Functional selectivity of adenosine A₁ receptor ligands?

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Abstract The concept of functional selectivity offers great potential for the development of drugs that selectively activate a specific intracellular signaling pathway. During the last few years, it has become possible to systematically analyse compound libraries on G protein-coupled receptors (GPCRs) for this ‘biased’ form of signaling. We screened over 800 compounds targeting the class of adenosine A₁ receptors using a β -arrestin-mediated signaling assay in U2OS cells as a G protein-independent readout for GPCR activation. A selection of compounds was further analysed in a G protein-mediated GTP γ S assay. Additionally, receptor affinity of these compounds was determined in a radioligand binding assay with the agonist [³H]CCPA. Of all compounds tested, only LUF5589 **9** might be considered as functionally selective for the G protein-dependent pathway, particularly in view of a likely overestimation of β -arrestin signaling in the U2OS cells. Altogether, our study shows that functionally selective ligands for the adenosine A₁ receptor are rare, if existing at all. A thorough analysis of biased signaling on other GPCRs also reveals that only very few compounds can be considered functionally selective. This might indicate that the concept of functional selectivity is less common than speculated.

Keywords Adenosine · Adenosine A₁ receptor · Functional selectivity · Biased signaling · GTP γ S · Beta-arrestin

Introduction

Adenosine receptors are G protein-coupled receptors (GPCR) that transduce an extracellular signal into the interior of the cell. Basically every mammalian cell expresses at least one of the four adenosine receptor subtypes (A₁R, A_{2A}R, A_{2B}R, and A₃R). Upon activation of the receptors by the ubiquitous endogenous ligand adenosine (**3** in Fig. 1), they engage classical G protein-mediated pathways, resulting in the production of second messengers and the activation of kinases [1]. Besides the well-described G protein-mediated signaling pathway, adenosine receptors, either engineered or wild type, recruit scaffold proteins such as β -arrestins [2].

Synthetic ligands for adenosine receptors are, like for other GPCRs, usually categorized as (partial) agonists, neutral antagonists, (partial) inverse agonists, as well as positive and negative allosteric modulators. Over the past decade, it has become clear that GPCR ligands exist that preferentially stimulate one signal transduction pathway over another. This phenomenon is referred to as functional selectivity or biased signaling [3–5]. Biased signaling implies that an agonist for one pathway may act as an antagonist or inverse agonist for another signal transduction cascade. Functional selectivity of compounds has been indicated as potentially highly interesting in reducing drug-induced side effects [4, 6]. When beneficial signaling pathways in a disease setting can be activated without simultaneous activation of receptor-mediated side effect-inducing pathways, this will improve the therapeutic window of the drug used. For instance, it has been suggested that adenosine A₁ receptor agonists exploiting the β -arrestin/ERK1/2 pathway would provide cardioprotection (for further refs., see [2]). Therefore, it is necessary to redefine the functional properties of currently known adenosine receptor ligands, which may indeed open possibilities for new

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Fig. 1 Structures of selected adenosine receptor ligands tested in G protein-dependent (GTP γ S) and independent (β -arrestin) signaling assays.

Ligands are indicated in the text by name and bold number.

Abbreviations: *CPA* **5**, *N*⁶-cyclopentyladenosine; *NECA* **4**, 5'-*N*-ethylcarboxamidoadenosine; *MECA* **12**, 5'-*N*-methylcarboxamidoadenosine. Adenosine **3**, derivatives LUF5580 **6** 5'-methyl-ether-*N*⁶-3-iodobenzyl adenosine, LUF5583 **7** 5'-methyl-ether-2-chloro-*N*⁶-3-iodobenzyl adenosine, LUF5586 **8** 5'-ethyl-ether-*N*⁶-3-iodobenzyl adenosine and LUF5589 **9** 5'-methyl-ether-2-chloro-*N*⁶-3-iodobenzyl-] adenosine. They all have an *N*⁶-3-iodobenzyl group, like 3-IB adenosine **10**, MRS542 **11**, IB-MECA **13**, and Cl-IB-MECA **14**. Reference compound MECA **12** lacks the *N*⁶-3-iodobenzyl substitution. Compounds **1** and **2**, both xanthine derivatives with in-house codes of AAN12 and AAN27, are further discussed in the text

and more selective ligands. With innovative and sensitive experimental tools, it has become possible to detect adenosine ligands that preferentially stimulate the β -arrestin pathway over the G protein-mediated signal transduction route, or vice versa. We recently reviewed the current knowledge of functionally selective adenosine receptor ligands and G protein-independent signaling of adenosine receptors through scaffold proteins [2]. Recent insight in signal transduction cascades teaches us that the current classification of receptor ligands (into agonists, antagonists, and inverse agonists) relies very much on the experimental setup, including receptor number and type of response measured [1]. Hence, a careful dissection of the nature of a ligand's efficacy for a given pathway is warranted.

Here, we report on the screening for functionally selective ligands for the A₁R, using G protein-independent β -

arrestin-mediated signaling as readout. Activation properties of A₁R ligands were investigated in a G_i protein-dependent assay as well. Although functional selectivity is not necessarily limited to this β -arrestin/G protein dichotomy, most studies so far, also on other GPCRs, have employed it (see also the "Discussion" section). A commercially available U2OS cell line, expressing an engineered adenosine A₁ receptor, was used to screen for β -arrestin-mediated signaling. More than 800 compounds were screened in this way. Several 3-iodobenzyl adenosine derivatives were studied in more detail. In addition, CHO cells expressing wild-type A₁R were used for affinity determination. G_i protein-mediated GTP γ S assays were performed on membranes from both cell types. Our results suggest that the plethora of A₁R ligands tested so far shows little functional selectivity. This might indicate that functionally selective ligands for the adenosine A₁ receptor are rare, if not absent.

Methods

Cell culture

CHO cells stably expressing the adenosine A₁ receptor were cultured in Dulbecco's modified Eagle medium/F12 with 10 % newborn calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin and 200 µg/ml G418. Tango™ ADORA1-bla U2OS cells (Invitrogen) were cultured in McCoy's 5a medium, supplemented with 10 % dialyzed fetal calf serum, 0.1 mM nonessential amino acids, 25 mM HEPES, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 µg/ml streptomycin, 100 µg/ml G418, 50 µg/ml hygromycin, 125 µg/ml zeocin, and 5 ml glutamax. Cell lines were incubated in a humidified atmosphere at 37 °C and 5 % CO₂.

β-arrestin-mediated signaling assay

Tango™ ADORA1-bla U2OS cells were plated (10,000 cells/well) in clear bottom 384-well plates (Corning, Lowell, MA) in 32 µl FreeStyle Expression medium (Invitrogen, Carlsbad, CA) and incubated for 24 h at 37 °C. Dilutions of compounds dissolved in DMSO were prepared in FreeStyle Expression medium, added to the cells in a total volume of 8 µl and incubated for 5 h at 37 °C. Next, the substrate of the Live-BLazer kit (Invitrogen, Carlsbad, CA) was used to detect beta-arrestin-mediated β-lactamase production. The loading solution for the substrate was prepared according to manufacturer's protocol and added in 8 µl/well. After 2 h incubation in the dark, samples were excited at 409 nm and fluorescence was measured at 460 and 535 nm using an Envision 2104 Multilabel reader (PE Waltham, MA). The 535/460-nm signal ratio was calculated for each well and used as a measure for β-arrestin-mediated signaling. For assays involving pertussis toxin (PTX), cells were plated in a total volume of 28 µl and 4 µl PTX was added after 8 h for a final concentration of 100 ng/ml after which the assay was continued as described.

Membrane preparation

Membranes from CHO cells stably expressing the adenosine A₁ receptor as well as from Tango™ ADORA1-bla U2OS cells were prepared as previously described [7]. Aliquots containing 0.8 U/ml adenosine deaminase (ADA) were stored at −80 °C. The protein concentration of the membranes was determined using the BCA protein assay reagent (Pierce Chemical Company, Rockford, IL, USA) with BSA as a standard.

Radioligand binding studies

For displacement studies, membranes from CHO cells stably expressing the adenosine A₁ receptor (4 µg) were

incubated for at least 1 h at 25 °C in 50 mM Tris–HCl (pH 7.4) in the presence of ADA (0.8 U/ml), approximately 2 nM [³H]CCPA, and increasing concentrations of A₁ receptor ligands in a total of 100 µl. Incubations were stopped by rapid dilution with 2 ml ice-cold 50 mM Tris–HCl buffer (pH 7.4), and bound radioactivity was subsequently recovered by filtration through Whatman GF/B filters using a Brandel harvester. Filters were washed three times with 2 ml ice-cold buffer. The retained radioactivity was measured after the addition of 3.5 ml Packard Emulsifier Safe scintillation liquid and counted in the Tri-Carb 2900TR spectrometer (PerkinElmer). Nonspecific binding of [³H]CCPA was measured in the presence of 10 µM CPA. No ligand depletion was observed when using <5-µg membranes.

[³⁵S]GTPγS binding assay

Membrane homogenates (CHO-hA₁ or Tango™ ADORA1-bla U2OS, 3 µg) were equilibrated in 80 µl total volume of assay buffer (50 mM Tris, 200 mM NaCl, 10 mM MgCl₂, pH 7.4, EDTA, DTT, and BSA) containing 3 µM GDP and a range of concentrations of ligand at 25 °C for 30 min. After this 20 µl of [³⁵S]GTPγS (final concentration 0.3 nM) was added and incubation continued for 90 min at 25 °C. Incubation was terminated by rapid filtration through a 96-well Unifilter GF/B (Perkin Elmer, NL) using a Filtermate Unifilter 96-well harvester (Perkin Elmer). Filters were washed six times with ice-cold assay buffer before drying. Microscint scintillation cocktail (37.5 µl) was added to each well, and plates were counted in a 1450 Trilux Microbeta liquid scintillation and luminescence counter (Perkin Elmer).

Data analysis

Data were analysed using the nonlinear regression curve-fitting program GraphPad Prism version 5.00 (GraphPad Software Inc., San Diego, CA). [³H]CCPA radioligand displacement curves were fitted to a one state/site binding model. Affinity data were analysed using nonlinear regression, and K_i values were calculated using a K_d value of 4.2 nM for the radioligand. Data shown are the mean ± SEM of at least three separate experiments performed in duplicate. In the functional [³⁵S]GTPγS assay, agonist concentration response curves were also analysed through nonlinear regression curve fitting. Data shown are the mean ± SEM of at least three separate experiments performed in duplicate.

Materials

CPA (N⁶-cyclopentyladenosine) was obtained from Research Biochemicals Inc. (Natick, MA, USA), NECA (5'-

N-ethylcarboxamido adenosine), IB-MECA (*N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide) and Cl-IB-MECA (2-chloro-*N*⁶-(3-iodobenzyl)adenosine-5'-*N*-methyluronamide) were obtained from Sigma-Aldrich (Zwijndrecht, the Netherlands). Compounds LUF5580, LUF5583, LUF5586, and LUF5589 were synthesized in our laboratory at the Leiden/Amsterdam Center for Drug Research (Leiden, The Netherlands), as described by Van Tilburg et al. [8]. MRS541 (*N*⁶-(3-iodobenzyl)adenosine) and MRS542 (2-chloro-*N*⁶-(3-iodobenzyl)adenosine) were synthesized at NIDDK, National Institutes of Health (Bethesda, MD, USA) and kindly donated by Dr. Ken Jacobson. In addition to these specific compounds, we tested a library of approx. 800 adenosine receptor ligands, available at the Leiden/Amsterdam Center for Drug Research and specified in the [Electronic supplementary material](#). All compounds were diluted from 10 mM stock solutions in DMSO. ADA was purchased from Roche Diagnostics (Mannheim, Germany). [³H]CCPA (2-chloro-*N*⁶-cyclopentyladenosine) was obtained from PerkinElmer Life Sciences with a specific activity of 42.6 Ci/mmol. All other compounds, reagents, or solvents were obtained from standard commercial sources and were of analytical grade.

Results

β-arrestin-mediated signaling screen

First, we confirmed that β-arrestin-mediated signaling in the Tango™ U2OS adenosine A₁ receptor expressing cells is independent of Gα_i. Cells were incubated with 100 ng/ml PTX overnight to abolish Gα_i protein activation, before stimulation with either NECA **4**, a nonselective full agonist reference compound, or CPA **5**, a A₁R-selective agonist. The structures of the compounds are shown in Fig. 1. Curves were superimposable in the presence and absence of PTX, indicating that β-arrestin-mediated signaling indeed is Gα_i protein independent (Fig. 2a). CPA **5** acted as a partial agonist with a maximal efficacy (*E*_{max}) of 85±3 % in the β-arrestin assay, when compared to the full agonist NECA **4** (100 %) with potencies in the low nanomolar ranges (Table 1). ADA is the purine-metabolizing enzyme that is routinely used to breakdown endogenous adenosine in assays involving the adenosine receptors. In this intact cell assay, we learned that the addition of ADA had a negligible effect on the pharmacological parameters (data not shown). Hence we decided not to routinely include ADA in the assay, which allowed us to record concentration–effect curves for the endogenous agonist adenosine (**3**).

Subsequently over 800 adenosine receptor ligands from our in-house collection were screened for the induction of beta-arrestin-mediated signaling at a final concentration of

10 μM (Supplemental Table 1). These ligands have been previously tested on adenosine receptor subtypes, in either radioligand binding studies (affinity), cAMP assays (activity), or both, providing a logical framework for an extension towards β-arrestin-mediated signaling. Among the ligands that were previously categorized as antagonist or inverse agonist, a few compounds enhanced β-arrestin-mediated signaling, indicative of functional selectivity. Examples of those are two caffeine-like structures, compounds **1** and **2** (Fig. 1). However, these compounds only induced β-arrestin-mediated signaling at 10 μM, with no response at lower concentrations. Upon chemical analysis we learned the compounds are fluorescent, which may have had a (nonspecific) impact on the TANGO assay, which is also fluorescence based. Hence, we decided to refrain from further investigations.

All known adenosine A₁ receptor agonists in the library were active in the β-arrestin assay. Scaffolds of compounds that were screened included ribose as well as non-ribose templates. From this initial screen, we learned that many agonists, when tested at a single high (10 μM) concentration, behave as full agonists in the β-arrestin-mediated signaling assay, i.e., their *E*_{max} value was approx. 100 % when compared to the agonist NECA. For further inspection we selected a number of closely related ribose-containing agonists (**6**–**14** in Fig. 1), for two reasons. First, despite their close structural resemblance, they displayed a spectrum of different *E*_{max} and EC₅₀ values in previously performed functional assays compared to the β-arrestin activation. Secondly, this congeneric set of compounds could provide insight in structure functional selectivity relationships (SFSR). These 3-iodobenzyl adenosine (3-IB) derivatives (LUF5583 **7**, LUF5586 **8**, LUF5589 **9**, all strongly resembling LUF5580 **6**) showed lower efficacy than the reference compound NECA in β-arrestin-mediated signaling (Fig. 2b) and were further investigated.

Additionally, MRS541 **10**, 3-IB adenosine (Fig. 2c) appeared as an interesting hit, since it showed strong partial agonism in β-arrestin-mediated signaling of 65±2 %. MRS542, the 2-chloro-containing version of 3-IB adenosine (**11**) showed an *E*_{max} value of 88±3 %, with a lower pEC₅₀ of 7.1 compared to 7.6 for **10**. Finally, MECA **12**, IB-MECA **13** and Cl-IB-MECA **14** were taken along for SFSR as well. While MECA **12**, 5' methyl-carboxamido-adenosine, showed full β-arrestin-mediated signaling (117±3 %), IB-MECA **13** and its 2-chloro derivative Cl-IB-MECA **14** were slightly less efficacious with *E*_{max} values in β-arrestin-mediated signaling of 94±5 and 82±13 %.

GTPγS binding assay

After we investigated the activation of β-arrestin-mediated signaling by these compounds, we determined their capacity

Fig. 2 Schematic representation of the two cell lines used in this study. For the β -arrestin-mediated signaling assay, the Tango-U2OS-ADORA1-bla cells (Invitrogen) contain the A_1 receptor with the vasopressin receptor (AVPR) C-tail, coupled via a protease site to the β -lactamase transcription factor (TF). Upon activation of the A_1 receptor,

GPCR-related kinases phosphorylate the C-tail of the GPCR, recruiting the β -arrestin. The protease linked to the β -arrestin cuts off the transcription factor, resulting in enhanced β -lactamase expression. In the CHO cells, the wild-type hA₁ receptor is expressed. Membranes from both cell types were compared in the G protein-mediated GTP γ S assay

to induce G protein-mediated signaling through GTP γ S activation (Fig. 3) as well as their affinity (Fig. 4) on CHO-hA1 membranes. By means of the [³⁵S]GTP γ S functional assay, we measured hA₁R-mediated G_i protein activation in membranes from both CHO-hA1 (Fig. 3a, c) and Tango U2OS-ADORA1-bla cells (Fig. 3b, d). We observed similar partial agonistic responses in the two types of membranes for the selection of A_1 R agonists LUF5580, LUF5583, LUF5586, and LUF5589 (6–9) when compared to the reference agonists NECA 4 and CPA 5 (Fig. 3a, b). Furthermore 6–9 showed a decrease in potency as well as in efficacy compared to the reference agonists. MRS541,

MRS542, MECA, IB-MECA, and CI-IB-MECA (10–14) showed a similar potency rank order in the two types of membranes tested (Fig. 3c, d; Table 1).

Affinity

For the compounds in Table 1, A_1 receptor affinity data had been obtained on rat brain membranes [8–10]. Therefore, we now examined their affinity at CHO membranes expressing human A_1 receptors. Both LUF5586 8 and LUF5589 9 had the lowest pK_i values of 6.4 (Fig. 4a, Table 1). The 5'-methylenemethoxy compounds LUF5580 6 and the 2-

Table 1 Potency and efficacy of adenosine receptor ligands compared to NECA or CPA at multiple effector pathways, including G protein-independent β -arrestin-mediated signaling and G protein-dependent GTP γ S binding, as well as affinity data (pK_i)

		Affinity	β -arrestin		GTP γ S			
		On CHO-hA1	On Tango-U2OS-A1		On Tango-U2OS-A1		On CHO-hA1	
			pK $_i$, $\pm$ SEM	pEC50, $\pm$ SEM	$E_{\max}$ (%NECA), $\pm$ SEM	pEC50, $\pm$ SEM	$E_{\max}$ (%CPA), $\pm$ SEM	pEC50, $\pm$ SEM
3	Adenosine	nd	7.2 $\pm$ 0.0	66 $\pm$ 2	nd	nd	nd	nd
4	NECA	8.3 $\pm$ 0.1	7.9 $\pm$ 0.1	100	8.1 $\pm$ 0.1	98 $\pm$ 2	8.4 $\pm$ 0.1	109 $\pm$ 26
5	CPA	8.8 $\pm$ 0.1	8.8 $\pm$ 0.3	85 $\pm$ 1	8.7 $\pm$ 0.04	100	8.8 $\pm$ 0.1	100
6	LUF5580	7.7 $\pm$ 0.01	6.9 $\pm$ 0.0	79 $\pm$ 1	7.8 $\pm$ 0.04	89 $\pm$ 6	7.8 $\pm$ 0.02	96 $\pm$ 4
7	LUF5583	7.1 $\pm$ 0.1	6.6 $\pm$ 0.0	49 $\pm$ 5	7.1 $\pm$ 0.1	83 $\pm$ 8	7.3 $\pm$ 0.1	90 $\pm$ 12
8	LUF5586	6.4 $\pm$ 0.01	6.5 $\pm$ 0.1	29 $\pm$ 5	7.0 $\pm$ 0.01	58 $\pm$ 6	7.1 $\pm$ 0.1	69 $\pm$ 8
9	LUF5589	6.4 $\pm$ 0.1	6.5 $\pm$ 0.3	14 $\pm$ 3	6.9 $\pm$ 0.1	51 $\pm$ 9	6.8 $\pm$ 0.1	56 $\pm$ 7
10	MRS541	7.8 $\pm$ 0.3	7.6 $\pm$ 0.2	65 $\pm$ 2	8.0 $\pm$ 0.1	93 $\pm$ 2	8.2 $\pm$ 0.1	92 $\pm$ 8
11	MRS542	7.6 $\pm$ 0.1	7.1 $\pm$ 0.0	88 $\pm$ 7	7.4 $\pm$ 0.1	95 $\pm$ 5	7.6 $\pm$ 0.1	82 $\pm$ 8
12	MECA	7.1 $\pm$ 0.02	6.7 $\pm$ 0.2	117 $\pm$ 4	7.0 $\pm$ 0.2	90 $\pm$ 4	7.3 $\pm$ 0.04	86 $\pm$ 8
13	IB-MECA	6.8 $\pm$ 0.1	6.3 $\pm$ 0.0	94 $\pm$ 5	6.8 $\pm$ 0.2	94 $\pm$ 3	6.9 $\pm$ 0.1	94 $\pm$ 10
14	CI-IB-MECA	6.5 $\pm$ 0.1	6.0 $\pm$ 0.1	82 $\pm$ 13	6.2 $\pm$ 0.1	105 $\pm$ 3	6.5 $\pm$ 0.1	94 $\pm$ 11

Affinity data were obtained using CHO cell membranes expressing the human A_1 receptor (CHO-hA1). β -arrestin-mediated signaling was measured using Tango-U2OS-ADORA1-bla cells (Invitrogen). GTP γ S data were obtained using membranes derived from both CHO-hA1 (wild type) and Tango U2OS-ADORA1-bla (vasopressin C-tail, see text) cells. nd not determined

Fig. 3 β -arrestin-mediated signaling assay on TangoTM U2OS-ADORA1-bla cells. All data are normalized to NECA 4. **a** PTX treatment does not affect NECA 4 and CPA 5 potency and efficacy in β -arrestin-mediated signaling. **b** Concentration–effect curves of 3-iodobenzyl adenosine derivatives LUF5580 6, LUF5583 7, LUF5586 8 and LUF5589 9. **c** Concentration–effect curves of 3-iodobenzyl adenosine derivatives 3-IB adenosine 10 and MRS542 11. Results are expressed as mean $\pm$ SEM from three experiments

chloro containing LUF5583 7 showed pK_i values of 7.7 ± 0.01 and 7.1 ± 0.1 . In fact all tested compounds showed affinities lower than the reference A_1R agonist CPA 5 (Fig. 4b). The 2-chloro containing bulkier compounds MRS542 11 and CI-IB-MECA 14 showed slightly lower affinities (7.5 ± 0.1 and 6.7 ± 0.03) than their non-C2-

substituted variants 3-IB adenosine 10 and IB-MECA 13 (7.8 ± 0.3 and 6.9 ± 0.01). MECA (12) had intermediate affinity ($pK_i = 7.1$). Due to the essential presence of ADA in both membrane-based assays (GTP γ S and affinity), we could not determine these pharmacological parameters for adenosine (3).

Fig. 4 [³⁵S]GTP γ S binding experiments on CHO-hA1 and TangoTM U2OS-ADORA1-bla membranes. The ability of increasing concentrations of several full and partial agonists (3-iodobenzyl adenosine derivatives) and inverse agonist DPCPX to stimulate or inhibit hA1R-mediated nucleotide exchange was compared on both CHO-hA1 (**a**, **b**) and TangoTM U2OS-ADORA1-bla (**c**, **d**) membranes. **a**, **c** Normalized data for CPA, LUF5580, LUF5583, LUF5586, and LUF5589 reveal LUF5586 8 and LUF5589 9 to be partial agonists at the hA1R in both membrane types. **b**, **d** NECA, 3-IB adenosine, MRS542, MECA, IB-MECA, and CI-IB-MECA act as full agonists in both membrane preparations. Results shown (mean $\pm$ SEM) are from experiments performed in duplicate and repeated on at least three independent occasions

Discussion

The aim of this study was to investigate β -arrestin-mediated signaling and to determine if known adenosine receptor ligands display functional selectivity at the adenosine A_1 receptor. For this purpose functional assays involving G protein-mediated and G protein-independent signaling were tested side by side. Screening of over 800 compounds was performed using the G protein-independent β -arrestin-mediated signaling assay using TangoTM U2OS-ADORA1-bla expressing cells. Within these 800 compounds, adenosine compound libraries as well as sets of compounds from previously published papers, representing a structurally diverse set of ligands, were taken along. Potential functionally selective ligands were identified as follows: when screening compounds were previously categorized as agonists for G protein-mediated pathways, these compounds were expected to show agonistic behaviour in β -arrestin-mediated signaling. Therefore, unexpected behaviour of a compound, such as low efficacy β -arrestin-mediated signaling, was thought to possibly represent a functionally selective compound. In case of screening compounds previously categorized as antagonists or inverse agonists, hits were defined as yielding a significant β -arrestin-mediated signal. For particular hits, the EC_{50} values for β -arrestin-mediated signaling were determined (Fig. 2, Table 1). An altered rank order of potency and efficacy in multiple signaling pathways is indicative for biased signaling or functional selectivity [5].

Several aspects of the β -arrestin-mediated signaling assay should be taken into account. First, the adenosine A_1 receptor in the TangoTM β -arrestin assay was engineered by the supplier to contain the final 26 amino acids of the V2 vasopressin receptor (AVPR2) instead of the final 15 amino acids of the A_1 receptor sequence (Fig. 5). This causes an increased coupling efficiency in the β -arrestin assay, which results in a larger assay window, as described in the manual. The most likely

reason is that the AVPR2-C-tail contains multiple phosphorylation sites, to enhance phosphorylation by GRKs and the subsequent β -arrestin recruitment [11]. Since the intrinsic internalization rate of the A_1 receptor is low (hours)—the wild-type A_1 receptor is known to be very slow in desensitization and degradation [12], the β -arrestin-mediated signaling seen in the TangoTM β -arrestin ADORA1-bla U2OS cells might be an overestimation. Nevertheless, the pEC_{50} values obtained with the β -arrestin and GTP γ S assay in the Tango U2OS-ADORA1-bla membranes show a highly significant correlation ($R^2=0.91$, Supplemental Fig. 1a). Similarly, E_{max} values obtained with these assays showed a lower but still significant correlation with R^2 of 0.71 (data not shown).

Due to the use of different cellular backgrounds for the β -arrestin-mediated signaling assay (Fig. 2) and the binding assay (Fig. 4), a direct comparison of the obtained potencies and efficacies in Table 1 might be ambiguous. Receptor expression levels as well as amounts of expressed GPCR-interacting proteins can differ greatly in the different cell types used. However, the rank order of activation potencies of series of compounds can be used to speculate on the functional selectivity displayed by these adenosine receptor ligands. Therefore, for the selection of interesting, and potentially functionally selective compounds, EC_{50} values in the G protein-dependent GTP γ S activation assay were determined in the two cellular backgrounds of CHO- and U2OS membranes expressing hA₁R (Fig. 3, Table 1). GTP γ S binding was comparable in membranes of both cell lines, displaying a similar potency rank order. A highly significant correlation between pEC_{50} values of GTP γ S data obtained in the two cell lines was observed ($R^2=0.97$, Supplemental Fig. 1b). Similarly, E_{max} values obtained with these assays showed a lower but still significant correlation with R^2 of 0.78 (data not shown). This indicates that the two cell lines can be used side by side in GTP γ S functional screening, without showing an intervening effect of the

Fig. 5 Displacement of agonist [³H]CCPA bound to hA₁R expressed on CHO cell membranes by increasing concentrations of 3-iodobenzyl adenosine derivatives. **a** CPA, LUF5580, LUF5583, LUF5586, and

LUF5589. **b** NECA, 3-IB adenosine, MRS542, IB-MECA, and CI-IB-MECA. Results shown (mean±SEM) are from independent experiments ($n=3$), performed in duplicate

vasopressin receptor C-tail in the Tango-U2OS-ADORA1-bla cell line.

Many of the compounds in Table 1 have been synthesized in the past as selective ligands for the adenosine A₃ receptor [1]. However, in the current A₁ receptor-based screening effort, they displayed a quite remarkable profile. The substituent pattern in adenosine analogs LUF5580–LUF5589 **6–9** shows that N⁶- and C2-substituents affect efficacy in both the β -arrestin-mediated signaling assay and the GTP γ S assay. Elongation at the ribose 5' position affects efficacy, as shown by the adenosine analogs LUF5580 **6** and LUF5586 **8**. LUF5580 **6** acts as a partial agonist in β -arrestin-mediated signaling (79 $\pm$ 1 %) with a pEC₅₀ of 6.9, while LUF5586 **8**, containing a 5' ethylether instead of a methylether, has a lower efficacy and potency (29 $\pm$ 5 % and pEC₅₀=6.5). Addition of a 2-chloro substituent to **8**, resulting in LUF5589 **9**, yielded an even weaker partial agonist in β -arrestin-mediated signaling (14 $\pm$ 5 %). Thus, elongation of the 5' substituent decreases the β -arrestin-mediated signaling significantly. In fact, of all compounds tested, **9** can be considered as functionally selective for the G protein-dependent pathway, particularly in view of the likely overestimation of β -arrestin signaling in the U2OS cells as discussed above. Still, even for **9**, one could argue that stimulus coupling efficiency rather than stimulus bias is the case. With respect to affinity, the 5'-methylene-methoxy compounds LUF5580 **6** and the 2-chloro containing LUF5583 **7** show pK_i values of 7.7 $\pm$ 0.01 and 7.1 $\pm$ 0.1, indicating the decrease of the 5'alkyl chain length in these compounds yields higher affinities than reached with the 5'-methylene-ethoxy compounds LUF5586 **8** and LUF5589 **9** (both 6.4).

Our study on the A₁R also included MRS541 **10** and MRS542 **11** [13], 3-IB adenosine and its 2-chloro derivative. MRS541 **10** acted as a strong partial agonist in β -arrestin-mediated signaling (Table 1 and Fig. 2c). The highly similar MRS542 **11** showed efficacy comparable to both MRS541 **10** and CPA **5**, although the potency of MRS542 **11** (7.1 $\pm$ 0.0) was lower than that of CPA **5** (8.8 $\pm$ 0.3). In all our other cases of the 2-chloro-addition, both efficacy and potency were either reduced or similar.

As was mentioned in the “Introduction” section, functional selectivity is emerging as a new concept in GPCR research and in drug discovery. It is important to be vigilant in the interpretation of pharmacological data from different assays, however [14]. Most prominently, partial agonists can have different efficacies in different assays, even in one “pathway” of, e.g., G protein coupling (e.g., GTP γ S activation vs cAMP accumulation), let alone in different pathways including β -arrestin signaling. Obviously, this differential behaviour does not point to ligand bias per se. Rajagopal et al. [15] therefore proposed to compare concentration–effect curves for series of ligands to quantitatively assess their individual bias factor and applied this approach to the β_2 -adrenergic and the angiotensin AT_{1A} receptor. There was

little evidence for biased signaling at the β_2 -adrenergic receptor, while some AT_{1A} receptor agonists appeared biased towards β -arrestin signaling. Likewise, Drake et al. [16] had identified limited ligand bias for one or two β_2 -adrenergic receptor agonists, albeit in differently formatted assays. Although other receptors have also been proposed as showing ligand bias (e.g., PTH and HCA2 receptors), these have not been tested in a similarly rigorous manner. Thus, also for these “established” receptors, only very few compounds appear to be able to act in a functionally selective manner. Overall, the amount of functionally selective compounds gathered so far might indicate that the concept of functional selectivity is less widespread than anticipated.

Conclusions

During the last years, it has become possible to screen compound libraries systematically using β -arrestin recruitment assays (e.g., confocal microscopy, fluorescence resonance energy transfer- or bioluminescence resonance energy transfer-based assays) or β -arrestin-mediated signaling (e.g., activation of a specific reporter gene) as readouts for GPCR activation. Although this new generation of high throughput screens might potentially lead to the identification of new pathway-selective drugs that have valuable therapeutic properties [17], in the case of the adenosine A₁ receptor, it did not yield the sought-for compounds. All together, our study indicates that functionally selective ligands for the adenosine A₁ receptor were not found within the β -arrestin-mediated signaling screen of over 800 compounds on Tango-U2OS-ADORA1-bla cells, with the possible exception of LUF5589 (**9**). This might indicate that the concept of functional selectivity is restricted to other GPCRs or less easily accessible than hoped. Identifying a functionally selective ligand for the other adenosine receptors might as well be a more challenging task than anticipated.

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