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Probing Biased/Partial Agonism at the G Protein-Coupled A_{2B} Adenosine Receptor[~]

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Abstract

G protein-coupled A_{2B} adenosine receptor (AR) regulates numerous important physiological functions, but its activation by diverse A_{2B}AR agonists is poorly profiled. We probed potential partial and/or biased agonism in cell lines expressing variable levels of endogenous or recombinant A_{2B}AR. In cAMP accumulation assays, both 5'-substituted NECA and C2-substituted MRS3997 are full agonists. However, only 5'-substituted adenosine analogs are full agonists in calcium mobilization, ERK1/2 phosphorylation and β -arrestin translocation. A_{2B}AR overexpression in HEK293 cells markedly increased the agonist potency and maximum effect in cAMP accumulation, but less in calcium and ERK1/2. A_{2B}AR siRNA silencing was more effective in reducing the maximum cAMP effect of non-nucleoside agonist BAY60-6583 than NECA's. A quantitative 'operational model' characterized C2-substituted MRS3997 as either balanced (cAMP accumulation, ERK1/2) or strongly biased agonist (against calcium, β -arrestin). N⁶-Substitution biased against ERK1/2 (weakly) and calcium and β -arrestin (strongly) pathways. BAY60-6583 is ERK1/2-biased, suggesting a mechanism distinct from adenosine derivatives. BAY60-6583, as A_{2B}AR antagonist in MIN-6 mouse pancreatic β cells expressing low A_{2B}AR levels, induced insulin release. This is the first relatively systematic study of structure-efficacy relationships of this emerging drug target.

Keywords

GPCR; adenosine receptor; purines; cyclic AMP; calcium; arrestin

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1. Introduction

Structure-efficacy relationships of the A₁, A_{2A} and A₃ adenosine receptor (AR) agonists have been studied extensively in recent years [1-5]. This knowledge has led to the identification of many partial/biased agonists, including 5'-truncated nucleosides as antagonists and partial agonists for the A₃AR [3,6], and as full agonists for A₁ [5] and A_{2A} ARs [4], which have potential clinical applications [5,7]. The structure-efficacy relationships of agonists at the A_{2B}AR have not been previously probed.

The A_{2B}AR plays a critical role in many physiological and pathophysiological conditions, such as taste [9,10], inflammation [11-17], cancer [18-22], ischemic conditions [23,24], erectile function [25], diabetes [26-28], stem cell differentiation [29] and oxidative stress [30]. Despite its recently demonstrated importance, the nature of A_{2B}AR activation is still poorly understood. The role of the A_{2B}AR has been reported controversially, e.g. both agonists and antagonists have been demonstrated to have anti-inflammatory effects [31-33]. Those seemingly contradictory results could be, at least in part, because of promiscuous G protein coupling of the A_{2B}AR depending on the tissues and cells investigated or pathways measured; the lack of A_{2B}AR selective agonists until recently; and most importantly, a severe deficiency of understanding of the pharmacological properties of A_{2B}AR agonists used in some early studies. It is known that an agonist in one biological system might be a partial agonist or even an antagonist in other systems. Also, it is possible that different agonists attain variable maximum effects in specific signaling pathways, or a specific agonist has variable maximum effects in different signaling pathways. Biased agonists have been reported for several AR subtypes, but not the A_{2B}AR [34,35]. In the present study we investigate various A_{2B}AR agonists of distinct structural classes (Fig. 1) in activating four signaling pathways under the direction of the A_{2B}AR using several cell lines, including T24 cells, a human bladder cancer cell line endogenously expressing the A_{2B}AR, and HEK293 cells expressing both endogenous and recombinant human A_{2B}ARs.

2. Materials and Methods

2.1. Materials

Agonists—We selected four chemical classes of synthetic A_{2B}AR agonists: **A.** 2-substituted adenosine analogs: MRS3997 [36], MRS3534 [37], CV1808 [38]. **B.** N⁶-substituted adenosine derivatives: MRS5911 [39], R-PIA [40]. **C.** 5'-substituted adenosine analogs, NECA (adenosine-5'-N-ethyluronamide) and CPCA (5-N'-cyclopropyl-carboxamidoadenosine). **D.** Non-nucleoside 3,5-dicyanopyridine agonists LUF5833 and BAY60-6583 (LUF6210, termed hereafter 'BAY') [41,42], which were synthesized at Leiden/Amsterdam Center for Drug Research (Leiden, The Netherlands). MRS3997, MRS3534 and MRS5911 [39] were synthesized at NIDDK, National Institutes of Health (Bethesda, MD, USA). Adenosine, NECA, CPA, CPCA, CV1808, R-PIA and probenecid were from Sigma (St. Louis, MO). CI-IB-MECA was from Tocris (Minneapolis, MN).

Cell lines—a. Human cell lines (ATCC, Manassas, VA) were selected for endogenous expression of the A_{2B}AR at variable levels: T24 bladder cancer cells; 1321N1 astrocytoma cells; WM266 melanoma cells; PC-3 prostate cancer cells and HEK293 cells expressing

both endogenous and recombinant human A_{2B}ARs. Other species were represented in: COS-7 monkey kidney cells endogenously expressing the A_{2B}AR (ATCC, Manassas, VA); MIN-6 mouse pancreatic β cells expressing the endogenous mouse A_{2B}AR (obtained from Jürgen Wess, NIDDK, NIH); and PathHunter CHO cells expressing the recombinant mouse A_{2B}AR and an engineered β -arrestin 2 (DiscoverX, Fremont, CA). The reason we used this mouse A_{2B}AR cell line was that a similar version of PathHunter CHO cells from DiscoverX expressing the human A_{2B}AR did not produce a robust response.

Additional materials and kits—Luminescence assay kit for β -arrestin2 translocation was from DiscoverX (Fremont, CA). Calcium dye kit was from Molecular Devices (Sunnyvale, CA). AlphaScreen cyclic AMP kits and SureFire p-ERK1/2 (Thr202/Tyr204) assay kits were from PerkinElmer (Boston, MA). Superscript III First Strand Synthesis Supermix kit was from Invitrogen (Carlsbad, CA). The gene-specific FAM-labeled MGB Taqman probes were from Applied Biosystems (Life Technologies, Grand Island, NY). All other materials are from commercial sources and are of analytical grade.

2.2. cAMP accumulation assay

Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 Units/ml penicillin, 100 μ g/ml streptomycin and 2 μ mol/ml glutamine. Cells were plated in 96-well plates in 100 μ l medium. After 24 h, the medium was removed and cells were washed three times with 100 μ l DMEM, containing 50 mM HEPES, pH 7.4. Cells were then treated for 30 min with agonist and/or test compound in the presence of rolipram (10 μ M) and adenosine deaminase (3 units/ml). The reaction was terminated by removal of the supernatant, and cells were lysed upon the addition of 100 μ l of lysis buffer (0.3% Tween-20). For determination of cAMP production, an AlphaScreen cAMP kit was used according manufacturer's instructions.

2.3. Intracellular calcium mobilization assay

Cells were grown overnight in 100 μ l of media in 96-well flat bottom plates at 37°C at 5% CO₂ or until approx. 90% confluency. The calcium assay kit was used as directed without washing cells, and with probenecid added to the loading dye at a final concentration of 2.5 mM to increase dye retention. Cells were incubated with 100 μ l dye/probenecid for 60 min at room temperature. The compound plate was prepared using dilutions of various compounds in Hank's Buffer (pH 7.4). Samples were run in duplicate using a FLIPR TETRA (Molecular Devices, Sunnyvale, CA) at room temperature. Cell fluorescence (Excitation = 485 and Emission = 525 nm) was monitored following exposure to the compound. Increases in intracellular calcium are reported as the maximum fluorescence value after exposure minus the basal fluorescence value before exposure.

2.4. ERK1/2 activation assay

The method used was essentially as previously described [43]. T24 or HEK293 cells (30,000 cells/100 μ l) were seeded in a 96-well plate in complete growth medium. After cell attachment, medium was removed and cells were serum-starved overnight in 90 μ l medium without fetal bovine serum. Agonists were stored cold as 5 mM stock solutions in DMSO and diluted in HANK's buffer. DMSO control (final concentration 0.1% corresponding to

the highest concentration of agonist that was used) and agonist (10 μ l) were added in a 10-fold concentrated solution in Hank's medium, and cells were stimulated for 5 min. Medium was removed and cells were lysed with 1 $\times$ Lysis buffer (20 μ l) (PerkinElmer AlphaScreen SureFire p-ERK1/2 (Thr202/Tyr204) Assay Kit). Lysate (4 μ l/well) was transferred to a 384 well ProxiPlate Plus (PerkinElmer). Acceptor Beads were diluted 1:50 in a 1:5 mixture of Activation buffer in Reaction Mix and added to the 384-well plate (5 μ l/well). The plate was sealed and incubated for 2 h at room temperature. Donor beads (2 μ l) diluted 1:20 in Dilution buffer were added, and the plate was incubated for another 2 h at room temperature. The plate was measured using an EnVision multilabel reader using standard AlphaScreen settings.

2.5. β -Arrestin2 translocation assay (mouse A_{2B}AR)

A β -arrestin2 translocation assay was performed using a PathHunter™ β -arrestin assay kit from DiscoverX (Fremont, CA) as previously described [6]. Briefly, PathHunter CHO cells expressing the mouse A_{2B}AR were grown in 384-well plates for 24 h in Hank's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 μ mol/ml glutamine. Cells were treated with agonists for 90 min before adding detection reagents (mixture of 1 part Galacton Star substrate with 5 parts Emerald II™ Solution, and 19 parts of PathHunter Cell Assay Buffer), and incubated at room temperature for 60 min before luminescence was measured.

2.6. Western blot analysis

Cells used in the assay were lysed at approximately 80% confluency using cell lysis buffer (Cell Signaling Technology, Danvers, MA), and the cell lysates were stored at -80 °C until the assay. Protein concentration was measured using a BCA protein assay kit (Thermo Scientific, Rockford, IL). Cell lysates (30 μ g protein/well) were analyzed under reducing conditions by SDS-PAGE and proteins were separated on 12 % Bis-Tris gel (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membrane by electroblotting. Membranes were blocked according to the manufacturer's instructions and probed with A_{2B}AR antibody (Alomone Labs, Jerusalem, Israel) overnight at 4 °C. Subsequently, blots were probed with IR dye-conjugated anti-rabbit secondary antibody for 1 h and then analyzed using an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). To check the specificity of the antibody, control antigen for the antibody was mixed with A_{2B} antibody and incubated for 1 h with constant agitation. This Ag–Ab mixture was also probed in parallel to antibody only blots as a control.

2.7. Insulin release in MIN6 cells

MIN6 cells were seeded in a 96-well plate at a density of 40,000 cells/well and cultured in DMEM at 37 °C. After 48 h, the medium was replaced with Krebs ringer bicarbonate buffer (KRBB) containing 3.3 mM glucose and 0.3% albumin, for 1 h. Insulin secretion was then induced by supplementing KRBB containing 16.7 mM glucose in the presence or absence of A_{2B}AR ligands in KRBB solution for 1 h at 37 °C. Insulin secretion was determined using an insulin ELISA kit with a mouse insulin standard (Crystal Chem, Inc., Downer's Grove, IL).

2.8. Detection of A_{2B}AR gene expression by quantitative real-time PCR (qRT-PCR)

Total mRNA was isolated following the protocol of the RNeasy Mini Kit (Qiagen, Valencia, CA). Reverse transcription was completed using Superscript III First Strand Synthesis Supermix kit (Invitrogen, Carlsbad, CA). The cDNA was then amplified by PCR with gene-specific FAM-labeled MGB Taqman probes (Applied Biosystems) in 96-well plates using a 7300 Real-Time PCR System (Applied Biosystems) and default thermocycler program. The expression levels of indicated genes were calculated by the 2^{-CT} method with GAPDH as the endogenous control.

2.9. Calculation of bias factors

To quantify this bias, a method based on the Black and Leff operational model [44] was used. We first determined τ and K_A values based on that model. τ ($\tau = [R_t]/K_E$), which is the ligand's efficacy toward a specific pathway, and is defined by the ratio of the total receptor density ($[R_t]$) over the general equilibrium dissociation constant (K_E). K_A is defined as the ligand equilibrium dissociation constant of the agonist-receptor complex. Using these parameters, the transduction ratios (τ/K_A) and the transduction coefficients [$\text{Log}(\tau/K_A)$] for agonists were deduced for each signaling pathway. Specifically, bias factors of NECA, MRS5911, MRS3997 and BAY for cAMP accumulation versus ERK1/2 phosphorylation, recruitment of β -arrestin and calcium mobilization were calculated essentially as previously described by Kenakin et al. [45] using the 'operational model' of Black and Leff [44]. τ and K_A values were calculated from dose-response curves fitted using the operational model-partial agonist function. To obtain the $\text{log}(\tau/K_A)$, which represents the relative ability of agonists to activate a given signaling pathway, the $\text{log}(\tau/K_A)$ values of agonists in each pathway were calculated and subtracted from the $\text{log}(\tau/K_A)$ of NECA. To determine the $\text{log}(\tau/K_A)$, representing the relative preference of a specific agonist for one pathway with respect to another pathway compared with NECA as standard, the $\text{Log}(\tau/K_A)$ values obtained for each signaling pathway (calcium, β -arrestin2) were subtracted from the $\text{Log}(\tau/K_A)$ values calculated for the reference pathway (G α i-related cAMP pathway). The bias factor is expressed as the anti-Log value of the $\text{Log}(\tau/K_A)$. Thus, if the calculated bias is $\gg 1$ for a given compound and associated pathway, it disfavors that particular pathway.

2.10. Statistical and data analyses

Functional parameters were calculated using Prism 6.0 software (GraphPAD, San Diego, CA). Data were expressed as mean $\pm$ standard error. Curves were fitted using least squares nonlinear regressions. Statistical significance of the differences was assessed using a Student t test (between two conditions) or a One-Way Analysis of Variance (ANOVA) followed by Bonferroni's multiple comparison tests (between multiple conditions). Differences yielding P values < 0.05 were considered as statistically significant.

3. Results

3.1 A_{2B}AR-mediated cAMP accumulation, intracellular calcium mobilization and ERK1/2 phosphorylation in various types of cell lines

For the assay of cAMP accumulation, all agonists were first tested in T24 cells endogenously expressing the human A_{2B}AR [46]. The 5'-substituted NECA and CPCA and

2-substituted MRS3997 were found to be the most efficacious (Table 1). The maximum effect values, relative to NECA set at 100%, of N^6 -substituted MRS5911 and two non-adenosine agonists LUF5833 and BAY are 77.7%, 52.8% and 62.9%, respectively. The N^6 -substituted R-PIA and 2-substituted MRS3534 (Table 1) and CV1808 ($EC_{50} = 9100 \pm 5800$ nM, maximum effect = 55% of NECA's) are somewhat weak, so it is not clear if they are partial agonists. The four representative agonists from each class (MRS3997, MRS5911, BAY and NECA) were further tested in HEK293 cells endogenously expressing the A_{2B} AR, the maximum effects of which show a pattern that is similar to that in T24 cells. However, all four agonists are full and potent agonists in HEK293 cells overexpressing the A_{2B} AR (Fig. 2A; Table 1), which suggests that some of these and other agonists tested in the A_{2B} AR-overexpressing cells may be partial or even antagonists in some natural systems depending on the expression level of the endogenous A_{2B} AR. The A_{2A} AR selective agonist CGS21680, which is weak at the A_{2B} AR [37], was shown to be nearly a full A_{2B} AR agonist, although still of low potency. The A_1 AR agonist CPA and A_3 AR agonist Cl-IB-MECA, although they stimulated cAMP accumulation at high concentrations via the A_{2B} AR, were inactive in inhibition of forskolin-stimulated cAMP accumulation, which excluded the involvement of A_1 and A_3 ARs.

In calcium mobilization assays in T24 cells, 5'-substituted NECA and CPCA are the most efficacious, followed by N^6 -substituted MRS5911 and R-PIA displaying maximum effects of 36.9% and 9.6%, respectively. However, the 2-substituted MRS3997, MRS3534, and CV1808, and the non-adenosine agonists LUF5833 and BAY are very weak and barely induce calcium mobilization. In HEK293 cells, the fluorescence signal induced by NECA and CPCA, although detectable, is weaker than that produced by NECA at T24 cells. In HEK293 cells overexpressing the A_{2B} AR, adenosine, CPCA and NECA are all full agonists in calcium mobilization with MRS5911 being partially efficacious (Table 1). Interestingly, overexpression of the A_{2B} AR did not substantially change the inability of the 2-substituted adenosine analogs and the non-adenosine derivatives to induce calcium mobilization (Fig. 2B), although it significantly enhanced both the potency and efficacy of these agonists in cAMP accumulation. This suggests potential biased agonism of these two classes of A_{2B} AR agonists (Table 1). It is noted that the potent non-adenosine agonist BAY and the C2-substituted adenosine derivative MRS3997 show little if any residual agonist (Ca^{2+}) activity even at high concentrations and in the A_{2B} AR-overexpressing cells.

Both cAMP accumulation and calcium mobilization induced by NECA in T24 cells were antagonized by the selective A_{2B} AR antagonist PSB603 (Fig. 3), suggesting that the effects of non-selective A_{2B} AR agonist are solely mediated by the A_{2B} AR. The K_B value of PSB603 to antagonize NECA-induced cAMP accumulation was calculated to be 0.31 nM, which is roughly consistent with its affinity for the A_{2B} AR.

The assay of ERK1/2 phosphorylation (Table 1) was initially performed in T24 cell endogenously expressing the human A_{2B} AR. Only NECA and CPCA induced a modest stimulation (within 2-fold of basal value). MRS5911 only produced a ~13% increase above basal value at 10 μ M, while MRS3997 and BAY did not produce any stimulation. In HEK293 cells, however, NECA was able to produce a relatively robust ERK1/2 response (over 3-fold above basal), and all other agonists also produced responses although variable

and to a lesser extent. The EC₅₀ values of various agonists are listed in Table 1. In HEK293 cells overexpressing the human A_{2B}AR, NECA was able to produce a much more robust response (10-fold). Interestingly, MRS3997, MRS5911 and BAY behave as partial agonists even in the A_{2B}AR-overexpressing cells (Table 1, Fig. 2C).

Additionally, in all three assays mentioned above, cAMP accumulation, calcium and ERK1/2 activation, the A_{2A} selective agonist CGS21680 was much weaker than NECA (Table 1), further demonstrating that the effect of NECA, CPCA and other agonists is solely via the A_{2B}AR.

3.2. Effect of A_{2B}AR specific siRNA knockdown on the efficacy and potency of agonists

In addition to overexpression of the A_{2B}AR, we also examined the effect of silencing the A_{2B}AR using specific siRNA on the maximum agonist effect in T24 cells. In cAMP accumulation, the knockdown has different effects on NECA and BAY, being more effective in reducing the maximum effect of BAY while only affecting the potency of NECA (Fig. 4), further indicating the partial agonist property of BAY. The maximum agonist effect of BAY in the absence of siRNA was $68 \pm 7\%$ (NECA as 100%), which was significantly different from that in the presence of 5, 50 and 500 nM siRNA ($54 \pm 4\%$, $48 \pm 6\%$ and $36 \pm 3\%$, respectively, $P < 0.05$ compared with the control group). The corresponding EC₅₀ values of BAY in the absence and presence siRNA are 98 ± 22 , 102 ± 17 , 127 ± 31 and 93 ± 19 nM, respectively. The maximum agonist effect of NECA was not altered by 50 nM A_{2B}AR siRNA ($100 \pm 5\%$ vs. $99 \pm 6\%$, $P > 0.05$). However, the potency of NECA was decreased ~3-fold by siRNA silencing (232 ± 37 vs. 724 ± 122 nM). In the calcium mobilization assay, A_{2B}AR siRNA also decreased the maximum effect of NECA ($P < 0.05$ compared with control), although it did not affect the EC₅₀ values (2310 ± 380 vs. 2120 ± 470 nM). This result suggests that even NECA may only produce a partial response in a system with low receptor density, depending on the pathway measured. Indeed, in cAMP accumulation with WM266 cells expressing a low level of the A_{2B}AR, NECA produced a response that was less than 50% of that of forskolin (data not shown). By contrast, in COS-7 cells expressing a relatively high level of the A_{2B}AR, NECA and forskolin are approximately equipotent (data not shown).

3.3. Agonism by various agonists in different cell lines endogenously expressing the A_{2B}AR

In addition to exploring the effect of overexpression and siRNA knockdown, we also tested the potency and maximum effect of representative agonists in various cells (PC-3, T24, COS-7, 1321N1, WM-266 and HEK293) predominantly expressing the A_{2B}AR endogenously, but with variable expression levels. Table 2 shows that the potency and maximum effect of four representative agonists assayed in several cell lines with an endogenous A_{2B}AR at different expression levels (the data from T24 and HEK293 cells are shown in Table 1). It was found that NECA and MRS3997 tend to be full agonists in all cells, while MRS5911 has a somewhat lower maximum effect (Table 2). The maximum effect of BAY is more sensitive to the A_{2B}AR expression level, which is consistent with the siRNA silencing results from T24 cells (Fig. 4).

The results above suggest that the maximum effects of some agonists are more sensitive than others to receptor expression levels, suggesting that some agonists such as BAY could be full or partial agonists or even antagonists depending on A_{2B}AR expression levels in cells or tissues. Indeed, in MIN-6 cells that express a low level A_{2B}AR both BAY and the antagonist PSB603 induced insulin secretion (Fig. 5), a demonstrated function of blockade of the A_{2B}AR [47]. Western blot analysis showed that the expression of the A_{2B}AR in MIN-6 cells is much lower than in other cells used, such as HEK293 and COS-7 cells (Fig. 5B).

3.4. A_{2B}AR-mediated β -arrestin translocation in PathHunter CHO cells expressing themouse A_{2B}AR

In a β -arrestin2 translocation assay, 5'-substituted NECA and CPCA are full agonists (Table 1; Fig. 2D), and N⁶-substituted MRS5911 is a partial agonist (40.9% compared with NECA as 100%). The maximum effects of 2-substituted MRS3997, and the two non-adenosine agonists BAY and LUF5833 were 28.1%, 26.6% and 9.7%, respectively. The order of maximum effect is similar to that from the calcium assay, but somewhat different from the cAMP assay in T24 cells, further suggesting potentially variable degrees of biased agonism for different agonists. The EC₅₀ and maximum effect values are listed in Table 1.

3.5. A_{2B}AR gene expression levels of various cell lines

Endogenous A_{2B}AR is highly expressed in T24, HEK293 and PC-3 cells (Fig. 6A). The A_{2B}AR expression level in 1321N1 astrocytoma cells is about 50% of that in T24 cells. In WM266 and MIN-6 cells, the A_{2B}AR expression levels are relatively low ($P < 0.05$, compared with T24 cells). The relative gene expression levels in comparison to T24 (1.00 ± 0.13) are 0.84 ± 0.08 , 0.74 ± 0.16 , 0.47 ± 0.06 , 0.10 ± 0.01 and 0.050 ± 0.003 for HEK293, PC-3, 1321N1, WM266 and MIN-6 cells, respectively. The results from the gene expression assay are roughly consistent with that partially obtained from the Western blot analysis of expressed receptor protein. The expression levels of three other ARs are significantly lower than that of the A_{2B}AR in all cells used (data not shown).

In the A_{2B}AR-overexpressing HEK293 cells, the mRNA expression is about 8-fold higher than the endogenous A_{2B}AR mRNA expression in HEK293 cells (9.02 ± 1.09 vs. 1.09 ± 0.19) (Fig. 6B). A_{2B} siRNA significantly and progressively diminished the A_{2B}AR expression in T24 cells (Fig. 6C). The relative gene expression levels in the absence and presence of siRNA (5, 50, 500 nM) were 1.0 ± 0.13 , 0.57 ± 0.14 , 0.32 ± 0.07 and 0.11 ± 0.03 , respectively.

3.6. Analysis of the partial agonism and/or degree of bias at different signaling pathways based on the operational model

As described above, we applied two methods, siRNA silencing and receptor overexpression, to accommodate the operational model of receptor activation and analyze the potential partial agonism. The analysis from both methods in two types of cells, T24 and HEK293, gave similar τ values for BAY (1.19 and 1.54), indicating the partial agonist activity in comparison to NECA ($\tau > 20$).

It is interesting to note that the 2-substituted adenosine derivative MRS3997 has a high maximum effect compared with the *N*⁶-substituted MRS5911 and the non-adenosine BAY in cAMP accumulation (in both T24 and HEK293 cells), but MRS5911 has a higher maximum effect than MRS3997 and BAY in the calcium mobilization assay. These three agonists are almost equally efficacious in ERK1/2 phosphorylation. Clearly, these results cannot be readily explained by partial agonism. Hence, we quantified the potential biased agonism at the A_{2B}AR-overexpressing HEK293 cells to distinguish it from the partial agonism in the low receptor-expression systems. The calculated bias factors of agonists in various signaling pathways, cAMP accumulation, calcium mobilization, ERK1/2 phosphorylation and β -arrestin translocation, are listed in Table 3. Results suggest that MRS3997 is a relatively balanced agonist for cAMP accumulation and ERK1/2 phosphorylation, but strongly biased against calcium mobilization and β -arrestin translocation. MRS5911 is weakly biased against ERK1/2 (with a bias factor of 1.86), but strongly biased against calcium mobilization and β -arrestin translocation. Interestingly, analysis suggests that BAY is an ERK1/2-biased agonist, weakly against cAMP accumulation but strongly against calcium mobilization and β -arrestin translocation (Table 3).

4. Discussion

In the present study, we demonstrated both partial agonism and biased agonism at the A_{2B}AR by various agonists of distinct chemical classes, which should be useful for understanding the important function of the A_{2B}AR and for explaining some of the contradictory and controversial results previously reported.

The A_{2B}AR has long been classified as a G_s-coupled receptor due to its ability to stimulate cAMP accumulation. The potential coupling to many other effectors, such as MAP kinases and calcium via other G proteins, has also been reported [48-50]. The A_{2B}AR has also been previously reported to mediate arrestin signaling [51]. However, agonists from different chemical classes have not been applied in those previous studies and thus, some A_{2B}AR functions reported previously may be due to the effect of a specific agonist in a specific type of cell or tissue and may be applicable only for a specific signaling pathway studied. Indeed, both agonists and antagonists have been demonstrated to have anti-inflammatory effects [31-33].

It is noted that the A_{2B}AR expression level clearly had an impact on maximum agonist effect or on the potency (EC₅₀ values). Considering this, we compared the agonist effects and analyzed the potential biased agonism using HEK293 cells overexpressing the A_{2B}AR. One of the important findings from the present study is that BAY, unlike NECA, MRS3997 and MRS5911, is an ERK1/2-biased agonist. This is in line with the earlier results from Yang et al [52] who showed that in HEK293 cells expressing the A_{2B}AR, the selective A_{2B}AR agonist BAY increased ERK phosphorylation and cAMP levels but did so via a distinct mechanism. Furthermore, it was also shown that in cardiomyocytes, BAY did not affect cAMP levels but blocked the increased superoxide generation mediated via ERK1/2 [52]. On the other hand, the lack of stimulation of ERK1/2 phosphorylation in T24 cells (in contrast to HEK 293 cells) by BAY and MRS3997 may also indicate a different mechanism

to induce ERK1/2 activation in this cell line than previously suggested [49,50], which should be a topic of interest to explore in the future. Of course, it should also be noted that all the assays were performed under somewhat different experimental conditions, which may have an impact on the results.

In the calcium mobilization assay, NECA and CPCA produced a robust response in T24 cells, but not in HEK293 cells, via an endogenous A_{2B}AR, which is consistent with an early report by Phelps et al. [44], although both express similar levels of the A_{2B}AR. This suggests that potentially different mechanisms are involved in A_{2B}AR-induced calcium mobilization in different cells. It seems that the calcium response is not only dependent on A_{2B}AR expression level, but also on the effector coupling. Even in the presence of siRNA, NECA can still induce a substantial, albeit lower, calcium response in T24 cells (Fig. 4C). Also, the overexpression of the A_{2B}AR in HEK293 cells enhanced the robustness of the calcium response induced by NECA and MRS5911, but failed to increase the agonist potency of NECA or the ability of MRS3997 and BAY to produce a robust response. This can be explained with the biased agonism but not the partial agonist activity of these compounds. In contrast to T24 cells, only a minimal response of calcium mobilization is observed in COS-7 and 1321N1 cells endogenously expressing the A_{2B}AR, or in CHO cells expressing the recombinant human A_{2B}AR (data not shown). This is possibly due to a different effector coupling and should be a topic of future investigation. Nevertheless, we demonstrated that both in T24 cells and in HEK293 cells overexpressing the A_{2B}AR, 5'-substituted NECA and CPCA are full agonists, while N⁶-substituted MRS5911 and R-PIA are partial agonists. The 2-substituted MRS3997 and MRS3534 and the non-adenosine agonists have little if any residual efficacy in calcium mobilization.

The class of non-adenosine agonists has been demonstrated to be partial agonists with a variable maximum agonist effect at the A_{2B}AR [41]. We noted in an earlier study that BAY may possess partial agonist activity in PC-3 cells expressing the A_{2B}AR [53]. Consistent with these early findings, a recent paper by Hinz et al. [42] confirmed the partial agonist activity of this agonist. Hinz et al. [42] further demonstrated that BAY could antagonize NECA-induced cAMP accumulation in Jurkat-T cells. However, the potential biased agonism of BAY and other agonists has not been previously explored.

Regarding N⁶-substituted adenosine derivatives, R-PIA has long been known as a low potency A_{2B}AR agonist. The present study demonstrated that the relatively more potent agonist MRS5911 is a full agonist in cAMP accumulation in HEK293 cells overexpressing the A_{2B}AR but is a partial agonist in calcium mobilization and β -arrestin translocation. The results from the present study are consistent with an earlier finding that the N⁶- and 5'-disubstituted N⁶-benzyl NECA showed partial agonist activity in calcium mobilization in HEK293 cells expressing the A_{2B}AR [54]. In addition, our preliminary data from label-free measurements in HEK293 cells, which is a kind of end point of detection of receptor activation, also suggest that NECA is more efficacious than BAY, MRS3997 and MRS5911 (unpublished results).

Although the choice of an appropriate method for quantification of biased agonism is still controversial [55,56], it seems that the results from analysis of biased agonism and partial

agonism using the operational model and the method described by Kenakin et al. [44,45] are quite reasonable. Concerning biased agonism, in some reported cases the clear dissection of partial and biased agonism is still quite ambiguous; the results from the present study evidently cannot be explained solely by either partial agonism or biased agonism. Additionally, our results suggest that each class of agonists may have a different activation profile in different cells or tissues, which may be related to the receptor expression level, down-stream signaling protein expression level, or different downstream signaling molecules.

In conclusion, both biased agonism and partial agonism at the A_{2B}AR are shown in the present study of four signaling pathways, which should be useful in the further demonstration and explanation of the important function of the A_{2B}AR. Effects on both potency and maximal efficacy were studied, and characteristic patterns were associated with each structural class of agonist. N⁶-Substituted agonists were biased in favor of the cAMP pathway and biased against calcium, β -arrestin and to a lesser extent ERK1/2. Non-nucleoside agonists appear to be ERK1/2-biased, and surprisingly, one such 3,5-dicyanopyridine displayed antagonism in pancreatic β -islet cells that express a A_{2B}AR associated with inhibiting insulin release. C2 substitution tends to preserve cAMP and ERK1/2 effects and to disfavor Ca²⁺ and arrestin. Only 5'-substituted adenosine analogs are full agonists in all pathways examined.

This signaling divergence has implications for various pathophysiological conditions (including cancer, diabetes, inflammation and hypoxia), for which treatment by A_{2B}AR agonists is being considered. The choice of agonist vs. antagonist of the A_{2B}AR for treatment of the associated disease states is still controversial, and we have added another important variable for agonists, i.e. signaling bias. These characteristics of standard compounds can now be used to probe the involvement of specific A_{2B}AR-related pathways in pathological states.

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References

1. Gao ZG, Kim SK, Biadatti T, Chen W, Lee K, Barak D, Kim SG, Johnson CR, Jacobson KA. Structural determinants of A₃ adenosine receptor activation: nucleoside ligands at the agonist/antagonist boundary. *J Med Chem.* 2002; 45:4471–84. [PubMed: 12238926]
2. Gao ZG, Blaustein JB, Gross AS, Melman N, Jacobson KA. N⁶-Substituted adenosine derivatives: selectivity, efficacy, and species differences at A₃ adenosine receptors. *J Med Chem.* 2003; 46:3775–7. [PubMed: 12930138]
3. Jeong LS, Pal S, Choe SA, Choi WJ, Jacobson KA, Gao ZG, Klutz AM, Hou X, Kim HO, Lee HW, Lee SK, Tosh DK, Moon HR. Structure-activity relationships of truncated D- and L-4'-thioadenosine derivatives as species-independent A₃ adenosine receptor antagonists. *J Med Chem.* 2008; 51:6609–13. [PubMed: 18811138]
4. Hou X, Kim HO, Alexander V, Kim K, Choi S, Park SG, Lee JH, Yoo LS, Gao ZG, Jacobson KA, Jeong LS. Discovery of new human A_{2A} adenosine receptor agonists: design, synthesis, and

- binding mode of truncated 2-hexynyl-4'-thioadenosine. *ACS Med Chem Lett.* 2010; 1:516–520. [PubMed: 21286238]
5. Tosh DK, Paoletta S, Deflorian F, Phan K, Moss SM, Gao ZG, Jiang X, Jacobson KA. Structural sweet spot for A₁ adenosine receptor activation by truncated (N)-methanocarba nucleosides: receptor docking and potent anticonvulsant activity. *J Med Chem.* 2012; 55:8075–90. [PubMed: 22921089]
 6. Gao ZG, Jacobson KA. Translocation of arrestin induced by human A₃ adenosine receptor ligands in an engineered cell line: comparison with G protein-dependent pathways. *Pharmacol Res.* 2008; 57:303–11. [PubMed: 18424164]
 7. Paoletta S, Tosh DK, Finley A, Gizewski ET, Moss SM, Gao ZG, Auchampach JA, Salvemini D, Jacobson KA. Rational design of sulfonated A₃ adenosine receptor-selective nucleosides as pharmacological tools to study chronic neuropathic pain. *J Med Chem.* 2013; 56:5949–63. [PubMed: 23789857]
 8. Sabbah HN, Gupta RC, Kohli S, Wang M, Rastogi S, Zhang K, Zimmermann K, Diedrichs N, Albrecht-Küpper BE. Chronic therapy with a partial adenosine A₁-receptor agonist improves left ventricular function and remodeling in dogs with advanced heart failure. *Circ Heart Fail.* 2013; 6:563–71. [PubMed: 23564604]
 9. Dando R, Dvoryanchikov G, Pereira E, Chaudhari N, Roper SD. Adenosine enhances sweet taste through A_{2B} receptors in the taste bud. *J Neurosci.* 2012; 32:322–30. [PubMed: 22219293]
 10. Kataoka S, Baquero A, Yang D, Shultz N, Vandenbeuch A, Ravid K, Kinnamon SC, Finger TE. A_{2B}R adenosine receptor modulates sweet taste in circumvallate taste buds. *PLoS One.* 2012; 7(1):e30032. [PubMed: 22253866]
 11. Gladwin MT. Sickle cell disease: is kind of inflammatory disease. Adenosine receptor crossroads in sickle cell disease. *Nat Med.* 2011; 17:38–40. [PubMed: 21217677]
 12. Belikoff BG, Vaickus LJ, Sitkovsky M, Remick DG. A_{2B} adenosine receptor expression by myeloid cells is proinflammatory in murine allergic-airway inflammation. *J Immunol.* 2012; 189:3707–13. [PubMed: 22956582]
 13. Kalla RV, Zablocki J, Tabrizi MA, Baraldi PG. Recent developments in A_{2B}adenosine receptor ligands. *Handb Exp Pharmacol.* 2009; (193):99–122.10.1007/978-3-540-89615-9_4 [PubMed: 19639280]
 14. Haskó G, Csóka B, Németh ZH, Vizi ES, Pacher P. A_{2B} adenosine receptors in immunity and inflammation. *Trends Immunol.* 2009; 30:263–70. [PubMed: 19427267]
 15. Ham J, Rees DA. The adenosine A_{2B} receptor: its role in inflammation. *Endocr Metab Immune Disord Drug Targets.* 2008; 8:244–54. [PubMed: 19075778]
 16. Haskó G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov.* 2008; 7:759–70. [PubMed: 18758473]
 17. Wei W, Du C, Lv J, Zhao G, Li Z, Wu Z, Haskó G, Xie X. Blocking A_{2B} adenosine receptor alleviates pathogenesis of experimental autoimmune encephalomyelitis via inhibition of IL-6 production and Th17 differentiation. *J Immunol.* 2013; 190:138–46. [PubMed: 23225885]
 18. Desmet CJ, Gallenne T, Prieur A, Reyat F, Visser NL, Wittner BS, Smit MA, Geiger TR, Laoukili J, Iskit S, Rodenko B, Zwart W, Evers B, Horlings H, Ajouaou A, Zevenhoven J, van Vliet M, Ramaswamy S, Wessels LF, Peeper DS. Identification of a pharmacologically tractable Fra-1/ADORA_{2B} axis promoting breast cancer metastasis. *Proc Natl Acad Sci U S A.* 2013; 110:5139–44. [PubMed: 23483055]
 19. Iannone R, Miele L, Maiolino P, Pinto A, Morello S. Blockade of A_{2B} adenosine receptor reduces tumor growth and immune suppression mediated by myeloid-derived suppressor cells in a mouse model of melanoma. *Neoplasia.* 2013; 15:1400–9. [PubMed: 24403862]
 20. Cekic C, Sag D, Li Y, Theodorescu D, Strieter RM, Linden J. Adenosine A_{2B} receptor blockade slows growth of bladder and breast tumors. *J Immunol.* 2012; 188:198–205. [PubMed: 22116822]
 21. Stagg J, Divisekera U, McLaughlin N, Sharkey J, Pommey S, Denoyer D, Dwyer KM, Smyth MJ. Anti-CD73 antibody therapy inhibits breast tumor growth and metastasis. *Proc Natl Acad Sci U S A.* 2010; 107:1547–52. [PubMed: 20080644]

22. Ryzhov S, Novitskiy SV, Zaynagetdinov R, Goldstein AE, Carbone DP, Biaggioni I, Dikov MM, Feoktistov I. Host A_{2B} adenosine receptors promote carcinoma growth. *Neoplasia*. 2008; 10:987–95. [PubMed: 18714400]
23. Methner C, Krieg T. A_{2B} adenosine receptors in cardioprotection: timing is everything. *J Mol Cell Cardiol*. 2011; 50:582–3. [PubMed: 21050857]
24. Grenz A, Bauerle JD, Dalton JH, Ridyard D, Badulak A, Tak E, McNamee EN, Clambey E, Moldovan R, Reyes G, Klawitter J, Ambler K, Magee K, Christians U, Brodsky KS, Ravid K, Choi DS, Wen J, Lukashev D, Blackburn MR, Osswald H, Coe IR, Nürnberg B, Haase VH, Xia Y, Sitkovsky M, Eltzschig HK. Equilibrative nucleoside transporter 1 (ENT1) regulates postischemic blood flow during acute kidney injury in mice. *J Clin Invest*. 2012; 122:693–710. [PubMed: 22269324]
25. Wen J, Grenz A, Zhang Y, Dai Y, Kellems RE, Blackburn MR, Eltzschig HK, Xia Y. A_{2B} adenosine receptor contributes to penile erection via PI3K/AKT signaling cascade-mediated eNOS activation. *FASEB J*. 2011; 25:2823–30. [PubMed: 21566208]
26. Csóka B, Koscsó B, Tőro G, Kókai E, Virág L, Németh ZH, Pacher P, Bai P, Haskó G. A_{2B} adenosine receptors prevent insulin resistance by inhibiting adipose tissue inflammation via maintaining alternative macrophage activation. *Diabetes*. 2013; 63:850–66. [PubMed: 24194503]
27. Johnston-Cox H, Koupenova M, Yang D, Corkey B, Gokce N, Farb MG, LeBrasseur N, Ravid K. The A_{2b} adenosine receptor modulates glucose homeostasis and obesity. *PLoS One*. 2012; 7(7):e40584. [PubMed: 22848385]
28. Figler RA, Wang G, Srinivasan S, Jung DY, Zhang Z, Pankow JS, Ravid K, Fredholm B, Hedrick CC, Rich SS, Kim JK, LaNoue KF, Linden J. Links between insulin resistance, adenosine A_{2B} receptors, and inflammatory markers in mice and humans. *Diabetes*. 2011; 60:669–79. [PubMed: 21270276]
29. Shih YR, Hwang Y, Phadke A, Kang H, Hwang NS, Caro EJ, Nguyen S, Siu M, Theodorakis EA, Gianneschi NC, Vecchio KS, Chien S, Lee OK, Varghese S. Calcium phosphate-bearing matrices induce osteogenic differentiation of stem cells through adenosine signaling. *Proc Natl Acad Sci U S A*. 2014; 111:990–5. [PubMed: 24395775]
30. van der Hoeven D, Wan TC, Gizewski ET, Kreckler LM, Maas JE, Van Orman J, Ravid K, Auchampach JA. A role for the low affinity A_{2B} adenosine receptor in regulating superoxide generation by murine neutrophils. *J Pharmacol Exp Ther*. 2011; 338:1004–12. [PubMed: 21693629]
31. Kolachala VL, Vijay-Kumar M, Dalmasso G, Yang D, Linden J, Wang L, Gewirtz A, Ravid K, Merlin D, Sitaraman SV. A_{2B} adenosine receptor gene deletion attenuates murine colitis. *Gastroenterology*. 2008; 135:861–70. [PubMed: 18601927]
32. Eckle T, Grenz A, Laucher S, Eltzschig HK. A_{2B} adenosine receptor signaling attenuates acute lung injury by enhancing alveolar fluid clearance in mice. *J Clin Invest*. 2008; 118:3301–15. [PubMed: 18787641]
33. Elzein E, Kalla RV, Li X, Perry T, Gimbel A, Zeng D, Lustig D, Leung K, Zablocki J. Discovery of a novel A_{2B} adenosine receptor antagonist as a clinical candidate for chronic inflammatory airway diseases. *J Med Chem*. 2008; 51:2267–78. [PubMed: 18321039]
34. Verzijl D, IJzerman AP. Functional selectivity of adenosine receptor ligands. *Purinergic Signal*. 2011; 7:171–92. [PubMed: 21544511]
35. Gao ZG, Jacobson KA. Allosteric modulation and functional selectivity of G protein-coupled receptors. *Drug Discov Today Technol*. 2013; 10:e237–e243.
36. Adachi H, Palaniappan KK, Ivanov AA, Bergman N, Gao ZG, Jacobson KA. Structure-activity relationships of 2,N⁶,5'-substituted adenosine derivatives with potent activity at the A_{2B} adenosine receptor. *J Med Chem*. 2007; 50:1810–27. [PubMed: 17378544]
37. Gao ZG, Mamedova LK, Chen P, Jacobson KA. 2-Substituted adenosine derivatives: affinity and efficacy at four subtypes of human adenosine receptors. *Biochem Pharmacol*. 2004; (68):1985–93. [PubMed: 15476669]
38. Brackett LE, Daly JW. Functional characterization of the A_{2b} adenosine receptor in NIH 3T3 fibroblasts. *Biochem Pharmacol*. 1994; 47:801–14. [PubMed: 8135856]

39. de Zwart M, de Groote M, van der Klein PAM, van Dun S, von Frijtag Drabbe Künzel JK, IJzerman AP. Phenyl-substituted N⁶-phenyladenosines and N⁶-phenyl-5'-N-ethylcarboxamidoadenosines with high activity at human adenosine A_{2B} receptors. *Drug Devel Res.* 2000; 49:85–93.
40. Ji X, Kim YC, Ahern DG, Linden J, Jacobson KA. [³H]MRS 1754, a selective antagonist radioligand for A_{2B} adenosine receptors. *Biochem Pharmacol.* 2001; 61:657–63. [PubMed: 11266650]
41. Beukers MW, Chang LCW, von Frijtag Drabbe Künzel JK, Mulder-Krieger T, Spanjersberg RF, Brussee J, IJzerman AP. New, non-adenosine, high-potency agonists for the human adenosine A_{2B} receptor with an improved selectivity profile compared to the reference agonist N-ethylcarboxamidoadenosine. *J Med Chem.* 2004; 47:3707–9. [PubMed: 15239649]
42. Hinz S, Lacher SK, Seibt B, Müller CE. BAY60-6583 acts as a partial agonist at adenosine A_{2B} receptors. *J Pharmacol Exp Ther.* 2014; 349:427–36. [PubMed: 24633424]
43. Gao ZG, Verzijl D, Zweemer A, Ye K, Göblyös A, IJzerman AP, Jacobson KA. Functionally biased modulation of A₃ adenosine receptor agonist efficacy and potency by imidazoquinolinamine allosteric enhancers. *Biochem Pharmacol.* 2011; 82:658–68. [PubMed: 21718691]
44. Black JW, Leff P. Operational models of pharmacological agonism. *Proc R Soc Lond B.* 1983; 220:141–162. [PubMed: 6141562]
45. Kenakin T, Watson C, Muniz-Medina V, Christopoulos A, Novick S. A simple method for quantifying functional selectivity and agonist bias. *ACS Chem Neurosci.* 2012; 3:193–203. [PubMed: 22860188]
46. Phelps PT, Anthes JC, Correll CC. Characterization of adenosine receptors in the human bladder carcinoma T24 cell line. *Eur J Pharmacol.* 2006; 536:28–37. [PubMed: 16581066]
47. Rüsing D, Müller CE, Verspohl EJ. The impact of adenosine and A_{2B} receptors on glucose homeostasis. *J Pharm Pharmacol.* 2006; 58:1639–45. [PubMed: 17331328]
48. Linden J, Thai T, Figler H, Jin X, Robeva AS. Characterization of human A_{2B} adenosine receptors: radioligand binding, western blotting, and coupling to G_q in human embryonic kidney 293 cells and HMC-1 mast cells. *Mol Pharmacol.* 1999; 56:705–13. [PubMed: 10496952]
49. Cohen MV, Yang XM, Liu Y, Solenkova NV, Downey JM. Cardioprotective PKG-independent NO signaling at reperfusion. *Am J Physiol Heart Circ Physiol.* 2010; 299:H2028–36. [PubMed: 20852051]
50. Schulte G, Fredholm BB. The G(s)-coupled adenosine A_{2B} receptor recruits divergent pathways to regulate ERK1/2 and p38. *Exp Cell Res.* 2003; 290:168–76. [PubMed: 14516797]
51. Mundell SJ, Matharu AL, Kelly E, Benovic JL. Arrestin isoforms dictate differential kinetics of A_{2B} adenosine receptor trafficking. *Biochemistry.* 2000; 39:12828–36. [PubMed: 11041847]
52. Yang X, Xin W, Yang XM, Kuno A, Rich TC, Cohen MV, Downey JM. A_{2B} adenosine receptors inhibit superoxide production from mitochondrial complex I in rabbit cardiomyocytes via a mechanism sensitive to Pertussis toxin. *Br J Pharmacol.* 2011; 163:995–1006. [PubMed: 21366548]
53. Wei Q, Costanzi S, Balasubramanian R, Gao ZG, Jacobson KA. A_{2B} adenosine receptor blockade inhibits growth of prostate cancer cells. *Purinergic Signal.* 2013; 9:271–80. [PubMed: 23315335]
54. Patel H, Porter RH, Palmer AM, Croucher MJ. Comparison of human recombinant adenosine A_{2B} receptor function assessed by Fluo-3-AM fluorometry and microphysiometry. *Br J Pharmacol.* 2003; 138:671–7. [PubMed: 12598421]
55. Rajagopal S. Quantifying biased agonism: understanding the links between affinity and efficacy. *Nat Rev Drug Discov.* 2013; 12(6):483. [PubMed: 23681006]
56. Kenakin T, Christopoulos A. Measurements of ligand bias and functional affinity. *Nat Rev Drug Discov.* 2013; 12:205–16. [PubMed: 23411724]

Abbreviations

BAY60-6583 (BAYLUF6210)	2-[[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]-2-pyridinyl]thio]acetamide
cyclic AMP (cAMP)	3',5'-cyclic adenosine monophosphate
CGS21680	2-[p-(2-carboxyethyl)phenylethylamino]-5'-N-ethylcarboxamidoadenosine
CHO	Chinese hamster ovary
CPA	N ⁶ -cyclopentyladenosine
CPCA	adenosine-5'-N-cyclopropyluronamide
CV1808	2-phenylaminoadenosine
DMEM	Dulbecco's modified Eagle's medium
ERK	extracellular-signal-regulated kinase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GPCR	G protein-coupled receptor
HEK	human embryonic kidney
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
IB-MECA	N ⁶ -(3-iodobenzyl)-adenosine-5'-N-methyluronamide
KRBB	Krebs ringer bicarbonate buffer
LUF5833	2-aminophenyl-6-(1 <i>H</i> -imidazol-2-ylmethylsulfanyl)pyridine-3,5-dicarbonitrile
MRS3534	2-(2-(indol-3-yl)ethyloxy)adenosine
MRS3997	2-(2-(6-bromo-indol-3-yl)ethyloxy)adenosine
MRS5911	N ⁶ -(4-iodophenyl)adenosine
NECA	adenosine-5'-N-ethyluronamide
R-PIA	R-N ⁶ -(phenylisopropyl)adenosine
PSB603	8-[4-[4-(4-chlorophenyl)piperazide-1-sulfonyl]phenyl]-1-propylxanthine

Fig. 1. Chemical structures of representative agonists used in the present study. Non-nucleoside agonists: BAY and LUF5833; 5'-substituted: NECA, CPCA, 2-substituted: MRS3997 and MRS3534. N⁶-substituted: MRS5911.

Fig. 2.

Agonist concentration-response curves in various signaling pathways. A. cAMP accumulation. B. Intracellular calcium mobilization. C. ERK1/2 phosphorylation. D. β -arrestin 2 translocation. Results shown in Figs. 2A, 2B and 2C are from assays in HEK293 cells overexpressing the human $A_{2B}AR$. Results shown in Fig. 2D are from PathHunter CHO cells expressing the mouse $A_{2B}AR$. Results are expressed as mean $\pm$ SEM and are from at least three independent experiments performed in triplicate. The values of EC_{50} and maximum effects are listed in Table 1.

Fig. 3. Effect of the A_{2B}AR antagonist PSB603 on NECA-induced cAMP accumulation (A) and calcium mobilization (B) in T24 bladder cancer cells. Cells were pretreated with PSB603 20 min before the addition of NECA. Results were expressed as mean $\pm$ SEM from 3-4 separate experiments performed in duplicate. PSB, PSB603.

Fig. 4. Distinct effects of receptor depletion by siRNA silencing on the $A_{2B}AR$ activation by different agonist in T24 cells. **A.** Effect on BAY-induced cAMP accumulation in T24 cells. **B.** Effect on the NECA-induced cAMP accumulation. **C.** Effect on calcium mobilization. The siRNA was transfected using lipofectamine 2000 as instructed by the manual. Results were expressed as mean $\pm$ SEM from three independent experiments performed in duplicate or triplicate. #Significant different from those values in the absence of siRNA ($P < 0.05$).

A.

B

Fig. 5.

A. Insulin release in MIN-6 cells. Glucose stimulated insulin secretion (GSIS) in MIN6 cells with or without various $A_{2B}AR$ ligands at 16.7 mM glucose concentration. Values are mean $\pm$ SEM, for $n=3$. * $P < 0.05$, when compared to 16.7 mM glucose samples. B. Western blot analyses of the expression of $A_{2B}AR$ in various cell lines. The left side panel was probed only with A_{2B} antibody and right side panel was probed with a mixture of A_{2B} antibody+ its specific antigen and used as a reference. *Lane 1*, Molecular weight marker; *Lane 2*, MIN6 cell lysate; *Lane 3*, 1321N1 astrocytoma cell lysate; *Lane 4*, COS7 cell lysate; *Lane 5*, HEK293 cell lysate.

Fig. 6.

Quantification of A_{2B}AR gene expression using qRT-PCR. A. A_{2B}AR mRNA expression level in various cells. B. Comparison of the A_{2B}AR gene expression levels of control HEK293 and A_{2B} overexpressing HEK293 cells. C. A_{2B}AR gene expression level in T24 cells in the absence and presence of various concentrations of A_{2B}AR specific siRNA. All data were normalized based on endogenous GAPDH expression level and were expressed as relative expression level. Data were from 3-4 separate experiments providing similar results performed in triplicate. [#]Significantly different from T24 or HEK293 control groups ($P < 0.05$ compared with control).

Table 1

Potency (EC_{50} , nM) and maximum effect (% of effect of NECA) of various agonists at four signaling pathways.^a

Ligand	cAMP		Calcium			ERK1/2		β -arrestin	
	T24	HEK	HEK-A _{2B}	T24	HEK	HEK-A _{2B}	T24	HEK	HEK-A _{2B}
MRS3534									
EC_{50}	2160 ±350	ND	ND	ND	ND	ND	ND	ND	ND
Max effect	82.0 ±5.9	ND	ND	-0.5 ±1.7	ND	-7.7 ±3.2	ND	ND	10.7 ±3.2
MRS3997									
EC_{50}	745 ±225	1020 ±250	62.9 ±7.6	ND	ND	ND	ND	166 ±32	203 ±48
Max effect	94.9 ±2.2	96.2 ±24.3	102 ±6.6	-2.7 ±3.2	1.6 ±2.2	7.8 ±1.3	-8.2 ±7.6	42.3 ±9.4	29.7 ±2.6
MRS5911									
EC_{50}	899 ±226	1130 ±340	25.3 ±7.8	6170 ±1990	ND	7150 ±2360	ND	1530 ±340	2680 ±460
Max effect	77.7 ±3.3	68.7 ±11.1	93.2 ±4.5	36.9 ±8.6	7.1 ±3.2	22.8 ±6.6	13.1 ±4.5	46.1 ±9.2	36.3 ±5.2
R-PIA									
EC_{50}	9750 ±2840	ND	ND	13,100 ±4800	ND	14,200 ±3600	ND	ND	ND
Max effect	68.6 ±7.1	ND	ND	9.6 ±4.1	0.6 ±1.4	18.2 ±4.9	ND	ND	13.2 ±1.1
LUF5833									
EC_{50}	9.82 ±2.43	ND	ND	NA	ND	NA	ND	ND	ND
Max effect	52.8 ±6.6	ND	ND	-5.6 ±3.8	ND	2.8 ±2.1	ND	ND	9.7 ±0.6
BAY									
EC_{50}	43.0 ±17.7	296 ±146	6.14 ±1.61	ND	ND	1100 ±390	ND	68.8 ±4.7	28.9 ±4.1
Max effect	62.9 ±9.6	73.0 ±12.1	102 ±1	2.3 ±1.5	-2.4 ±1.7	9.7 ±2.9	-9.4 ±3.8	38.3 ±5.9	38.4 ±3.3
CPCA									
EC_{50}	177 ±67	267 ±32	32.8 ±4.2	1930 ±680	2620 ±460	1490 ±360	369 ±32	1030 ±230	1860 ±240
Max effect	98.5 ±1.9	102 ±9	98.9 ±6.6	101 ±7	98.7 ±6.6	102 ±4	101 ±8	99.3 ±2.4	100 ±6
NECA									
EC_{50}	242 ±72	331 ±146	21.0 ±2.5	1480 ±210	2460 ±1240	1850 ±450	478 ±66	787 ±168	2360 ±370
Max effect	100	100	100	100	100	100	100	100	100
CGS21680									

Ligand	cAMP			Calcium			ERK1/2			β-arrestin
	T24	HEK	HEK-A _{2B}	T24	HEK	HEK-A _{2B}	T24	HEK	HEK-A _{2B}	
EC ₅₀	>10,000	>10,000	2920 ±570	ND	ND	ND	ND	ND	ND	ND
Max effect	-7.6 ±6.9	2.7 ±2.4	107 ±9	-3.4±2.7	0.8 ±1.4	4.3 ±2.8	2.2 ±0.8	ND	ND	ND
Adenosine										
EC ₅₀	4620 ±680	ND	ND	11,400 ±5000	ND	28,400 ±5700	ND	ND	ND	ND
Max effect	97.1 ±3.8	ND	ND	101 ±9	ND	98.8 ±5.6	ND	ND	ND	ND

^aResults were expressed as mean±SEM from at least three independent experiments performed in duplicate or triplicate. Data related to cAMP, calcium and ERK1/2 were from assays in T24 cells and HEK293 cells expressing the human A_{2B}AR. Data related to the β-arrestin2 translocation were from PathHunter CHO cells expressing the mouse A_{2B}AR, as the corresponding human version did not produce a robust response.

ND, not determined or there was not a sufficient effect to determine the parameters.

Table 2

Potency (EC_{50} , nM) and maximum effect (%) of agonist-induced cAMP accumulation in various cells endogenously expressing the $A_{2B}AR$.^a

Ligand	1321N1	COS-7	PC-3	WM266
MRS3997				
EC_{50}	1260±180	226±32	480±32	1870±320
Max effect	98.4±3.1	100±6	98.2±7.7	96.2±1.8
MRS5911				
EC_{50}	1010±180	418±22	321±66	1270±220
Max effect	89.8±9.2	99.3±2.1	89.4±2.6	48.3±4.9
BAY				
EC_{50}	113±17	7.9±1.2	45.3±5.9	ND
Max effect	52.6±3.1	98.3±3.7	72.6±5.1	9.8±1.7
NECA				
EC_{50}	258±25	28.5±3.1	147±33	1280±360
Max effect	100	100	100	100

^aData for other 2 cell lines endogenously expressing the human $A_{2B}AR$, T24 and HEK293, are included in Table 1. Results are expressed as mean ±SEM from at least three independent experiments performed in triplicate.

ND, EC_{50} could not be determined due to insufficient effect.

Table 3

Calculation of bias factors of four representative agonists (5', C2, and N⁶ modified and nonnucleoside) at different signaling pathways.^a

Pathway/agonist	τ	K_A (M)	$\log(\tau/K_A)$	$\log(\tau/K_A)$	$\log(\tau/K_A)$	Bias
cAMP						
NECA	238	9.07E-6	7.42 ± 0.24	0.00 ± 0.21		
MRS3997	45.1	6.70E-6	6.83 ± 0.45	0.59 ± 0.08		
MRS5911	116	7.76E-6	7.17 ± 0.68	-0.25 ± 0.02		
BAY60-6583	100	7.05E-7	8.15 ± 0.38	0.73 ± 0.04		
ERK1/2						
NECA	25.0	5.87E-5	5.63 ± 0.31	0.00 ± 0.02	0.00	1.00
MRS3997	0.44	3.62E-7	6.08 ± 0.89	0.45 ± 0.03	0.14	1.38
MRS5911	0.68	5.26E-6	5.11 ± 0.52	-0.52 ± 0.04 [*]	0.27	1.86
BAY60-6583	0.62	5.95E-8	7.02 ± 0.44	1.39 ± 0.17 [*]	-0.66	0.22
Calcium						
NECA	39.0	3.56E-5	5.04 ± 0.21	0.00 ± 0.09	0.00	1.0
MRS3997 ^b	0.02	7.10E-6	3.35 ± 0.44	-3.47 ± 0.05	4.06	11500
MRS5911	0.59	8.47E-5	3.84 ± 0.19	-1.20 ± 0.13 [*]	1.93	85
BAY60-6583 ^b	0.08	1.67 E-6	4.66 ± 0.35	-3.49 ± 0.38	4.22	16600
β-Arrestin						
NECA	38.9	7.32E-5	5.73 ± 0.51	0.00 ± 0.11	0.00	1.00
MRS3997	0.27	1.81E-5	4.17 ± 0.23	-1.56 ± 0.18 [*]	2.15	141
MRS5911	1.04	8.34E-5	4.10 ± 0.22	-1.63 ± 0.20 [*]	1.38	24
BAY60-6583	0.25	1.23E-5	4.30 ± 0.17	-1.43 ± 0.09 [*]	2.16	145

^a Results are expressed as mean ± SEM from at least three independent experiments.

^b The respective maximum agonist efficacies of MRS3997 and BAY are only about 8% and 10% in comparison to NECA (100%).

^{*} Significantly different from control ($P < 0.05$). The calculation was as described in references 43 and 44.