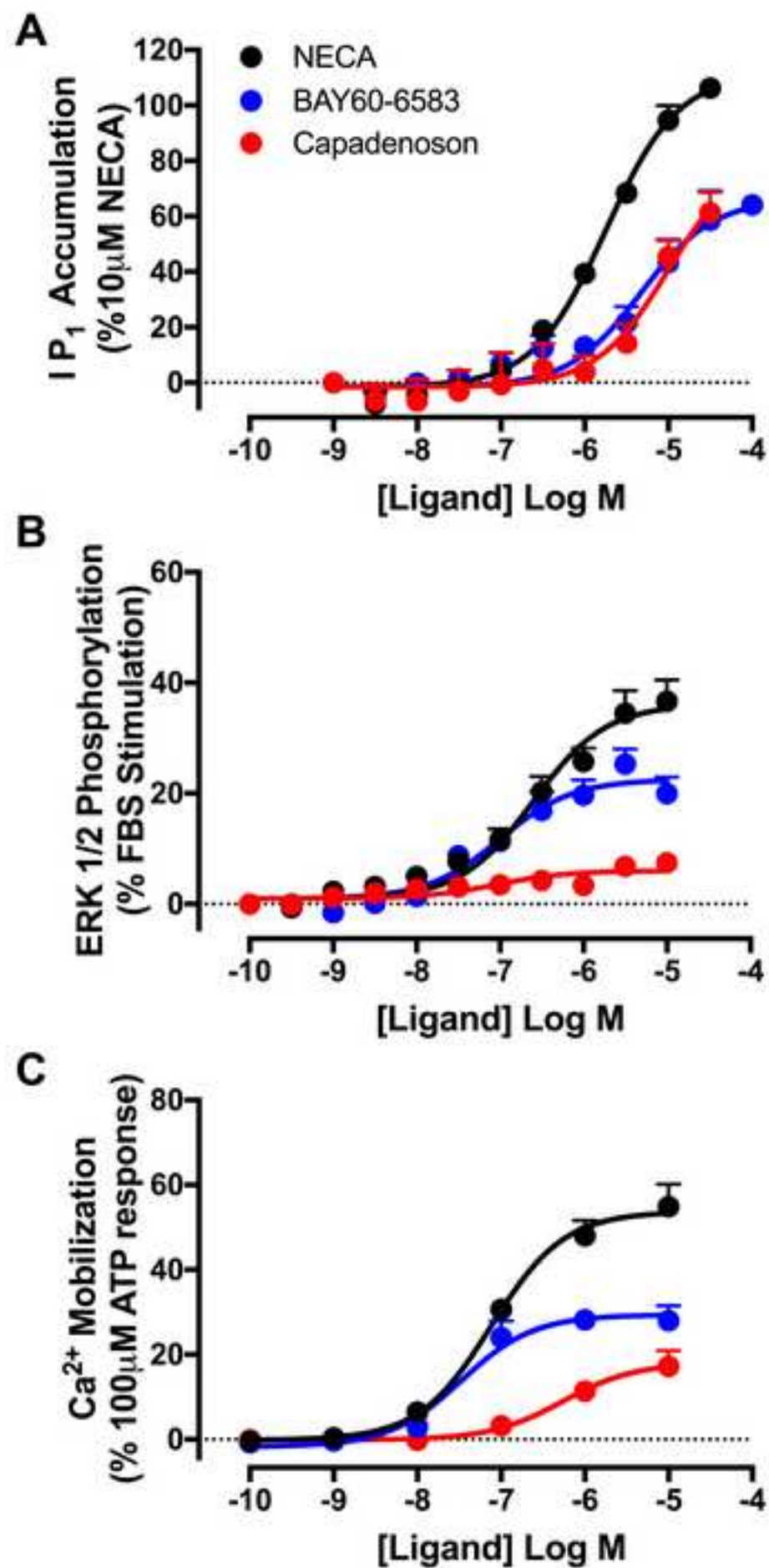


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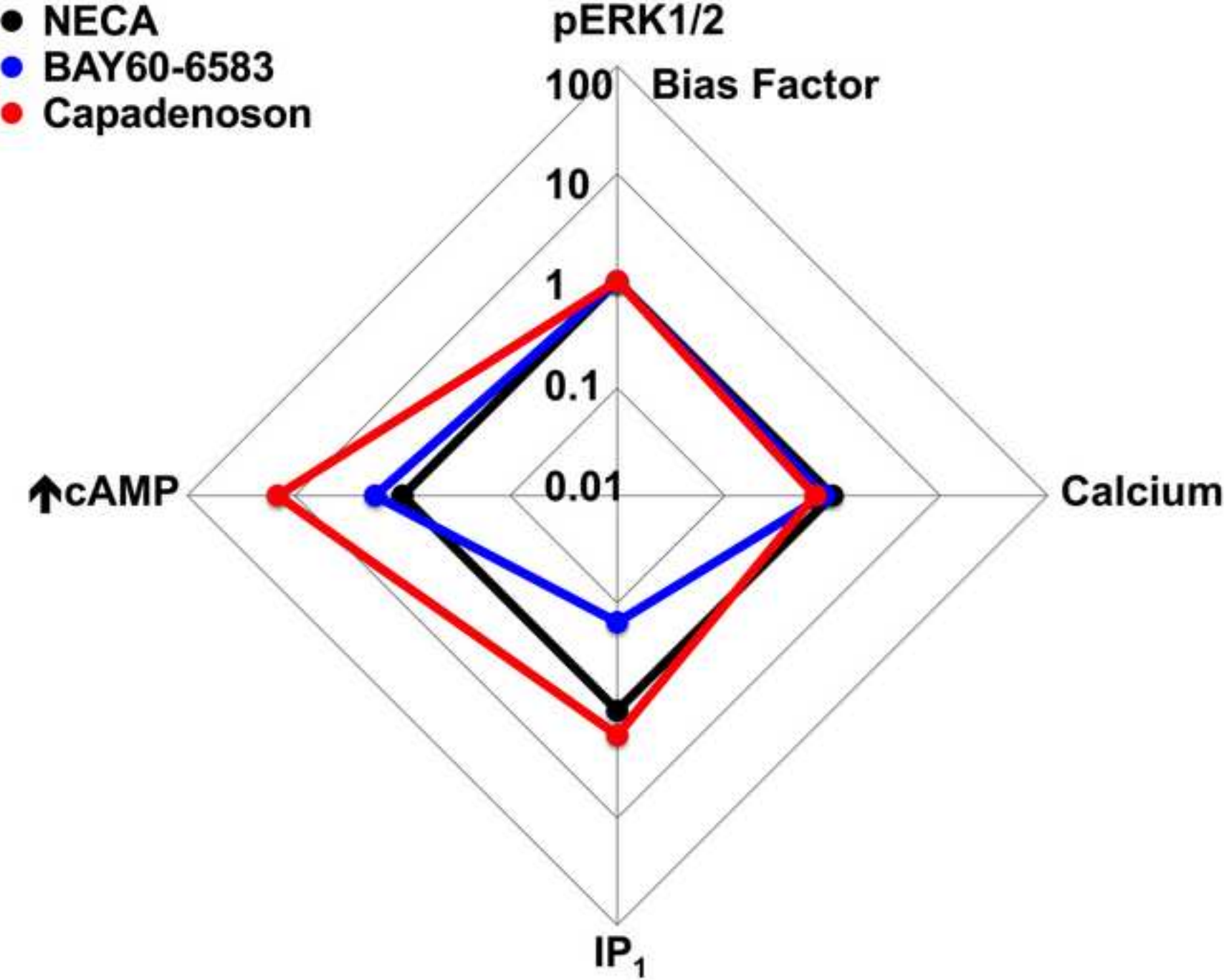


Figure 5_Color

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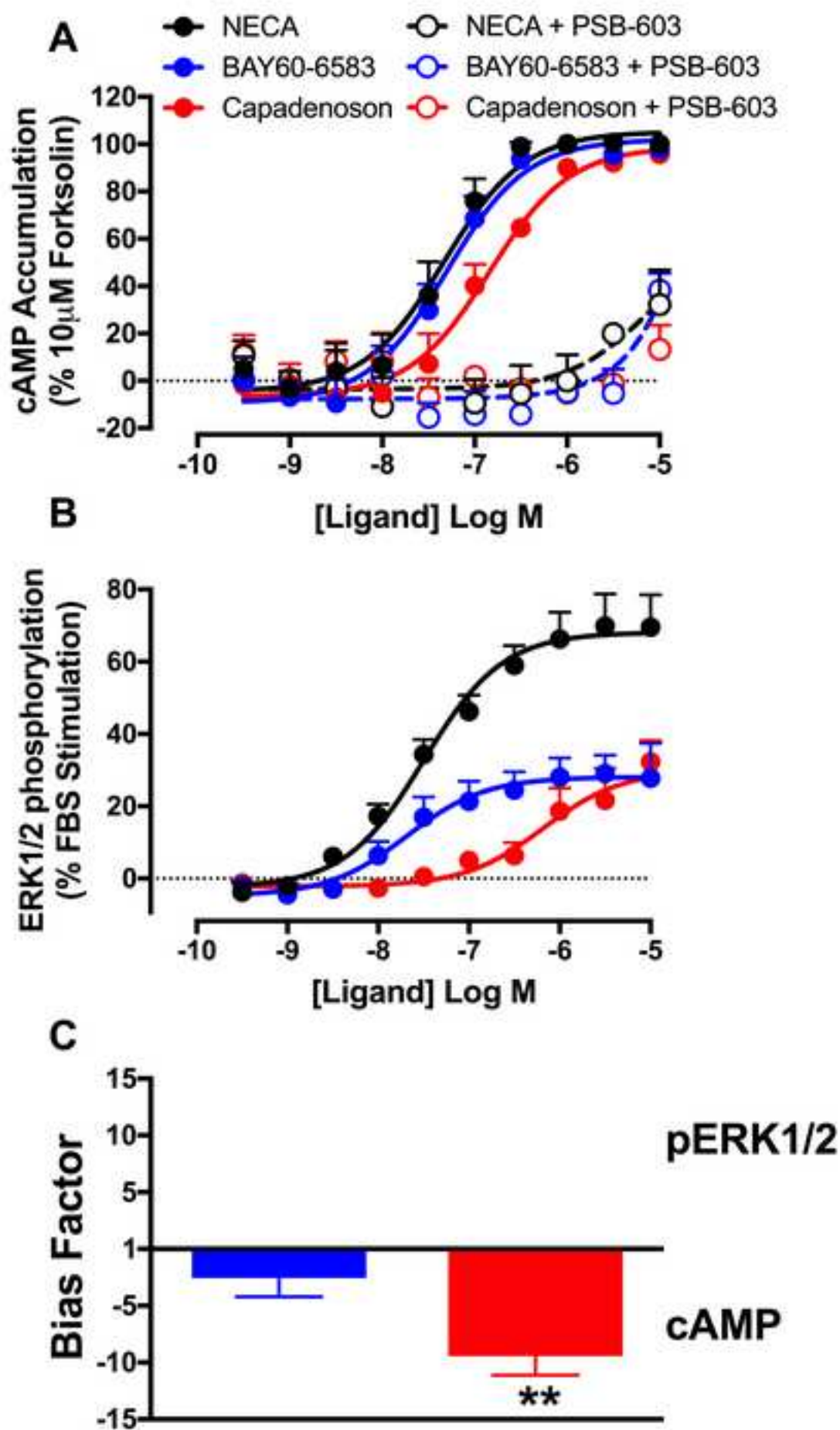


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