



HHS Public Access

Author manuscript

Biochem Pharmacol. Author manuscript; available in PMC 2019 December 01.

Published in final edited form as:

Biochem Pharmacol. 2018 December ; 158: 103–113. doi:10.1016/j.bcp.2018.10.003.

Probing Structure-Activity Relationship in β -Arrestin2 Recruitment of Diversely Substituted Adenosine Derivatives

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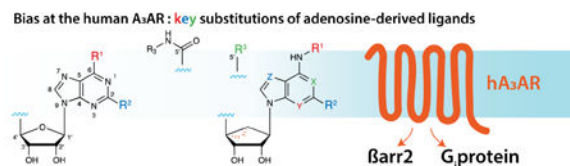
Abstract

In the adenosine receptor (AR) subfamily of G protein-coupled receptors (GPCRs), biased agonism has been described for the human A₁AR, A_{2B}AR and A₃AR. While diverse A₃AR agonists have been evaluated for receptor binding and Gi-mediated cAMP signalling, the β -arrestin2 (β arr2) pathway has been left largely unexplored. We screened nineteen diverse adenosine derivatives for β arr2 recruitment using a stable hA₃AR-NanoBit[®]- β arr2 HEK293T cell line. Their activity profiles were compared with a cAMP accumulation assay in stable hA₃AR CHO cells. Structural features linked to β arr2 activation were further investigated by the evaluation of an additional ten A₃AR ligands. The A₃AR-selective reference agonist 2-Cl-IB-MECA, which is a full agonist in terms of cAMP inhibition, only showed partial agonist behaviour in β arr2 recruitment. Highly A₃AR-selective (N)-methanocarba 5'-uronamide adenosine derivatives displayed higher potency in both cAMP signalling and β arr2 recruitment than reference agonists NECA and 2-Cl-IB-MECA. Their A₃AR-preferred conformation tolerates C2-position substitutions, for increased β arr2 efficacy, better than the flexible scaffolds of ribose derivatives. The different amino functionalities in the adenosine scaffold of these derivatives each seem to be important for signalling as well. In conclusion, we have provided insights into ligand features that can help to guide the future therapeutic development of biased A₃AR ligands with respect to G-protein and β arr2 signalling.

Graphical Abstract

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Keywords

G protein-coupled receptor; A₃ adenosine receptor; β -arrestin2; structure-activity relationship; biased signaling

1. INTRODUCTION

The family of purinergic adenosine receptors (ARs) is involved in different (patho)physiological processes in the human body via modulation of the nervous system, immune response, vascular function and metabolism. The endogenous agonist adenosine, at resting physiological concentrations of less than 1 μ M, may bind the four AR subtypes (A₁, A_{2A}, A_{2B} and A₃). The elevation of adenosine levels due to acute stress or ischemic conditions can benefit cellular adaptation. Under pathological conditions, sustained levels of excess adenosine can contribute to the development and/or progression of various diseases [1–3]. The human A₃AR (hA₃AR) is highly expressed in lung, liver and immune cells, with lower expression levels in the heart, brain and eye [4]. In recent years, the A₃AR has attracted interest as a therapeutic target due to its role in the pathogenesis of heart and vascular diseases, autoimmune inflammatory disorders, COPD and asthma, along with different types of cancer [5–8]. A₃AR expression is upregulated in various tumour cells, modulating tumour growth depending on the tumour type, the other AR subtypes present, the surrounding immune cells and micro-environmental conditions involved [9, 10]. More recently, targeting of the A₃AR has been suggested as a promising, safe and effective therapeutic approach for the management of chronic neuropathic pain of various etiologies [11–13].

Given the delicate role of adenosine in tissue homeostasis and the plethora of therapeutic opportunities, significant work has been done in synthetic ligand design to target the human A₃AR. Structure-based molecular modeling has led to the rational design of an impressive panel of potent, highly-selective A₃AR ligands [8]. For therapeutic application, these synthetic ligands must display good pharmacokinetic properties, as well as excellent A₃AR binding affinity, selectivity, efficacy and potency [14, 15]. Currently, functional evaluation of these A₃AR ligands relies mainly on the measurement of G_i protein-mediated signalling using cAMP accumulation assays. However, as with all GPCRs, the A₃AR couples to other downstream signalling proteins besides the G protein. There has been increased interest in designing and developing GPCR agonists that show biased signalling towards certain signalling pathway(s). In recent years, one of the most studied non-G-protein signalling pathways in GPCR systems has been that involving the adaptor protein β -arrestin2 (β arr2) [16, 17]. β arr2 can induce both downstream signalling and GPCR desensitization, thus influencing the duration of a therapeutic effect and/or tolerance in a physiological setting. The biased activation of G_i-dependent or β arr2-dependent signalling pathways in A₃AR

agonism has remained insufficiently explored. The application of functional assays to expand the structure-activity relationships of A₃AR ligands may help link desired therapeutic profiles to *in vivo* models in the future. Furthermore, this could allow for the reorientation of the therapeutic profiles of previously synthesized ligands.

Here, we report on the development of a stable hA₃AR HEK293T cell assay system, which allows real-time monitoring of β arr2 recruitment by application of NanoBit[®] technology (Promega). This technology relies on the functional complementation of the NanoLuc luciferase enzyme. The applicability of the system to study hA₃AR- β arr2 interaction was previously demonstrated by our research group, using transiently transfected HEK293T cells [18]. After establishing that the stable cell line provided a reproducible and concentration-dependent response with a known A₃AR agonist (2-Cl-IB-MECA), we compared the β arr2 activity profiles of nineteen compounds with those for cAMP signalling, which were obtained with a cAMP accumulation assay performed in CHO cells stably transfected with the hA₃AR. Structural features linked to β arr2 activity were further investigated via the evaluation of an additional panel of ten A₃AR ligands, providing insight into the structure-activity relationship of A₃AR agonists with respect to β arr2 recruitment.

2. MATERIALS & METHODS

2.1 Chemicals and reagents

HEK293T cells (passage 20) were kindly provided by Prof. O. De Wever (Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, Belgium). The human A₃AR construct (**NM_000677.2**, transcript variant 2 of the *ADORA3* gene) and human β arr2 construct (**NM_004313**) were purchased from Origene Technologies (Rockville, MD, USA). DOTAP Liposomal Transfection Reagent was purchased from Sigma Aldrich (Steinheim, Germany), and the anti-dNGFR antibody was purchased from Chromaprobe (Maryland Heights, MO, USA). Reference agonists 2-Cl-IB-MECA, IB-MECA, CGS21680 and NECA were purchased from Tocris Bioscience (Bio-technie, Abingdon, UK). All other chemicals and reagents used were purchased from the same suppliers as described previously [18] or synthesized at NIDDK, National Institutes of Health as reported [19–33].

2.2 Development of a stable A₃AR-NanoBit[®]- β arr2 HEK293T cell line by retroviral transduction

2.2.1 Production of retrovirus using the PhoenixA packaging cell line—To generate retroviral expression vectors, the coding sequences for the A₃AR-LgBit and SmBit- β arr2 fusion proteins (reported in [18]) were transferred to the retroviral vectors pLZRS-IRES-EGFP and pLZRS-pBMN-link-I-dNGFR, respectively, using standard cloning procedures, as described previously [18]. Primers, PCR conditions and restriction enzymes used are given in Table 1. The resulting retroviral expression vectors lead to co-expression of the fusion proteins A₃AR-LgBit and SmBit- β arr2 with enhanced green fluorescent protein (EGFP) and truncated human nerve growth factor (dNGFR), respectively, in the cell line of choice (in this case HEK293T cells).

The retroviral vectors, as well as the protocol for production of retrovirus, were adapted from the research group of Prof. Bruno Verhasselt (Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University, Belgium). The Phoenix-AMPHO (Φ NX-A) packaging cell line was cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Thermo Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 IU/mL penicillin, 100 μ g/mL streptomycin and 0.25 μ g/mL amphotericin B (full IMDM) under humidified atmosphere at 37°C, 5% CO₂. Twenty-four h before transfection, cells were seeded at a density of 106 cells/6 cm dish in full IMDM. The next day, cells were transiently transfected using the Calcium Phosphate Transfection method (Thermo Fisher Scientific, Pittsburgh, PA, USA) according to the manufacturer's instructions, using 20 μ g of retroviral expression vector DNA. Five min prior to transfection, chloroquine (Sigma Aldrich, Steinheim, Germany) was added to the cells at a final concentration of 25 μ M, followed by dropwise addition of the transfection mixture. After overnight incubation, the medium was refreshed. Forty-eight h after transfection, the first viral supernatant was harvested, centrifuged (10 min, 350 \times g, 4°C), aliquoted on ice without disturbing the pellet, and stored at -80°C. After the first harvest, puromycin selection (2 μ g/mL) (Sigma Aldrich, Steinheim, Germany) was carried out for 3 rounds of 48 h, punctuated each time with a 48-h incubation in puromycin-free full IMDM. The day before the last viral harvest, the medium was again refreshed. The second viral supernatant was harvested, centrifuged and stored as was done for the first harvest.

2.2.2 Retroviral transduction of HEK293T cells—HEK293T cells were seeded in a 96-well plate at 104 cells/well in full DMEM and incubated overnight. For retroviral transduction, the medium was replaced by retroviral supernatant, pre-incubated with DOTAP Liposomal Transfection Reagent according to the manufacturer's instructions, consisting of a 1:1 mixture of both A₃AR- and β arr2-sequence containing retroviruses. Subsequently, the plate was centrifuged for 90 min at 950 \times g (32°C) to increase transduction efficiency. After overnight incubation, the medium was refreshed, and cells were further cultured in full DMEM. Forty-eight h after transduction, expression efficiency was evaluated with flow-cytometry by measuring the level of EGFP or dNGFR (by pre-incubation with a Allophycocyanin-linked anti-dNGFR antibody), which are co-expressed with the A₃AR- and β arr2-fusion proteins, respectively.

2.2.3 Cell sorting of stably transduced HEK293T cells—After routine culture of the stably transduced HEK293T cells for 3 passages, cell sorting was performed with a BDFACSaria III cell sorter (BD Biosciences). In this stage, co-expression of EGFP and dNGFR was used to select a subpopulation of cells with the desired expression levels of A₃AR- and β arr2 fusion proteins, respectively. Cells were maintained in full DMEM medium. Stability of the cell line was monitored every 3 to 5 passages by flow cytometry, as described above.

2.3 Screening of synthetic A₃AR ligands

2.3.1 A₃AR NanoBit[®] β arr2 assay—In total, a panel of twenty-nine synthetic ligands was subjected to the stable A₃AR-NanoBit[®]- β arr2 HEK293T cell line, using a 2-day assay protocol. On the first day, cells were seeded on PDL-pre-coated, white 96-well plates at

5×10⁴ cells/well and incubated overnight (37°C, 5% CO₂). On day 2, the assay read-out was performed at room temperature, as described previously [18]. Briefly, following 2 washing steps, 90 µL of Opti-MEM I reduced serum medium was added to the cells, followed by addition of 25 µL of freshly prepared detection reagent and monitoring of luminescence equilibration. Once a stable signal was observed, 20 µL of 6.75× concentrated agonist solution in Opti-MEM I was added, and luminescence monitored for at least 90 min. All stock solutions of synthetic ligands were DMSO-based. The final in-well concentrations were (100 pM) - (500 pM) - 1 nM - (2 nM) - (5 nM) - 10 nM - 50 nM - 100 nM - 1 µM - (2 µM) - 5 µM - (10 µM) - (25 µM) agonist (maximum 0.5% DMSO). A solvent control (blank sample) of 0.001–0.5% DMSO in Opti-MEM I was included each time.

2.3.2 cAMP assay—The levels of intracellular 3',5'-cyclic AMP (cAMP) were measured by modification of the originally described competitive protein binding method [34, 35], which is widely used. Briefly, CHO cells stably expressing the human A₃AR were treated with the synthetic ligands in the presence of rolipram (10 µM) and adenosine deaminase (3 units/ml). After 30 min, forskolin (10 µM) was added to the medium, and incubation was continued for an additional 15 min. The reaction was terminated by removing the supernatant, and cells were lysed by addition of 0.1 M ice-cold HCl. The cell lysate was resuspended and stored at –20°C until analysis by enzyme immunoassay.

2.3.3 Binding assay—Binding experiments were performed as described previously [26, 28, 36], using [¹²⁵I]-N⁶-(4-amino-3-iodobenzyl)adenosine-5'-N-methyluronamide ([¹²⁵I]I-AB-MECA) as a radioligand. Briefly, membranes were prepared from CHO cells stably expressing the human A₃AR. Cells were detached and resuspended in 50 mM Tris HCl buffer (pH 8.0) containing 10 mM MgCl₂, and 1 mM EDTA prior to homogenization with an electric homogenizer (10s), and re-centrifugation at 20,000g for 20 min (4 °C). The membrane pellets were resuspended in buffer in the presence of adenosine deaminase (3 U/mL), and suspensions were stored at –80°C until the binding assay. For competition experiments, each tube contained 100 µL of membrane suspension (±20 µg protein), 50 µL of [¹²⁵I]I-AB-MECA (concentration 0.2 – 1 nM), and 50 µL of increasing concentrations of compounds in Tris–HCl buffer (50 mM, pH 8.0) containing 10 mM MgCl₂, 1 mM EDTA. Non-specific binding was determined using 10 µM 2-Cl-IB-MECA. The mixtures were incubated at 25°C for 60 min. Binding reactions were terminated by filtration through Whatman GF/B filters under reduced pressure using an MT-24 cell harvester (Brandell). Filters were washed three times with ice-cold buffer. Radioactivity was determined in a Beckman 5500B γ-counter. IC₅₀ values were obtained from competition curves and converted to K_i values using the Cheng-Prusoff equation [37].

2.4 Data analysis

Concentration-responses (area under the curve; AUCs) were calculated, where the absolute signals were corrected for solvent control, and for inter-well variability. For calculation of logEC₅₀ values, a sigmoidal curve was fitted to the normalized responses by analysing dose-response data using GraphPad Prism software (San Diego, CA, USA). Data points for the highest agonist concentrations were excluded when the signal height showed more than 20%

reduction compared to the maximum signal of the closest lower concentration. A non-linear regression model (Hill Slope 1) was fitted to the normalized responses.

3. RESULTS

3.1 Development of a stable A₃AR reporter assay for real-time monitoring of β arr2 recruitment

The originally-described reporter system for evaluation of β arr2 coupling to A₃AR [18] was characterized by a transient assay set-up with a 4-day protocol, in which the transfection efficiency varied between different assays, complicating inter-day comparisons. In order to obtain a system that could serve high-throughput screening and would more easily allow comparative analysis of a panel of compounds, a stable A₃AR- β arr2 reporter cell line was developed by retroviral transduction, reducing the assay protocol to 2 days. Moreover, a stable expression level of both fusion proteins can be ensured by monitoring the level of co-expressed markers (EGFP and dNGFR) using flow cytometry. If needed, an additional advantage is that cells can be sorted into subpopulations with certain expression levels of both constructs. A subpool was selected with the highest expression levels of both A₃AR and β arr2 fusion proteins. A clear concentration-dependent response to the reference agonist 2-Cl-IB-MECA, starting at ~2 nM and reaching a maximum at 1 μ M, was obtained with this cell line (Figure 1).

3.2 Screening of A₃AR ligands for β arr2 recruitment and cAMP signalling

A panel of nineteen synthetic A₃AR ligands was evaluated for β arr2 recruitment, using the stable hA₃AR-NanoBit[®]- β arr2 HEK293T cell line, and G-protein dependent cAMP signalling, using a cAMP accumulation assay in stable hA₃AR CHO cells. In Table 2, the maximal efficacy (E_{max}) and potency ($\log EC_{50}$) of these compounds is shown for both signalling pathways, as well as their A₃AR binding K_i values and structures. To gain more insight into their β arr2-related activity profiles, an additional ten structural analogues were tested with the hA₃AR- β arr2 cell line (Table 3). The sigmoidal dose-response curves depicted in Figure 2 show different extents of maximal β arr2 recruitment for all compounds tested, relative to reference agonists NECA and 2-Cl-IB-MECA (black curves).

4. DISCUSSION

The human A₃ adenosine receptor is gaining interest in the field of drug discovery as a therapeutic target for inflammatory diseases and cancer [4]. Two A₃AR agonists, IB-MECA (CF101, Piclidenoson) and 2-Cl-IB-MECA (CF102, Namodenoson) have shown safety and efficacy in Phase I and II clinical trials. The former is entering Phase III for treatment of rheumatoid arthritis and psoriasis [38], while its 2-chloro analogue is currently in Phase II for treatment of nonalcoholic steatohepatitis (NASH) and hepatocellular carcinoma [39, 40]. Given the A₃AR's therapeutic potential, highly selective and potent ligands have been synthesized, and their structure-activity relationship (SAR) has been extensively explored and reviewed [8, 19, 41–43]. This SAR is based on an evaluation of the ligands' binding affinities, as well as their efficacies and potencies in activating downstream A₃AR signalling pathways. Radioligand binding assays are originally carried out with membranes from AR-

overexpressing mammalian cells, from which the affinities at different AR species homologues can be compared [36]. The availability of an A₃AR homology model, which uses an agonist-bound A_{2A}AR X-ray structure [44], but not the recently solved antagonist-bound A₁AR [45], as a template, as well as opsin and the β₂AR receptor, has enabled additional prediction of nucleoside binding interactions at the A₃AR [46–48]. However, how this binding is translated into differential signalling has remained elusive.

Although the A₃AR generally couples to the G_i protein, inhibiting adenylate cyclase and leading to a decrease in cAMP levels, there is substantial coupling to G_{βγ} and possibly G_q, leading to activation of phospholipase C (PLC) and a downstream rise in intracellular calcium [4, 8]. Furthermore, other signalling mediators have been described, such as the nuclear factor kappa B (NF-κB) and the Wnt signalling pathways. For multiple other GPCRs, biased ligands have been developed that preferentially activate a certain subset of signalling pathway(s) in order to obtain a desired therapeutic profile. One of the most explored crossroads for signalling bias in GPCR drug development is the one between the G protein and βarr2 [49–55].

In the AR field, the first steps towards a favoured profile of biased agonism have recently been set for the A₁AR, the A_{2B}AR, and A₃AR [56–60]. However, so far, evaluation of the βarr2 pathway and a possible bias versus the G-protein pathway, has largely remained unexplored for the human A₃AR. First reports of the interaction of the human A₃AR with βarr2 were based on experiments using the PathHunter system (DiscoverX), which records activity at a single time point after 2 h of agonist incubation [61, 62]. We recently reported on the development of a live-cell assay system for real-time monitoring of βarr2 recruitment, based on the functional complementation of the Nanoluc luciferase enzyme [18]. Here, we report on the development of a stable hA₃AR-NanoBit[®]-βarr2 HEK293T cell line that was used to screen a panel of nineteen synthetic ligands for βarr2 recruitment to the hA₃AR. The resulting βarr2-recruitment activity profiles and those obtained for G_i protein coupling (Table 2), using the cAMP accumulation assay in stable A₃AR CHO cells, were compared (Figure 3). Ten additional structural analogues of this parent compound panel were evaluated for the confirmation or more in-depth analysis of SAR features related to the βarr2 pathway (Table 3, Figure 2). The efficacies reported are expressed relative to the non-selective AR reference agonist NECA, for consistency with respect to previous results reported for A₃AR signalling. NECA is considered to be a full agonist in multiple A₃AR signalling pathways — including the cAMP- and βarr2-pathways, as well as A₃AR mediated membrane hyperpolarization and intracellular Ca²⁺ mobilization [62]. The A₃AR-selective reference agonist 2-Cl-IB-MECA has also been reported as a full agonist in the cAMP- and βarr2-pathways based on the aforementioned PathHunter system, but as a partial agonist in membrane hyperpolarization and Ca²⁺ mobilization (58% and 55% relative to NECA) [62]. Interestingly, in this study, 2-Cl-IB-MECA displayed a partial agonist E_{max} of only 56% relative to NECA in the βarr2-recruitment assay. The difference in E_{max} for βarr2 recruitment, when comparing these results with previously published data, might arise from several factors. First, previous data relate to a single time-point readout (PathHunter system), while this study implements a more comprehensive kinetic readout using AUCs (NanoBit[®] system). Second, we cannot dismiss the possibility that A₃AR expression levels in the different assays may have varied and influenced the signal measured. However, with the

NanoBit[®] system, we also evaluated β arr2 recruitment for 2-Cl-IB-MECA in CHO-K1 cells; potencies did not differ substantially between HEK293T cells ($\log EC_{50}$ of -7.53 ± 0.04) and transiently transfected ($\log EC_{50}$ of -7.26 ± 0.08) as well as stably-transduced ($\log EC_{50}$ of -7.24 ± 0.05) CHO-K1 cells. The E_{\max} of 2-Cl-IB-MECA in CHO-K1 cells was 61% relative to NECA. Whilst the similar results obtained in different cell lines for 2-Cl-IB-MECA do indicate that the data obtained for our β arr2 recruitment assay are not confined to a single cell system, it is difficult to predict to what extent this can be extrapolated to the complete panel of compounds. The β arr2-recruitment activity profiles of the ligands tested here were generally similar to the structure-activity relationship for cAMP activity, which has been fine-tuned over years by step-wise structural modification, with differences as described below. In general, we observed a consistent difference of one log unit between $\log EC_{50}$ s for cAMP signalling and β arr2 recruitment (Figure 3B).

Several N^{σ} substituted adenosine derivatives were tested, as they are known to be quite potent A_3 AR ligands from G-protein-dependent signalling experiments [61, 63]. N^{σ} -cyclopentyladenosine (CPA), an A_1 AR-selective agonist, and the N^{σ} -phenylisopropyl R- and S-diastereomers R-PIA and S-PIA, which have demonstrated stereoselectivity in binding at the A_1 AR, A_2A AR and A_3 AR [19], show only partial agonist efficacy in β arr2 recruitment, in contrast to full agonism in cAMP signaling (Table 2; Figure 2A; Figure 3A). These compounds also have a low potency in β arr2 recruitment ($\log EC_{50}$ s of around -5.8), although R-PIA is more potent than S-PIA ($\log EC_{50}$ of -6.35), as is also the case in cAMP signalling (Table 2; Figure 3B). The 2-chloro analogue of N^{σ} -cyclopentyladenosine (CCPA) is a full antagonist in the cAMP assay, but a partial agonist in β arr2 recruitment (E_{\max} of 34%; Table 2). The 1-deaza analogue of CCPA, DCCA, showed a further reduced 6% efficacy in β arr2 recruitment (Table 3), suggesting an importance of the 1-position ring nitrogen in β arr2-pathway activation. Furthermore, substitution of N^{σ} with a benzyl or phenyl group instead of a cyclopropyl group (as in CPA) reduces or stimulates β arr2 recruitment, respectively (Table 3). The N^{σ} -chain elongated adenosine congener N^{σ} -[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]-carbonyl]methyl]phenyl]adenosine (ADAC), an agonist with selectivity for human A_1 AR and A_3 AR, has been studied for enhanced A_3 AR-selectivity upon conjugation to a nanocarrier [64]. Amongst the ribose-5'-OH analogues, this compound performs substantially well in β arr2 recruitment (Table 2; Figure 3A). Overall, the N^{σ} -substituted adenine 9-ribose ($4'$ -CH₂OH) derivatives mentioned here all have intermediate potency for β arr2 recruitment ($\log EC_{50}$ s of around -6) compared to reference agonists NECA or 2-Cl-IB-MECA ($\log EC_{50}$ s of around -7).

A series of 5'-alkyluronamide adenosine derivatives was developed as selective A_3 AR agonists, and substitutions at the C2- and/or N^{σ} - positions were explored [20]. Focusing on the structure of NECA, these substitutions preserved efficacy and influenced potency of cAMP signalling. The A_2A AR-selective agonist CGS21680 [61] contains an extended C2-substituent, decreasing the potency in cAMP signalling, by roughly one log unit ($\log EC_{50}$ from -7.42 to -6.62), as well as β arr2 recruitment ($\log EC_{50}$ from -6.99 to -5.87) (Table 2; Figure 2B). Introduction of a shorter methyluronamide group was reported to favour A_3 AR binding compared to larger alkyl groups. An N^{σ} -halobenzyl group maintains affinity at the A_3 AR while reducing A_1 AR and A_2A AR affinity, to increase A_3 AR selectivity. Examples

include the prototypical agonists IB-MECA [8] and 2-Cl-IB-MECA (Figure 2B; Figure 3, black curves) [21]. These agonists display high potency in both cAMP signalling and β arr2 recruitment ($\log EC_{50}$ s of around -8.5 and -7.5 , respectively) (Table 2). However, the combination of the N^6 -halobenzyl group in IB-MECA with a C2-substituent, as in 2-Cl-IB-MECA, reduces the E_{max} of β arr2 recruitment (Table 2; Figure 2B). Thus, for (5'-uronamide) adenosine derivatives, the E_{max} of β arr2 recruitment can be fine-tuned by substitution of the N^6 - and C2-positions.

Numerous highly A_3AR -selective (N)-methanocarba nucleoside ligands were developed that include a rigid bicyclo[3.1.0]hexane ring system in place of the flexible ribose, stabilizing the favoured adenosine receptor-bound isomer conformation [22]. Combining this (N)-methanocarba modification with a 5'-uronamide group has been reported to yield a good efficacy and potency in A_3AR activation, which are otherwise reduced when utilizing selectivity-enhancing N^6 - and/or C2-substituents [23, 24]. This was observed for MRS3558, which shows high efficacy and potency in both cAMP signalling and β arr2 recruitment ($\log EC_{50}$ s of -9.42 and -8.12 , respectively) (Table 2). Exchanging the 5'-uronamide group, as in the adenosine-like 4'-CH₂OH derivative MRS1873 [19] and the truncated 4'-H MRS5127, drastically lowered the E_{max} and potency towards both signalling pathways (Table 2; Figure 2C). MRS5127 has well-suited antagonist-like properties for radioligand binding assays but partial agonist activity in some functional assays [25, 26, 65]. The N^6 -iodobenzyl group of MRS5127 tends to preserve potency more than, for example, the N^6 -dicyclopropylmethyl group of MRS5474 [27] in β arr2 recruitment as well as in cAMP signalling (Table 2; Figure 2C; Figure 3, blue curves).

In SAR studies with (N)-methanocarba 5'-uronamide derivatives, the A_3AR binding site appeared to be very flexible in its ability to accommodate extended C2-substituents, such as C2-ethynyl and arylethynyl groups that further increased A_3AR selectivity [8, 26, 28]. This is demonstrated with MRS5698, which contains a 3,4-difluorophenylethynyl group at the C2 position [28, 29], notwithstanding a somewhat reduced signalling potency compared to MRS3558 (Table 2; Figure 2D; Figure 3, red curves). To further evaluate C2-extension, the β arr2 activity profile of analogue MRS5679, containing a biphenyl substituent at the C2-position, was tested. Interestingly, this compound showed much lower potency ($\log EC_{50}$ of -5.92), and only half the E_{max} of MRS5698 (Table 3; Figure 2D). This might be explained by the characteristics of transmembrane helix 2 (TM2). When relying solely on the A_2AAR structure as a template for the generation of an A_3AR homology model, there are three cysteine bridges present in the extracellular regions, which restrict the flexibility of the TM helices. However, there is only one cysteine bridge present in the A_3AR and the extracellular part of TM2 is expected to be more flexible. Sensibly, a hybrid model basing the TM2 conformation on the activated β 2-adrenergic receptor and rhodopsin, better accommodated the ligands with extended C2-substituents [28, 30, 47]. The proposed outer displacement of TM2 logically requires overcoming a greater energy barrier, which might be reflected here in the lower potency of MRS5679. Once this has occurred, a maximal effect can still be obtained, at least comparable to that of 2-Cl-IB-MECA (47%). Known to favour human A_3AR association [28, 30], the N^6 -methyl substitution in derivatives MRS5967 and MRS5663 also promoted β arr2 recruitment (Table 3; Figure 2D). Both compounds, only differing slightly at the ortho-position of the C2-phenylethynyl group, display equally good

efficacy and potency as the highly potent MRS3558 ($\log EC_{50}$ s of around -8.3). They have more pronounced activities than compounds bearing N^6 -benzyl substitutions, a modification that is mostly made to obtain species-independent A₃AR selectivity. Thus, in contrast to ribose analogs, (N)-methanocarba 5'-uronamide adenosine derivatives seem to occupy the A₃AR binding site in a manner that maintains efficacy for β arr2 recruitment when certain C2 modifications are present.

(N)-Methanocarba 5'-methyluronamide adenosine derivatives containing 2-arylethynyl groups have been screened in an *in vivo* mouse pain model, surpassing the effect of MRS5698 [30]. Compound MRS5917 showed very high potency in both cAMP signalling and β arr2 recruitment ($\log EC_{50}$ of -9.38 and -8.5 , respectively) (Table 2; Figure 2D). By testing two additional 5-chlorothien-2-yl analogues, MRS5980 and MRS7154 [31], it was confirmed that a small methyl group at the N^6 -position favours β arr2 signalling (Table 3). MRS5917 and its arylethynyl congeners are highly promising orally active A₃AR agonists for the treatment of chronic pain, displaying a prolonged *in vivo* effect [30], independent of endogenous opioid or endocannabinoid pathways [12]. In cell systems, the A₃AR is subject to desensitization and downregulation [66], which is known to be correlated with β arr2 recruitment. However, protection in animal models of pain results from prolonged A₃AR agonist action, which can be prevented by coadministration of an A₃AR antagonist [12]. Despite potential A₃AR downregulation, animal models have already revealed the persistent anti-inflammatory and anticancer effects of A₃AR agonists. Thus, the roles of G-protein versus β arr2- recruitment signalling pathways and receptor desensitization in the downstream inhibition of key regulatory proteins involved in inflammation/tumour growth and in pain, i.e. the targeted mechanism of action, warrant further investigation [13].

From the structures of the above-mentioned compounds, some final SAR conclusions can be drawn. The 1-deaza analogue MRS7144 shows that the N1-group is more dispensable for β arr2 recruitment, although it is characterized by a somewhat reduced but still substantially good $\log EC_{50}$ of approximately -7.3 (Table 2; Figure 2E; Figure 3, purple curve). Thus, the more stabilized A₃AR interaction of (N)-methanocarba 5'-methyluronamide adenosine derivatives tends to compensate for the loss of N1 in β arr2 recruitment, as well as cAMP signalling [31]. Also, the additionally tested 3-deaza analogue MRS7173 was fully efficacious in recruiting β arr2 (Table 3). Replacement of the 5'- N -methyluronamide with an ethyl ester group, as in the 7-deaza analogue MRS7299, completely abolishes cAMP signalling as well as β arr2 recruitment (Table 2; Figure 2E; Figure 3, bright blue curve) [33]. When re-introducing the amino group (MRS7232), β arr2 recruitment was partially restored (Table 3; Figure 2E). The necessity of N^7 is a well-described feature in literature concerning A₃AR binding activation, reflecting its proposed function as a H-bond acceptor with Asn250 (6.55). The same residue accepts a H-bond from the 6-amino group (bidentate ligand coordination). However, it was shown that a suitable combination of stabilizing interactions in these kind of hypermodified A₃AR-selective ligands can partially compensate for the lack of an exocyclic amine, an otherwise important contributor to recognition in the A₃AR binding site [32]. This is demonstrated by the sustained A₃AR activity of MRS7195 and MRS7220 in cAMP signalling as well as in β arr2 recruitment (Table 2; Figure 2F; Figure 3, mustard curves), although MRS7220 has a substantially reduced E_{max} and $\log EC_{50}$ compared to those of other (N)-methanocarba 5'-methyluronamide derivatives.

In summary, the screening of a panel of synthetic nucleosides as A₃AR ligands using a β arr2-recruitment assay, and comparison with the G_i-mediated cAMP-pathway, has provided us insight into ligand features that can be of meaning for future development of biased A₃AR ligands. A next step will be to elucidate which of these pathways is key for a certain therapeutic profile that is (mostly) devoid of side effects; in doing so, the role of G-protein versus β arr2 signalling, as well as A₃AR desensitization, remains to be determined for the different therapeutic applications.

ACKNOWLEDGMENTS

The authors acknowledge support from the Ghent University (Belgium) Special Research Council (BOF) for granting a Ph.D. fellowship to J. Storme (application number 01D31913), as well as the NIDDK Intramural Research Program (ZIADK31117). Edward Will is acknowledged for editing the manuscript.

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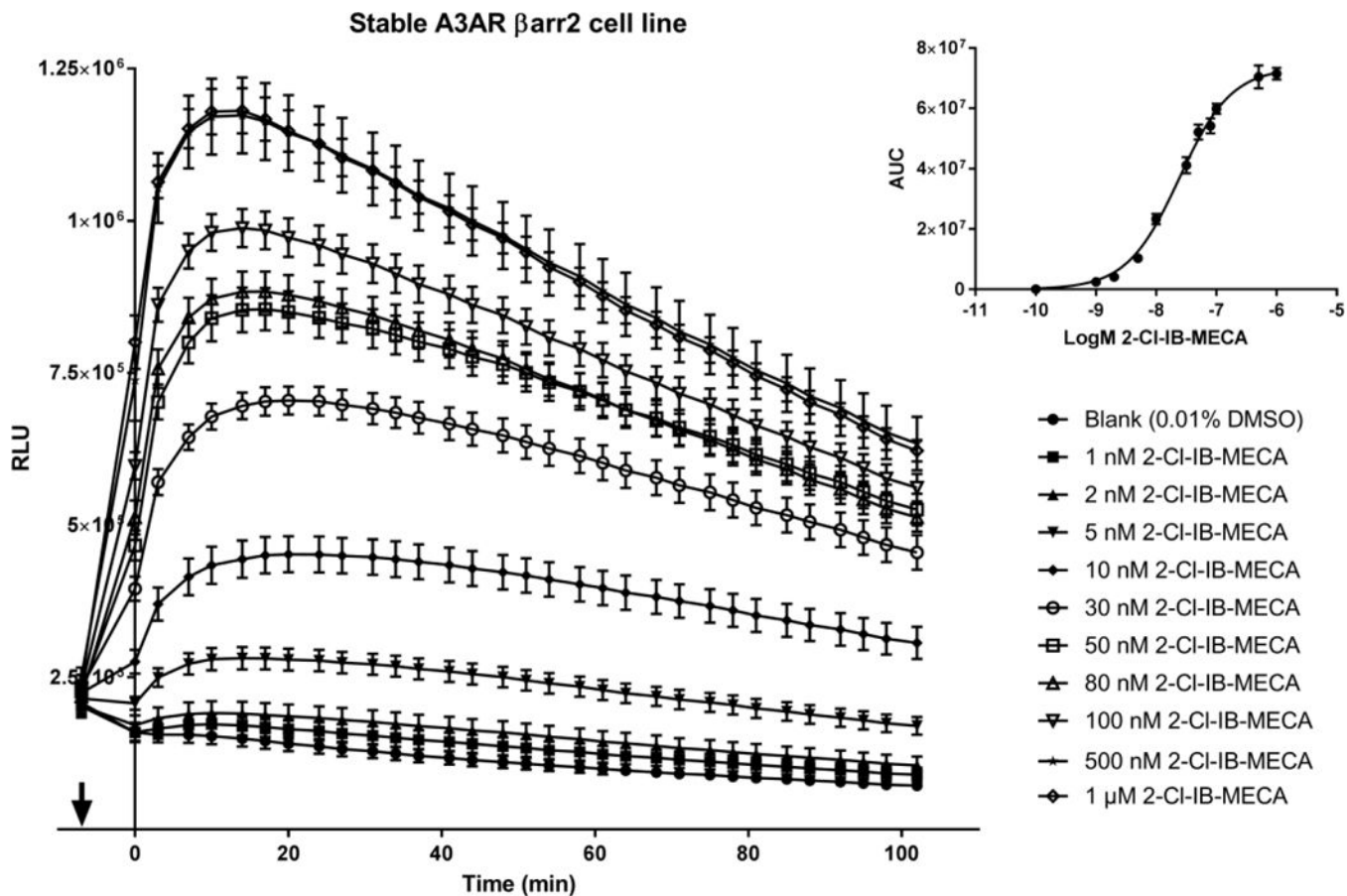


Figure 1: Concentration-dependent effect of reference A₃AR agonist 2-Cl-IB-MECA on β arr2 recruitment in a stable hA₃AR-NanoBit[®]- β arr2 HEK293T cell line. 1, 2, 5, 10, 30, 50, 80, 100, 500 nM and 1 μ M 2-Cl-IB-MECA was added at the time point indicated by the arrow, and luminescence was measured for >90 min. A solvent control of 0.01% DMSO was included. Insert: Concentration-response (AUCs). AUCs (\pm SEM) of quadruplicate wells are shown for a representative experiment (n=3).

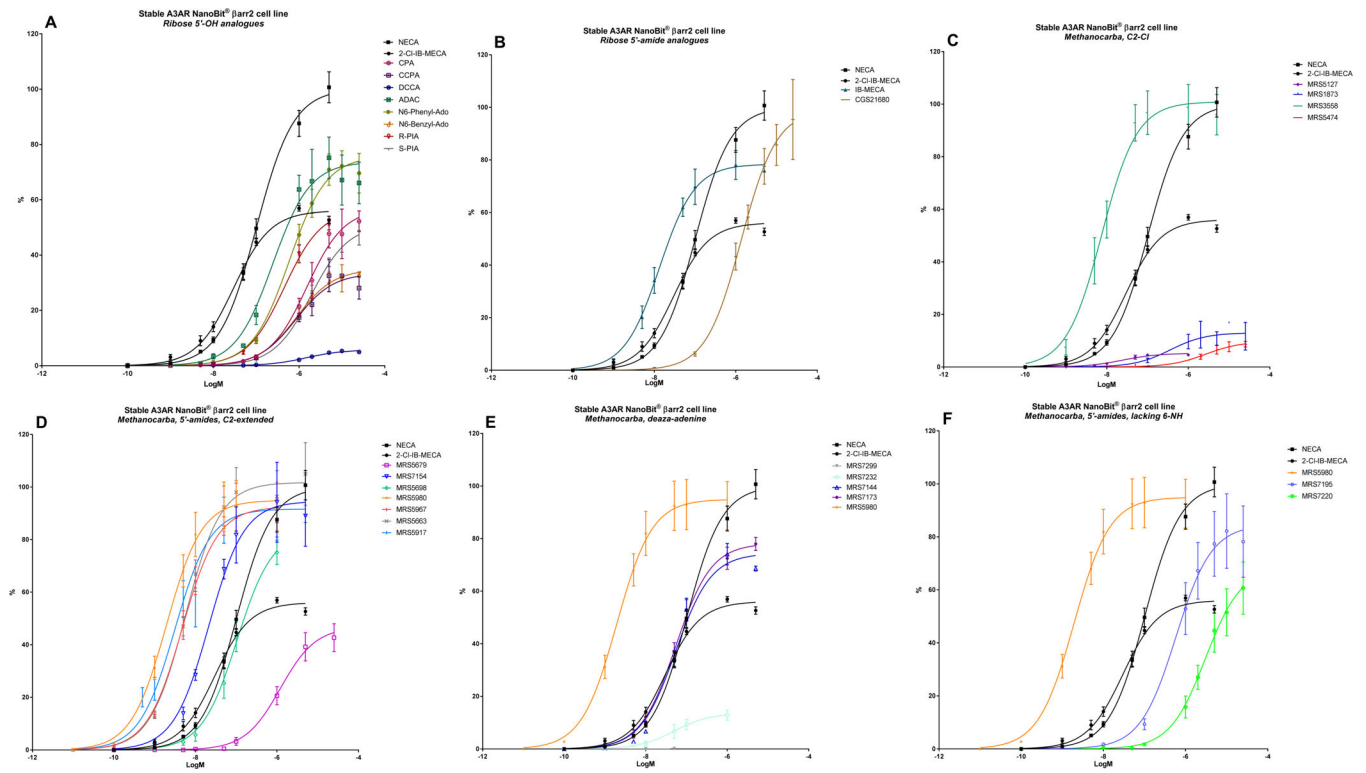
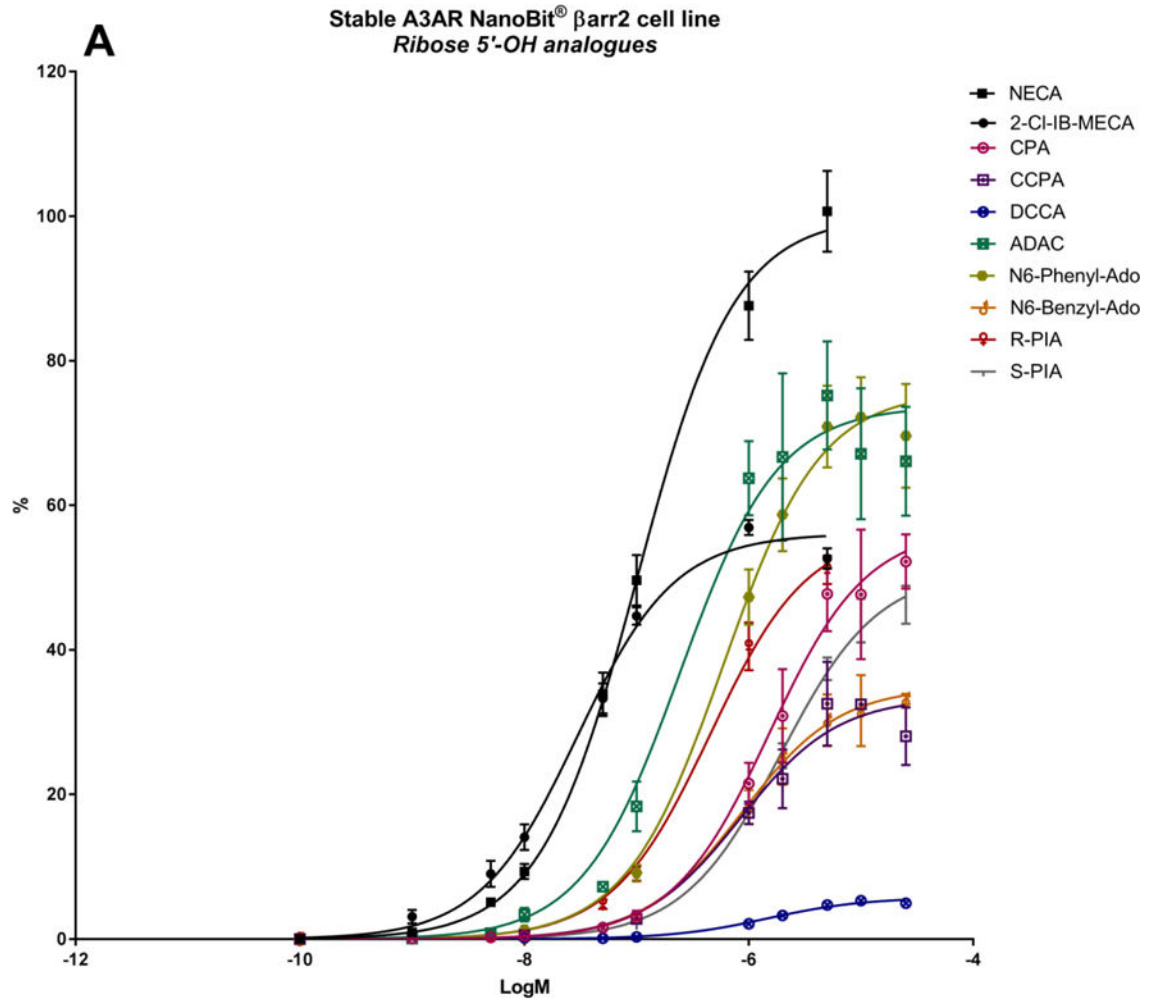
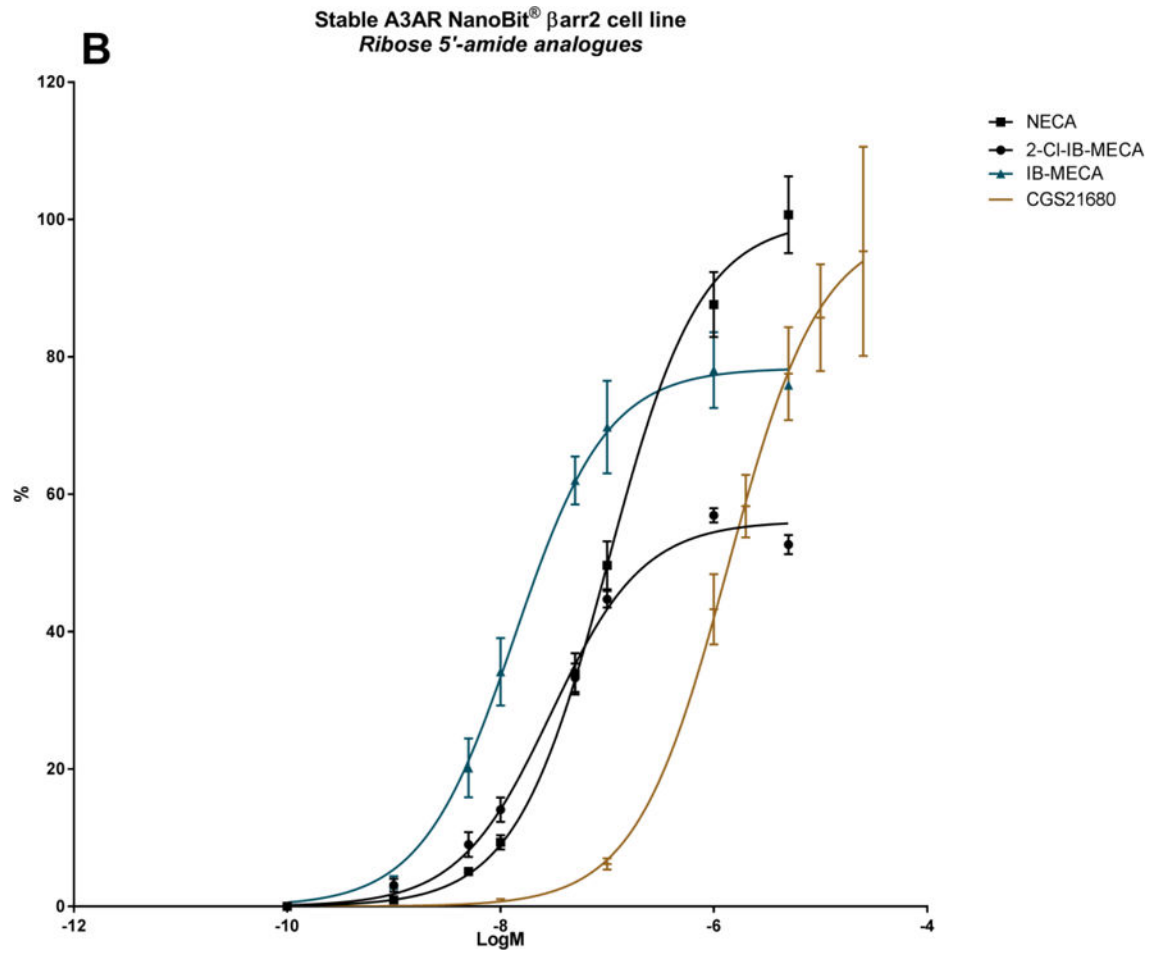


Figure 2,
 A-F: Non-linear regression analysis of the sigmoidal dose-response curves for all twenty-nine synthetic A₃AR ligands tested in the stable hA₃AR-NanoBit[®]-βarr2 cell line. The curves were normalized to that of reference agonist NECA. AUCs (± SEM) were calculated based on at least 3 experiments performed in duplicate or triplicate.



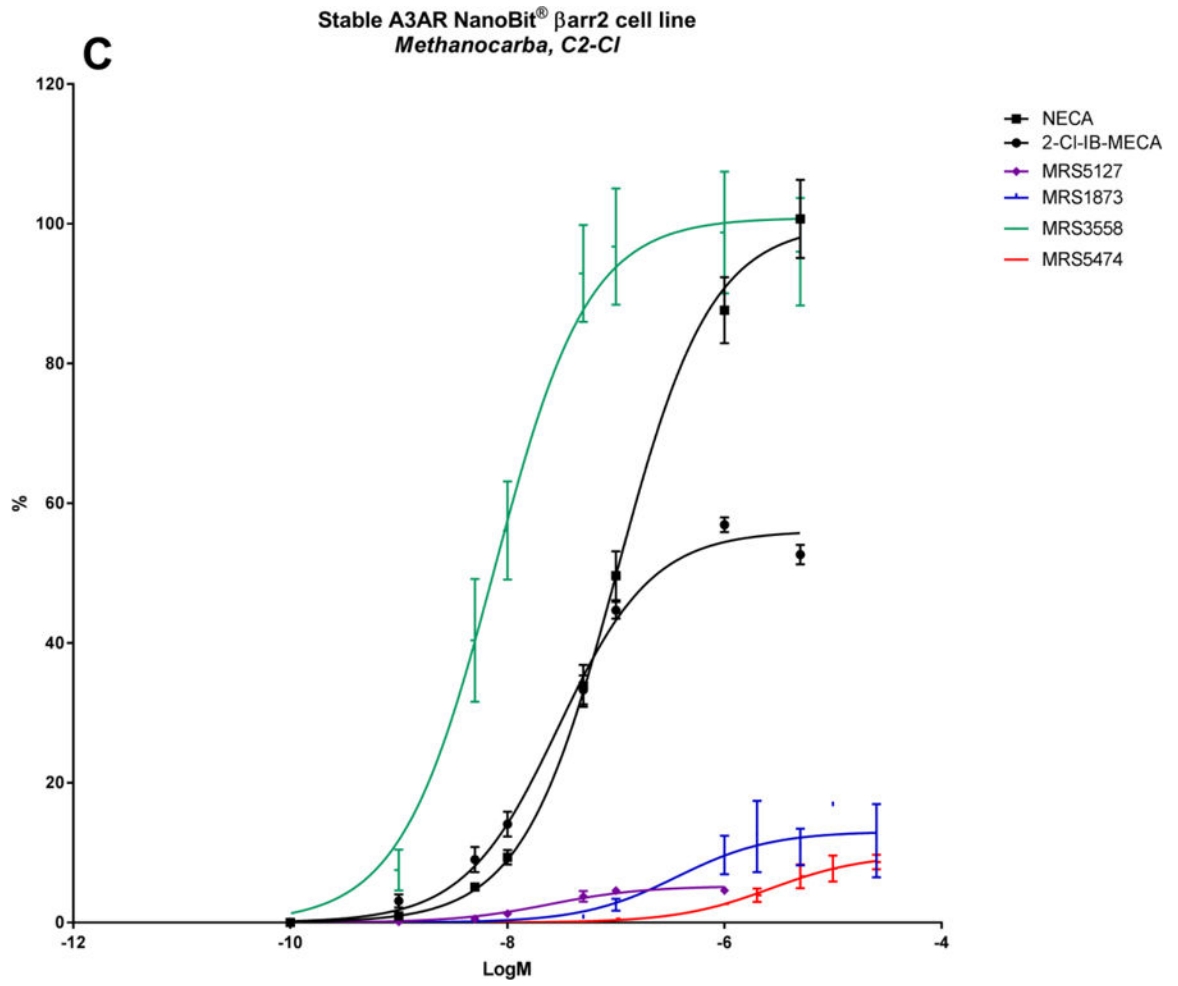


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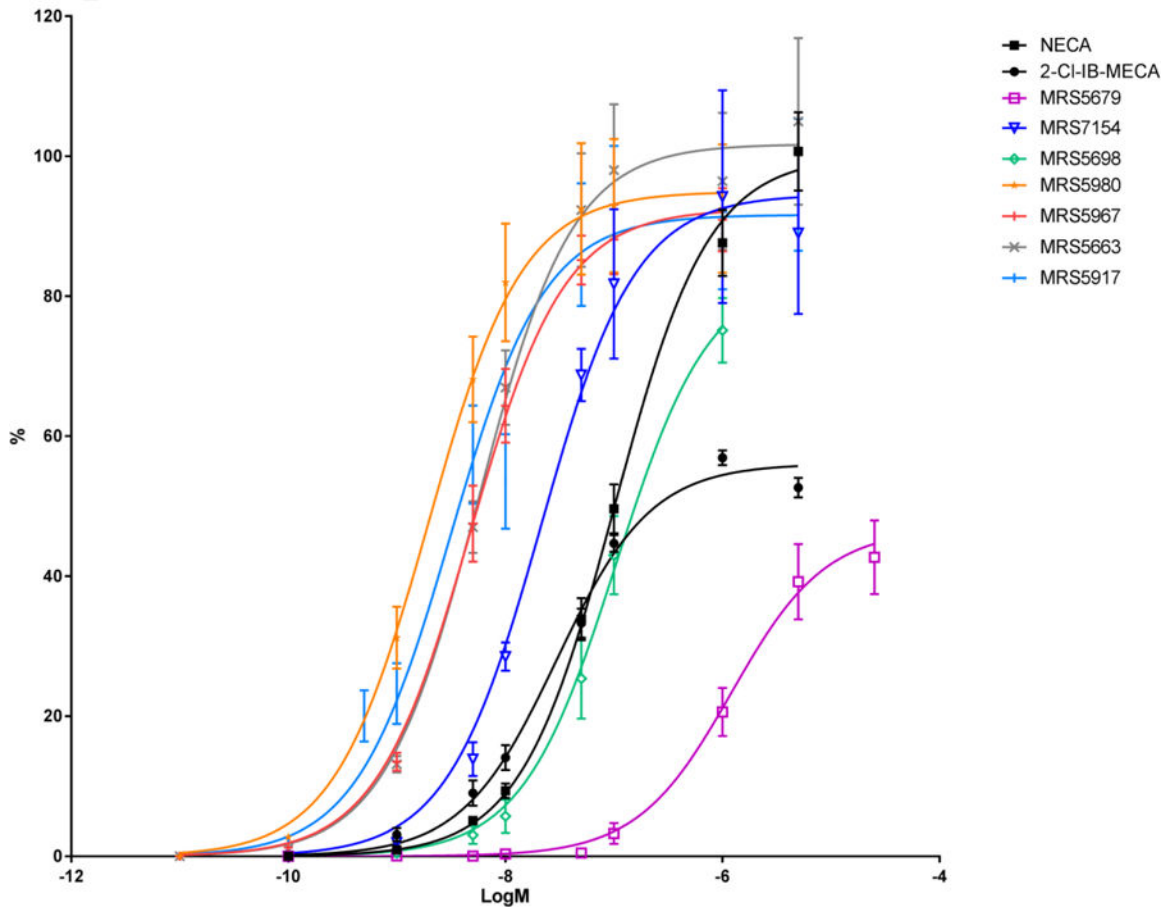
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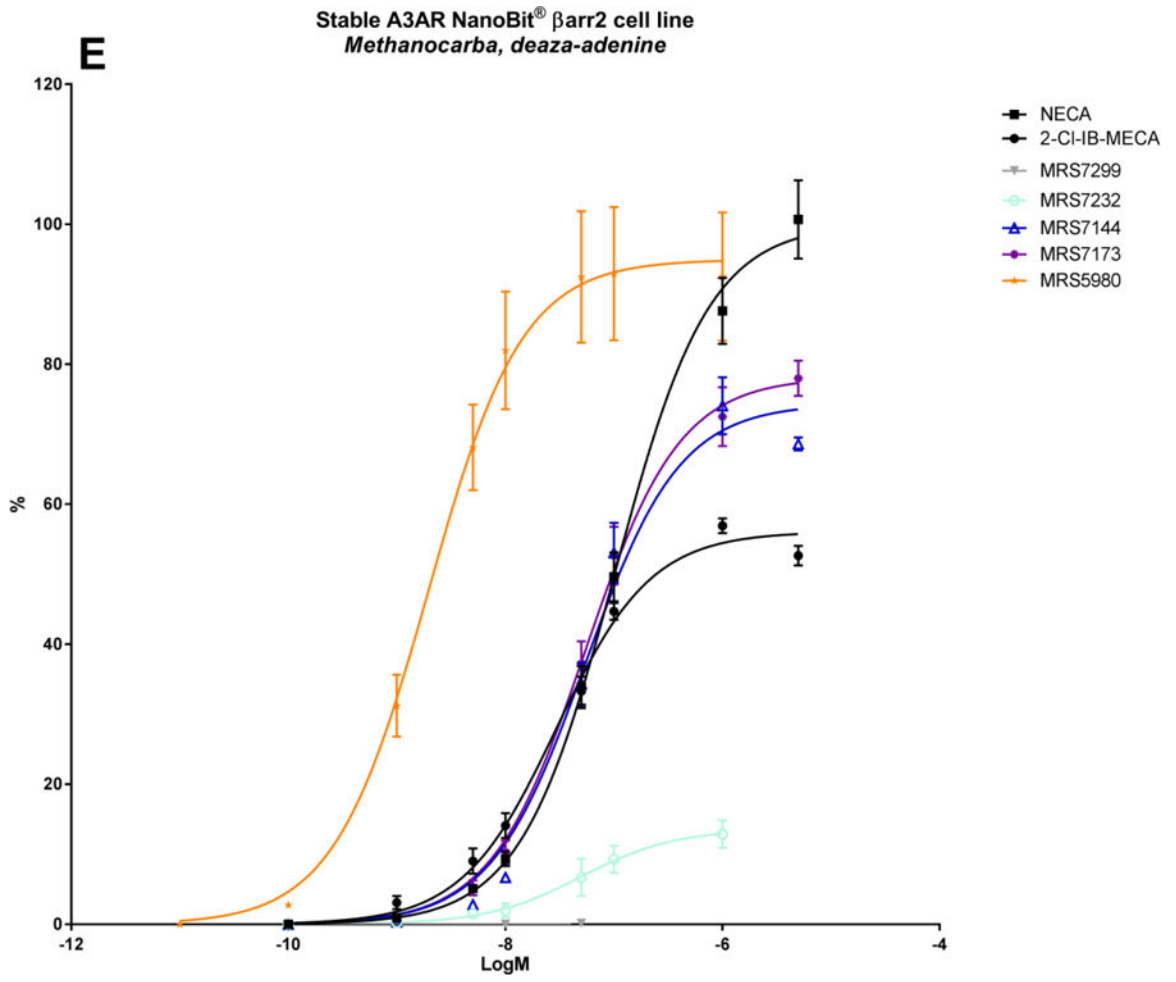
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**Stable A3AR NanoBit® β arr2 cell line
Methanocarpa, 5'-amides, C2-extended**





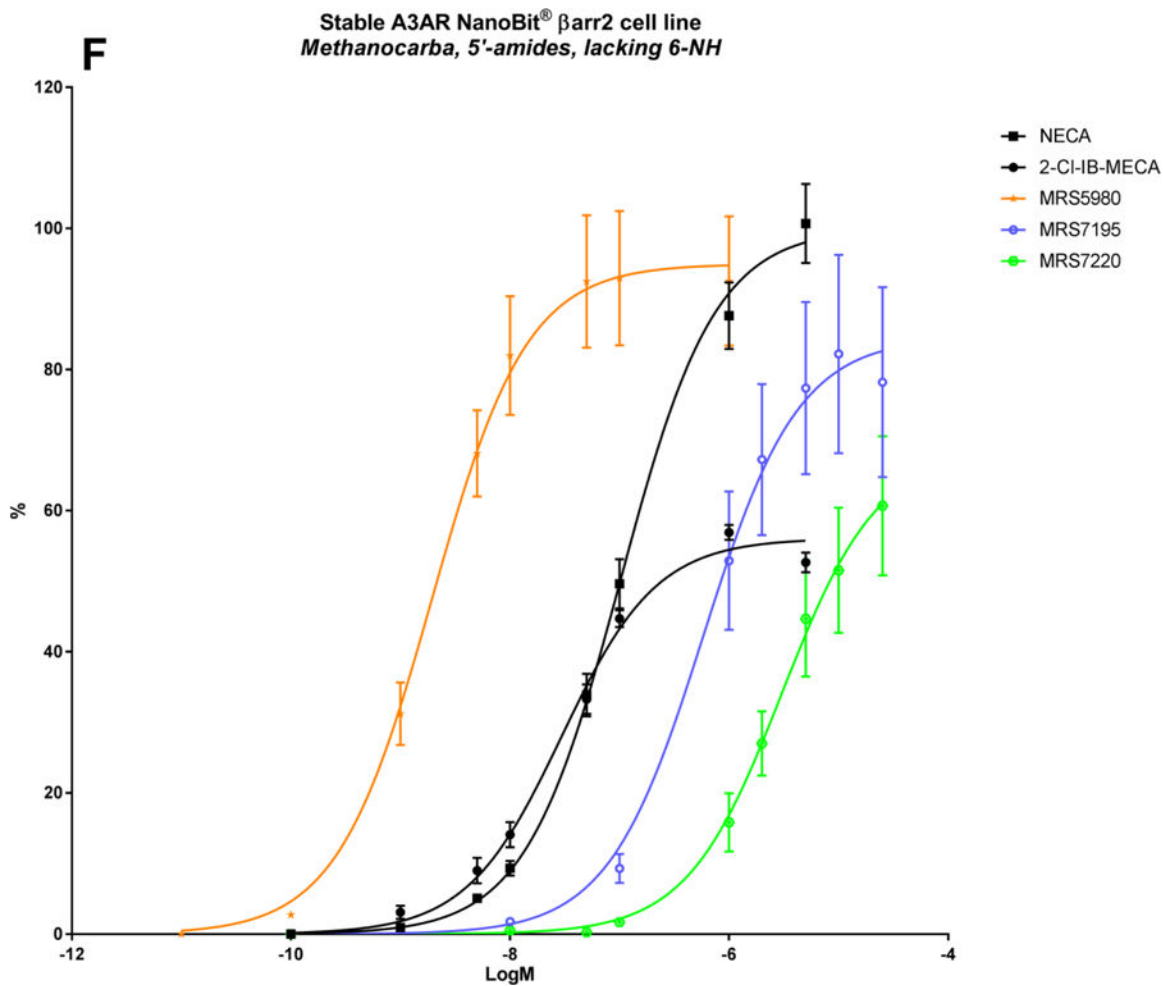


Figure 3:

Comparison of the E_{max} relative to that of NECA (arbitrarily set at 100%) (A) and negative $\log EC_{50}$ (B) of nineteen synthetic A_3AR ligands tested with the stable hA_3AR -NanoBit®- β arr2 HEK293T cell line (Barr2) versus the stable hA_3AR CHO cell line (cAMP).

PCR conditions (a-c) and restriction enzymes (re; d) used to construct the retroviral expression vectors*: a: primers containing the specific restriction site (underlined), kozak sequence (bold italic) and coding sequence of interest (italic). A stop codon (bold) to ensure a correct reading frame was added where necessary.

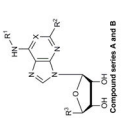
Table 1:

Coding Sequence	Primers (F: forward – R: reverse) ^a	T _m (°C) ^b	Ext. time (s) ^c	RE ^d	Retroviral expression vector*
A ₃ AR-LgBit	F ACTCAAGGATCCACC ATGCCCAACAACAGC R ACTCAATACGTATTA GCTGTTGATGTTACTCGG	69.5	40	BamHI - SnaBI	pLZRS-A ₃ AR-LgBit-IRES-EGFP
SmBit-βarr2	F ACTCAAGGATCCACC ATGGTGACCGGCTACCG R ACTCAA GCGGCCGCTCAGCAGATTGATCATCATAGTCG	74	38	BamHI - NotI	pLZRS-SmBit-βarr2-IRES-dNGFR

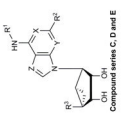
Structures, efficacies (E_{max}), potencies (logEC₅₀), and hA₃AR binding affinities (K_i) of nineteen known A₃AR ligands. Data for βarr2 recruitment were obtained with the stable hA₃AR-NanoBit®-βarr2 HEK293T cell line, and those for cAMP inhibition were obtained with a stable hA₃AR CHO cell line.

Table 2:

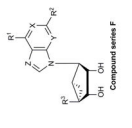
Compound	R ¹	R ²	R ³	X	Y	Z	Stable A ₃ AR		Stable A ₃ AR CHO cell line ^B		Ki (nM) ± SEM
							NanoBit® HEK293T cell line ^A	LogEC50	E _{max} (%)	LogEC50	
<i>A. Ribose 5'-OH analogues</i>											
CPA		H	CH ₂ OH	N	-	-	56.9 ± 3.7	-5.82 ± 0.11	97 ± 4	-6.91	72 ± 12
CCPA		Cl	CH ₂ OH	N	-	-	33.5 ± 2.7	-6.08 ± 0.15	0	NA	38 ± 6
R-PIA		H	CH ₂ OH	N	-	-	56.6 ± 1.9	-6.35 ± 0.06	102 ± 6	-7.57	8.7 ± 0.9
S-PIA		H	CH ₂ OH	N	-	-	51.0 ± 1.4	-5.72 ± 0.04	97 ± 3	-6.68	68 ± 12
ADAC		H	CH ₂ OH	N	-	-	73.7 ± 3.1	-6.61 ± 0.12	103 ± 4	-7.00	13.3 ± 3.0
<i>B. Ribose 5'-amide analogues</i>											
NECA	H	H	CONHC ₂ H ₅	N	-	-	100 ± 2.5	-6.99 ± 0.05	103	-7.42	35 ± 12
CGS21680	H		CONHC ₂ H ₅	N	-	-	99.0 ± 5.5	-5.87 ± 0.10	98 ± 5	-6.62	114 ± 16
IB-MECA	3-I-benzyl	H	CONHCH ₃	N	-	-	78.4 ± 2.5	-7.87 ± 0.07	100	-8.44	1.8 ± 0.7
2-Cl-IB-MECA	3-I-benzyl	Cl	CONHCH ₃	N	-	-	56.1 ± 1.1	-7.53 ± 0.04	100	-8.55	1.4 ± 0.3
<i>C. Methanocarba, C2-C1</i>											
MRS3558	3-Cl-benzyl	Cl	CONHCH ₃	N	N	N	100.9 ± 3.7	-8.12 ± 0.08	103 ± 7	-9.42	0.29 ± 0.04
MRS1873	H	Cl	CH ₂ OH	N	N	N	13.0 ± 1.6	-6.44 ± 0.31	41 ± 5	-6.1	353 ± 54
MRS5127	3-I-benzyl	Cl	H	N	N	N	5.2 ± 0.4	-7.61 ± 0.13	44 ± 6	-7.9	1.44 ± 0.6



Compound series A and B



Compound series C, D and E



Compound series F

Compound	R ¹	R ²	R ³	X	Y	Z	Stable A ₃ AR		Stable A ₃ AR		Ki (nM) ± SEM	
							NanoBit® HEK293T cell line ^A	CHO cell line ^B	Emax (%)	LogEC50		
MRS5474	dicyclopropylmethyl	Cl	H	N	N	N	9.8 ± 1.2	-5.57 ± 0.17	56.0 ± 4.8	-5.86 ± 0.15	470 ± 15	
<i>D. Methanocarba, 5'-amides, C2-extended</i>												
MRS5698	3-Cl-benzyl	(3,4-F ₂ -phenyl)ethyl	CONHCH ₃	N	N	N	83.5 ± 4.6	-6.98 ± 0.07	95.7 ± 6.4	-8.3	3.49 ± 1.84	
MRS5917	CH ₃	(thien-2-yl)ethyl	CONHCH ₃	N	N	N	91.6 ± 3.4	-8.51 ± 0.10	97.3 ± 2.9	-9.38	0.57 ± 0.10	
<i>E. Methanocarba, deaza-adenine</i>												
MRS7144	CH ₂ CH ₃	(5-Cl-thien-2-yl)ethyl	CONHCH ₃	CH	N	N	74.4 ± 2.3	-7.27 ± 0.06	95.5 ± 2.3	-8.82	1.7 ± 0.4	
MRS7299	CH ₃	(5-Cl-thien-2-yl)ethyl	CO ₂ C ₂ H ₅	N	CH	NA	NA	NA	1 ± 7	NA	448 ± 13	
<i>F. Methanocarba, 5'-amides, lacking 6-NH</i>												
MRS7195	CH ₃	(5-Cl-thien-2-yl)ethyl	CONHCH ₃	N	N	N	84.5 ± 6.4	-6.24 ± 0.19	107 ± 26	-7.9	42.2 ± 17.3	
MRS7220	H	(5-Cl-thien-2-yl)ethyl	CONHCH ₃	N	N	N	68.4 ± 5.3	-5.52 ± 0.11	97.8 ± 0.1	-7.57	60 ± 19	

^AEmax is relative to that of reference agonist NECA

^BEmax is relative to that of reference agonist NECA at 10 μM

NA, not significantly active at 10 μM

Table 3:

Structures, efficacies (E_{max}), potencies ($\log EC_{50}$), and hA_3AR binding affinities (K_i) of ten additional structural analogs tested in the stable hA_3AR -NanoBit[®]- β arr2 HEK293T cell line.

Compound	R ¹	R ²	R ³	X	Y	Z	Stable A_3AR		Ki (nM) \pm SEM
							NanoBit [®] HEK293T cell line ^A		
							E_{max} (%)	$\log EC_{50} \pm$ SEM	
<i>A. Ribose 5'-OH analogues</i>									
DCCA	cyclopentyl	Cl	CH ₂ OH	CH	-	-	5.8 \pm 0.2	-5.83 \pm 0.07	244 \pm 37
N ⁶ -benzyl-adenosine	benzyl	H	CH ₂ OH	N	-	-	34.9 \pm 2.3	-6.07 \pm 0.13	41.7 \pm 5.3
N ⁶ -phenyl-adenosine	phenyl	H	CH ₂ OH	N	-	-	75.8 \pm 3.0	-6.23 \pm 0.0	14.9 \pm 3.1
<i>D. Methanocarba, 5'-amides, C2-extended</i>									
MRS5679	3-Cl-benzyl	(4-phenyl-phenyl)ethynyl	CONHCH ₃	N	N	N	46.8 \pm 3.3	-5.92 \pm 0.11	3.06 \pm 1.35
MRS5967	CH ₃	(2-CH ₃ O-phenyl)ethynyl	CONHCH ₃	N	N	N	92.5 \pm 2.4	-8.33 \pm 0.06	0.77 \pm 0.17
MRS5663	CH ₃	(2-Cl-phenyl)ethynyl	CONHCH ₃	N	N	N	101.7 \pm 3.6	-8.25 \pm 0.08	0.58 \pm 0.04
MRS5980	CH ₃	(5-Cl-thien-2-yl)ethynyl	CONHCH ₃	N	N	N	95.0 \pm 4.0	-8.71 \pm 0.11	0.7 \pm 0.11
MRS7154	(CH ₂) ₂ CH ₃	(5-Cl-thien-2-yl)ethynyl	CONHCH ₃	N	N	N	94.5 \pm 4.8	-7.67 \pm 0.11	1.1 \pm 0.3
<i>E. Methanocarba, deaza-adenine</i>									
MRS7173	CH ₃	(5-Cl-thien-2-yl)ethynyl	CONHCH ₃	N	CH	N	78.2 \pm 1.8	-7.26 \pm 0.04	1.56 \pm 0.2
MRS7232	CH ₃	(5-Cl-thien-2-yl)ethynyl	CO ₂ C ₂ H ₅	N	N	N	13.6 \pm 1.6	-7.30 \pm 0.17	5.38 \pm 0.03

^A E_{max} is relative to that of reference agonist NECA

