FACULTY OF PHARMACEUTICAL SCIENCES

β-Arrestin coupling to Purinergic Receptors: Glo with the Flow?

Jolien STORME

Promoter: Prof. Dr. Christophe Stove

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't Leven zit vol met bochten,
en soms, moet ge gewoon meegaan.

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2A3BTs	4-substituted 2-amino-3-benzoylthiophenes
2-Cl-IB-MECA	2-chloro-N^6-(3-iodobenzyl)-5'-*N*-methyl-carboxamidoadenosine
2-MeSADP	2-methylthio-ADP
7TMR	seven transmembrane receptor
(h)A_3AR	(human) A3 adenosine receptor
AC	adenylate cyclase
ACE	angiotensin converting enzyme
AD	Alzheimer's disease
ADP	adenosine 5'-diphosphate
AngII	Angiotensin II
AP-2	clathrin adaptor complex
Ap4A	P^1,P^4-di(adenosine-5′)-tetraphosphate
AR(s)	adenosine receptor(s)
ARB	angiotensin receptor blocker
AT1R	angiotensin II type 1 receptor
ATP	adenosine 5'-triphosphate
β1AR	β1-adrenergic receptor
β2AR	β2-adrenergic receptor
βARK	βAR kinase
βarr(s)	β-arrestin(s)
βarr1	β-arrestin1
βarr2	β-arrestin2
BAY60-6583	2-{[6-Amino-3,5-dicyano-4-(4-(cyclopropylmethoxy)phenyl)pyridin-2-yl]thio}acetamide
BiFC	bimolecular fluorescence complementation
bla	β-lactamase
BPTU	1-(2-(2-(t*ert*-butyl)phenoxy)pyridin-3-yl)-3-(4-(trifluoromethoxy)-phenyl)urea
BRET	bioluminescence resonance energy transfer
cAMP	3',5'-cyclic adenosine monophosphate
CB1	cannabinoid receptor 1

CB2	cannabinoid receptor 2
CCP(s)	clathrin-coated pit(s)
CCPA	2-chloro-N^6-(cyclopentyl)-adenosine
CCS(s)	clathrin-coated structure(s)
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
CPA	N^6-cyclopentyladenosin*e*
CPCPA	8-cyclopentyl-N^6-cyclopentyladenosine
cryo-EM	cryo-electron microscopy
D2R	D2 dopamine receptor
DAG	diacylglycerol
DBXRM	1,3-dibutylxanthine-7-riboside-5′-N-methylcarboxamide
DNA	deoxyribonucleic acid
ERK	extracellular-signal regulated kinase
FL	Firefly luciferase
FRET	fluorescence resonance energy transfer
GFP	green fluorescent protein
GL	Gaussia luciferase
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HEMADO	2-(1-hexynyl)-N-methyladenosine
IB-MECA	N^6-(3-iodobenzyl)-5’-*N*-methyl-carboxamidoadenosine
IL	intracellular loop
IP(3)	inositol (1,4,5-tris)phosphate
IRI	ischemia-reperfusion injury
IV	intravenous
JNK	c-Jun N-terminal kinase
LUF6000	*N*-(3,4-dichlorophenyl)-2-cyclohexyl-1*H*imidazo[4,5-*c*]quinolin-4-amine

μOR	μ-opioid receptor
MAPK	mitogen-activated protein kinase
MeCPA	8-methylamino- N^6-cyclopentyladenosine
miRNA	microRNA
MRS542	2-chloro-N^6-(3-iodobenzyl)-adenosine
MRS5679	(1S,2R,3S,4R,5S)-4-(2-(Biphenyl-4-ylethynyl)-6-(3-chlorobenzylamino)-9H-purin-9-yl)-2,3-dihydroxy-N-methylbicyclo[3.1.0]hexane-1-carboxamide
NECA	5'-*N*-ethyl-carboxamidoadenosine
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cell
NHERF	Na^+/H^+ exchanger regulatory factor
NL	NanoLuc luciferase
P2Y2R	P2Y2 receptor
PAM	positive allosteric modulator
PDEase	phosphodiesterease
PDZ	Type II postsynaptic density, disc large and zo-1 protein
PINC	platelet-induced neutrophil chemotaxis
PKA	protein kinase A
PKC	protein kinase C
PM	plasma membrane
PMA	phorbol 12-myristate 13-acetate
PTX	pertussis toxin
RA	rheumatoid arthritis
RacGEF	Rac guanine nucleotide exchange factor
RA_i	intrinsic relative activity
RL	Renilla luciferase
R-PIA	R-N^6-(phenylisopropyl)adenosine
SAH	S-adenosylhomocysteine
SAR	structure-activity relationship
SFSR	structure functional selectivity relationship
TIRF	total internal reflection fluorescence
TM	transmembrane domain

UDP	uridine 5’-diphosphate
Up4U	P^1,P^4-di(uridine-5′)-tetraphosphate
UTP	uridine 5’-triphosphate
V2R	vasopressin receptor
VCP746	4-(5-amino-4-benzoyl-3-(3-(trifluoromethyl)phenyl)thiophen-2-yl)-N-(6-(9-((2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)tetrahydrofuran-2-yl)-9H-purin-6-ylamino)hexyl)benzamide
vYFP	venus YFP
XFEL	X-ray free electron laser
YFP	yellow fluorescent protein

Chapter 1:
General Introduction

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1.1 Introduction on GPCR signalling and the regulation by arrestins in the light of drug discovery

1.1.1 Introduction on G protein-coupled receptors (GPCRs)

G protein-coupled receptors (GPCRs) consist of seven membrane-spanning α-helices and are therefore often referred to as seven transmembrane receptors (7TMRs). They represent the largest and most diverse group of membrane proteins. More than 800 different GPCRs have been identified, encoded by about 4% of the genes in the human genome. Roughly half of these 800 GPCRs represent olfactory and other chemosensory GPCRs, the other half are targeted by endogenous ligands; they represent the target of nearly one third of the drugs available on the market today [1, 2]. A 'phylogenetic classification' divides the human GPCR superfamily into five main GPCR families – *Rhodopsin* (class A), *Secretin* (class B), *Adhesion* (class B), *Glutamate* (class C) and *Frizzled/Taste2* – also shortened to the acronym GRAFS. The *Rhodopsin* family represents the largest class and comprises GPCRs that generally bind their ligands via a binding pocket in the TM regions, with participation of the extracellular loops and/or the N-terminus [3]. There are multiple examples of GPCRs that have an important, fundamental role in the homeostasis or regulation of different physiological processes. An altered regulation and/or expression of these GPCRs is indicative for pathological conditions, and they can represent biomarkers as well as drug targets for a variety of disease states [4]. The most 'famous' of all is rhodopsin, the prototypical member of the class A GPCR family, which is activated by light [5]. The β2-adrenergic receptor (β2AR) was cloned a few years later and belongs to the adrenergic family of GPCRs, together with the β1AR. They modulate heart rate and contractility, as well as bronchodilation in the lung, and are involved in the pathogenesis of heart diseases and asthma. Others include the vasopressin receptor (V2R) and angiotensin II type 1 receptor (AT1R), involved in the regulation of blood pressure, being the targets of blood-pressure regulating drugs. Furthermore, opioid receptors, such as the μ-opioid receptor (μOR), are involved in pain regulation and are the target of opiate analgesics and opioid drugs. In the brain, the D2 dopamine receptor (D2R) is one of the major neurological targets of antipsychotic drugs for the treatment of Parkinson's disease, schizophrenia and other affective disorders. Cannabinoid receptors (CB1 and CB2) are drug targets for regulation of pain and

inflammation, and are most known as targets for (synthetic) cannabinoids as drugs of abuse [6, 7]. The class of purinergic GPCRs, which will be the focus of this thesis, is activated by the endogenous nucleoside adenosine and nucleotides ATP, ADP, UTP and UDP, which represent both the building blocks of DNA as regulators of energy metabolism [8]. Throughout years, GPCRs have become prototypical examples for molecular and biochemical research on GPCR structure and signalling, nurturing future drug research on this highly interesting superclass of membrane receptors.

As the name reveals, all G protein-coupled receptors couple to an intracellular signalling transducer molecule, named G protein, which is a heterotrimeric protein consisting of a G_{α} subunit in complex with a $G_{\beta}G_{\gamma}$ dimeric subunit. The G_{α} subunit has GTPase activity and upon GPCR activation, GDP in its catalytic domain is exchanged for GTP, upon which the $G_{\beta}G_{\gamma}$ dimeric subunit dissociates from G_{α}, and each of the subunits can evoke downstream signalling [9, 10]. Many different types of G protein subunits exist, of which the G_{α} subunits are generally classified into four groups – $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$ and $G_{\alpha 12/13}$. The G_{α} subunits, as well as the $G_{\beta\gamma}$ subunits, couple to a variety of membrane-bound and intracellular primary effector molecules. Examples are adenylate cyclase (AC; activated or inhibited by $G_{\alpha s}$ and $G_{\alpha i}$, respectively), phospholipase C (PLC; activated by $G_{\alpha q}$) and Rho guanine nucleotide exchange factors (coupling to $G_{\alpha 12/13}$). These effector molecules can activate or inhibit the production of second messengers such as cAMP (by AC), inositol(1,4,5)-trisphosphate and diacylglycerol (by PLC), eventually leading to an intracellular rise of Ca^{2+}, to the activation/generation of transcription factors or the closing/opening of ion channels, influencing cellular behaviour. GPCR signalling also includes signalling via other proteins than G proteins. Many other protein cascades link the GPCR to the nucleus, such as phosphoinositide-3 kinases (PI3Ks) and the family of mitogen-activated protein kinases (MAPKs) – which are involved in cell cycle progression, cell survival and proliferation – non-receptor tyrosine kinases such as Src, and receptor tyrosine kinases [11, 12]. From a drug discovery point of view, a key point of interest has been to elucidate how GPCRs signal to the inside of the cell via these different signalling pathways after binding of an endogenous- or drug molecule. For many of the GPCRs mentioned above, crystal structures have become available. These show the GPCR in complex with a ligand, revealing important features of structural rearrangements in the GPCR molecule upon ligand binding. However, equally

important for drug discovery is the structural change that is brought about in the signalling complex consisting of a GPCR and signalling molecule(s). Structures of the GPCR-G protein complex have been reported and complexes with other signalling proteins, such as arrestins, are only just now being revealed (Figure 1.1).

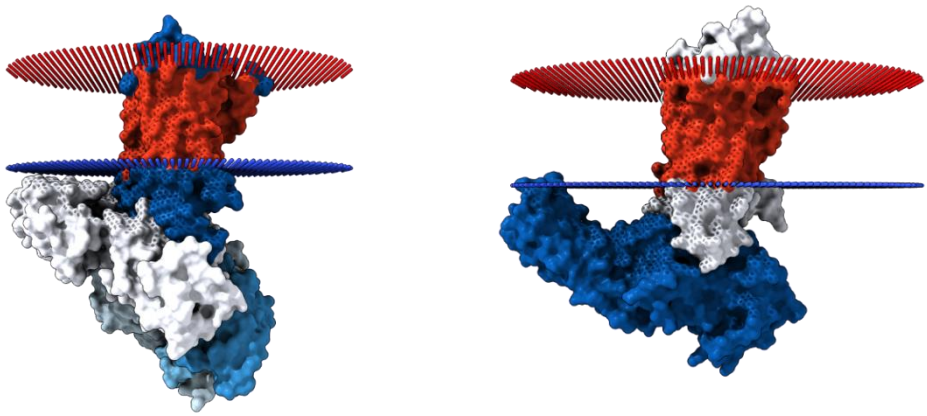


Figure 1.1: Structures of GPCR signalling complexes: (A) Structure of β2AR with G_s (Protein Data Bank (PDB) ID: 3SN6; [13]) - (B) Structure of rhodopsin with arrestin-1 (PDB ID: 4ZWJ; [14]). Red and blue grid represent upper and lower side of the plasma membrane. Image adopted from the RCSB PDB (www.rcsb.org).

1.1.2 GPCR signalling: from lock-key model to functional selectivity and signalling bias

The conceptual framework underlying the well-established pharmacology of GPCRs has been evolving; during the last decade, distinct molecular features of GPCR signalling have attracted close attention concerning drug discovery research. Questions are now being raised on how we can exploit differential GPCR signalling for the development of new and better therapeutics.

The effect of an external stimulus (light, odour) or extracellular ligand (hormone, neurotransmitter, drug molecule) on cellular behaviour via a GPCR membrane protein has classically been described by a ternary complex model. In this model, the stimulus or ligand binds to the GPCR, evoking a conformational rearrangement in its structure, leading to the association with and thus the activation of an intracellular G-protein, by its contact with the cytoplasmic side of the GPCR. In this way, GPCR activation has been looked at for decades as an ‘on-off phenomenon’; a ligand

evokes intracellular G-protein mediated signal transduction, leading to an altered level of second messenger molecules, which ultimately leads to an altered pattern of enzyme activation, protein expression, or ion levels by affecting ion channels. In that scenario, if a ligand shows sufficient GPCR selectivity, it binds and activates the GPCR in a linear way, either inhibiting or stimulating all downstream responses. This general image has now evolved, or one should rather say surpassed, although the classical concept still stands to some extent [15].

It is now believed that a ligand, besides showing selectivity for a GPCR, displays an additional selectivity on the functional level, by stimulating/activating/inhibiting certain (subsets of) signalling pathways upon receptor binding. By making crucial contact points with extracellular and/or transmembrane residues of the GPCR, a ligand may stabilize a very particular GPCR conformation. There is thus not only one 'active state' of a GPCR, but rather a ligand-induced conformational change towards one of many active or inactive conformational states [2, 16]. Furthermore, a kinetic interconversion between these different conformational states might exist, rendering regulation of the interaction with a preferred cytosolic binding partner even more complex [17]. Ligands may bind to a GPCR and (in)activate a certain signalling pathway to a certain extent, while leaving other pathways less stimulated or even untouched. Hence, an agonist may be biased towards a signalling pathway, displaying selectivity in its signalling (**functional selectivity**) [16]. Therefore, classical terms as agonist, antagonist and inverse agonist that were suited for the original model, must now be interpreted relative to the signalling pathway that is affected. In the ligand-induced active GPCR conformation, the transmembrane helices are rearranged, resulting in the opening of a cytoplasmic cavity in the GPCR, which can accommodate multiple signalling proteins. These mainly belong to three families; G-proteins, receptor kinases and (β-)arrestins [2]. Functional selectivity can exist towards each of these – as well as towards other – proteins [18-21].

Functional selectivity is not solely orchestrated by the conformation of the receptor induced by extracellular ligands, but also by intracellular as well as intra-membrane components that can influence signalling [19]. The expression level of the receptor as well as its downstream signalling molecules, which can vary depending on cellular context and under various (patho)physiological states, is also of relevance (see

below). Besides, functional selectivity can be influenced by allosteric modulation. Allosteric modulators bind to a site on the GPCR that is different from the orthosteric site, occupied by the endogenous ligand. An allosteric modulator can negatively or positively influence the binding of the orthosteric ligand and/or the activity of this ligand in different signalling pathways, which is referred to as negative or positive allosteric modulation, respectively. Besides, allosteric ligands might (in)activate the receptor independent of orthosteric ligand binding, displaying allosteric (ant)agonism as such [22]. Allosteric modulators have several advantages over orthosteric ligands. As the orthosteric binding site in GPCRs is well conserved across family members, design of selective ligands for a certain member of a subfamily is often tedious. Allosteric modulators with deviating structures can sometimes provide this selectivity, while potentially displaying better pharmacokinetic properties than the orthosteric ligands. Further, they often provide a more physiologically-like effect as they can modulate the function of endogenous ligands, without causing side effects due to overstimulation of the receptor (as with overdose) or due to receptor desensitization or downregulation under sustained stimulation, as with chronic therapies [23]. As a matter of fact, one could even state that G proteins, arrestins and other adaptor molecules function as allosteric modulators towards the binding of the orthosteric/allosteric ligand and thus influence functional selectivity in a reciprocal way [24]. From a drug development point of view, the possibility to activate one (or certain) pathway(s), with less activation of other pathways, offers unique possibilities to obtain a beneficial therapeutic profile, possibly reducing or preventing side effects.

1.1.3 How to evaluate signalling bias

1.1.3.1 True bias versus apparent bias

For the evaluation of signalling bias, keeping in mind pharmacological relevance, we have to describe the consequence of ligand-induced differential receptor conformations in terms of functional outcome. This has mainly been achieved by using *in vitro* functional assays or *in vivo* animal models. In the *in vitro* context, ‘bias’ - in the broad sense of the term - occurs when a ligand exhibits different efficacies or potencies, relative to a reference ligand, for two different signalling pathways. Care should be taken when interpreting results from assays that monitor different

signalling pathways, in such a way that true bias should be clearly distinguished from apparent bias, which can occur under multiple circumstances. E.g., apparent bias may be observed for partial agonists, which can appear as biased agonists when not strong enough to stimulate a given pathway to a detectable extent, or when different receptor expression levels are involved. This has to do with the phenomenon of receptor reserve; only a fraction of the whole receptor population present in a tissue or cell is necessary to evoke the maximal effect, or there is an excess of receptors on the cell surface. This means that a partial agonist in a system with low receptor expression levels can become a full agonist at high receptor expression levels. Vice versa, a partial agonist at high receptor levels may display insufficient intrinsic activity to even evoke a response at low receptor expression levels. These considerations should be taken into account when interpreting results. As a general rule, one can state that ligand bias can be observed when there is a reversal of the rank order of efficacy and/or potency between two pathways measured [18]. However, things are somewhat more complicated than that; quantification of bias by using a system-independent parameter is important to exclude the system- and observational component (i.e. the apparent bias) out of the total observed bias. In doing so, only true ligand bias will be left [25].

1.1.3.2 How to detect and quantify bias?

The answer to the question how to detect signalling bias and how to quantify it in a reliable and reproducible way, is of relevance for the efforts that are being made in research on biased signalling. As already mentioned above, the observation of signalling bias may be influenced by the methods used to characterize this signalling. For the canonical G protein pathway there's a multitude of sensitive and selective functional assays, which are often based on monitoring of the second messengers downstream of the specific G protein subtype [26, 27]. However, for the second most studied signalling pathway, via arrestins, there are far fewer functional assays [28, 29]. Generally, these rely on measurement of GPCR phosphorylation, arrestin functions (i.e. desensitization, internalization and signalling) or arrestin trafficking. Interpretation of results from desensitization, internalization and signalling, such as activation of the extracellular-signal regulated kinase (ERK) pathway, is often tedious as these events can also occur independent of arrestin activation. Therefore,

arrestin trafficking is the most popular assay. General methods include arrestin tracking with fluorescence microscopy via tagged arrestins (redistribution assays). To obtain a more quantifiable result, proximity assays such as fluorescence resonance energy transfer (FRET) or bioluminescence resonance energy transfer (BRET) might be used, which detect the interaction between a GPCR and arrestin. One caveat with these assays is that they suffer from limited sensitivity. This is often a problem for arrestin measurements because, unlike G protein measurements, there is no signal amplification, but a rather stoichiometric binding of arrestin to the GPCR. It must be noted that this signal amplification aspect can also complicate the interpretation for G protein assays; for example a $GTP_{\gamma}S$ binding assay versus a cAMP accumulation assay can give different results [15, 30]. More sensitive protein fragment complementation assays have now been developed.

In order to eliminate cell-type dependent confounding factors - such as the level of receptors and/or downstream signalling partners present (e.g; G protein or arrestin) - in the evaluation of functional selectivity, distinct quantitative methods have been developed to identify and quantify true biased signalling based on mathematical models. In these models, a ternary signalling complex between ligand, receptor and transducer/signalling molecule is used, in which the ligand has two primary properties; its affinity for the receptor, and its efficacy at the signalling complex, i.e. the ability of the ligand-receptor complex to induce signalling. An example of an increasingly used mathematical model is the operational model of Black & Leff [31], in which the pharmacological response of a ligand (A) is expressed in terms of unconditional ligand affinity (K_A; the equilibrium dissociation constant of ligand-receptor complex) and intrinsic ligand efficacy (τ) (Eq. 1).

$$\text{response} = \frac{E_m[A]^n\tau^n}{[A]^n\tau^n + ([A] + K_A)^n} \qquad \text{(Eq. 1)}$$

This equation is actually a mathematical rearrangement of two equations; that of agonist concentration by receptor occupancy and that of receptor occupancy by pharmacological effect. The parameter τ is determined by the ratio $[R_t]/K_e$; the receptor density ($[R_t]$) and the intrinsic efficacy of the agonist to activate a particular signalling pathway (K_e; the receptor occupancy needed to produce 50% of the

system's maximal effect). E_m represents the maximum response of the system and *n* the transducer slope of the concentration-response curve. The factors E_m and *n* are cell-specific and thus shared for all agonists tested in a certain cell type at a certain pathway. The factor K_A is ligand-receptor specific, while τ is both receptor- as well as cell-specific. For the quantification of functional selectivity, Kenakin and colleagues assumed that ligand bias is characterized by different affinities (K_A) and efficacies (K_e or thus τ) [22, 32]. By putting ligand functional dose-response data into this model, the ratio $\log(\tau/K_A)$ was characterized, which has been referred to as the 'transduction coefficient'. This ratio embodies ligand activity in a system-independent way, i.e. independent of receptor density and/or receptor reserve (i.e. tissue or cell sensitivity), signal amplification, and assay sensitivity. The relative activity of agonists – for example a test ligand compared to a reference ligand – in a certain signalling pathway can be quantified with a 'normalized' transduction coefficient $\Delta\log(\tau/K_A)$. Furthermore, a 'bias factor' can be determined, which can be used to quantitatively express 'ligand bias', as defined as in Eq. 2-4.

$$\text{bias factor} = \Delta\Delta\log(\tau/K_A) \quad \text{(Eq. 2)}$$

$$= \Delta\log(\tau/K_A)_{\text{pathway 1}} - \Delta\log(\tau/K_A)_{\text{pathway 2}} \quad \text{(Eq. 3)}$$

$$\text{ligand bias} = 10^{\Delta\Delta\log(\tau/K_A)} \quad \text{(Eq. 4)}$$

It is important to note that the transduction coefficient $\log(\tau/K_A)$ is linear with changing receptor density (which is cell-type or tissue dependent), and this for different transducer slopes (*n*). This means that $\Delta\log(\tau/K_A)$ for two agonists is constant for different receptor densities and thus can be used as a measure for comparison of relative agonism activities between different cell types. Furthermore, if the biased profile of a ligand between two pathways, shown by $\Delta\Delta\log(\tau/K_A)$, has an established link with a certain therapeutic effect, this scale can allow medicinal chemists to optimize the bias in a delicate way. In contrast, the most commonly used measure of ligand activity, i.e. the negative logarithm of the EC_{50}, pEC_{50}, is only linear for full agonists with varying receptor densities, but not for partial agonists. Hence, ΔpEC50 is not constant over changing receptor densities when one of both of the compared

ligands shows partial agonism, which hampers comparison of ligand activities between different cell-systems. Yet, another measure of ligand activity, known as Ehlert's activity [33] or 'intrinsic relative activity' (RA_i), i.e. the ratio $\log(E_{max}/EC_{50})$, is also influenced by receptor densities. However, $\Delta\log(E_{max}/EC_{50})$ is constant for full as well as partial agonists when the transducer slope n = 1. This means that this factor can also be used for comparison of relative agonist activities, at least when the Hill slope of the concentration-response curve is not different from unity [32, 34].

1.1.4 β-arrestins as regulators of GPCR signalling: from desensitizing adaptor to scaffolding protein for downstream biased signalling

The by any means best studied interaction partner of G protein-coupled receptors, besides the G-protein, is the arrestin family of adaptor proteins [2]. This family of GPCR regulatory molecules has originally been identified as inhibitory adaptor proteins for the termination of G protein mediated signalling. Their discovery dates from thirty years ago, in the context of regulation of rhodopsin and adrenergic receptor signalling. Light converts rhodopsin to photoexcited rhodopsin (R*), which allows binding of transducin, the G protein of visual cells, thereby activating cGMP phosphodiesterease (PDEase). In 1986 it was shown that the activation of transducin and PDEase by phosphorylated R* was quenched, or thus "arrested" by a 48 kDa protein, rather than via spontaneous slow decay of the photoproduct or hydrolysis of the transducin-bound GTP [35]. Therefore, this protein was given the name arrestin. In 1990, similar research was performed for the β-adrenergic receptor (βAR) by the group of the 2012 Noble Prize in Chemistry Laureate Robert Lefkowitz [36]. In the human body, the cellular response to neurotransmitters and hormones, such as the endogenous catecholamines (nor)epinephrine for the βAR system, generally wanes rapidly despite continuous presence of these stimuli. This was referred to as 'desensitization' of the receptor. βAR desensitization after stimulation was shown to rely on uncoupling of the receptor from stimulatory G_s protein, involving receptor phosphorylation by protein kinase A (PKA) and βAR kinase (βARK), and an 'additional cytosolic factor'. The kinetics of the process appeared to be different from receptor sequestration to vesicles avoid of G_s, or receptor downregulation to lysosomes. This desensitization causes cAMP levels to plateau or even return to basal levels within minutes, and substantially diminishes AC activity upon re-

stimulation with the desensitizing ligand. The additional cytosolic factor involved is now known as **β-arrestin (βarr)** [37] and is distinct from visual arrestin. Throughout the years, the arrestin isoform repertoire has emerged as visual arrestins 1 and 4, and ubiquitously expressed arrestin 2 (β-arrestin1; βarr1) and arrestin 3 (β-arrestin2; βarr2), of which the β-arrestins (βarrs) are more highly expressed in sympathically nerved areas of the brain, in heart and lung, with the link to the aforementioned discovery. β-arrestins share 78% identity, with main differences in their C-terminal region [38]. To characterize the role of βarrs *in vivo*, knock-out mice have been generated. βarr1/2 double knock-out results in embryonic lethality in mice, while single knock-outs did not show very abnormal phenotypes, pointing at considerable redundancy [39].

The recruitment of arrestins to the GPCR is believed to depend on the phosphorylation of serine and/or threonine residues that are located in the cytoplasmic sites of the GPCR, i.e. the cytoplasmic loops and/or the C-terminus. These residues are phosphorylated by kinase enzymes that can be divided into two groups, related to the desensitization process; second-messenger regulated receptor kinases, e.g. cAMP dependent protein kinase A (PKA) and protein kinase C (PKC), and a family of G protein-coupled receptor kinases (GRKs), consisting of 7 subtype members (GRK1-7). Second-messenger regulated kinases can be activated by any GPCR (or other process) in the cell, leading to agonist-independent phosphorylation of different GPCRs, thus leading to an altered/diminished cell signalling (heterologous desensitization). In contrast, GRKs selectively phosphorylate ligand-activated GPCRs, leading to a more focused, diminished response for that specific GPCR (homologous desensitization). It is known that GPCRs can also display a constitutive level of activation, leading to phosphorylation by GRKs independent from ligand binding. **In this thesis, the main focus will be on β-arrestins**, as these have a prominent role in the regulation of non-visual GPCRs [38-41].

Once the β-arrestin molecule is recruited to the GPCR, it is activated by its interaction with the GPCR, causing a structural rearrangement in the arrestin molecule and the release of its C-terminal tail. The structural details of this activation process have been studied extensively and have been subject to discussion; this will

be described in detail in the next section. Once activated, arrestin can interact with adaptor proteins of the endocytic machinery; in the 410 amino acid long βarr2 molecule, the clathrin binding region concerns a C-terminal motif (L-I-E-F; residues 373-376 in human βarr2), which binds the clathrin heavy chain, while two arginine residues upstream of this region bind the β2-adaptin subunit of the clathrin adaptor (AP-2) complex [42, 43]. This interaction directs the GPCR-arrestin complex towards clathrin-coated pits for endocytosis. In this way, the GPCR is either internalized (receptor sequestration) and recycled back to the plasma membrane or is degraded into lysosomes (receptor downregulation) (Figure 1.2) [39, 40].

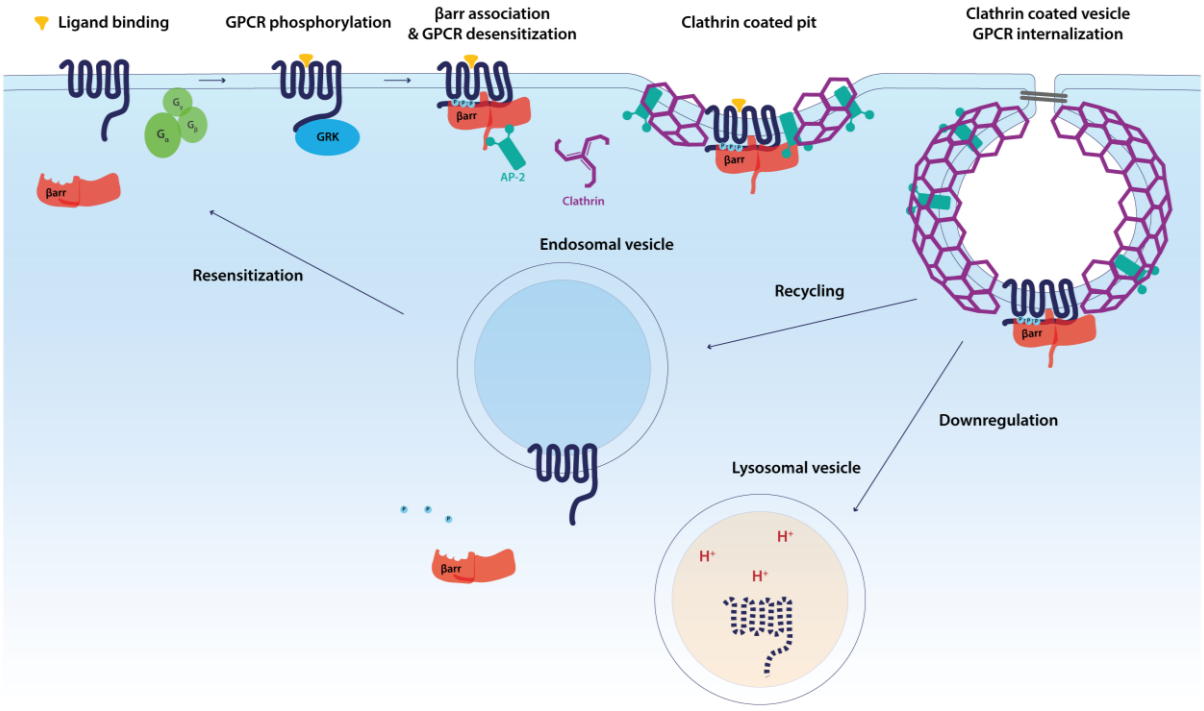


Figure 1.2: β-arrestin dependent GPCR desensitization, internalization, downregulation and/or recycling. Upon ligand binding and G protein activation, GRKs (or other kinases) phosphorylate the GPCR at cytoplasmic regions (C-terminus and/or intracellular loops) and βarr is recruited. βarr activation and liberation of its C-tail allows association with AP-2 and clathrin, targeting the GPCR-βarr complex to clathrin-coated pits. The GTPase dynamin pinches off the clathrin-coated pit, forming a clathrin-coated vesicle. The GPCR can be internalized into endosomes to be dephosphorylated and recycled to the cell surface, or can get degraded in lysosomes.

The trafficking of the GPCR-arrestin complex has been reported to occur with different kinetics, leading to the classification of GPCRs into two classes. Class A receptors (for example β2AR, μOR, D2R, CBs) show preferential binding to βarr2

compared with βarr1, and the interaction is rather transient, with a fast dissociation of βarr from the receptor, and thus fast recycling. Class B receptors (for example V2R, AT1R) bind βarr1 and βarr2 with equal affinity, giving a more stable receptor complex. Based on studies with chimeric GPCRs in which the C-terminal tails of two GPCRs were switched, it has been assumed that this C-terminus is determining for the behaviour of the GPCR; clusters of serine/threonine would evoke class B behaviour (see **Chapter 3**) [44, 45].

Besides targeting the GPCR for endocytosis, the activated arrestin molecule also exposes adaptor sites for interaction with signalling proteins, and thus can initiate its own distinct downstream G protein-independent signalling. An ever-increasing list of proteins downstream of βarr has been identified: interaction with Src-family tyrosine kinases, scaffolding of ERK1/2 [46], c-Jun N-terminal kinase (JNK) and p38 MAPK cascades, regulation of the NF-κB pathway [47], scaffolding of PDEases, just to name a few [48]. Class B GPCRs would give more pronounced cytosolic retention of ERK than class A GPCRs [30, 38, 39, 49-51]. For non-visual GPCRs, βarr2 is most involved in GPCR signalling [52]. Moreover, arrestins not only mediate numerous signalling pathways initiated by activated GPCRs, they also participate in receptor-independent signalling processes, affecting cell adhesion, motility, survival, and apoptotic death. Given this role in signal transduction, arrestins have been put on an equal footing with G proteins, representing a parallel class of GPCR interacting signalling proteins. However, the structural basis of the interaction of arrestin with various downstream signalling partners involved in GPCR-(in)dependent arrestin signalling remains to be elucidated [2].

Given all the above, it is not surprising that in recent years, the arrestin pathway is by far the most extensively studied G protein-independent signal transduction pathway for which biased agonism has been thoroughly explored. In the light of drug discovery, there is a high interest in the challenging concept of biased signalling for the synthesis of biased drug molecules that specifically (in)activate those signalling pathways that lead to a desired therapeutic profile with reduced side effects. Furthermore, the arrestin pathway is also highly interesting with respect to its GPCR desensitization properties; opportunities may lie here in terms of overdose and/or drug tolerance [15, 30, 53, 54].

1.1.4.1 GPCR-βarr contact points

1.1.4.1.a GPCR C-terminal phosphorylation triggers βarr recruitment: catching a tiger by the tail?

As evolution created GRKs and arrestins to suppress GPCR signalling by G proteins, it seems convenient that an overlap exists in the cytoplasmic side of the GPCR that can accommodate all three GPCR interaction partners. Moreover, there are only a limited number of G protein, GRK and arrestin subtypes that bind hundreds of different GPCRs. However, there must also be a difference, induced by the binding of the ligand itself and/or subsequent phosphorylation, creating a non-overlapping pattern, which distinguishes between G proteins, GRKs and arrestins, and their subtypes. The structural motifs involved are highly promising to be exploited for therapeutic purposes. At this very moment, we still don't know which exact GPCR conformations or patterns in the cytosolic part of the GPCR favour binding to each interaction partner. Also important to note is that we do not know if these different conformations are really fixed, exploitable for biased signalling, or if some state of equilibrium exists between different conformational states depending on the environmental and cellular conditions involved [2].

The recruitment of arrestin to a GPCR is generally believed to be triggered, or to (partly) rely on the phosphorylation of serine/threonine residues in the cytoplasmic exposed sites - the second and third intracellular loops (IL), or the C-terminus - of the activated GPCR by receptor kinases [55-57]. In contrast to other kinases (PKA, PKC), GRKs do not recognize a consensus phosphorylation sequence in the multitude of different GPCRs they phosphorylate. Therefore, it is tempting to speculate that the specific position of the serine/threonine residues determines a pattern of phosphorylation by GRKs. For example, for rhodopsin, the β2AR, the V2R and the AT1R it was shown that different GRKs generate distinct phosphorylation patterns [58-61]. Many studies have reported on key phosphorylation sites in the intracellular loops and/or C-terminus, by mutation and truncation of GPCRs. However, the results are often highly divergent and not indicative of a consensus phosphorylation pattern in GPCRs in their contact with arrestins; e.g. the D1 dopamine receptor displays a sequential phosphorylation of the C-terminus, and 3rd

IL [62]. The μOR is also C-terminally phosphorylated [63-65]. The β1AR contains multiple phosphorylation sites in the 3rd IL and C-terminus, of which two crucial serine residues in the distal C-terminus that determine βarr2 recruitment [66]. Yang *et al.* ([67]) have compiled a non-exhaustive list of studies mapping phosphorylation sites on the C-terminus and/or ILs using different techniques. Based on these observations, the phospho-barcode hypothesis came to light; ligand-induced patterns of phosphorylation may constitute a barcode that dictates the interaction with arrestin, its conformation, and subsequent GPCR downstream signalling. However, it is still not known how this barcode is deciphered exactly by the arrestin molecule, because there is so little primary phosphorylation pattern identity among different receptors. However, there must be some consensus pattern, as there is only a definite number of arrestin isoforms. For the majority of GPCRs, the C-terminus has been described as the key site for phosphorylation. However, this is not supported by all studies; the interaction between the human lutropin receptor and βarr2 is independent of phosphorylation [68], and the substance P receptor still recruits βarr1 and βarr2, independent of C-terminal truncation [69]. For the A3 adenosine receptor, neither the C-terminus nor potential phosphorylation sites in the 3rd IL are crucial for recruitment of βarr2, as described in detail in **Chapter 3**. Therefore, the view on the role of GPCR phosphorylation in the regulation of arrestin-based desensitization and signalling has evolved to some kind of controversy in latest years.

The relevance of phosphorylation as an activator of arrestin recruitment and signalling can be found in the arrestin structure, which can accommodate phosphorylated residues by an extensively studied mechanism. Crystal structures of all four arrestin isoforms in their basal conformation (i.e. not bound to a GPCR) have been available for a while ([70] visual arrestin, [71] βarr1, [72] cone arr, [73] βarr2) and provided early insights into the conformational changes upon phosphate recognition. Arrestin is an elongated molecule consisting of an N-terminal and C-terminal domain, each consisting of a sandwich of 7 β-strands, connected via a hinge-region. The relative orientation of these domains in the basal arrestin conformation is supported by two intra-molecular interactions between groups of key residues, which are conserved in all animal arrestins. These two interactions include the polar core, a network of five interacting virtually solvent-excluded charged

residues between the domains, and the three-element interaction, relying on hydrophobic interactions between the β-strand I, α-helix I and the C-terminus, which strongly connects the C-terminus with the amino-terminal domain (Figure 1.3). These two intramolecular 'locks' are believed to be unfastened by receptor attached phosphates, evoking a global change in the arrestin molecule by release of its C-terminal tail and a rearrangement of the 2 domains relative to each other [48, 55, 57, 74, 75].

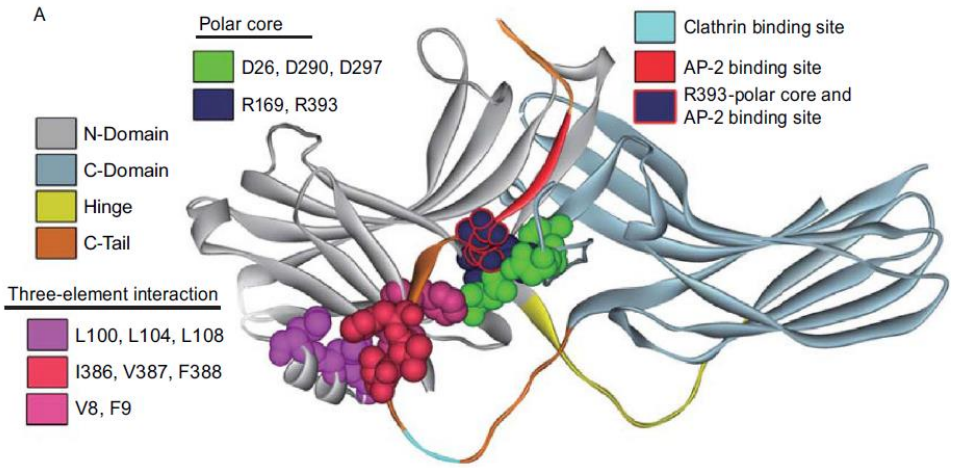


Figure 1.3: Intra-molecular interactions in βarr1 (PDB ID 1G4M) holding the molecule in a basal conformation. Figure adopted from [76].

More valuable information on the receptor-engaged arrestin structure came with the elucidation of the interaction of a β2AR-V2Rtail chimeric receptor with βarr1 by cryo-electron microscopy (cryo-EM) [77]. This study showed that arrestin could be 'hanging' to the GPCR, solely attached via the phosphorylated GPCR C-terminus, or could fully engage the GPCR by binding to additional cytoplasmic GPCR sites [19]. This was in line with previous observations for the rhodopsin receptor [78]. Thus, it was assumed that GPCR-arrestin interaction is biphasic, involving two contact sites; one between the phosphorylated sites of the GPCR and the N-domain of arrestin (partially engaged complex), and one involving the activated receptor, i.e. exposed transmembrane helices and cytoplasmic loops (fully engaged complex). It seems that the fully engaged complex is not always present, and that the partially engaged complex is already sufficient for some functional outcomes of GPCR-βarr interaction such as endocytosis and scaffolding of ERK. For example, the arr-biased βAR ligand carvedilol has only partial association with the GPCR C-terminus (no full

engagement), while still being of pharmacological relevance as there is no G protein activation [79]. Furthermore, in 2015, a higher resolution X-ray free electron laser (XFEL) structure of constitutively active human rhodopsin bound to a pre-activated mouse visual arrestin was solved [14]. However, the phosphorylated rhodopsin C-terminus was missing in this structure and was added later [80]. This revealed a phosphorhodopsin-arrestin interface displaying an intermolecular β sheet together with an extensive network of electrostatic interactions between positive pockets at the N-terminal domain of arrestin and phosphates and/or negatively charged side chains in the C-terminus of rhodopsin; arrestin 'reads' the phosphorylation/negative code on the GPCR through three code-sensing pockets. These positive pockets are highly conserved among the different arrestin isoforms (also between species), but show some differences as well, which might contribute to the specificity for GPCRs. By comparison of the phosphorylation pattern of the rhodopsin and the V2R C-terminus, two types of 'phosphorylation codes' were identified in which the phosphates should be separated by certain distances (or a certain number of residues); PxPxxP/E/D (short code) or PxxPxxP/E/D (long code), in which P is a phospho-serine/threonine and x is any amino acid (except proline in the second xx occurrence). These structural features were validated by biochemical and biophysical experiments and computer modelling [80]. The authors noted that certain phosphorylated residues that had been identified with mass spectrometry, were not present in the crystal structure. This can be explained by the fact that crystal structures give a 'fixed' image of a GPCR, often stabilized by adaptory proteins, and thus, for the best possible interpretation, also data from other biochemical experiments should be taken into account.

Based on the aforementioned resolved structures of different arrestin isoforms, as well as the pioneering studies on GPCR-arrestin complexes, a theory has emerged on the structural features of the GPCR-arrestin interaction process. Hereby, inactive arrestin has its positively charged N-terminal region hidden by intramolecular N-C domain interactions. The first step upon GPCR activation is the interaction of this positively charged region with phosphates in the C-terminus or other loops of the receptor. This breaks the intramolecular N-C lock, thereby releasing the C-terminus of arrestin, which is now available for interaction with adaptor proteins, and triggering a 20° rotation between the N and C domains. By this rearrangement, a concave

surface in arrestin is exposed, which binds the GPCR core more tightly by repositioning multiple arrestin loops (finger loop, lariat loop, middle loop) in the GPCR cavity [2, 67, 80-82]. Hence, in this theory, the formation of the GPCR-arrestin complex is believed to be largely driven by the phosphorylated GPCR C-terminus.

1.1.4.1.b Recent view on arrestin activation

Some recent papers further complexed the view on arrestin activation. In a very recent study of Eichel *et al.* ([83]), a distinct mechanism of βarr activation has been reported for βARs, being possibly C-terminus independent. It involves a transient engagement with the GPCR core; this destabilizes a conserved inter-domain charge network in βarr and promotes its capture at the plasma membrane and subsequent accumulation in clathrin-coated endocytic structures (CCSs). The experiments in this study were based on total internal reflection fluorescence (TIRF) microscopy. Upon stimulation with isoproterenol, βarr2 showed rapid accumulation in CCSs, without co-accumulation of β1AR. In contrast, β2AR did co-accumulate with βarr2 in CCSs, but this was not solely receptor-dependent, as βarr2 also trafficked into CCSs upon receptor immobilization. The C-terminus of the β1AR as well as that of the β2AR was not required to induce βarr2 trafficking. Other GPCRs tested, among which the D2R and μOR also showed a discrete trafficking of βarr2. The authors hypothesized that a transient interaction with the GPCR core is sufficient to activate βarr trafficking; in line with this, a core mutation of the D2R, which has a short C-terminus, prevented βarr2 trafficking. They stated that three charged residues in the 'finger-loop-proximal' region of arrestin function as part of an extensive network of polar residues that form an N- and C- intradomain network. This network stabilizes arrestin in its inactive conformation (as mentioned above), and is destabilized by interaction with the GPCR core, activating arrestin. After dissociation from the GPCR, it was shown that arrestin is stabilized by non-GPCR interactions with membrane phosphoinositides and CCS-lattice proteins. Thus, the GPCR core seems to act catalytically in arrestin activation. Remarkably, this phosphoinositide stabilizing determinant was not required for accumulation of βarr2 in CCSs by a β2AR-V2R chimeric receptor. For this receptor, the phosphorylated C-terminus sufficiently stabilized the arrestin molecule at the plasma membrane. Thus, phosphoinositide binding seems crucial for βarr2 trafficking

after GPCR dissociation, but not when βarr is bound in a sufficiently stable complex by a highly phosphorylated GPCR C-terminus. This 'action at a distance' behaviour seems widespread and introduced a totally new concept in arrestin activation; transient GPCR core engagement can act catalytically or scaffold-driven. The tendency of a GPCR to act by either of the two mechanisms (catalytically or scaffold-driven, to a certain extent) is regulated by the state of phosphorylation of the GPCR C-terminus.

In fact, and even stronger, it was shown by Latorraca *et al.* ([84]) that the activated GPCR core ànd cytoplasmic tail can each independently and individually stabilize the active arrestin conformation, and binding of both further stabilizes this, which is indicative of an allosteric interaction between both. Thus, not both core and C-terminal sites have to be engaged simultaneously to activate arrestin **(**Figure 1.4). These authors tried to reveal the rhodopsin-arrestin1 interaction through extensive atomic-level simulations from the crystal structure, in which either the GPCR phosphorylated C-terminus (Rp) was removed, (leaving only the GPCR core), or the receptor core (leaving only the Rp tail). The 20° twist angle between the arrestin N- and C-domain, amongst other conformational changes, was used as a measure of arrestin activation. It was concluded that the GPCR core cytoplasmic loops (mainly IL2 and IL3) seem to be the main determinants for arrestin activation by interaction with the arrestin 'body' (N-C domain interaction), rather than the rearrangement of the arrestin finger loop in the GPCR core, which was until now believed to be a major trigger for arrestin activation. They also found that arrestin frequently adopts the active arrestin conformation in absence of an activating GPCR, which might explain the arrestins' ability to remain active after GPCR dissociation. This supports the above catalytically-driven theory as well.

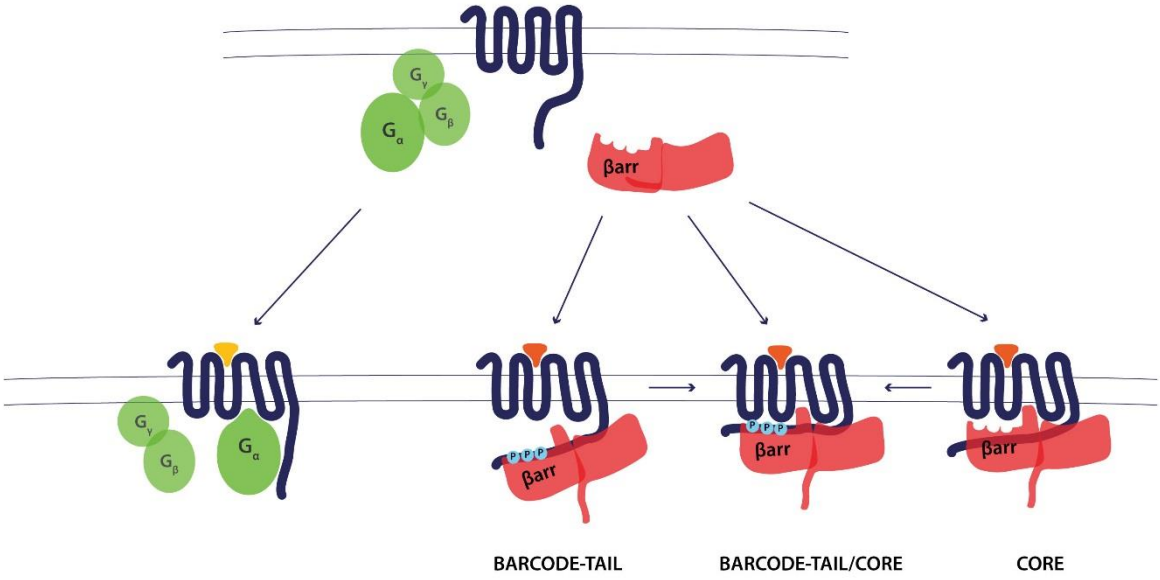


Figure 1.4: Schematic representation of G protein and arrestin interaction with the activated GPCR. Both signalling proteins seem to require an outward shift of GPCR TM6 to accommodate a cavity in the GPCR transmembrane bundle. The paradigm on arrestin activation has changed a lot throughout recent years. Phosphorylated residues in the C-terminus (or cytoplasmic loops) have always been considered as a major trigger for arrestin activation; this led to the phospho-barcode-hypothesis. During the last few years, a more important role has been assigned to the activated GPCR core in arrestin activation. Initially, it was thought that both phospho-sites and core were required to obtain high affinity binding, in a step-wise manner. However, the most recent studies (mutational functional studies – non-exhaustive overview given in [84] –, structural studies and computer modelling) state that both the GPCR core and the C-terminus are able to independently activate arrestin. Until now, it is not known if there are functional consequences of the arrestin binding/activation mode on GPCR trafficking or arrestin downstream signalling.

1.1.4.2 GPCR-βarr biased signalling

There has been an increasing focus in GPCR drug discovery on the identification of biased ligands that selectively activate the G protein- or β-arrestin signalling pathway [85, 86]. Most research has been on ligands that show an extreme case of ligand bias: herein, stimulation of one pathway occurs without (detectable) stimulation of the other pathway. Some important discoveries have been made for the angiotensin receptor system, the β-adrenergic receptor system, the dopaminergic receptor system, the opioid receptor system and the cannabinoid receptor system [15, 30, 53]. These are highlighted in depth in **Chapter 6**. Biased ligands that exhibit bias to a

certain degree (imperfect bias) are also of high interest. Unfortunately, there is no such thing as a pattern in receptor features that are required for stimulating G protein without or with less activation of β-arrestin, or vice versa. The aforementioned studies on the GPCR-arrestin interaction highlight the knowledge gaps and the relevance of ongoing research on this topic with the eye on elucidating key molecular features for biased signalling [54, 87, 88].

While the exact molecular mechanism of G protein versus arrestin biased GPCR signalling is still not clear, it has recently been explored by comparison of the crystal structure of the β2AR-G_s protein complex [13] with that of the rhodopsin-arr1 complex [14, 81]. For class A GPCRs, the GPCR-G protein interface has been reported for the β2AR (with G_s) and the A_{2A}AR (with mini-G_s) [13, 89]. Both structures show the interaction of the C-terminal domain of the $G_{\alpha s}$ subunit with the cavity that opens up on the cytoplasmic side of the activated GPCR by the outward movement of the cytoplasmic end of (TM5), TM6 and IL3. The crystal structure of pre-activated βarr1 with a C-terminal phosphopeptide of the V2R revealed an interaction between the receptor C-terminus and the N-terminal β strand of arrestin, and additional charge interactions between the phosphates and the positive residues in both strands, respectively [90]. Upon agonist binding, there is a C-terminal extension of TM5, accompanied by an outward movement of TM6 and IL3, which opens an intracellular pocket in the GPCR transmembrane bundle, which can accommodate both G protein and arrestin. It is observed that for accommodating G protein there is a larger outward movement of TM6 (14 Å) than for arrestin (8 Å); thus, remarkably, the difference in GPCR conformation between G protein- and arrestin-bound state is relatively small. However, it is the phosphorylation and conformational change in the GPCR C-terminus that is characteristic for arrestin binding [80]. It also seems that, at least in the case of rhodopsin, the interaction of the GPCR C-terminus to the N domain of arrestin results in a conformational change, which may help TM7 and helix 8 in their association with arrestin to achieve full arrestin engagement [81].

In the meantime, structures of GPCR-G protein complexes have increasingly been reported; the structure of the β2AR-G_s protein complex [13] has been supplemented with the structure of the A_{2A}AR in complex with mini-$G_{\alpha s}$, resolved by crystallography

[89], as well as in complex with an engineered heterotrimeric G protein complex, including mini-$G_{\alpha s}$ and a stabilizing nanobody, resolved by cryo-EM [91]. With respect to G_i-coupling GPCRs, the structures of μOR-G_{i1} [92], A_1AR-G_{i2} [93], the 5HT1B receptor-G_o [94], and light-rhodopsin-G_{i1} [95] have been reported. All these structures reveal a GPCR-G protein interface consisting of the outward movement of TM6 to a larger (for G_s) or smaller (for G_i) extent, via which the GPCR pocket can accommodate the G protein C-terminal α5 helix. Based on these data, it has been suggested that GPCR specificity towards signalling proteins, either G protein, arrestin, or other signalling molecules, might rely on 'pocket complementarity' rather than on the interaction with specific conserved amino acids. In this model, the structural rearrangement in the GPCR upon activation induces a pocket region that is conducive to the binding of G protein subtypes or arrestin subtypes [96].

We are very close to the elucidation of key structural features of the GPCR binding interface that distinguishes between selectivity towards G protein and arrestin, but at the same time, a lot of structural similarities are found as well. Fitting all these structural studies together with functional studies on GPCR-G protein and -arrestin coupling will provide new insights that could help in the design of biased ligands for drug development. Obviously, assessment of candidate biased ligands as therapeutic leads will still require testing in the biological setting (*in vivo* models).

1.1.4.3 G protein- and βarr-dependent signalling: do these pathways cross again?

Despite the efforts to dissect G protein- versus arrestin-dependent pathways with the eye on biased signalling, it was not clear if both signalling pathways could occur fully independently, i.e. if arrestin signalling is possible without any G protein activity present or vice versa. This has been addressed by recent studies [97-101]. Grundmann *et al.* ([99]) used the genome-editing CRISPR/Cas9 technology for depletion of βarrs or G proteins, in combination with pharmacological G protein inhibition by specific G protein inhibitors (such as pertussis toxin for $G_{i/o}$), creating "zero G protein" or "zero arrestin" HEK293 cells. Special emphasis was placed on ERK1/2 phosphorylation, as this pathway downstream of G protein or arrestin is known to be critical for cell survival, growth and proliferation, and generally receives

considerable attention in high-throughput drug screens for biased ligands that display arrestin-(in)dependent signalling. A sustained βarr recruitment, but a loss of ERK1/2 phosphorylation, was observed upon genomic elimination of G protein signalling. Therefore, it was concluded that G proteins and not arrestins act as main drivers for ERK signalling, and this for the β2AR, the AT1R and the V2R, as well as for three other GPCRs. For the β2AR, it had already been shown that initiation of ERK signalling was rather βarr-independent, with critical involvement of G_s protein, and that depletion of βarr enhanced ERK signalling [98]. These studies mainly point at arrestins acting as scaffolds for stabilization of the ERK signalling cascade, critically regulating ERK signal amplitude and duration; an action that implies arrestin activation downstream – but not independent – of G proteins.

Mechanistically, the existence of so-called ternary 'supercomplexes', including the GPCR, arrestin and G protein, fit in this view of interplay between G proteins and arrestins in GPCR signalling [97]. Within this conceptual framework, it is possible that a biased ligand induces a receptor conformation that can simultaneously accommodate both G protein and arrestin, and for which all interacting partners in the complex allosterically influence the GPCR conformation. Concrete studies on this have appeared for the β1AR and the AT1R [102, 103]. The use of genome-edited cell lines might be of relevance for future assessment of the relative contribution of certain signalling pathways to the variable(s) ultimately measured when evaluating a biased ligand. However, it seems that the effects of arrestins on GPCR desensitization, internalization and (ERK) signalling are different concerning G protein-(in)dependence, and also differ between GPCRs and cellular backgrounds. Additionally, deletion of both βarrs is possible in an *in vitro* setting, but not readily in an *in vivo* setting, again questioning the relevance of genome-edited cell systems. Therefore, one must stay cautious with extrapolating *in vitro* results to other GPCRs, and definitely to native systems [101].

1.2 Aims and Outline of this thesis

Biased signalling has emerged as a new, challenging concept in current drug discovery research targeting G protein-coupled receptors (GPCRs). Tremendous efforts have been made during the last decade to discover the molecular features of G protein versus arrestin coupling, and to evaluate if there is a consensus in the binding sites for all or subsets of GPCRs. Knowledge on this will aid in the directed design of highly selective, biased ligands, introducing a new chapter in drug discovery. These biased ligands could represent safer therapeutics, with less on-target side effects, or with less risk for drug tolerance or drug overdose. Besides the synthesis of new compounds, existing drug molecules might be fine-tuned according to these new findings. At the moment, there is no comprehensive molecular model available for the prediction of specificity towards G protein or arrestin signalling. However, continuous research has been performed for prototypical GPCR models, including rhodopsin, the adrenergic receptors, opioid receptors, the vasopressin receptor and purinergic receptors. The structural paradigm on arrestin activation has evolved substantially during the last decade; the phosphorylated GPCR C-terminus as a trigger for arrestin coupling has now been supplemented with a role for the GPCR core. The structural basis relies on pioneer studies on crystallography, cryo-EM, computer homology modelling, and many more techniques. Besides, functional assays have shown to be highly valuable as they evaluate confounding factors such as receptor expression level and cellular context.

For the aforementioned prototypical GPCR systems, biased ligands that exploit or avoid the arrestin pathway have shown promising results for future drug therapies with possible clinical relevance (discussed in **Chapter 6**). However, for purinergic receptors, functional selectivity is only just being reported – an overview is given in **Chapter 2** – but is scarce with respect to **β-arrestin (βarr)** signalling. Therefore, the aim of **Chapter 3** and **Chapter 5** was to explore **the human A_3 adenosine receptor (A_3AR)** and **the P2Y2 receptor**, members of the adenosine receptor (P1) and P2Y receptor subfamily, respectively, for their **contact sites with βarr2**, to see if we could find any similarities with the current view on arrestin coupling. β-arrestin coupling is evaluated in a HEK293T cell line, using a **live-cell reporter system based on the**

functional complementation of the bioluminescent nanoluciferase (NanoLuc) enzyme (Figure 1.5).

In **Chapter 4**, the assay set-up is transferred to a stable HEKT293T cell line for **screening of a panel of (newly synthesized) synthetic A_3AR ligands**. The activity profiles obtained for βarr2 recruitment are compared to those for G_i protein-dependent cAMP signalling in order to **elucidate a possible functional selectivity relationship**.

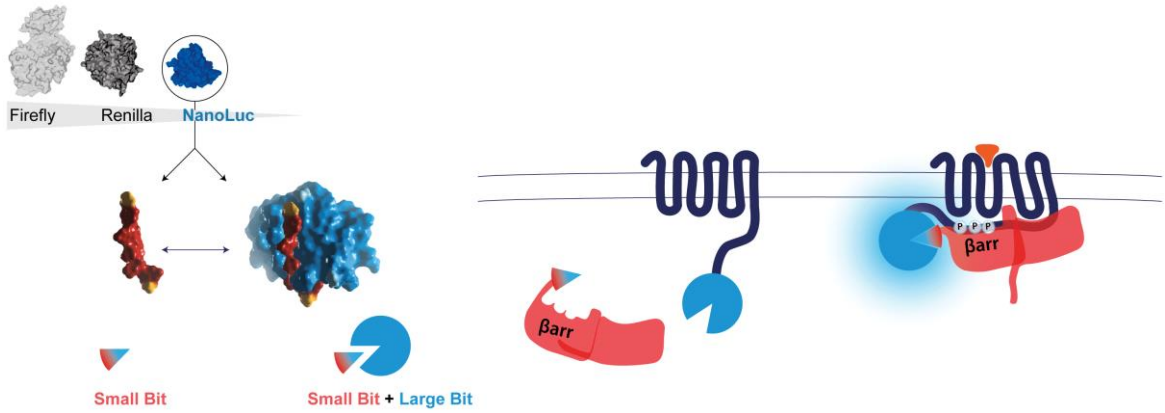


Figure 1.5: Depiction of the small NanoLuc luciferase (19 kDa) - compared to Firefly luciferase (61 kDa) and Renilla luciferase (36 kDa) - and its splitted parts for fusion to two interacting proteins of interest: the GPCR and βarr2. Picture partly altered from Promega Company and [104] Copyright © 2015 American Chemical Society

References

[1] Rosenbaum DM, Rasmussen SGF, Kobilka BK. The structure and function of G-protein-coupled receptors. Nature 2009;459:356-63.

[2] Gurevich VV, Gurevich EV. Molecular Mechanisms of GPCR Signaling: A Structural Perspective. Int J Mol Sci 2017;18(12):2519.

[3] Lagerstrom MC, Schioth HB. Structural diversity of G protein-coupled receptors and significance for drug discovery. Nat Rev Drug Discov 2008;7:339-57.

[4] Stoy H, Gurevich VV. How genetic errors in GPCRs affect their function: Possible therapeutic strategies. Genes Dis 2015;2:108-32.

[5] Ovchinnikov YA. Rhodopsin and Bacteriorhodopsin - Structure-Function-Relationships. Febs Lett 1982;148:179-91.

[6] Mechoulam R, Parker LA. The Endocannabinoid System and the Brain. Annu Rev Psychol 2013;64:21-47.

[7] Tan L, Yan W, McCorvy JD, Cheng J. Biased Ligands of G Protein-Coupled Receptors (GPCRs): Structure-Functional Selectivity Relationships (SFSRs) and Therapeutic Potential. J Med Chem 2018.

[8] Burnstock G. Purinergic signalling: from discovery to current developments. Exp Physiol 2014;99:16-34.

[9] Gether U. Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. Endocr Rev 2000;21:90-113.

[10] Hermans E. Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. Pharmacol Therapeut 2003;99:25-44.

[11] Marinissen MJ, Gutkind JS. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 2001;22:368-76.

[12] Magalhaes AC, Dunn H, Ferguson SS. Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. Brit J Pharmacol 2012;165:1717-36.

[13] Rasmussen SGF, DeVree BT, Zou YZ, Kruse AC, Chung KY, Kobilka TS, et al. Crystal structure of the beta(2) adrenergic receptor-Gs protein complex. Nature 2011;477:549-U311.

[14] Kang YY, Zhou XE, Gao X, He YZ, Liu W, Ishchenko A, et al. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. Nature 2015;523:561-7.

[15] Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 2007;28:416-22.

[16] Kenakin T. Functional Selectivity and Biased Receptor Signaling. J Pharmacol Exp Ther 2011;336:296-302.

[17] Manglik A, Kim TH, Masureel M, Altenbach C, Yang ZY, Hilger D, et al. Structural Insights into the Dynamic Process of beta(2)-Adrenergic Receptor Signaling. Cell 2015;161.5:1101-11.

[18] Violin JD, Crombie AL, Soergel DG, Lark MW. Biased ligands at G-protein-coupled receptors: promise and progress. Trends Pharmacol Sci 2014;35:308-16.

[19] Shukla AK, Singh G, Ghosh E. Emerging structural insights into biased GPCR signaling. Trends Biochem Sci 2014;39:594-602.

[20] Rankovic Z, Brust TF, Bohn LM. Biased agonism: An emerging paradigm in GPCR drug discovery. Bioorg Med Chem Lett 2016;26:241-50.

[21] Bologna Z, Teoh JP, Bayoumi AS, Tang Y, Kim IM. Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology. Biomol Ther 2017;25:12-25.
[22] Kenakin T, Miller LJ. Seven Transmembrane Receptors as Shapeshifting Proteins: The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. Pharmacol Rev 2010;62:265-304.
[23] Gao ZG, Jacobson KA. Allosteric modulation and functional selectivity of G protein-coupled receptors. Drug Discov Today Technol 2013;10:e237-43.
[24] Edelstein SJ, Changeux JP. Biased Allostery. Biophys J 2016;111:902-8.
[25] Kenakin T, Christopoulos A. OPINION Signalling bias in new drug discovery: detection, quantification and therapeutic impact. Nat Rev Drug Discov 2013;12:205-16.
[26] Thomsen W, Frazer J, Unett D. Functional assays for screening GPCR targets. Curr Opin Biotech 2005;16:655-65.
[27] Michelini E, Cevenini L, Mezzanotte L, Coppa A, Roda A. Cell-based assays: fuelling drug discovery. Anal Bioanal Chem 2010;398:227-38.
[28] Martins SAM, Trabuco JRC, Monteiro GA, Chu V, Conde JP, Prazeres DMF. Towards the miniaturization of GPCR-based live-cell screening assays. Trends Biotechnol 2012;30:566-74.
[29] Zhang R, Xie X. Tools for GPCR drug discovery. Acta Pharmacol Sin 2012;33:372-84.
[30] Rajagopal S, Rajagopal K, Lefkowitz RJ. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov 2010;9:373-86.
[31] Black JW, Leff P. Operational Models of Pharmacological Agonism. Proc R Soc Ser B-Bio 1983;220:141-62.
[32] 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.
[33] Ehlert FJ. Analysis of allosterism in functional assays. Journal of Pharmacol Exp Ther 2005;315:740-54.
[34] Kenakin T. A Scale of Agonism and Allosteric Modulation for Assessment of Selectivity, Bias, and Receptor Mutation. Mol Pharmacol 2017;92:414-24.
[35] Wilden U, Hall SW, Kuhn H. Phosphodiesterase Activation by Photoexcited Rhodopsin Is Quenched When Rhodopsin Is Phosphorylated and Binds the Intrinsic 48-Kda Protein of Rod Outer Segments. Proc Natl Acad Sci USA 1986;83:1174-8.
[36] Hausdorff WP, Caron MG, Lefkowitz RJ. Turning Off the Signal - Desensitization of Beta-Adrenergic-Receptor Function. Faseb J 1990;4:2881-9.
[37] Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ. beta-Arrestin: a protein that regulates beta-adrenergic receptor function. Science 1990;248:1547-50.
[38] DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. beta-arrestins and cell signaling. Annu Rev Physiol 2007;69:483-510.
[39] Shenoy SK, Lefkowitz RJ. Multifaceted roles of beta-arrestins in the regulation of seven-membrane-spanning receptor trafficking and signalling. Biochem J 2003;375:503-15.

[40] Ferguson SSG. Evolving concepts in G protein-coupled receptor endocytosis: The role in receptor desensitization and signaling. Pharmacol Rev 2001;53:1-24.
[41] Shenoy SK, Lefkowitz RJ. beta-Arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci 2011;32:521-33.
[42] Krupnick JG, Goodman OB, Keen JH, Benovic JL. Arrestin/clathrin interaction - Localization of the clathrin binding domain of nonvisual arrestins to the carboxyl terminus. J Biol Chem 1997;272:15011-6.
[43] Beautrait A, Paradis JS, Zimmerman B, Giubilaro J, Nikolajev L, Armando S, et al. A new inhibitor of the beta-arrestin/AP2 endocytic complex reveals interplay between GPCR internalization and signalling. Nat Commun 2017;8:15054.
[44] Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem 2000;275:17201-10.
[45] Kohout TA, Lin FT, Perry SJ, Conner DA, Lefkowitz RJ. beta-arrestin 1 and 2 differentially regulate heptahelical receptor signaling and trafficking. Proc Natl Acad Sci USA 2001;98:1601-6.
[46] Eichel K, Jullie D, von Zastrow M. beta-Arrestin drives MAP kinase signalling from clathrin-coated structures after GPCR dissociation. Nat Cell Biol 2016;18:303-10.
[47] Witherow DS, Garrison TR, Miller WE, Lefkowitz RJ. beta-Arrestin inhibits NF-kappa B activity by means of its interaction with the NF-kappa B inhibitor I kappa B alpha. Proc Natl Acad Sci USA 2004;101:8603-7.
[48] Gurevich EV, Gurevich VV. Arrestins: ubiquitous regulators of cellular signaling pathways. Genome Biol 2006;7:236.
[49] Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. Science 2005;308:512-7.
[50] Lefkowitz RJ, Rajagopal K, Whalen EJ. New roles for beta-arrestins in cell signaling: Not just seven-transmembrane receptors. Mol Cell 2006;24:643-52.
[51] Peterson YK, Luttrell LM. The Diverse Roles of Arrestin Scaffolds in G Protein-Coupled Receptor Signaling. Pharmacol Rev 2017;69:256-97.
[52] Spiller BW, Zhan X, Gurevich VV. Arrestin-3: The Structural Basis of Lower Receptor Selectivity. The Structural Basis of Arrestin Functions: Springer, 2017. p. 59-67.
[53] Whalen EJ, Rajagopal S, Lefkowitz RJ. Therapeutic potential of beta-arrestin- and G protein-biased agonists. Trends Mol Med 2011;17:126-39.
[54] Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular Mechanism of beta-Arrestin-Biased Agonism at Seven-Transmembrane Receptors. Annu Rev Pharmacol 2012;52:179-97.
[55] Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol Therapeut 2006;110:465-502.
[56] Gurevich VV, Gurevich EV. Structural Determinants of Arrestin Functions. Prog Mol Biol Transl 2013;118:57-92.
[57] Ostermaier MK, Schertler GFX, Standfuss J. Molecular mechanism of phosphorylation-dependent arrestin activation. Curr Opin Struc Biol 2014;29:143-51.

[58] Vishnivetskiy SA, Ostermaier MK, Singhal A, Panneels V, Homan KT, Glukhova A, et al. Constitutively active rhodopsin mutants causing night blindness are effectively phosphorylated by GRKs but differ in arrestin-1 binding. Cell Signal 2013;25:2155-62.
[59] Nobles KN, Xiao KH, Ahn S, Shukla AK, Lam CM, Rajagopal S, et al. Distinct Phosphorylation Sites on the beta(2)-Adrenergic Receptor Establish a Barcode That Encodes Differential Functions of beta-Arrestin. Sci Signal 2011;4:ra51.
[60] Ren XR, Reiter E, Ahn S, Kim J, Chen W, Lefkowitz RJ. Different G protein-coupled receptor kinases govern G protein and beta-arrestin-mediated signaling of V2 vasopressin receptor. Proc Natl Acad Sci USA 2005;102:1448-53.
[61] Kim J, Ahn S, Ren XR, Whalen EJ, Reiter E, Wei HJ, et al. Functional antagonism of different G protein-coupled receptor kinases for beta-arrestin-mediated angiotensin II receptor signaling. Proc Natl Acad Sci USA 2005;102:1442-7.
[62] Kim OJ, Gardner BR, Williams DB, Marinec PS, Cabrera DM, Peters JD, et al. The role of phosphorylation in D-1 dopamine receptor desensitization - Evidence for a novel mechanism of arrestin association. J Biol Chem 2004;279:7999-8010.
[63] Chen YJ, Oldfield S, Butcher AJ, Tobin AB, Saxena K, Gurevich VV, et al. Identification of phosphorylation sites in the COOH-terminal tail of the mu-opioid receptor. J Neurochem 2013;124:189-99.
[64] DeWire SM, Yamashita DS, Rominger DH, Liu GD, Cowan CL, Graczyk TM, et al. A G Protein-Biased Ligand at the mu-Opioid Receptor Is Potently Analgesic with Reduced Gastrointestinal and Respiratory Dysfunction Compared with Morphines. J Pharmacol Exp Ther 2013;344:708-17.
[65] Birdsong WT, Arttamangkul S, Bunzow JR, Williams JT. Agonist Binding and Desensitization of the mu-Opioid Receptor Is Modulated by Phosphorylation of the C-Terminal Tail Domain. Mol Pharmacol 2015;88:816-24.
[66] Hinz L, Ahles A, Ruprecht B, Kuster B, Engelhardt S. Two serines in the distal C-terminus of the human beta(1)-adrenoceptor determine beta-arrestin2 recruitment. Plos One 2017;12:e0176450.
[67] Yang Z, Yang F, Zhang DL, Liu ZX, Lin A, Liu C, et al. Phosphorylation of G Protein-Coupled Receptors: From the Barcode Hypothesis to the Flute Model. Mol Pharmacol 2017;92:201-10.
[68] Min L, Galet C, Ascoli M. The association of arrestin-3 with the human lutropin/choriogonadotropin receptor depends mostly on receptor activation rather than on receptor phosphorylation. J Biol Chem 2002;277:702-10.
[69] Richardson MD, Balius AM, Yamaguchi K, Freilich ER, Barak LS, Kwatra MM. Human substance P receptor lacking the C-terminal domain remains competent to desensitize and internalize. J Neurochem 2003;84:854-63.
[70] Hirsch JA, Schubert C, Gurevich VV, Sigler PB. The 2.8 angstrom crystal structure of visual arrestin: A model for arrestin's regulation. Cell 1999;97:257-69.
[71] Han M, Gurevich VV, Vishnivetskiy SA, Sigler PB, Schubert C. Crystal structure of beta-arrestin at 1.9 angstrom: Possible mechanism of receptor binding and membrane translocation. Structure 2001;9:869-80.

[72] Sutton RB, Vishnivetskiy SA, Robert J, Hanson SM, Raman D, Knox BE, et al. Crystal structure of cone arrestin at 2.3 angstrom: Evolution of receptor specificity. J Molecular Biol 2005;354:1069-80.
[73] Zhan XZ, Gimenez LE, Gurevich VV, Spiller BW. Crystal Structure of Arrestin-3 Reveals the Basis of the Difference in Receptor Binding Between Two Non-visual Subtypes. J Mol Biol 2011;406:467-78.
[74] Gurevich VV, Gurevich EV. The molecular acrobatics of arrestin activation. Trends Pharmacol Sci 2004;25:105-11.
[75] Gurevich VV, Gurevich EV. Extensive shape shifting underlies functional versatility of arrestins. Curr Opin Cell Biol 2014;27:1-9.
[76] Gurevich VV, Gurevich EV, Uversky VN. Arrestins: structural disorder creates rich functionality. Protein & cell 2018:1-18.
[77] Shukla AK, Westfield GH, Xiao KH, Reis RI, Huang LY, Tripathi-Shukla P, et al. Visualization of arrestin recruitment by a G-protein-coupled receptor. Nature 2014;512:218-22.
[78] Gurevich VV, Hanson SM, Song XF, Vishnivetskiy SA, Gurevich EV. The functional cycle of visual arrestins in photoreceptor cells. Prog Retin Eye Res 2011;30:405-30.
[79] Kumari P, Srivastava A, Banerjee R, Ghosh E, Gupta P, Ranjan R, et al. Functional competence of a partially engaged GPCR-beta-arrestin complex. Nat Commun 2016;7:13416.
[80] Zhou XE, He YZ, de Waal PW, Gao X, Kang YY, Van Eps N, et al. Identification of Phosphorylation Codes for Arrestin Recruitment by G Protein-Coupled Receptors. Cell 2017;170:457-+.
[81] Zhou XE, Melcher K, Xu HE. Understanding the GPCR biased signaling through G protein and arrestin complex structures. Curr Opin Struc Biol 2017;45:150-9.
[82] Chen QY, Iverson TM, Gurevich VV. Structural Basis of Arrestin-Dependent Signal Transduction. Trend Biochem Sci 2018;43:412-23.
[83] Eichel K, Jullie D, Barsi-Rhyne B, Latorraca NR, Masureel M, Sibarita JB, et al. Catalytic activation of beta-arrestin by GPCRs. Nature 2018;557:381-6.
[84] Latorraca NR, Wang JK, Bauer B, Townshend RJL, Hollingsworth SA, Olivieri JE, et al. Molecular mechanism of GPCR-mediated arrestin activation. Nature 2018;557:452-6.
[85] Shukla AK, Xiao KH, Lefkowitz RJ. Emerging paradigms of beta-arrestin-dependent seven transmembrane receptor signaling. Trends Biochem Sci 2011;36:457-69.
[86] Wisler JW, Xiao KH, Thomsen ARB, Lefkowitz RJ. Recent developments in biased agonism. Curr Opin Cell Biol 2014;27:18-24.
[87] Draper-Joyce CJ, Christopoulos A. Strength in numbers—an arrestin interaction code. Nat Struct Mol Biol 2018;25:437.
[88] Sente A, Peer R, Srivastava A, Baidya M, Lesk AM, Balaji S, et al. Molecular mechanism of modulating arrestin conformation by GPCR phosphorylation. Nat Struct Mol Biol 2018;25:538.
[89] Carpenter B, Nehmé R, Warne T, Leslie AG, Tate CG. Structure of the adenosine A 2A receptor bound to an engineered G protein. Nature 2016;536:104.
[90] Shukla AK, Manglik A, Kruse AC, Xiao KH, Reis RI, Tseng WC, et al. Structure of active beta-arrestin-1 bound to a G-protein-coupled receptor phosphopeptide. Nature 2013;497:137-41.

[91] Garcia-Nafria J, Lee Y, Bai XC, Carpenter B, Tate CG. Cryo-EM structure of the adenosine A(2A) receptor coupled to an engineered heterotrimeric G protein. Elife 2018;7:e35946.
[92] Koehl A, Hu H, Maeda S, Zhang Y, Qu Q, Paggi JM, et al. Structure of the micro-opioid receptor-Gi protein complex. Nature 2018;558:547-52.
[93] Draper-Joyce CJ, Khoshouei M, Thal DM, Liang YL, Nguyen ATN, Furness SGB, et al. Structure of the adenosine-bound human adenosine A1 receptor-Gi complex. Nature 2018;558:559-63.
[94] Garcia-Nafria J, Nehme R, Edwards PC, Tate CG. Cryo-EM structure of the serotonin 5-HT1B receptor coupled to heterotrimeric Go. Nature 2018;558:620-3.
[95] Kang YY, Kuybeda O, de Waal PW, Mukherjee S, Van Eps N, Dutka P, et al. Cryo-EM structure of human rhodopsin bound to an inhibitory G protein. Nature 2018;558:553-58.
[96] Capper MJ, Wacker D. A complex story of receptor signalling. Nature 2018;558:529-30.
[97] Thomsen ARB, Plouffe B, Cahill TJ, 3rd, Shukla AK, Tarrasch JT, Dosey AM, et al. GPCR-G Protein-beta-Arrestin Super-Complex Mediates Sustained G Protein Signaling. Cell 2016;166:907-19.
[98] O'Hayre M, Eichel K, Avino S, Zhao X, Steffen DJ, Feng X, et al. Genetic evidence that beta-arrestins are dispensable for the initiation of beta2-adrenergic receptor signaling to ERK. Sci Signal 2017;10.484:eaal3395.
[99] Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, et al. Lack of beta-arrestin signaling in the absence of active G proteins. Nat Commun 2018;9:341.
[100] Gutkind JS, Kostenis E. Arrestins as rheostats of GPCR signalling. Nat Rev Mol Cell Biol 2018;19:615-6.
[101] Luttrell LM, Wang J, Plouffe B, Smith JS, Yamani L, Kaur S, et al. Manifold roles of beta-arrestins in GPCR signaling elucidated with siRNA and CRISPR/Cas9. Sci Signal 2018;11.549:eaat7650.
[102] Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, et al. Galphai is required for carvedilol-induced beta1 adrenergic receptor beta-arrestin biased signaling. Nat Commun 2017;8:1706.
[103] Wang J, Hanada K, Gareri C, Rockman HA. Mechanoactivation of the angiotensin II type 1 receptor induces beta-arrestin-biased signaling through Galphai coupling. J Cell Biochem 2018;119:3586-97.
[104] Dixon AS, Schwinn MK, Hall MP, Zimmerman K, Otto P, Lubben TH, et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS Chem Biol 2016;11:400-8.

Chapter 2:
Focus on Purinergic Signalling

A role for functional selectivity between G protein- and β-arrestin dependent signalling pathways in future purinergic drug development

2.1 Introduction on purinergic receptors

2.1.1 Classification and endogenous ligands

2.1.2 (Patho)physiological role as drug targets

2.1.2.1 Adenosine receptors

2.1.2.2 P2Y receptors

2.1.3 Structure of purinergic receptor ligands

2.1.3.1 Adenosine receptors

2.1.3.2 P2Y receptors

2.2 Functional selectivity in purinergic receptor signalling

2.2.1 Adenosine receptors

2.2.2 P2Y receptors

2.3 β-arrestins in functional selectivity of purinergic receptor signalling

2.3.1 Adenosine receptors

2.3.2 P2Y receptors

2.4 Conclusive thoughts

2.1 Introduction on purinergic receptors

2.1.1 Classification and endogenous ligands

Purinergic receptors are generally divided into two major classes; P1 receptors, better known as adenosine receptors (ARs), which contain four members that are activated by the nucleoside adenosine, and P2 receptors, which are activated by adenosine 5'-triphosphate (ATP), and related nucleotides (ADP, UTP, UDP). The P2 receptors are subdivided into the subclass of ligand-gated ion channels or ionotropic P2X receptors, and the subclass of metabotropic P2Y receptors (P2YRs). The P2Y receptors and P1 adenosine receptors belong to the superclass of G protein-coupled receptors (GPCRs) [1] (Figure 2.1). This chapter provides an overview on the functional selectivity of the signalling by these G protein-coupled purinergic receptors, with a particular focus on the β-arrestin (βarr) pathway.

Adenosine is an endogenous molecule that is present in each cell and in all extracellular fluids of the human body. It has a classical "helper" role in the protection of cells against stressful conditions, by enabling them to reduce energy-consuming activities. Under normal physiological conditions, extracellular adenosine levels are low (20 - 300 nM), rising to low μM levels under extreme physiological situations (e.g. intensive exercise) and about 30 μM levels under conditions of cellular stress: oxidative stress, inflammation, ischemia, hypoxia, or when cell damage occurs. When present extracellularly, adenosine exerts its effect mainly by binding to ARs at the cell surface. These are widespread throughout the human body, both in the central nervous system (CNS) and in peripheral tissues. The main effect evoked by adenosine has led to a sub-classification into four subtypes of ARs; the A_1AR and A_3AR, which mainly couple to G_i protein, while the A_{2A}AR and A_{2B}AR mainly couple to G_s protein [2-4]. The sequence similarity between ARs, both intra- and inter-species, is relatively high; human A_{2A}AR/A_{2B}AR have a sequence identity of 46%, and the similarity between A_{2A} and A_1 receptors is 37%, and 31% for A_3AR. However, the difference in amino acid sequence between human and rat A_3AR is 30% [1].

ATP is released into the extracellular space as an autocrine/paracrine molecule in response to neuronal stimulation, platelet aggregation, stress, mechanical stimulation

and other mechanisms. The concentration of nucleotides required in the extracellular space for P2Y receptor activation ranges from 0.1 to 10 μM. In contrast, millimolar concentrations exist intracellularly, but because of their net negative charge, nucleotides require active transport over the plasma membrane (PM). Extracellular nucleotides trigger numerous (patho)physiological functions, including neurotransmission, (cardiac) muscle contraction, inflammation, pain initiation, vascular tone, platelet activation in haemostasis and thrombosis, and more.

The P2Y purinergic receptor class consists of eight GPCR subtypes; P2Y1R, P2Y2R, P2Y4R, P2Y6R, P2Y11R, P2Y12R, P2Y13R, and P2Y14R, in which the numbering reflects the chronological order of cDNA cloning. These subtypes can be divided pharmacologically into: (i) adenine nucleotide-preferring receptors, responding to ATP and/or ADP (P2Y1R, P2Y11R, P2Y12R, and P2Y13R), (ii) uracil nucleotide-preferring receptors, responding to UTP and/or UDP (P2Y4R and P2Y6R), (iii) a receptor of mixed selectivity, responding to UTP and ATP (P2Y2R), and (iv) receptors responding to sugar nucleotides UDP-glucose and -galactose (P2Y14R). Besides, a division can be made based on sequence similarity, presence of amino acid motifs important for ligand binding, and primary G protein coupling. The P2Y1R, P2Y2R, P2Y4R, P2Y6R, and P2Y11R share moderate (28-52%) sequence homology and principally couple to $G_{q/11}$, whereas the P2Y12R, P2Y13R, and P2Y14R share 45-50% sequence homology and principally couple to $G_{i/o}$ [1, 5, 6].

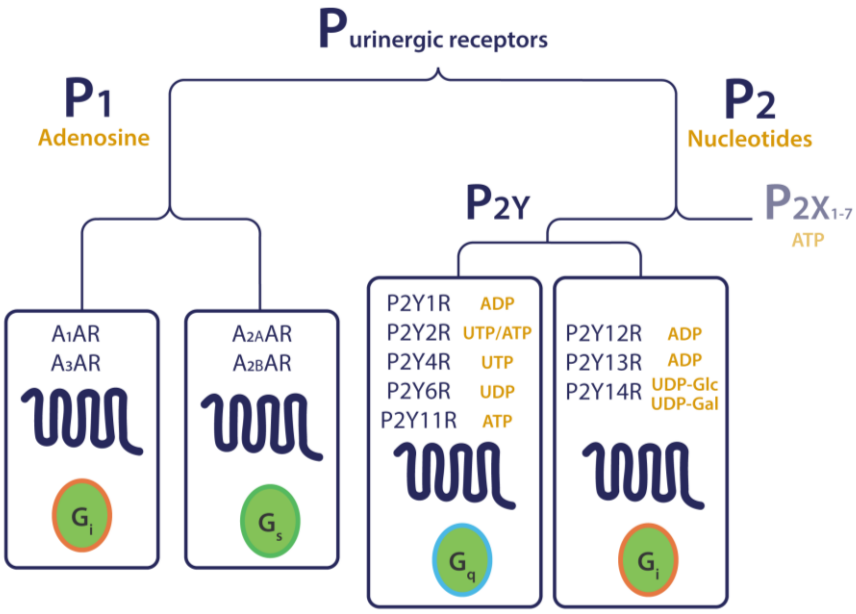


Figure 2.1: Classification of purinergic receptors into P1 or adenosine receptors and P2 receptors. P2 receptors are subdivided into P2X receptors (ligand-gated ion channels) and P2Y receptors. The P1 and P2Y subfamilies are GPCRs, activated by endogenous adenosine or (sugar-) nucleotides.

2.1.2 (Patho)physiological role as drug targets

The (patho)physiological role of the different adenosine and P2Y receptors in their native expression systems, and their potential as biomarkers and/or drug targets for various states of disease has been the topic of several excellent recent reviews. Multiple ligands with potential therapeutic value have been developed, which are currently entering/in clinical trials and/or are already used in the clinical setting [4, 7-9]. We only provide a sneak-peak on the topic, emphasizing the (patho)physiological role of the receptors that has (possible) relevance for therapeutic application.

2.1.2.1 Adenosine receptors

In the atria of the heart, the A_1AR has a cytoprotective role and holds therapeutic potential for the treatment of cardiac diseases. Adenosine as such is a long-term clinically used drug in the diagnosis and treatment of paroxysmal supraventricular tachycardia, and neladenoson is in Phase II clinical trials for the treatment of heart failure [10]. In white adipose tissue, the A_1AR inhibits lipolysis and insulin secretion, and stimulates glucose uptake in adipocytes. In airway epithelium and smooth muscle the A_1AR evokes bronchoconstriction; clinically used antagonists include theophylline, doxofylline, and bamifylline [4]. The central A_{2A}AR is involved in Parkinson's, Huntington's, and Alzheimer's disease (AD); the antagonist istradefylline is available in Japan as co-adjuvant in the treatment of Parkinson's disease. In the periphery, the A_{2A}AR is expressed in leucocytes and blood platelets, where it regulates the onset of vasodilation and inhibits platelet aggregation. Adenosine as such is used for coronary artery imaging, based on its action via this receptor subtype [4]. The A_{2B}AR is present at high levels in the periphery in the intestine and bladder, but at very low levels in the CNS. Not much is known about the functional significance of this receptor; it has been implied in allergic and inflammatory disorders, with some controversy about the pro- versus anti-inflammatory effects of A_{2B}AR stimulation. The **A_3AR, topic of Chapter 3 and 4,** is highly expressed in various inflammatory cells (mast cells, eosinophils, neutrophils, monocytes, macrophages, dendritic cells, lymphocytes), where its anti-inflammatory effects can aid in the treatment of inflammatory disorders. Clinically relevant agonists, such as the prototypical IB-MECA (CF101; Piclidenoson), have been developed for the

treatment of psoriasis and rheumatoid arthritis (RA). Upregulation of A_3AR that occurs in RA [11] likely represents an endogenous compensatory mechanism to counteract the inflammatory status [12, 13]. The A_3AR also mediates airway inflammation; antagonists have been developed for the treatment of asthma and chronic obstructive pulmonary disease (COPD). Together with the A_1AR, the A_3AR has been identified as an interesting drug target to address cardiac/cerebral ischemia, although its exact location in the heart has not yet been reported. As the A_3AR is highly expressed in tumour cells versus normal cells, it might represent a valuable target for the treatment of cancer, or a promising cancer biomarker. The agonist 2-Cl-IB-MECA (CF102; Namodenoson) has been developed for the treatment of hepatocellular carcinoma [3, 14-17].

2.1.2.2 P2Y receptors

Because of diverse expression in brain, immune cells, epithelial and endothelial cells, (vascular) smooth muscle and many other tissues, P2YRs receptors hold therapeutic potential for the treatment of neurological [18], inflammatory, cardiovascular disorders [19, 20], and cancer [21].

Both the P2Y1R and P2Y12R are expressed in platelets [22]. Platelets express four P2R subtypes – P2Y1R, P2Y12R, P2Y14R, and P2X1R; the former two synergize to achieve platelet activation by the action of endogenous ADP; the P2Y1R initiates platelet activation, while the P2Y12R is responsible for completion of the aggregation. The P2Y12R is the target for antithrombotic drugs in clinical use, while the P2Y1R and P2X1R are at a preclinical stage [23]. The P2Y12R is the target of the thienopyridine class of antithrombotic drugs, such as the prodrugs clopidogrel, ticlopidine, and prasugrel, which irreversibly bind the P2Y12R, as well as of two new (classes of) molecules; the orally active, reversible nucleoside antagonist ticagrelor and the intravenous short-lived ATP-derived antagonist cangrelor [24]. Moreover, it is now recognised that platelets have functions important for haemostasis as well as for inflammatory processes; purinergic signalling might be involved in platelet activation during atherosclerosis, sepsis, asthma and other inflammatory conditions [25]. Multiple studies have pointed at the role of the **P2Y2R** in the pathogenesis and/or treatment of inflammatory (autoimmune) conditions, atherosclerosis, cystic fibrosis

[26, 27], dry eye disease [28], cancer, and neurodegenerative disorders such as AD [5, 23, 29]. Up4U (INS365; diquafosol) was the first P2Y2R agonist to be approved (April 2010) and was launched in Japan [30]. In the intestine, the epithelial response to nucleotides is mainly mediated by the P2Y4R, with a contribution of the P2Y2R, whereas in the airways it involves mainly the P2Y2R [1, 23].

2.1.3 Structure of purinergic receptor ligands

Newly synthesized ligands are often evaluated via receptor binding experiments and functional assays of GPCR signalling. Nowadays, these are often preceded and/or accompanied by computer-based modelling and ligand docking, using resolved GPCR structures or homology models based on closely related GPCRs. The structure of quite some members of the purinergic receptor family has been resolved during the last decade; the A_{2A}AR [31-33], the P2Y12R [34], the P2Y1R [35], and the recently resolved A_1AR [36]. These can serve homology modelling purposes for ligand discovery of other purinergic receptor subtypes and will undoubtedly nourish the development of new therapeutic agents targeting purinergic receptors [37, 38].

2.1.3.1 Adenosine receptors

The structure-activity relationship (SAR) of high affinity agonists, antagonists and allosteric modulators for each of the AR subtypes has been extensively reviewed [39-45], which provides a fundament for the rational design of (ant)agonists with high affinity binding to certain AR subtypes, and high efficacy and/or potency in downstream AR signalling pathways. Most prototypical AR agonists are analogues of the endogenous ligand adenosine, and thus contain a ribose group connected to a purine ring. The ribose group (mainly the hydroxyl functions) is especially important for stabilization of the agonist in the adenosine receptor binding pocket and subsequent receptor activation. By modification of the adenosine backbone structure – e.g. substitutions at the adenine base and/or ribose group, and/or introduction of a rigid bicyclo[3.1.0]hexane (methanocarba) ring system instead of the ribose moiety – the affinity and selectivity for the different AR subtypes has been fine-tuned (Figure 2.2 upper panel). Not surprisingly, most AR antagonists thus lack the ribose group and generally possess a mono-, bi- or tricyclic core structure, with caffeine as a

prototypical non-selective antagonist having a xanthine as a core structure. Furthermore, AR agonists have been developed with a non-nucleoside structure (Figure 2.2 mid panel) that bind the orthosteric site or an allosteric site on the receptor. Also, bitopic ligands have been developed which combine structural features of orthosteric and allosteric ligands (Figure 2.2 lower panel). Members of each of the ligand groups depicted in Figure 2.2 have been reported with respect to (therapeutically relevant) functional selectivity at ARs (see section 2.2).

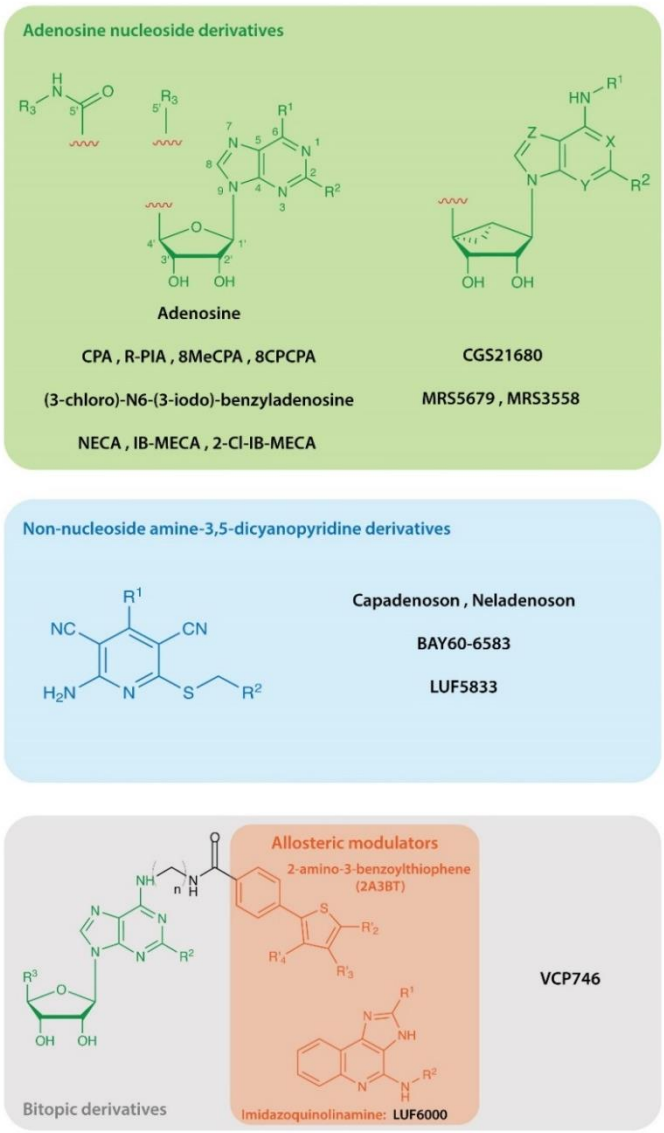


Figure 2.2: Overview of 3 common structural classes of AR ligands that have been reported with respect to functional selectivity: prototypical adenosine derivatives in green, non-nucleoside derivatives in blue, bitopic ligands (combination of an orthosteric pharmacophore via a linker with an allosteric pharmacophore) in grey, allosteric agonists /modulators in orange. Ligands discussed in this chapter are subdivided in their respective group.

2.1.3.2 P2Y receptors

Considerable progress has been made regarding the SAR for ligands of the P2Y1R and P2Y12R, and to a lesser extent for P2Y2R, P2Y4R, P2Y6R and P2Y13R [18, 40, 46-48]. ADP is the preferred ligand at the P2Y1R, P2Y12R and P2Y13R; ATP is a

weak partial agonist at P2Y1R and P2Y13R, and has been reported as an antagonist in platelets at the P2Y12R [25]. Molecular modelling and SAR studies have allowed the generation of ATP-derived, as well as non-nucleotide P2Y12R antagonists [49, 50]. P2Y1R antagonism only results in moderate prolongation of bleeding time, which might represent an option to achieve greater therapeutic safety [25]. At these ADP-preferring P2Y receptors, nucleotide modifications are generally tolerated at the 2- and 8-position (e.g. 2-methylthio-ADP (2-MeSADP)), and ring-constrained (N)-methanocarba nucleotides confer high potency and selectivity at multiple subtypes [51], e.g. MRS2365, the (N)-methanocarba analogue of 2-MeSADP, which is a potent P2Y1R agonist [1, 5, 52].

The **P2Y2R** is fully activated by equivalent concentrations of UTP and ATP, but not by the corresponding diphosphates UDP and ADP. UTPγS and ATPγS also act as full agonists at this receptor; terminal thiophosphate modification likely increases stability towards enzymatic degradation by ectonucleotidases. UTP analogues with modifications at the ribose and base moiety have been developed to distinguish between P2Y2R and P2Y4R. 2-thio-UTP and analogues are highly selective and potent P2Y2R agonists [30, 53-55]. Besides, dinucleoside polyphosphates such as Ap4A and Up4U (diquafosol) are used clinically as agonists for the P2Y2R [30]. UTP is an agonist at the human P2Y4R, but ATP acts as an antagonist. However, the rat P2Y4R is activated equipotently by UTP and ATP; this challenges pharmacological discrimination between the P2Y2R and P2Y4R. The P2Y6R is activated by UDP, much more than UTP. The potency of ATP at the P2Y11R is relatively low (EC_{50} in the 5-100 µM range) compared to the potency of other natural ligands at P2YRs (EC_{50} in the 10-500 nM range) [1, 23].

The SAR of purinergic receptors is mainly expressed with respect to their primary, best-studied G protein dependent signalling pathways, i.e. cAMP measuring assays for G_s- and G_i coupled receptors, or IP and/or Ca^{2+} accumulation assays for G_q coupled receptors. For drug discovery purposes, this has been the general way of screening and for ranking compound libraries, or hits from molecular docking studies, according to efficacy and potency. Actually, it may be more appropriate to refer to a ‘structure functional selectivity relationship’ (SFSR), which includes the evaluation of agonist activity in a specific pathway.

2.2 Functional selectivity in purinergic receptor signalling

Since purinergic receptors are constantly influenced by endogenous levels of adenosine and nucleotides, there is high interest in their downstream signalling and the processes that regulate this signalling. During the last decade, it has become clear that besides classical coupling to G proteins, GPCRs can additionally couple to a variety of adaptor proteins. This means that besides the selectivity for the receptor, ligands may display an extra selectivity towards certain signalling pathways; a concept known as functional selectivity or biased signalling [56, 57] (see **Chapter 1** for an introduction on functional selectivity). Knowledge about biased signalling at purinergic receptors may serve as a basis for the design of new molecules with therapeutic potential that make use of or avoid certain receptor signalling pathways in a selective way, to obtain a therapeutic profile which is mostly devoid of side effects. Furthermore, existing molecules can be re-evaluated in order to fine-tune their therapeutic properties.

Excellent reviews exist on the signalling properties of purinergic receptors; for ARs [3, 4, 16, 58, 59], as well as for P2Y receptors [5, 6]. These describe the canonical G protein coupling, as well as the coupling to other G protein (in)dependent pathways, pointing at functional diffraction downstream of each receptor. Below, we will discuss what is known about functional selectivity for purinergic receptors, in particular with therapeutic relevance. In section 2.3, we will focus on biased signalling with respect to the arrestin pathway.

2.2.1 Adenosine receptors

The A_1AR and **A_3AR** mainly couple to pertussis toxin (PTX) sensitive G_i protein, giving an inhibition of adenylate cyclase (AC) and a decrease in 3',5'-cyclic adenosine monophosphate (cAMP) levels. The A_{2A}AR and A_{2B}AR couple to G_s protein, which stimulates AC and gives an increase in cAMP. The A_1AR additionally couples to G_s and G_q protein. Coupling to G_q leads to activation of phospholipase C (PLC), producing the second messengers inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), giving an increase in Ca^{2+} and an activation of protein kinase C (PKC), respectively [4, 16]. The A_{2B}AR also couples to G_q and G_i proteins. The **A_3AR**

additionally couples to G_q, especially at high agonist concentrations. The G_i mediated reduction in cAMP inhibits the nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) pathway**.** This has been shown to mediate the anti-inflammatory and anticancer effects of A_3AR agonists [4, 16]. All ARs have been shown to stimulate extracellular signal-regulated kinases (ERK1/2), members of the mitogen-activated protein kinase (MAPK) family. ERK1/2 can be activated by both G protein-dependent – A_3AR ERK1/2 activation is G_i-mediated in CHO cells – and G protein-independent pathways, such as the one mediated by β-arrestin (βarr) [60]. This can happen with different kinetics; the G protein pathway seems to give rapid and transient ERK1/2 activation, while that via βarr is more delayed and sustained [16, 61-63].

Functional selectivity at ARs has been extensively reviewed in the past [16, 64], and more recently for biased modulation [65]. With the emergence of a substantial number of studies indicative of AR functional selectivity, the question arises as to where we stand with the observation of biased signalling, and whether this observed bias has (already) been linked to certain therapeutic effects?

<u>The A_1AR</u>

In the past decade, subtle patterns of functional selectivity have been observed for A_1AR ligands in the additional coupling to PTX-insensitive G_s and $G_{q/11}$ proteins, besides the classical G_i coupling. The non-selective agonist NECA showed higher intrinsic efficacy, although with low potency, compared to A_1-selective agonists CPA and R-PIA in G_s mediated cAMP accumulation and G_q mediated formation of inositol phosphates (IP). A_1AR expression levels needed to be high to observe this bias, as under low expression levels A_1AR selective agonists were not able to activate G_s- and G_q proteins, and NECA seemed to 'additionally' activate these G proteins, distorting the functional selectivity pattern [66]. A series of CPA and NECA analogues were identified which even broadened the G protein activation potential. 8-alkylamino-substituted CPA analogues (MeCPA and CPCPA) were almost completely inactive in $G_{s/q}$ protein signalling, while still being full agonists for G_i. These partial – or better said biased – agonists were developed as potential anti-lipolytic agents with reduced cardiovascular side effects for the treatment of non-insulin-dependent diabetes mellitus. They were readily tested two decades ago *in vivo* [67, 68]. Substituting the 5'N-position of NECA with cycloalkyl groups of

increasing size, gave a sequential reduction in efficacy at PTX-insensitive G proteins pathways. Hence, a small change in substituents of the adenosine backbone gives a pathway-specific reduction in efficacy [69] (Figure 2.2 & 2.3).

Functional selectivity for the A_1AR with more therapeutic relevance is being explored with regard to the cytoprotective effects of A_1AR ligands in ischemia-reperfusion injury (IRI), where a decreased blood supply in combination with pro-inflammatory reperfusion can cause serious tissue damage. Until now, therapeutic targeting of A_1AR for treatment of IRI has been largely unsuccessful, as high concentrations of the prototypical A_1AR agonists evoke significant haemodynamic side effects, particularly bradycardia. Partial A_1AR ligands have been developed in the past, which rather seem to hold a pattern of functional selectivity; this has mostly been explored for the ERK1/2 pathway, previously reported to be involved in cardioprotective A_1AR effects [70, 71]. For the A_1AR, it is not yet known if the ERK1/2 pathway is activated by G protein-(in)dependent mechanisms. Besides, cross regulation of other (adenosine) receptors is involved [72-75]. Different classes of allosteric modulators (e.g. 2A3BTs) have been shown to act as biased allosteric agonists as such, displaying a differential G_i- versus ERK1/2 mediated signalling compared to the orthosteric ligand R-PIA, and to allosterically modulate the signalling of R-PIA in different pathways [76, 77] (Figure 2.2 & 2.3).

Baltos *et al.* (2016) provided proof of concept of potential therapeutically useful biased A_1AR agonists with the atypical, bitopic (hybrid orthosteric/allosteric) agonist VCP746 and the non-nucleoside agonist capadenoson (BAY 68-4986); agonists that both mediate cardioprotection with minimal effects on the heart rate. VCP746 was synthesized prospectively with the purpose of biased signalling [78], while the signalling profile of capadenoson had not been investigated yet [79]. Capadenoson has previously entered Phase II clinical trials for treatment of angina pectoris [80], and atrial fibrillation [81] but failed to reach its primary endpoint of heart rate reduction. Upon testing the ability of ligands to modulate ERK1/2 phosphorylation, G_i mediated inhibition of cAMP accumulation and G_q mediated Ca^{2+} mobilization, Baltos *et al*. found that VCP746 and capadenoson were significantly biased away from A_1AR-mediated Ca^{2+} mobilization, relative to the reference agonist NECA, while prototypical A_1AR agonists CPA and R-PIA were not biased at any of the

investigated signalling pathways [82]. The bitopic VCP746 is a bivalent molecule consisting of an orthosteric adenosine pharmacophore, connected via a linker to an allosteric 2A3BT pharmacophore (Figure 2.2). Thus, a biased agonist profile was generated by combining the aforementioned allosteric bias potential with orthosteric ligand activity. Recently, modifications were made to the orthosteric adenosine pharmacophore, the linker, and the allosteric 2A3BT pharmacophore to further explore the SFSR of these clinically promising bitopic biased A_1AR ligands [83]. For capadenoson, a structure-based mechanism of bias has not yet been elucidated; its structure, containing an amino 3,5-dicyanopyridine core, differs from classical nucleoside-based AR agonists. It is not known if it has allosteric or bitopic properties. Meibom *et al*. (2017) synthesized a prodrug which is a derivative of capadenoson; neladenoson bialanate hydrochloride is now in clinical trials for heart failure without giving central side effects or reduction of the heart rate [84] (Figure 2.2 & 2.3).

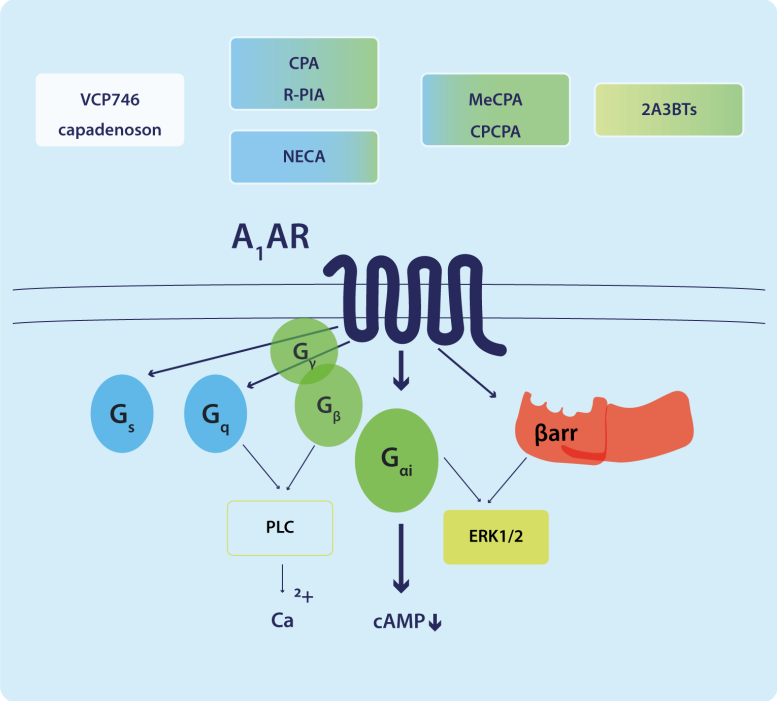


Figure 2.3: Functional selectivity at the A_1AR: the A_1AR classically couples to G_i protein, and besides to G_s and G_q. G_q and the $G_{\beta\gamma}$-subunits from G_i activate PLC, giving an increase in Ca^{2+}. Activation of ERK1/2 occurs via G protein- as well as βarr dependent pathways. NECA is more biased towards $G_{s/q}$ than A_1AR-selective CPA and R-PIA. 8-alkylamino-substituted CPA analogues MeCPA and CPCPA are biased to G_i and away from $G_{s/q}$. 2A3BTs both act as allosteric agonists and as allosteric modulators. The bitopic agonist VCP746 and non-nucleoside capadenoson are biased away from Ca^{2+} compared to NECA.

The A_{2A}AR and A_{2B}AR

Although it is speculated that the A_{2A}AR holds potential to engage different G protein-(in)dependent signalling pathways due to its remarkably long C-terminus (see below), no (pharmacologically relevant) biased ligands for the A_{2A}AR have been identified that might regulate these pathways [16].

Biased A_{2B}AR ligands have long stayed unidentified, until recently. Because both pro- and anti-inflammatory effects have been associated with the A_{2B}AR, the possible link with functional selectivity has been explored [16]. Gao *et al*. (2014) evaluated four prototypical classes of AR agonists – three groups of substituted adenosine derivatives and one group of non-nucleoside amino 3,5-dicyanopyridine compounds, including the agonists LUF5833 and BAY60-6583 (Figure 2.2) – for biased signalling in four signalling pathways downstream of the A_{2B}AR, and this in multiple cell lines [85]. Pathways evaluated were G_s mediated cAMP accumulation, G_q mediated Ca^{2+} mobilization, ERK1/2 activation, and βarr2 recruitment (see section 2.3.1). Of all ligands tested, the non-nucleoside agonists LUF5833 and BAY60-6583 were more ERK1/2-biased (Figure 2.4). The authors noted that the observed activation (potency and especially efficacy) was influenced by the cell line used, i.e. receptor expression levels (biased agonism was mainly evaluated using HEK293 cells overexpressing A_{2B}AR), levels of downstream signalling proteins as well as coupling efficiency.

Especially BAY60-6583 was identified as a A_{2B}AR agonist with a unique signalling profile relative to the reference agonist NECA; it had already been reported with a partial agonist profile [86], which was confirmed (in cells with low receptor expression) and supplemented with a biased agonist profile (in cells with high receptor expression) in the study of Gao *et al.* [85]. It is the only available potent and selective hA_{2B}AR agonist so far, now reaching preclinical-phase investigation for treatment of angina pectoris. Other non-nucleoside derivatives based on an amino 3,5-dicyanopyridine core are also being explored [87] and seem to display less species-dependent AR stimulation. These compounds are highly promising for the treatment of different heart diseases.

The above mentioned bitopic ligand VCP746 and the non-nucleoside based capadenoson have now also been identified as biased agonists for the A_{2B}AR.

VCP746 was characterized by a higher affinity and potency than BAY60-6583; it activates both G_s and G_q downstream of A_{2B}AR, rather than the naturally seen preference of G_s over G_q signalling of orthosteric ligands [88]. Capadenoson was biased towards cAMP signalling compared to the reference agonist NECA [89]. These ligands appear to stimulate anti-fibrotic A_{2B}AR signalling and could serve further investigation of the cardioprotective role of the A_{2B}AR in the attenuation of myocardial fibrosis in the treatment of heart failure [90]. Therefore, the dual A_1/A_{2B}AR agonism of VCP746 and capadenoson may represent a new therapeutic approach for modulating both myocardial fibrosis and hypertrophy in the treatment of heart failure [88, 89]. In the lung, the A_{2B}AR could represent an interesting 'biasable' target for the treatment of lung diseases [91] (Figure 2.4).

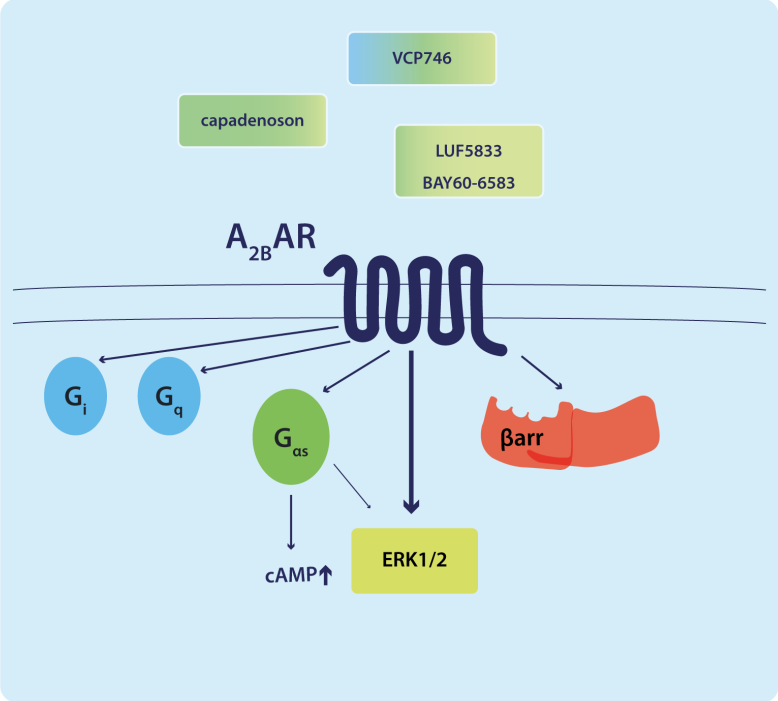


Figure 2.4: Functional selectivity for the A_{2B}AR: the A_{2B}AR generally couples to G_s, and besides to G_q (and possibly G_i). There is substantial ERK1/2 activation. Prototypical agonists signal to G_s and ERK1/2, but give less pronounced activation of G_q and G_i. The non-nucleoside amino 3,5-dicyanopyridine agonists LUF5833 and BAY60-6583 are ERK1/2-biased, capadenoson is more G_s-biased, and VCP746 activates both G_s and G_q, as well as ERK1/2.

The A_3AR

Bias at the A_3AR has been reported for G protein-dependent pathways [92], and with respect to the βarr pathway (see section 2.3.1), for ligands that bind the orthosteric site, as well as for allosteric modulators [93, 94] (Figure 2.5).

Clear patterns of biased agonism for the **human A_3AR (hA_3AR)** have long remained unknown, until recently. Baltos *et al.* (2016) reported on the assessment of a panel of agonists for their ability to promote cell survival, phosphorylate ERK1/2, stimulate Ca^{2+} mobilization, and inhibit cAMP signalling in CHO cells stably expressing the hA_3AR [92]. They quantified the bias profile for a number of prototypical adenosine derivatives and (N)-methanocarba 5'-uronamide adenosine derivatives, containing modifications at the N^6- and/or C2 positions (Figure 2.2). Compared to the reference agonist IB-MECA, each compound behaved as a full agonist in the inhibition of cAMP signalling. The majority of compounds also were full agonists in ERK1/2 phosphorylation and Ca^{2+} mobilization; an example of an exception is the partial agonism of MRS5679, an (N)-methanocarba derivative containing an extended C2-biphenyl-substituent. A significant positive correlation was found between the length of the C2-substituent and the bias towards cell survival. Homology models, based on hybrid A_{2A}AR-β2AR or A_{2A}AR-opsin templates, suggest that extended C2-substituents promote a progressive outward displacement of transmembrane helix 2, likely to be involved in the stabilization of a unique A_3AR conformation responsible for a pattern of biased signalling towards cell survival. The bias conferred by N^6 substitution is more complex. Although a bias towards cell survival was observed in this study, there was no preferential coupling to ERK1/2 pathway, which has relevance with respect to cytoprotection for other AR subtypes. The authors suggested a possible involvement for the βarr pathway. Very recently, this pathway has been explored in HEK293T cells for a panel of ((N)-methanocarba) adenosine derivatives [95], amongst which MRS5967 behaved as a partial agonist for βarr2 recruitment (see **Chapter 4**).

The discovery of ligands with bias towards cell protection/survival might be of clinical interest in the protection of cardiac and lung IRI [96, 97]. Paradoxically, there have been reports about a biphasic effect of A_3AR activation on cell growth and survival; inhibiting apoptosis at low concentrations, while promoting apoptosis and having anti-proliferative effects at high concentrations. Since very high ligand concentrations are

often used in studies on cell growth, care must be taken when making conclusions about biased effects. It is unclear if the observations at higher concentrations are due to stimulation of different A_3AR pathways, rapid receptor desensitization, non-selective activation of other ARs, or a combination of these [16]. Therefore, ligands that are biased towards pro-survival pathways may promote cell-survival and prevent apoptosis at higher concentrations.

The positive allosteric modulator (PAM) LUF6000 (Figure 2.2 and section 2.3.1), acted as an allosteric agonist and showed anti-inflammatory effects, mediated by the NF-κB pathway, similarly as described for other A_3AR agonists that are used for inflammatory disorders (e.g. IB-MECA) [98]. Furthermore, it was shown that the anti-rheumatic drug methotrexate enhanced A_3AR levels in RA, sensitizing the effect of A_3AR agonists [99].

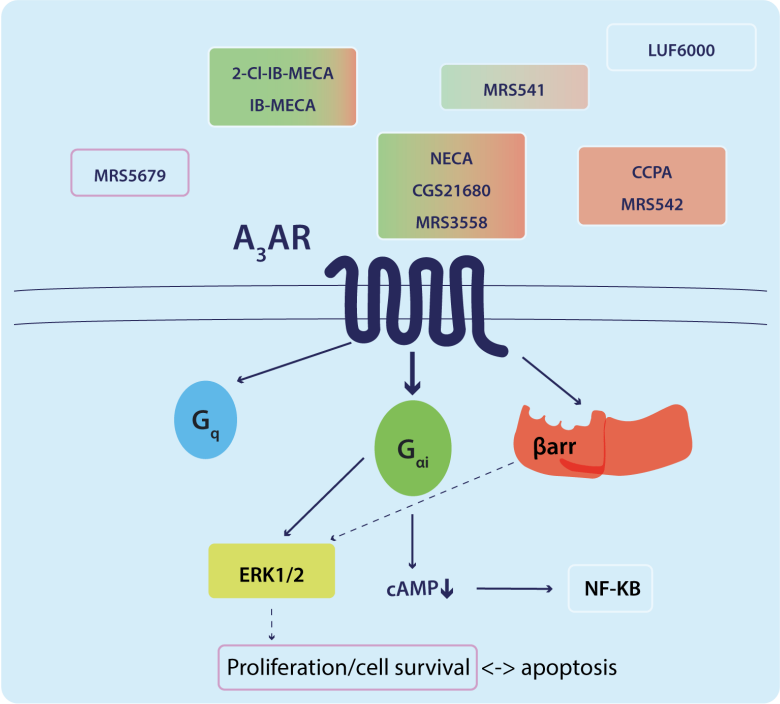


Figure 2.5: Functional selectivity for the A_3AR: the A_3AR generally couples to G_i, and besides to G_q. Adenosine derivative MRS5679 is biased towards cell survival; a feature that might be attributed to the extended C2-substituent on the adenosine scaffold. LUF6000 acts as an allosteric agonist, having anti-inflammatory effects via the NF-κB pathway, and as a PAM. NECA, CGS21680, and highly potent MRS3558 are full agonists in both the G_i and βarr2 pathway, while MRS541 is a partial agonist in both pathways. A_3AR selective agonist 2-Cl-IB-MECA and IB-MECA have partial activity in the βarr2 pathway, in contrast to the G_i pathway. CCPA and MRS542 are antagonists in the G_i pathway, but partial agonists in the βarr2 pathway. DBXRM was a full agonist in the G_i pathway, but a partial agonist in the βarr2 pathway.

2.2.2 P2Y receptors

The P2Y1,2,4,6,11 receptors couple to $G_{q/11}$, activating PLCβ, leading to an increase in Ca^{2+} and an activation of PKC. On the other hand, P2Y12,13,14 receptors couple to $G_{i/o}$, inhibiting AC and decreasing cAMP levels. The P2Y2R additionally couples to PTX-sensitive $G_{i/o}$ protein; studies have reported that coupling to G_o requires interaction with $\alpha_v\beta_{3/5}$ integrins via a three amino acid integrin-binding domain (RGD) in the first extracellular loop [100, 101]. Furthermore, this integrin-interaction is also needed for coupling to G_{12} [102]. Both G_o and G_{12} are involved in cytoskeletal rearrangements that regulate cell migration and chemotaxis, which could be of relevance in P2Y2R-mediated inflammatory responses [1, 6, 23]. Few reports exist about biased signalling for P2Y receptors, especially when considering therapeutic relevance.

The P2Y1R

Biased allosteric activation/modulation has been reported for the purinergic receptor family. However, biased (allosteric) antagonism is a less explored phenomenon. Gao & Jacobson (2017) reported biased antagonism by an orthosteric antagonist (MRS2500) and a negative allosteric modulator (BPTU) [103]. The influence on the agonism of three structurally diverse agonists was evaluated at various signalling pathways; G_q-mediated IP production, $GTP_\gamma S$ binding to G_q, G_q- or βarr2-mediated (by adding a PKC-inhibitor) ERK1/2 stimulation, βarr2 recruitment, and βarr2-mediated P2Y1R internalization. BPTU evoked a rightward shift in the concentration response curves of two agonists, without affecting the agonist E_{max}, in ERK1/2 stimulation, but suppressed the E_{max} in $GTP_\gamma S$ binding, βarr2 recruitment and receptor internalization. However, when using a different agonist, BPTU suppressed the E_{max} insurmountably at all signalling pathways. By comparison, the orthosteric antagonist MRS2500 behaved as a surmountable antagonist, shifting concentration-response curves of all agonists in all signalling pathways. Hence, allosteric antagonism might be surmountable (competitive) or insurmountable (non-competitive), depending on the signalling pathway and agonist evaluated, representing a hallmark for functional selectivity. The authors suggested that the different degree of antagonism at different signalling pathways, induced by different

agonists, renders BPTU with functional selective allosteric modulating antagonist properties, which might be interesting for therapeutic purposes.

The group of Page & Pitchford were the first to identify 'biased' signalling for the P2Y1R that controls leucocyte recruitment during inflammation, but that does not affect haemostasis. Purinergic nucleotides have shown signalling at P2Y1Rs to Rho-GTPases under inflammatory conditions, evoking formation of platelet-leukocyte complexes and migration/recruitment of inflammatory cells [104, 105]. Rho-GTPases seem to play a minor role in platelet aggregation, but do link P2Y1R activation to platelet functions in inflammatory processes. The authors evaluated the contribution of PLC- compared to Rho-GTPase signalling in P2Y1R-induced platelet aggregation (applicable to haemostasis), and in platelet-motility and platelet-induced neutrophil chemotaxis (PINC) (immune regulating). The P2Y1R selective agonist MRS2365 had a strongly induced platelet chemotaxis and PINC in comparison with the endogenous agonist ADP. Hence, it seems that canonical G_q mediated PLC activation leads to platelet aggregation, while Rho-GTPase activation evokes motility and interactions with neutrophils [106]. The manner of P2Y1R signalling might depend on the inflammatory conditions involved, illustrating the influence of micro-environmental conditions on functional selectivity.

The P2Y2R

Gabl *et al.* (2015) showed that ATP induces different signalling downstream of the **human P2Y2R (hP2Y2R)** in neutrophils, depending on its interaction with the intracellular actin cytoskeleton; the conformational change in the P2Y2R upon activation by ATP only gave a transient Ca^{2+} response, and other signalling pathways were blocked [107]. Disruption of the actin cytoskeleton leads to a Ca^{2+} response concomitant with activation of the superoxide-generating NADPH oxidase. It remains to be determined if this is effectively occurring in human cells. Hence, this is an example of functional selectivity for the P2Y2R conveyed by an intracellular component, which might serve future research on the design of drugs affecting immune reactivity and/or inflammatory processes for the P2Y2R.

The P2Y11R

White *et al.* (2003) suggested that UTP might be a biased agonist at the P2Y11R, increasing cytosolic Ca^{2+} but not IP, whereas ATP increases both [108]. However, how UTP evoked this differential signalling remained elusive. Hence, Morrow *et al*. (2014) tried to characterize Ca^{2+} and IP signalling of ATP and UTP at the P2Y11R in 1321N1 cells [109]. UTP did not evoke a concentration dependent rise in Ca^{2+} and IPs, not even at high concentrations. Furthermore, UTP also did not have any antagonist properties. Therefore, UTP cannot be considered as a biased P2Y11R (ant)agonist, putting a question mark behind its P2Y11R signalling properties.

2.3 β-arrestins in functional selectivity of purinergic receptor signalling

Quite some studies on purinergic receptor signalling have focused on receptor phosphorylation, desensitization and internalization; these processes are involved in the regulation of different signalling pathways. The attenuation of receptor signalling in either pathway can be of particular relevance when there is a continuous, high level of receptor stimulation, which is likely to occur in a physiological setting (sustained levels of adenosine or nucleotides), as well as in the therapeutic setting, for example in the case of chronic drug therapy or drug overdose. Many of these studies were performed before the concept of functional selectivity was introduced. Within this concept, quite some attention has been given towards the inhibitory adaptor protein β-arrestin (βarr), originally characterized for its role in inhibitory GPCR-regulating processes. In this section, we mention early reports on βarr interaction for the different subclasses of purinergic receptors, and the link with patterns of phosphorylation/internalization for regulation of receptor signalling. Besides, we discuss more recent studies that explore biased signalling for the βarr pathway and G protein-(in)dependent signalling pathways, and we provide a view on the techniques and assay systems used to study the interplay between these receptors and βarr.

2.3.1 Adenosine receptors

Studies on the regulation of AR signalling by receptor phosphorylation, desensitization, and internalization have been summarized in extensive reviews [15,

16, 110]; discussing this in detail would be beyond the scope of this section. However, some observations are worth mentioning because they have relevance with respect to βarr signalling.

The A_1AR and A_3AR

The inhibitory A_1AR and A_3AR have subtype-specific kinetics of receptor phosphorylation, desensitization and internalization, suggested to occur by their differential sensitivity for phosphorylation of their C-terminus by G protein-coupled receptor kinases (GRKs). Studies have been performed for the human A_1AR (hA_1AR) and the rat A_3AR (rA_3AR), which differ substantially in terms of the presence of potential C-terminal phosphorylation sites; the rA_3AR contains six serine/threonine residues, while these are absent in the hA_1AR C-terminus (Figure 2.6). The rA_3AR shows much faster desensitization of G_i-mediated AC inhibition (order of minutes) than the hA_1AR (several hours); switching the C-terminal part of the hA_1AR, distal to its predicted palmitoylation site, with the corresponding part of the rA_3AR, results in a chimeric hA_1AR-CT_{rA3AR} with equally fast phosphorylation and desensitization as the native rA_3AR [111-113]. It was shown that threonine residues 307, 318 and 319 – especially the latter two – in the rA_3AR were crucial for this rapid phosphorylation, desensitization and internalization [114, 115] (Figure 2.6). This chimeric hA_1AR-CT_{rA3AR} also showed βarr2-GFP recruitment that was indistinguishable from the rA_3AR upon 30 min stimulation with 1μM R-PIA in CHO cells. Surprisingly, the internalized receptor showed no colocalization with βarr2. A $Cys^{302,305}$ rA_3AR mutant, in which the potential palmitoylation sites are removed, showed significantly faster kinetics of internalization and was able to recycle to the PM, but showed no difference in βarr2 translocation pattern. A Cys^{309} hA_1AR mutant had an unchanged, lack of internalization. The authors suggested that receptor palmitoylation might control the accessibility of the C-terminus to phosphorylation by GRKs [116]. However, in a rat basophilic leukaemia cell 2H3 cell line, endogenously expressing the rA_3AR, neither of both βarrs redistributed upon stimulation with 100 μM NECA [117]. The authors noted that they could not exclude βarr recruitment below the limit of detection or that the recruitment might be agonist-selective. Hence, the distinct results for rA_3AR-βarr interaction might be due to the fact that different agonists were used (R-PIA versus NECA); this might point at functional selectivity for this pathway.

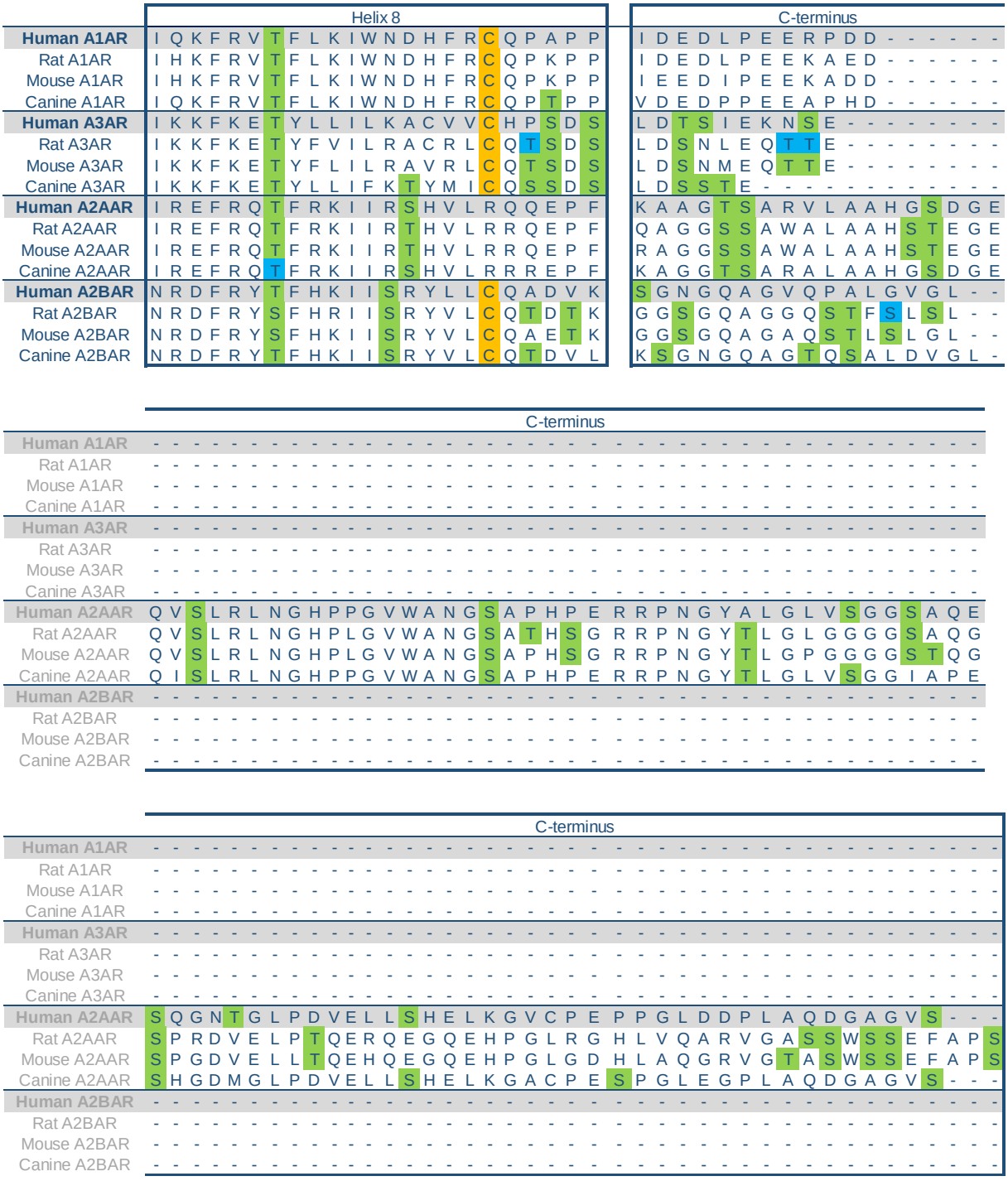


Figure 2.6: Alignment of the C-terminal part (Helix 8 and C-terminus) of ARs from different species: predicted palmitoylation cysteines are marked in yellow, serine and threonine residues are marked in green and the ones discussed in the text are marked in blue.

Similar rapid kinetics of desensitization and internalization were shown for the **hA$_3$AR**, stably expressed in CHO cells, as well as natively expressed in human astrocytoma cells [118, 119]. All these early reports suggested that phosphorylation, desensitization, internalization, as well as βarr recruitment is different for hA$_1$AR and

rA$_3$AR because of their sensitivity for C-terminal phosphorylation – or thus the presence of S/T residues – and possibly the presence of palmitoylation sites. Although the rA$_3$AR and hA$_3$AR C-terminus are quite alike (Figure 2.6), none of the rat or human A$_3$AR S/T residues is located within a fixed distance to the palmitoylation Cys. Therefore, the importance of C-terminal phosphorylation might be found in a pattern of phosphorylation, rather than in the specific location of the residues.

For the **hA$_3$AR** it is only more recently that structural patterns have been identified which have a role in βarr recruitment and/or -coupling. Stoddart *et al*. (2014) showed that Trp (W6.48) in TM6 – known to act as an activating switch for the movement of TM6 upon receptor activation [120] – is important for βarr2 interaction and receptor internalization [121]. This group also reported on the use of a new fluorescent agonist for monitoring receptor internalization and colocalization with βarr2 [122]. Very recently, we reported that C-terminal truncation of the **hA$_3$AR** did not hamper βarr2 recruitment, pointing at the fact that additional motifs, such as the conserved 'DRY' motif at the boundary of TM3 and IL2, are important for βarr2 recruitment in a complementary or synergistic way [123] (see **Chapter 3**).

The ERK1/2 pathway might be involved in the regulation of inhibitory AR signalling. In stably transfected CHO cells, stimulation with 10 μM NECA showed that ERK1/2 might regulate **hA$_3$AR** receptor desensitization and internalization by positive feedback of GRK2 activity, probably controlling its association with the receptor and receptor phosphorylation [124]. In Syrian hamster ductus deferens smooth muscle tumour (DDT1 MF-2) cells, stimulation of the A$_1$AR with 1 μM R-PIA resulted in rapid (within 30 minutes) PM translocation of βarr1, accompanied by transient ERK1/2 activation. The authors suggested that the βarr/ERK pathway is involved in the physiological role of the A$_1$AR, the termination of A$_1$AR signalling (desensitization and downregulation), and receptor synthesis as a positive feedback regulation of A$_1$AR expression [125].

Since βarr-mediated ERK1/2 signalling might contribute to the cytoprotective effect of the A$_1$AR after IRI [70, 71], Langemeijer *et al*. (2013) explored functional selectivity for the hA$_1$AR by screening over 800 known AR ligands for βarr recruitment, and comparing the activity with the G protein pathway [126]. For the measurement of βarr

recruitment, a commercially available Tango™ADORA1-*bla* U2OS cell line was used. This reporter assay cell system contains an engineered A_1AR, fused at its C-terminus to an exogenous transcription factor via a protease cleavage site, and contains a protease-tagged βarr. The cell line also contains the β-lactamase *(bla)*-reporter gene responsive to the transcription factor. Upon βarr recruitment, the cleavage site is recognized by the protease, the transcription factor is liberated and expression of β-lactamase occurs, which can cleave a FRET-enabled substrate that will produce blue fluorescence instead of green. Compared to previous studies, mainly using microscopy for evaluation of βarr trafficking, this system more readily allows quantification of βarr recruitment. GTPγS assays were performed on membranes of both CHO cells and U2OS cells, giving a similar rank order of EC_{50s} as well as E_{max}. Modification of adenosine derivatives at the N^6 and C2 position affected efficacy in both βarr and G protein pathways, and elongation of the 5'C position decreased βarr recruitment significantly. Interestingly, these positions were also crucial for βarr2 and G protein activity in studies by Gao *et al.* [93] and Storme *et al.* [95] (see **Chapter 4**). However, Langemeijer *et al.* concluded that all tested A_1AR ligands showed little to no functional selectivity, and that functionally selective ligands for the hA_1AR must be rare, if not absent. However, some aspects of the Tango™ βarr assay set-up should be taken into account. To start with, ligands were initially screened at a concentration of 10 μM, and cells were incubated for 5 hours, with an additional 2 hours of substrate incubation. Although ligand panels for ARs are routinely screened at concentrations as high as 10 μM (especially for G protein mediated signalling), this concentration might be too high to observe differences in βarr recruitment, especially when aimed at being representative for the physiological setting. It is also important to note that there was no real-time measurement of βarr recruitment, but rather a cumulative image over several hours was monitored. This is in contrast with other βarr recruitment assays, discussed below. Second, the A_1AR in the assay was engineered by the supplier to contain the final 26 amino acids of the V2 vasopressin receptor, instead of the final 15 amino acids of the A_1AR; the A_1AR C-terminus went from containing none to 11 S/T residues. The authors note that there is likely to be an overestimation of βarr recruitment. Hence, one may wonder to what extent these observations represent what is happening at the native A_1AR, taking into account the aforementioned observations for the chimeric hA_1AR-CT_{rA3AR}.

Gao *et al.* (2008) were the very first to show the effective coupling of the **hA$_3$AR** to βarr2 and to explore biased signalling for this pathway; they tested a structurally diverse panel of ligands for βarr2 recruitment to the hA$_3$AR and compared activities at this pathway with previously reported activity for G$_i$-mediated inhibition of cAMP in CHO cells [93]. They used a PathHunter™ (DiscoverX) CHO cell reporter assay system, which relies on the functional complementation of the β-galactosidase enzyme. It contains the A$_3$AR, fused at its C-terminus to a small enzyme donor fragment (called ProLink™), and a fusion protein of βarr2 with a larger, N-terminal deletion mutant of β-galactosidase (the enzyme acceptor). Activation of the A$_3$AR results in recruitment of βarr2 and subsequent functional complementation of the two enzyme fragments, with formation of an active β-galactosidase enzyme. Cells are lysed for read-out by addition of a buffer containing a substrate, which is hydrolysed by the β-galactosidase enzyme to generate a chemiluminescent signal. In this assay system, cells are treated with agonists for 60 minutes (or time points chosen), followed by an additional 60 minutes of incubation with detection reagent at room temperature. Most of the tested compounds were full agonists in both pathways, with similar although not identical potency and efficacy; examples are the non-selective reference agonists NECA and IB-MECA, A$_3$AR selective reference agonist 2-Cl-IB-MECA, CGS21680, CPA, and the highly potent compound MRS3558. *N*^{6}-benzyl- and *N*^{6}-(3-iodobenzyl)-adenosine (MRS541) were partial agonists in both assays. However, differences between the two pathways (G$_i$-mediated inhibition of cAMP versus βarr2 recruitment) were found as well. Several compounds that were antagonists in the cAMP assay, amongst which CCPA, and MRS542, behaved as partial agonists for βarr2 recruitment. Conversely, the xanthine 7-riboside DBXRM, a full agonist in the G$_i$-mediated pathway, was only partially efficacious in βarr2 recruitment. The (to a certain degree) reversed activities in the two pathways examined here are clear indications of functional selectivity for the hA$_3$AR.

Recently, we tested a panel of A$_3$AR ligands – amongst which some that had been tested by Gao *et al.* (2008) [93] – using a NanoBit® reporter assay system (Promega) in a stable HEK293T cell system [95] (see **Chapter 4**). The NanoBit® reporter assay system is somewhat analogous to the functional complementation used in the PathHunter™ system, but makes use of the NanoLuc luciferase enzyme [127]. This is a small 19 kDa enzyme, producing high intensity, glow-type luminescence by

conversion of the cell-permeable substrate furimazine. The hA_3AR is fused at its C-terminus to the large part of nanoluciferase (LgBit; 18 kDa) and the βarr2 at its N-terminus to the small part (SmBit; 1 kDa). The interaction between the hA_3AR and βarr2 can be monitored in a kinetic, real-time, live-cell set-up. In this assay system, luminescence is measured during substrate equilibration before agonist addition, and continuously after agonist addition for the desired time points at room temperature. The tested ligand panel consisted of (N)-methanocarba adenosine derivatives, in which the N^6-, C2, and 5'C-positions were explored for substitution. The efficacy of βarr2 recruitment was mainly dependent on substitution of the N^6- and C2-positions. (N)-methanocarba 5'-uronamide adenosine derivatives reached very high potency in cAMP signalling as well as βarr2 recruitment compared to reference agonists NECA and 2-Cl-IB-MECA. Remarkably, none of these compounds surpassed the efficacy of NECA in any of the pathways.

When comparing the PathHunter™ (Gao et al 2008) and NanoBit® systems ([95], see **Chapter 4**), the results were quite similar, except for the reference agonist 2-Cl-IB-MECA and IB-MECA; these agonists showed only partial agonist efficacy in the NanoBit® system, compared to full efficacy, equal to that of NECA, in the PathHunter™ system. This was rather surprising and might be related to receptor expression levels. Presumably, both the receptor and βarr2 are over-expressed in the PathHunter™ as well as the NanoBit® system. The artificial nature of the systems might also affect A_3AR-mediated βarr recruitment, and it is difficult to make a statement as to how these findings may translate to the real physiologic situation. Therefore, it will be important to examine if the patterns of biased agonism observed here are consistent in cells or tissues endogenously expressing the A_3AR. Then, these patterns could be used as a starting point for the rational design of novel A_3AR agonists. Additionally, it is highly important that the pharmacological actions of these A_3AR ligands can be reconciled with particular signalling pathways, which requires the use of *in vivo* experiments.

In the aforementioned study of Stoddart *et al.* (2014), signalling bias was observed upon W243F mutation in TM6 of the hA_3AR in CHO-K1 cells for cAMP signalling, ERK1/2 phosphorylation, βarr2 interaction and receptor internalization, by agonists NECA and HEMADO [121]. βarr2 interaction was evaluated using a venus YFP (vYFP) based bimolecular fluorescence complementation (BiFC) approach, in which the interaction of βarr2-vYnL (residues 1-173 of vYFP) with A_3AR-vYc (residues 155-

238 of vYFP) was monitored with confocal microscopy. The complementation between the two fragments is regarded as irreversible; the authors note that this could lead to 'trapping' of the A_3AR-βarr2 complex, stimulating receptor internalization. NECA was able to induce W243F-A_3AR internalization, while HEMADO did not evoke any internalization, but was still able to bind the mutated receptor. Furthermore, HEMADO had a lower efficacy than NECA for A_3AR-βarr2 interaction, and W243F mutation further reduced this efficacy. In the ERK1/2 and cAMP pathway, both NECA and HEMADO had a reduced efficacy at the W243F-A_3AR compared to wild type A_3AR. Hence, the W243F-A_3AR mutant shows functional selectivity in the signalling of different agonists. The authors therefore suggest that conformational changes in TM6 in the region of W6.48 might have a link with functional selectivity for the A_3AR.

Functional selectivity in allosteric modulation was evaluated for the PAM LUF6000 [128]. In a GTPγS binding assay – which uses cell membranes from CHO cells stably expressing the A_3AR (reconstituted with $G_{\alpha i}$ subunits) – the E_{max} of 2-Cl-IB-MECA was only half that of NECA. LUF6000 enhanced the E_{max} of 2-Cl-IB-MECA to a larger extent than that of NECA, likely because of the intrinsic efficacy of NECA already being high. In a follow-up study, the agonist-enhancing effects of LUF6000 seemed to differ for the various signalling pathways evaluated (cAMP, membrane hyperpolarization, Ca^{2+} mobilization assay). However, in βarr2 translocation, the agonist-enhancing effect of LUF6000 was not pronounced [129].

The A_{2A}AR and A_{2B}AR

The A_{2A}AR has a 122 amino acid long C-terminus, which is remarkably long compared to the C-termini of other ARs, which are usually about 30-40 amino acids in length; it represents the perfect anchoring spot for intracellular signalling proteins [16, 130, 131]. The engagement of this C-terminus in A_{2A}AR phosphorylation, desensitization and internalization has been addressed by different groups. Deleting the last 96 amino acids of the canine A_{2A}AR C-terminus, containing 11 possible phosphorylation sites, did not hamper desensitization of the A_{2A}AR upon treatment with NECA in CHO cells [132]. However, a T298A mutation just upstream of the deleted part (see Figure 2.6) attenuated receptor short-term (30 min) but not long-term (24h) agonist-induced desensitization. This was rather surprising, as βarr

recruitment is more reported to require clusters of phosphates [133] (see **Chapter 1**). However, phosphorylation of T298 might be sufficient to cause a receptor conformational change, disrupting G protein coupling but not influencing βarr recruitment; hence, no definitive conclusions can be made with respect to βarr contact. Early studies also provided evidence for the involvement of GRK2 in the desensitization of the rat A_{2A}AR (rA_{2A}AR) [134, 135]. Receptor internalization has been reported for the rA_{2A}AR [136] and human A_{2A}AR [137]. Important to note is that, while the rA_{2A}AR adenosine receptor has 19 potential phosphorylation sites in its C-terminus (of which multiple are clustered), the human and canine A_{2A}AR have only 13 potential phosphorylation sites in their C-terminus. It is conceivable that desensitization and internalization of the A_{2A}AR require different regions in the C-terminus, or that the regulation of these processes is species-dependent, involving βarr-dependent as well as βarr-independent processes.

The effective A_{2A}AR-βarr interaction has been reported for the rA_{2A}AR in a study evaluating the role of the actin cytoskeleton in rA_{2A}AR internalization [136]. Both βarr1-GFP and βarr2-GFP showed rapid (2 min) translocation to the cell surface upon stimulation with 200 nM of the A_{2A}AR selective agonist CGS21680 in HEK293 cells. Also, a truncated A_{2A}AR failed to cluster and internalize upon agonist exposure, suggesting that association with actinin is a prerequisite for internalization. Interaction of βarrs with the truncated receptor was not explored. βarr recruitment to the hA_{2A}AR has only been shown by Khoa *et al.* (2006) [138]; the effect of cytokine TNF-α pretreatment on CGS21680-dependent A_{2A}AR desensitization and redistribution of GRK2 and βarr1 was examined. Cytokine pre-treatment diminished agonist-dependent translocation of GRK2 as well as βarr1 to the PM.

For the rat A_{2B}AR (rA_{2B}AR), evidence has been provided for agonist-induced desensitization, although with differential results for involvement of GRK2 [110, 134]. Mathura *et al.* (2001) explored desensitization, internalization, and recruitment of βarr2 to the rA_{2B}AR by step-wise truncation and mutation of the receptor [139]. Phe328 and Gln325 stop mutants did neither desensitize nor internalize and showed no recruitment of βarr2 upon stimulation with 10 μM NECA compared to native A_{2B}AR. The authors concluded that a single S329G point mutation was critical for rapid (<1h) agonist-induced desensitization and internalization, and that the rate,

extent and mechanism (e.g. arrestin dependence) of internalization depends on specific sites in the C-terminus. A C-terminal 'Type II postsynaptic density, disc large and zo-1 protein' (PDZ) motif would have an influence on the trafficking of the rA_{2B}AR [140]. This x-Ø-x-Ø motif – in which x stands for any amino acid and Ø for a bulky hydrophobic reside – includes the last 4 amino acids of the A_{2B}AR (Ser-Leu-Ser-Leu in rat and Gly-Val-Gly-Leu in human), and binds to proteins that have a role in the assembly of signalling complexes, controlling GPCR trafficking. Removal of the PDZ motif (i) switched internalization from an arrestin/clathrin-dependent pathway to an arrestin/clathrin-independent pathway, and (ii) inhibited receptor recycling.

Gao *et al.* (2014) explored functional selectivity for the mouse A_{2B}AR (see section 2.2.1) and evaluated the βarr2 pathway using an aforementioned PathHunter™ (DiscoverX) CHO cell system, expressing mouse A_{2B}AR. The authors comment their choice for the mouse receptor because the human A_{2B}AR did not produce a robust response. βarrs were involved in the desensitization and trafficking of the human A_{2B}AR, endogenously expressed in HEK293 cells, upon stimulation with 100 μM NECA [141]**.** The βarr isoforms translocate with different kinetics to the receptor in HEK293 cells [142] and in airway smooth muscle cells, which endogenously express the receptor [143].

2.3.2 P2Y receptors

Studies on the involvement of βarrs in P2YR internalization, desensitization and signalling are scarce compared to those on ARs. Hoffmann *et al.* (2008) investigated six fluorescently tagged human P2YRs for internalization and recruitment of fluorescently tagged βarr1 and βarr2 with confocal microscopy upon stimulation with agonists (100 μM, 15 min) [144]. The agonists used were ADP (P2Y1R), UTP (P2Y2R), UTP (P2Y4R), UDP (P2Y6R), ATP (P2Y11R) and ADP (P2Y12R). Internalization was observed for all six receptors, but for the P2Y6R, P2Y11R and P2Y12R only upon co-transfection of GRK2. The P2Y1R, **P2Y2R** and P2Y4R showed rapid translocation of βarr1 and βarr2 to the PM. The P2Y1R showed pronounced recruitment of βarr2, and only modest recruitment of βarr1. The P2Y4R exhibited equally strong translocation for βarr1 and βarr2. The P2Y6R, P2Y11R and

P2Y12R did not show any translocation of βarrs. When GRK2 was co-transfected, only the P2Y6R and P2Y11R showed some recruitment of βarr2, and even slighter recruitment of βarr1. The P2Y12R did not show any recruitment of βarrs, although βarr2 interaction was observed with FRET, pointing at the possibly higher sensitivity of this detection method for GPCR-βarr interaction. Based on these results, P2Y1R, P2Y6R and P2Y11R would be classified as class A GPCRs with respect to βarr recruitment, and the P2Y4R as a class B GPCR [145]. Interestingly, the **P2Y2R** showed a βarr recruitment depending on the agonist used (see below)**.**

The P2Y1R and P2Y12R

P2Y1R and P2Y12R desensitization and internalization seem to be regulated by different kinases introducing different patterns of phosphorylation. The group of Poole and Mundell showed that the P2Y1R and P2Y12R are internalized via clathrin-coated pits (CCPs) in human 1321N1 astrocytoma cells. The P2Y1R showed PKC-dependent internalization and recycling that was not dependent on βarr1 nor GRKs, the P2Y12R trafficked to a different type of CCPs, in a βarr1- and GRK dependent way [146-148]. For the P2Y1R, these findings were in contrast with studies showing internalization of the human P2Y1R in a βarr-dependent way, with more involvement of βarr2 than βarr1 [144, 149]. For the P2Y12R, Hoffmann *et al.* (2008) did observe receptor internalization and a very slight interaction with βarr2, but not with βarr1, upon co-transfection of GRK2, suggesting at least some involvement of the phosphorylation status of the receptor [144].

P2Y1R desensitization and internalization was shown to be mediated by differential C-terminal phosphorylation [149]. Human P2Y1R and βarr2 (βarr1 was not tested; the authors alluded to [144]) were C-terminally fused to a fluorescent protein, and trafficking of both was monitored with confocal microscopy in HEK293 cells upon stimulation with 100 μM ADP. Rapid receptor internalization, accompanied by βarr2 trafficking was observed for wild type P2Y1R and P2Y1Rs lacking potential phosphorylation sites in IL3 and in the proximal C-terminus, but not when lacking phosphorylation sites in the distal C-terminus (Ser352 and Thr358; see Figure 2.7). In contrast, PKC-mediated receptor desensitization (based on the PKC activator phorbol 12-myristate 13-acetate (PMA)) was not affected by mutation of the distal

phosphorylation sites, but was affected by mutation of the proximal phosphorylation sites. Qi *et al.* (2011) confirmed this for endogenous canine P2Y1R as well as for recombinant hP2Y1R (by retroviral transduction), which internalized in a PKC-independent manner, relying on C-terminal Ser352 and Ser354 (Figure 2.7). The levels of recombinant P2Y1R did not adversely affect the internalization machinery [150].

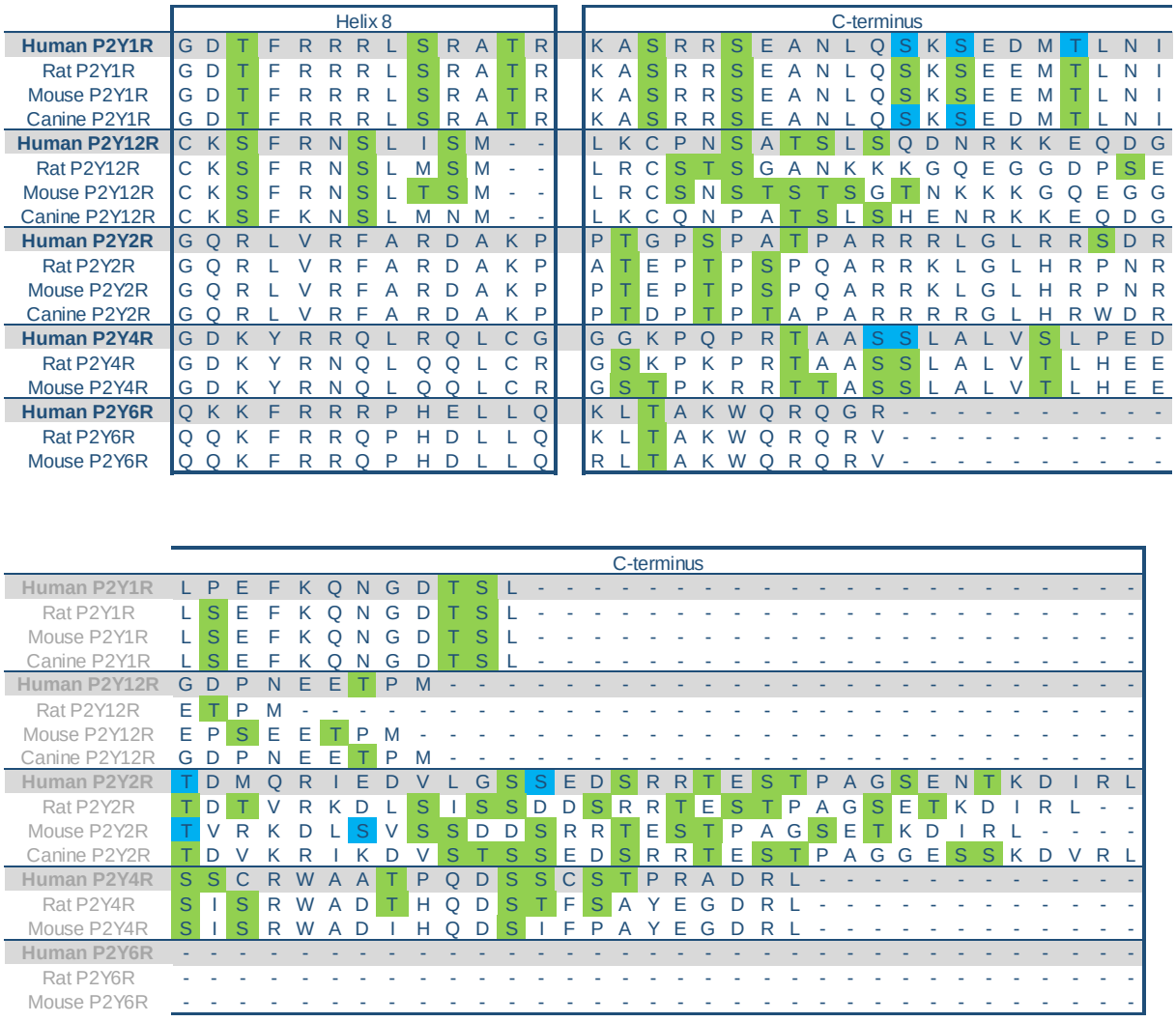


Figure 2.7: Alignment of the C-terminal part (Helix 8 and C-terminus) of selected P2YRs from different species: serine and threonine residues are marked in green and the ones discussed in the text are marked in blue.

In a study by Nisar *et al.* (2012), it was shown that an interaction with the PDZ-domain containing Na^+/H^+ exchanger regulatory factor (NHERF) is required for P2Y12R internalization [151]. Prior to agonist stimulation, there already is a direct

interaction of NHERF1 with a PDZ-motif in the C-terminus of P2Y12R. Upon agonist stimulation, arrestin serves as an adaptor to further promote NHERF1 interaction and P2Y12R internalization. Thus, NHERF1 is required for P2Y12R internalization by forming a tertiary complex consisting of P2Y12R, arrestin and NHERF1.

Platelets activated by ADP become refractory to re-stimulation; this has been attributed to P2Y1R desensitization and internalization [152, 153]. Besides, it was shown that P2Y1Rs and P2Y12Rs on human platelets rapidly resensitize, which allows platelets to maintain responsiveness [154]. Schaff *et al.* (2012) evaluated the role of βarr1 and βarr2 in agonist-induced platelet activation, P2Y1R and P2Y12R desensitization, haemostasis and experimental thrombosis. Although βarr1 and βarr2 have been shown to interact with the P2Y1R and P2Y12R, it was not clear whether they regulate desensitization in platelets. The results showed that βarr1 and βarr2 did not directly regulate P2Y1R/P2Y12R desensitization. P2Y1R desensitization and platelet refractoriness to ADP still occurred in βarr1-/- and βarr2-/- platelets – although a compensatory mechanism between the two isoforms could not be excluded. It was observed that platelets activated by ADP were unable to fully re-aggregate in response to a second challenge, as a result of P2Y1R but not P2Y12R desensitization [155]. Hence, P2Y1R desensitization might occur by an arrestin-independent mechanism, for example by phosphorylation by PKC, as mentioned earlier.

Gao & Jacobson (2017) (see section 2.2.2) evaluated the effect of orthosteric and allosteric antagonism on agonist-induced βarr2 recruitment to the P2Y1R, as well as βarr2 dependent receptor internalization, using two PathHunter™ U2OS cell systems (DiscoverX) [103]. Each cell line expressed a fusion protein of βarr2 to the enzyme acceptor. For the βarr2 recruitment assay, the cell line additionally contained the P2Y1R, fused at its C-terminus to a ProLink™ tag, and for the internalization assay the cell line contained untagged P2Y1R and a ProLink™ tag that was localised to endosomes. Cells were treated with antagonists (20 min) prior to addition of agonists (60 min), subsequently followed by incubation with detection reagent (60 min) at room temperature. For internalization assays, the agonist incubation time was 180 min instead of 60 min. Interestingly, the negative allosteric modulator BPTU showed different potencies and patterns of antagonism in different βarr2-mediated events

induced by the agonists tested. The authors suggested that this modulator must influence the P2Y1R conformation in such a way that different arrestin conformations are induced that have different functional outcomes. This is an example of functional selectivity even within the arrestin pathway and nicely demonstrates the multiple functions of the arrestin molecule.

The P2Y2R

In a study of Otero *et al.* (2000)**,** P2Y2R desensitization was investigated in human 1321N1 astrocytoma cells, transfected with murine P2Y2R, and human colonic adenocarcinoma HT-29 cells, endogenously expressing **hP2Y2R** [156]. A 5 min exposure to UTP caused P2Y2R desensitization in both cell lines. Full receptor responsiveness returned 5-10 min after UTP removal. A C-terminal P2Y2R truncation mutant, which had two of three potential consensus phosphorylation sites for PKC eliminated (344Thr and 350Ser in murine P2Y2R; see Figure 2.7) still showed desensitization by the PKC activator PMA but did no longer show UTP-induced desensitization. This suggested that phosphorylation of the murine P2Y2R C-terminus by kinases other than (PMA-sensitive) PKCs mediate agonist-induced receptor desensitization. These findings were in line with those of Flores *et al.* (2005) in human 1321N1 cells; mutation of three potential GRK/PKC phosphorylation sites in IL3 and the C-terminus of the P2Y2R (S243A, T344A, and S356A) did not affect Ca^{2+} mobilization, though extinguished UTP-induced receptor phosphorylation, reduced the efficacy of UTP to desensitize the P2Y2R, and impaired receptor internalization**.** Activation of PKC isoforms with PMA that caused heterologous receptor desensitization did not increase levels of P2Y2R phosphorylation [157]. These results indicate that agonist-mediated (homologous) P2Y2R desensitization must involve protein kinases other than PMA-activated PKC isoforms (such as GRKs or PMA-insensitive PKC isoforms), and that PKC-mediated (heterologous) P2Y2R desensitization might involve phosphorylation of signalling molecules other than the receptor.

Regarding the involvement of βarrs, Hoffmann *et al.* (2008) showed a βarr recruitment pattern to the **hP2Y2R** that was dependent on the agonist used; UTP gave pronounced βarr1 and βarr2 recruitment (class B behaviour), while ATP

recruited βarr2 more than βarr1 (class A behaviour). This was also reflected in ERK1/2 signalling; ATP-induced ERK1/2 phosphorylation was prolonged, while UTP gave transient stimulation [144]. The authors state that this was the first notion of biased signalling reported for the P2Y2R. We recently explored the coupling of the hP2Y2R with βarr2 using the NanoBit® reporter assay in HEK293T cells (see **Chapter 5**). C-terminal truncation of the P2Y2R did not hamper βarr2 recruitment upon stimulation of the receptor with UTP, ATP and the P2Y2R-selective agonist 2-thio-UTP, though seemed to have an influence on the kinetics of βarr2 recruitment. In studies by Morris *et al.* (2011, 2012), endogenous P2Y2Rs in rat arterial smooth muscle cells (ASMC) showed involvement of βarr1 and GRK2 in desensitization and agonist-stimulated ASMC migration, a key process in vascular remodeling and progression of vascular disease [158, 159]. Recently, it was shown that P2Y2R homodimer formation in transfected HEK293T and 1321N1 cells influenced βarr2 recruitment; certain cysteine to alanine mutants in the extracellular domains of the P2Y2R abrogated βarr2 recruitment. The assay used was based on functional complementation of β-galactosidase; cells were stimulated with UTP for 1h at 37°C, followed by 40 min incubation with reagents [160].

The P2Y4R and P2Y6R

In an early study by Robaye *et al*. (1997), the P2Y4R and the P2Y6R were reported to desensitize fast upon stimulation with UTP, or slow upon stimulation with UDP, respectively, in transfected human 1321N1 astrocytoma cells [161]. The authors noted that this might be due to the presence of four potential phosphorylation sites in the P2Y4R, compared to only one in the P2Y6R. The fast kinetics of P2Y4R desensitization were accompanied by pronounced receptor internalization, but also by fast receptor recovery on the cell surface after removal of agonist. In contrast, the P2Y6R only internalized upon extended agonist incubation, but hardly showed receptor recovery. The processes appear to occur independent of PKC or other second messenger related kinases. Regarding potential phosphorylation sites, it was shown that two serine residues in the P2Y4R C-terminus (Ser333 and Ser334; see Figure 2.7) are involved in agonist-dependent phosphorylation, desensitization and internalization of the P2Y4R [162]. Comparing these findings with those from Hoffmann *et al.* (2008), in which βarr recruitment to and internalization of the P2Y6R

could only be observed upon co-transfection of GRK2, the above-mentioned slow kinetics of P2Y6R internalization might be because of a lack of sufficient GRK2 [144].

The P2Y11R

The P2Y11R has shown internalization when forming a heterodimer with the P2Y1R [163]; P2Y11R dimerizes with P2Y1R when both are expressed recombinantly in 1321N1 astrocytoma cells, but also when P2Y1R is present at endogenous levels in HEK293 cells. Moreover, pharmacological analysis revealed that ligand specificity of the P2Y11R differed when only P2Y11R was expressed in 1321N1 cells, compared to when both receptors are present in HEK293 cells. This might be indicative of a role for receptor dimerization in functional selectivity.

2.4 Conclusive thoughts

For P1 or adenosine receptors (ARs) and P2Y receptors (P2YRs) – the GPCRs of the purinergic receptor family – first steps have been taken in the field of functional selectivity towards the development of biased ligands with a possibly beneficial pharmacological profile for application in the clinical setting. These ligands could separate wanted, on-target pharmacological effects from on-target but unwanted side effects when acting on their purinergic receptor. Molecular determinants that govern this bias have remained unknown for a long time and are only just now arising. For ARs, biased ligands have been developed for the A_1AR and/or A_{2B}AR with a promising therapeutic profile for the treatment of heart failure and angina pectoris. Also, for the A_3AR there is arising research exploring biased signalling profiles of existing and newly developed ligands. Some of these biased AR ligands bind the orthosteric binding site of the receptor, others bind an allosteric site, showing signalling bias as such (allosteric agonists) or modulating the signalling profile of orthosteric ligands (allosteric modulators). Besides, bitopic ligands have been developed that combine orthosteric and allosteric binding features. The advantage of functional selectivity by allosteric modulation is the capability to target areas where levels of the endogenous ligand are increased; in the case of adenosine, this involves areas of inflammation, ischemia/hypoxia or tumour sites, while areas with low adenosine levels are refractory to allosteric modulation. Fewer reports exist on functional selectivity for P2YRs. Biased signalling might hold therapeutic potential at

platelet-P2YRs; differential signalling at P2Y1R and P2Y12R might regulate platelet refractoriness after ADP stimulation, platelet aggregation and/or platelet-induced migration of inflammatory cells under inflammatory conditions.

The coupling to β-arrestin (βarr) is still one of the most popular pathways for which functional selectivity is explored. This pathway might hold therapeutic potential in the light of drug tolerance and/or overdose, although a clear link with the pathophysiological setting is still missing for purinergic GPCRs. This chapter shows a growing body of evidence for both P1 and P2Y receptors on the role of βarrs in receptor desensitization, internalization, as well as βarr-dependent signalling. However, some of these processes (densensitization, internalization) might also occur in a βarr-independent manner. Therefore, functional assays that directly evaluate the interaction of the GPCR with βarr are of equal or even bigger importance; when applicable in the high throughput setting, these can foster future development of biased purinergic ligands. In general, distinct techniques are used to study the βarr pathway; fluorescence microscopy-based βarr or receptor trafficking, as well as proximity assays and protein complementation assays, the latter often allowing more sensitive monitoring/quantification of signal. Most of the data originate from experiments in cell lines (e.g. CHO, 1321N1 and HEK293 cell lines) using (often) single, very high agonist concentrations up to 10 µM or 100 µM for P1 and P2Y receptors, respectively. Although concentrations that high may arise in the human body under stressful or pathologic conditions, it is also expedient that (lower) concentration ranges are tested to evaluate dose-dependent effects.

References

[1] Burnstock G, Verkhratsky A. Receptors for purines and pyrimidines. Purinergic Signalling and the Nervous System: Springer, 2012. p. 119-244.

[2] Fredholm BB. Adenosine, an endogenous distress signal, modulates tissue damage and repair. Cell Death Differ 2007;14:1315-23.

[3] Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Muller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine Receptors-An Update. Pharmacol Rev 2011;63:1-34.

[4] Borea PA, Gessi S, Merighi S, Vincenzi F, Varani K. Pharmacology of Adenosine Receptors: The State of the Art. Physiol Rev 2018;98:1591-625.

[5] Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International union of pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: From molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 2006;58:281-341.

[6] Erb L, Weisman GA. Coupling of P2Y receptors to G proteins and other signaling pathways. Wiley Interdiscip Rev Membr Transp Signal 2012;1:789-803.

[7] Burnstock G. Purinergic Signalling: Therapeutic Developments. Front Pharmacol 2017;8:661.

[8] Burnstock G. The therapeutic potential of purinergic signalling. Biochem Pharmacol 2018;151:157-65.

[9] Gessi S, Merighi S, Varani K. Adenosine Receptors: The Status of the Art. The Adenosine Receptors: Springer, 2018. p. 1-11.

[10] Voors AA, Dungen HD, Senni M, Nodari S, Agostoni P, Ponikowski P, et al. Safety and Tolerability of Neladenoson Bialanate, a Novel Oral Partial Adenosine A1 Receptor Agonist, in Patients With Chronic Heart Failure. J Clin Pharmacol 2017;57:440-51.

[11] Madi L, Cohen S, Ochayin A, Bar-Yehuda S, Barer F, Fishman P. Overexpression of A(3) adenosine receptor in peripheral blood mononuclear cells in rheumatoid arthritis: Involvement of nuclear factor-kappa B in mediating receptor level. J Rheumatol 2007;34:20-6.

[12] Varani K, Padovan M, Vincenzi F, Targa M, Trotta F, Govoni M, et al. A(2A) and A(3) adenosine receptor expression in rheumatoid arthritis: upregulation, inverse correlation with disease activity score and suppression of inflammatory cytokine and metalloproteinase release. Arthritis Res Ther 2011;13:R197.

[13] Ravani A, Vincenzi F, Bortoluzzi A, Padovan M, Pasquini S, Gessi S, et al. Role and Function of A(2A) and A(3) Adenosine Receptors in Patients with Ankylosing Spondylitis, Psoriatic Arthritis and Rheumatoid Arthritis. Int J Mol Sci 2017;18:697.

[14] Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 2006;5:247-64.

[15] Klaasse EC, Ijzerman AP, de Grip WJ, Beukers MW. Internalization and desensitization of adenosine receptors. Purinerg Signal 2008;4:21-37.

[16] Verzijl D, Ijzerman AP. Functional selectivity of adenosine receptor ligands. Purinerg Signal 2011;7:171-92.

[17] Muller CE, Jacobson KA. Recent developments in adenosine receptor ligands and their potential as novel drugs. Biochim Biophys Acta 2011;1808:1290-308.

[18] Jacobson KA, Muller CE. Medicinal chemistry of adenosine, P2Y and P2X receptors. Neuropharmacology 2016;104:31-49.
[19] Djerada Z, Feliu C, Richard V, Millart H. Current knowledge on the role of P2Y receptors in cardioprotection against ischemia-reperfusion. Pharmacol Res 2017;118:5-18.
[20] Nishimura A, Sunggip C, Oda S, Numaga-Tomita T, Tsuda M, Nishida M. Purinergic P2Y receptors: Molecular diversity and implications for treatment of cardiovascular diseases. Pharmacol Therapeut 2017;180:113-28.
[21] Burnstock G, Di Virgilio F. Purinergic signalling and cancer. Purinerg Signal 2013;9:491-540.
[22] Gachet C. Regulation of platelet functions by P2 receptors. Annu Rev Pharmacol Toxicol 2006;46:277-300.
[23] von Kugelgen I, Hoffmann K. Pharmacology and structure of P2Y receptors. Neuropharmacology 2016;104:50-61.
[24] Cattaneo M. The platelet P2Y12 receptor for adenosine diphosphate: congenital and drug-induced defects. Blood 201;117:2102-2112.
[25] Hechler B, Gachet C. P2 receptors and platelet function. Purinerg Signal 2011;7:293-303.
[26] Fajac I, De Boeck K. New horizons for cystic fibrosis treatment. Pharmacol Therapeut 2017;170:205-11.
[27] Marson FAL. Disease-modifying genetic factors in cystic fibrosis. Curr Opin Pulm Med 2018;24:296-308.
[28] Lau OC, Samarawickrama C, Skalicky SE. P2Y2 receptor agonists for the treatment of dry eye disease: a review. Clin Ophthalmol 2014;8:327.
[29] Weisman GA, Ajit D, Garrad R, Peterson TS, Woods LT, Thebeau C, et al. Neuroprotective roles of the P2Y(2) receptor. Purinerg Signal 2012;8:559-78.
[30] Xu PF, Feng X, Luan HY, Wang JB, Ge RL, Li ZY, et al. Current knowledge on the nucleotide agonists for the P2Y2 receptor. Bioorgan Med Chem 2018;26:366-75.
[31] Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, et al. The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. Science 2008;322:1211-7.
[32] Xu F, Wu HX, Katritch V, Han GW, Jacobson KA, Gao ZG, et al. Structure of an Agonist-Bound Human A(2A) Adenosine Receptor. Science 2011;332:322-7.
[33] Piirainen H, Ashok Y, Nanekar RT, Jaakola VP. Structural features of adenosine receptors: From crystal to function. BBA-Biomembranes 2011;1808:1233-44.
[34] Zhang K, Zhang J, Gao ZG, Zhang D, Zhu L, Han GW, et al. Structure of the human P2Y12 receptor in complex with an antithrombotic drug. Nature 2014;509:115-8.
[35] Zhang D, Gao ZG, Zhang K, Kiselev E, Crane S, Wang J, et al. Two disparate ligand-binding sites in the human P2Y1 receptor. Nature 2015;520:317-21.
[36] Glukhova A, Thal DM, Nguyen AT, Vecchio EA, Jorg M, Scammells PJ, et al. Structure of the Adenosine A1 Receptor Reveals the Basis for Subtype Selectivity. Cell 2017;168:867-77 e13.
[37] Ciancetta A, Jacobson KA. Structural Probing and Molecular Modeling of the A(3) Adenosine Receptor: A Focus on Agonist Binding. Molecules 2017;22:449.

[38] Ciancetta A, Jacobson KA. Breakthrough in GPCR Crystallography and Its Impact on Computer-Aided Drug Design. Methods Mol Biol 2018;1705:45-72.
[39] Jacobson KA, Klutz AM, Tosh DK, Ivanov AA, Preti D, Baraldi PG. Medicinal chemistry of the A_3 adenosine receptor: agonists, antagonists, and receptor engineering. Handb Exp Pharmacol 2009:123-59.
[40] Jacobson KA, Balasubramanian R, Deflorian F, Gao ZG. G protein-coupled adenosine (P1) and P2Y receptors: ligand design and receptor interactions. Purinerg Signal 2012;8:419-36.
[41] Jacobson KA, Merighi S, Varani K, Borea PA, Baraldi S, Aghazadeh Tabrizi M, et al. A_3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy. Med Res Rev 2018:38:1031-72.
[42] Jacobson KA, Tosh DK, Gao Z-G, Yu J, Suresh RR, Rao H, et al. Medicinal Receptor Chemistry of the A_3 Adenosine. 2018;34:169.
[43] Gao Z-G, Tosh DK, Jain S, Yu J, Suresh RR, Jacobson KA. A 1 Adenosine Receptor Agonists, Antagonists, and Allosteric Modulators. The Adenosine Receptors: Springer, 2018. p. 59-89.
[44] Baraldi S, Baraldi PG, Oliva P, Toti KS, Ciancetta A, Jacobson KA. A 2A Adenosine Receptor: Structures, Modeling, and Medicinal Chemistry. The Adenosine Receptors: Springer, 2018. p. 91-136.
[45] Müller CE, Baqi Y, Hinz S, Namasivayam VJTAR. Medicinal Receptors Chemistry of A2B Adenosine. The adenosine receptors 2018;34:137.
[46] Jacobson KA, Jarvis MF, Williams M. Purine and pyrimidine (P2) receptors as drug targets. J Med Chem 2002;45:4057-93.
[47] Jacobson KA, Paoletta S, Katritch V, Wu BL, Gao ZG, Zhao Q, et al. Nucleotides Acting at P2Y Receptors: Connecting Structure and Function. Mol Pharmacol 2015;88:220-30.
[48] Rafehi M, Müller CE. Tools and drugs for uracil nucleotide-activated P2Y receptors. Pharmacol Therapeut 2018;190:24-80.
[49] Liu HB, Ge H, Peng Y, Xiao PG, Xu J. Molecular mechanism of action for reversible P2Y(12) antagonists. Biophys Chem 2011;155:74-81.
[50] Paoletta S, Sabbadin D, von Kugelgen I, Hinz S, Katritch V, Hoffmann K, et al. Modeling ligand recognition at the P2Y(12) receptor in light of X-ray structural information. J Comput Aid Mol Des 2015;29:737-56.
[51] Kim HS, Ravi RG, Marquez VE, Maddileti S, Wihlborg AK, Erlinge D, et al. Methanocarba modification of uracil and adenine nucleotides: High potency of northern ring conformation at P2Y(1), P2Y(2), P2Y(4), and P2Y(11) but not P2Y(6) receptors. J Med Chem 2002;45:208-18.
[52] Chhatriwala M, Ravi RG, Patel RI, Boyer JL, Jacobson KA, Harden TK. Induction of novel agonist selectivity for the ADP-activated P2Y1 receptor versus the ADP-activated P2Y12 and P2Y13 receptors by conformational constraint of an ADP analog. J Pharmacol Exp Ther 2004;311:1038-43.
[53] Ivanov AA, Ko H, Cosyn L, Maddileti S, Besada P, Fricks I, et al. Molecular modeling of the human P2Y(2) receptor and design of a selective agonist, 2 '-amino-2 '-deoxy-2-thiouridine 5 '-triphosphate. J Med Chem 2007;50:1166-76.
[54] El-Tayeb A, Qi AD, Muller CE. Synthesis and structure-activity relationships of uracil nucleotide derivatives and analogues as agonists at human P2Y(2), P2Y(4), and P2Y(6) receptors. J Med Chem 2006;49:7076-87.
[55] El-Tayeb A, Qi AD, Nicholas RA, Muller CE. Structural Modifications of UMP, UDP, and UTP Leading to Subtype-Selective Agonists for P2Y(2), P2Y(4), and P2Y(6) Receptors. J Med Chem 2011;54:2878-90.

[56] Kenakin T, Miller LJ. Seven Transmembrane Receptors as Shapeshifting Proteins: The Impact of Allosteric Modulation and Functional Selectivity on New Drug Discovery. Pharmacol Rev 2010;62:265-304.
[57] Kenakin T. Functional Selectivity and Biased Receptor Signaling. J Pharmacol Exp Ther 2011;336:296-302.
[58] Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527-52.
[59] Merighi S, Gessi S, Borea PA. Adenosine Receptors: Structure, Distribution, and Signal Transduction. The Adenosine Receptors: Springer, 2018. p. 33-57.
[60] Luttrell LM, Spiller BW. Arrestin-Dependent ERK Activation and Its Disruption. The Structural Basis of Arrestin Functions: Springer, 2017. p. 199-217.
[61] Schulte G, Fredholm BB. Human adenosine A(1), A(2A), A(2B), and A(3) receptors expressed in Chinese hamster ovary cells all mediate the phosphorylation of extracellular-regulated kinase 1/2. Mol Pharmacol 2000;58:477-82.
[62] Schulte G, Fredhohn BB. Signalling from adenosine receptors to mitogen-activated protein kinases. Cell Signal 2003;15:813-27.
[63] Rajagopal S, Rajagopal K, Lefkowitz RJ. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov 2010;9:373-86.
[64] Gao ZG, Jacobson KA. Allosteric modulation and functional selectivity of G protein-coupled receptors. Drug Discov Today Technol 2013;10:e237-43.
[65] Vecchio EA, Baltos JA, Nguyen ATN, Christopoulos A, White PJ, May LT. New paradigms in adenosine receptor pharmacology: allostery, oligomerization and biased agonism. Br J Pharmacol 2018;175:4036-46.
[66] Cordeaux Y, Briddon SJ, Megson AE, McDonnell J, Dickenson JM, Hill SJ. Influence of receptor number on functional responses elicited by agonists acting at the human adenosine A(1) receptor: Evidence for signaling pathway-dependent changes in agonist potency and relative intrinsic activity. Mol Pharmacol 2000;58:1075-84.
[67] Van Schaick E, Mathôt R, Gubbens-Stibbe J, Langemeijer M, Roelen H, IJzerman A, et al. 8-Alkylamino-substituted analogs of N6-cyclopentyladenosine are partial agonists for the cardiovascular adenosine A1 receptors in vivo. J Pharmacol Exp Ther 1997;283:800-8.
[68] Van Schaick E, Tukker H, Roelen H, IJzerman A, Danhof M. Selectivity of action of 8-alkylamino analogues of N6-cyclopentyladenosine in vivo: haemodynamic versus anti-lipolytic responses in rats. Brit J Pharmacol 1998;124:607-18.
[69] Cordeaux Y, IJzerman AP, Hill SJ. Coupling of the human A1 adenosine receptor to different heterotrimeric G proteins: evidence for agonist-specific G protein activation. Brit J Pharmacol 2004;143:705-14.
[70] Reid EA, Kristo G, Yoshimura Y, Ballard-Croft C, Keith BJ, Mentzer RM, et al. In vivo adenosine receptor preconditioning reduces myocardial infarct size via subcellular ERK signaling. Am J Physiol-Heart C 2005;288:H2253-H9.
[71] Germack R, Dickenson JM. Adenosine triggers preconditioning through MEK/ERK1/2 signalling pathway during hypoxia/reoxygenation in neonatal rat cardiomyocytes. J Mol Cell Cardiol 2005;39:429-42.
[72] Peart JN, Headrick JP. Adenosinergic cardioprotection: Multiple receptors, multiple pathways. Pharmacol Therapeut 2007;114:208-21.

[73] Zhan EB, McIntosh VJ, Lasley RD. Adenosine A(2A) and A(2B) receptors are both required for adenosine A(1) receptor-mediated cardioprotection. Am J Physiol-Heart C 2011;301:H1183-H9.
[74] Williams-Pritchard G, Knight M, Hoe LS, Headrick JP, Peart JN. Essential role of EGFR in cardioprotection and signaling responses to A(1) adenosine receptors and ischemic preconditioning. Am J Physiol-Heart C 2011;300:H2161-H8.
[75] Lasley RD. Adenosine Receptor-Mediated Cardioprotection-Current Limitations and Future Directions. Front Pharmacol 2018;9:310.
[76] Aurelio L, Valant C, Flynn BL, Sexton PM, Christopoulos A, Scammells PJ. Allosteric Modulators of the Adenosine A(1) Receptor: Synthesis and Pharmacological Evaluation of 4-Substituted 2-Amino-3-benzoylthiophenes. J Med Chem 2009;52:4543-7.
[77] Valant C, Aurelio L, Urmaliya VB, White P, Scammells PJ, Sexton PM, et al. Delineating the Mode of Action of Adenosine A(1) Receptor Allosteric Modulators. Mol Pharmacol 2010;78:444-55.
[78] Valant C, May LT, Aurelio L, Chuo CH, White PJ, Baltos JA, et al. Separation of on-target efficacy from adverse effects through rational design of a bitopic adenosine receptor agonist. Proc Natl Acad Sci USA 2014;111:4614-9.
[79] Albrecht-Kupper BE, Leineweber K, Nell PG. Partial adenosine A1 receptor agonists for cardiovascular therapies. Purinerg Signal 2012;8:S91-S9.
[80] Tendera M, Gaszewska-Zurek E, Parma Z, Ponikowski P, Jankowska E, Kawecka-Jaszcz K, et al. The new oral adenosine A1 receptor agonist capadenoson in male patients with stable angina. Clin Res Cardiol 2012;101:585-91.
[81] Study to Investigate the Effect of the A1 Agonist Capadenoson on Ventricular HR in Patients With Persistent or Permanent Atrial Fibrillation. https://clinicaltrials.gov/ct2/show/NCT00568945
[82] Baltos JA, Gregory KJ, White PJ, Sexton PM, Christopoulos A, May LT. Quantification of adenosine A(1) receptor biased agonism: Implications for drug discovery. Biochem Pharmacol 2016;99:101-12.
[83] Aurelio L, Baltos JA, Ford L, Nguyen ATN, Jorg M, Devine SM, et al. A Structure-Activity Relationship Study of Bitopic N-6-Substituted Adenosine Derivatives as Biased Adenosine A(1) Receptor Agonists. J Med Chem 2018;61:2087-103.
[84] Meibom D, Albrecht-Kupper B, Diedrichs N, Hubsch W, Kast R, Kramer T, et al. Neladenoson Bialanate Hydrochloride: A Prodrug of a Partial Adenosine A(1) Receptor Agonist for the Chronic Treatment of Heart Diseases. Chemmedchem 2017;12:728-37.
[85] Gao ZG, Balasubramanian R, Kiselev E, Wei Q, Jacobson KA. Probing biased/partial agonism at the G protein-coupled A(2B) adenosine receptor. Biochem Pharmacol 2014;90:297-306.
[86] Hinz S, Lacher SK, Seibt BF, Muller CE. BAY60-6583 Acts as a Partial Agonist at Adenosine A(2B) Receptors. J Pharmacol Exp Ther 2014;349:427-36.
[87] Betti M, Catarzi D, Varano F, Falsini M, Varani K, Vincenzi F, et al. The aminopyridine-3,5-dicarbonitrile core for the design of new non-nucleoside-like agonists of the human adenosine A(2B) receptor. Eur J Med Chem 2018;150:127-39.

[88] Vecchio EA, Chuo CH, Baltos JA, Ford L, Scammells PJ, Wang BH, et al. The hybrid molecule, VCP746, is a potent adenosine A(2B) receptor agonist that stimulates anti-fibrotic signalling. Biochem Pharmacol 2016;117:46-56.
[89] Baltos JA, Vecchio EA, Harris MA, Qin CX, Ritchie RH, Christopoulos A, et al. Capadenoson, a clinically trialed partial adenosine A1 receptor agonist, can stimulate adenosine A2B receptor biased agonism. Biochem Pharmacol 2017;135:79-89.
[90] Vecchio EA, White PJ, May LT. Targeting Adenosine Receptors for the Treatment of Cardiac Fibrosis. Front Pharmacol 2017;8:243.
[91] Bessa-Goncalves M, Braganca B, Martins-Dias E, Correia-de-Sa P, Fontes-Sousa AP. Is the adenosine A(2B) 'biased' receptor a valuable target for the treatment of pulmonary arterial hypertension? Drug Discov Today 2018;23:1285-92.
[92] Baltos JA, Paoletta S, Nguyen AT, Gregory KJ, Tosh DK, Christopoulos A, et al. Structure-Activity Analysis of Biased Agonism at the Human Adenosine A3 Receptor. Mol Pharmacol 2016;90:12-22.
[93] Gao ZG, Jacobson KA. Translocation of arrestin induced by human A(3) adenosine receptor ligands in an engineered cell line: comparison with G protein-dependent pathways. Pharmacol Res 2008;57:303-11.
[94] Gao ZG, Verzijl D, Zweemer A, Ye K, Goblyos A, Ijzerman AP, et al. Functionally biased modulation of A(3) adenosine receptor agonist efficacy and potency by imidazoquinolinamine allosteric enhancers. Biochem Pharmacol 2011;82:658-68.
[95] Storme J, Tosh DK, Gao ZG, Jacobson KA, Stove CP. Probing Structure-Activity Relationship in β-Arrestin2 Recruitment of Diversely Substituted Adenosine Derivatives. Biochem Pharmacol 2018; 158: 103-113.
[96] Matot I, Weiniger CF, Zeira E, Galun E, Joshi BV, Jacobson KA. A 3 adenosine receptors and mitogen-activated protein kinases in lung injury following in vivo reperfusion. Critical Care 2006;10:R65.
[97] Headrick JP, Lasley RD. Adenosine receptors and reperfusion injury of the heart. Handb Exp Pharmacol 2009:189-214.
[98] Cohen S, Barer F, Bar-Yehuda S, IJzerman AP, Jacobson KA, Fishman P. A(3) Adenosine Receptor Allosteric Modulator Induces an Anti-Inflammatory Effect: In Vivo Studies and Molecular Mechanism of Action. Mediat Inflamm 2014.
[99] Ochaion A, Bar-Yehuda S, Cohn S, Del Valle L, Perez-Liz G, Madi L, et al. Methotrexate enhances the anti-inflammatory effect of CF101 via up-regulation of the A(3) adenosine receptor expression. Arthritis Res Ther 2006;8:R169.
[100] Erb L, Liu J, Ockerhausen J, Kong QM, Garrad RC, Griffin K, et al. An RGD sequence in the P2Y(2) receptor interacts with alpha(V)beta(3) integrins and is required for G(0)-mediated signal transduction. J Cell Biol 2001;153:491-501.
[101] Bagchi S, Liao ZJ, Gonzalez FA, Chorna NE, Seye CI, Weisman GA, et al. The P2Y(2) nucleotide receptor interacts with alpha(v) integrins to activate G(o) and induce cell migration. J Biol Chem 2005;280:39050-7.
[102] Liao ZJ, Seye CI, Weisman GA, Erb L. The P2Y(2) nucleotide receptor requires interaction with alpha v integrins to access and activate G12. J Cell Sci 2007;120:1654-62.
[103] Gao ZG, Jacobson KA. Distinct Signaling Patterns of Allosteric Antagonism at the P2Y(1) Receptor. Mol Pharmacol 2017;92:613-26.

[104] Page G, Pitchford S. Neutrophil and platelet complexes and their relevance to neutrophil recruitment and activation. Int Immunopharmacol 2013;17:1176-84.
[105] Pan DX, Amison RT, Riffo-Vasquez Y, Spina D, Cleary SJ, Wakelam MJ, et al. P-Rex and Vav Rac-GEFs in platelets control leukocyte recruitment to sites of inflammation. Blood 2015;125:1146-58.
[106] Amison RT, Jamshidi S, Rahman KM, Page CP, Pitchford SC. Diverse signalling of the platelet P2Y(1) receptor leads to a dichotomy in platelet function. Eur J Pharmacol 2018;827:58-70.
[107] Gabl M, Winther M, Welin A, Karlsson A, Oprea T, Bylund J, et al. P2Y(2) receptor signaling in neutrophils is regulated from inside by a novel cytoskeleton-dependent mechanism. Exp Cell Res 2015;336:242-52.
[108] White PJ, Webb TE, Boarder MR. Characterization of a Ca2+ response to both UTP and ATP at human P2Y(11) receptors: Evidence for agonist-specific signaling. Mol Pharmacol 2003;63:1356-63.
[109] Morrow GB, Nicholas RA, Kennedy C. UTP is not a biased agonist at human P2Y 11 receptors. Purinerg Signal 2014;10:581-5.
[110] Mundell S, Kelly E. Adenosine receptor desensitization and trafficking. Biochim Biophys Acta 2011;1808:1319-28.
[111] Palmer TM, Benovic JL, Stiles GL. Agonist-dependent phosphorylation and desensitization of the rat A_3 adenosine receptor. Evidence for a G-protein-coupled receptor kinase-mediated mechanism. J Biol Chem 1995;270:29607-13.
[112] Palmer TM, Benovic JL, Stiles GL. Molecular basis for subtype-specific desensitization of inhibitory adenosine receptors. Analysis of a chimeric A1-A3 adenosine receptor. J Biol Chem 1996;271:15272-8.
[113] Ferguson G, Watterson KR, Palmer TM. Subtype-specific kinetics of inhibitory adenosine receptor internalization are determined by sensitivity to phosphorylation by G protein-coupled receptor kinases. Mol Pharmacol 2000;57:546-52.
[114] Palmer TM, Stiles GL. Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor. Mol Pharmacol 2000;57:539-45.
[115] Palmer TA, Ferguson G, Watterson KR. Dissecting the regulatory mechanisms controlling inhibitory adenosine receptor signaling. Drug Develop Res 2003;58:302-14.
[116] Ferguson G, Watterson KR, Palmer TM. Subtype-specific regulation of receptor internalization and recycling by the carboxyl-terminal domains of the human A_1 and rat A_3 adenosine receptors: consequences for agonist-stimulated translocation of arrestin3. Biochemistry 2002;41:14748-61.
[117] Santini F, Penn RB, Gagnon AW, Benovic JL, Keen JH. Selective recruitment of arrestin-3 to clathrin coated pits upon stimulation of G protein-coupled receptors. J Cell Sci 2000;113:2463-70.
[118] Trincavelli ML, Tuscano D, Cecchetti P, Falleni A, Benzi L, Klotz KN, et al. Agonist-induced internalization and recycling of the human A(3) adenosine receptors: Role in receptor desensitization and resensitization. J Neurochem 2000;75:1493-501.
[119] Trincavelli ML, Tuscano D, Marroni M, Falleni A, Gremigni V, Ceruti S, et al. A(3) adenosine receptors in human astrocytoma cells: Agonist-mediated desensitization, internalization, and down-regulation. Mol Pharmacol 2002;62:1373-84.

[120] Gao ZG, Chen A, Barak D, Kim SK, Muller CE, Jacobson KA. Identification by site-directed mutagenesis of residues involved in ligand recognition and activation of the human A(3) adenosine receptor. J Biol Chem 2002;277:19056-63.
[121] Stoddart LA, Kellam B, Briddon SJ, Hill SJ. Effect of a toggle switch mutation in TM6 of the human adenosine A(3) receptor on Gi protein-dependent signalling and Gi-independent receptor internalization. Brit J Pharmacol 2014;171:3827-44.
[122] Stoddart LA, Vernall AJ, Briddon SJ, Kellam B, Hill SJ. Direct visualisation of internalization of the adenosine A(3) receptor and localization with arrestin3 using a fluorescent agonist. Neuropharmacology 2015;98:68-77.
[123] Storme J, Cannaert A, Van Craenenbroeck K, Stove CP. Molecular dissection of the human A3 adenosine receptor coupling with beta-arrestin2. Biochem Pharmacol 2018;148:298-307.
[124] Trincavelli ML, Tuscano D, Marroni M, Klotz KN, Lucacchini A, Martini C. Involvement of mitogen protein kinase cascade in agonist-mediated human A(3) adenosine receptor regulation. BBA-Mol Cell Res 2002;1591:55-62.
[125] Jajoo S, Mukherjea D, Kumar S, Sheth S, Kaur T, Rybak LP, et al. Role of beta-arrestin1/ERK MAP kinase pathway in regulating adenosine A1 receptor desensitization and recovery. Am J Physiol Cell Physiol 2010;298:C56-65.
[126] Langemeijer EV, Verzijl D, Dekker SJ, Ijzerman AP. Functional selectivity of adenosine A1 receptor ligands? Purinerg Signal 2013;9:91-100.
[127] Dixon AS, Schwinn MK, Hall MP, Zimmerman K, Otto P, Lubben TH, et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS Chemical Biology 2016;11:400-8.
[128] Gao ZG, Ye K, Goblyos A, Ijzerman AP, Jacobson KA. Flexible modulation of agonist efficacy at the human A3 adenosine receptor by the imidazoquinoline allosteric enhancer LUF6000. BMC pharmacology 2008;8:20.
[129] Gao ZG, Verzijl D, Zweemer A, Ye K, Goblyos A, Ijzerman AP, et al. Functionally biased modulation of A(3) adenosine receptor agonist efficacy and potency by imidazoquinolinamine allosteric enhancers. Biochem Pharmacol 2011;82:658-68.
[130] Zezula J, Freissmuth M. The A(2A)-adenosine receptor: a GPCR with unique features? Brit J Pharmacol 2008;153:S184-S90.
[131] Keuerleber S, Gsandtner I, Freissmuth M. From cradle to twilight: The carboxyl terminus directs the fate of the A(2A)-adenosine receptor. BBA-Biomembranes 2011;1808:1350-7.
[132] Palmer TM, Stiles GL. Identification of an A(2) adenosine receptor domain specifically responsible for mediating short-term desensitization. Biochemistry 1997;36:832-8.
[133] Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol Therapeut 2006;110:465-502.
[134] Mundell SJ, Benovic JL, Kelly E. A dominant negative mutant of the G protein-coupled receptor kinase 2 selectively attenuates adenosine A(2) receptor desensitization. Mol Pharmacol 1997;51:991-8.
[135] Mundell SJ, Luty JS, Willets J, Benovic JL, Kelly E. Enhanced expression of G protein-coupled receptor kinase 2 selectively increases the sensitivity of A(2A) adenosine receptors to agonist-induced desensitization. Brit J Pharmacol 1998;125:347-56.

[136] Burgueno J, Blake DJ, Benson MA, Tinsley CL, Esapa CT, Canela EI, et al. The adenosine A(2A) receptor interacts with the actin-binding protein alpha-actinin. J Biol Chem 2003;278:37545-52.
[137] Brand F, Klutz AM, Jacobson KA, Fredholm BB, Schulte G. Adenosine A(2A) receptor dynamics studied with the novel fluorescent agonist Alexa488-APEC. Eur J Pharmacol 2008;590:36-42.
[138] Khoa ND, Postow M, Danielsson J, Cronstein BN. Tumor necrosis factor-alpha prevents desensitization of G alpha(s)-coupled receptors by regulating GRK2 association with the plasma membrane. Mol Pharmacol 2006;69:1311-9.
[139] Matharu AL, Mundell SJ, Benovic JL, Kelly E. Rapid agonist-induced desensitization and internalization of the A(2B) adenosine receptor is mediated by a serine residue close to the COOH terminus. J Biol Chem 2001;276:30199-207.
[140] Mundell SJ, Matharu AL, Nisar S, Palmer TM, Benovic JL, Kelly E. Deletion of the distal COOH-terminus of the A(2B) adenosine receptor switches internalization to an arrestin- and clathrin-independent pathway and inhibits recycling. Brit J Pharmacol 2010;159:518-33.
[141] Mundell SJ, Loudon RP, Benovic JL. Characterization of G protein-coupled receptor regulation in antisense mRNA-expressing cells with reduced arrestin levels. Biochemistry 1999;38:8723-32.
[142] Mundell SJ, Matharu AL, Kelly E, Benovic JL. Arrestin isoforms dictate differential kinetics of A(2B) adenosine receptor trafficking. Biochemistry 2000;39:12828-36.
[143] Penn RB, Pascual RM, Kim YM, Mundell SJ, Krymskaya VP, Panettieri RA, et al. Arrestin specificity for G protein-coupled receptors in human airway smooth muscle. J Biol Chem 2001;276:32648-56.
[144] Hoffmann C, Ziegler N, Reiner S, Krasel C, Lohse MJ. Agonist-selective, Receptor-specific Interaction of Human P-2Y Receptors with beta-Arrestin-1 and-2. J Biol Chem 2008;283:30933-41.
[145] Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem 2000;275:17201-10.
[146] Hardy AR, Conley PB, Luo JS, Benovic JL, Poole AW, Mundell SJ. P2Y(1) and P2Y(12) receptors for ADP desensitize by distinct kinase-dependent mechanisms. Blood 2005;105:3552-60.
[147] Mundell SJ, Jones ML, Hardy AR, Barton JF, Beaucourt SM, Conley PB, et al. Distinct roles for protein kinase C Isoforms in regulating platelet purinergic receptor function. Mol Pharmacol 2006;70:1132-42.
[148] Mundell SJ, Luo JS, Benovic JL, Conley PB, Poole AW. Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. Traffic 2006;7:1420-31.
[149] Reiner S, Ziegler N, Leon C, Lorenz K, von Hayn K, Gachet C, et al. beta-Arrestin-2 interaction and internalization of the human P2Y1 receptor are dependent on C-terminal phosphorylation sites. Mol Pharmacol 2009;76:1162-71.
[150] Qi AD, Houston-Cohen D, Naruszewicz I, Harden TK, Nicholas RA. Ser352 and Ser354 in the carboxyl terminus of the human P2Y(1) receptor are

required for agonist-promoted phosphorylation and internalization in MDCK cells. Brit J Pharmacol 2011;162:1304-13.
[151] Nisar SP, Cunningham M, Saxena K, Pope RJ, Kelly E, Mundell SJ. Arrestin Scaffolds NHERF1 to the P2Y(12) Receptor to Regulate Receptor Internalization. J Biol Chem 2012;287:24505-15.
[152] Baurand A, Eckly A, Bari N, Leon C, Hechler B, Cazenave JP, et al. Desensitization of the platelet aggregation response to ADP: Differential down-regulation of the P2Y(1) and P2cyc receptors. Thromb Haemostasis 2000;84:484-91.
[153] Baurand A, Eckly A, Hechler B, Kauffenstein G, Galzi JL, Cazenave JP, et al. Differential regulation and relocalization of the platelet P2Y receptors after activation: A way to avoid loss of hemostatic properties? Mol Pharmacol 2005;67:721-33.
[154] Mundell S, Barton J, Mayo-Martin M, Hardy A, Poole A. Rapid resensitization of purinergic receptor function in human platelets. J Thromb Haemost 2008;6:1393-404.
[155] Schaff M, Receveur N, Bourdon C, Ohlmann P, Lanza F, Gachet C, et al. beta-arrestin-1 participates in thrombosis and regulates integrin alpha(IIB)beta(3) signalling without affecting P2Y receptors desensitisation and function. Thromb Haemostasis 2012;107:735-48.
[156] Otero M, Garrad RC, Velazquez B, Hernandez-Perez MG, Camden JM, Erb L, et al. Mechanisms of agonist-dependent and -independent desensitization of a recombinant P2Y2 nucleotide receptor. Mol Cell Biochem 2000;205:115-23.
[157] Flores RV, Hernandez-Perez MG, Aquino E, Garrad RC, Weisman GA, Gonzalez FA. Agonist-induced phosphorylation and desensitization of the P2Y(2) nucleotide receptor. Mol Cell Biochem 2005;280:35-45.
[158] Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RAJ, et al. G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoceptor signalling. Cardiovasc Res 2011;89:193-203.
[159] Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RAJ, Willets JM. Arrestins 2 and 3 differentially regulate ETA and P2Y(2) receptor-mediated cell signaling and migration in arterial smooth muscle. Am J Physiol-Cell Ph 2012;302:C723-C34.
[160] Abe M, Watanabe K, Kuroda Y, Nakagawa T, Higashi H. Homodimer formation by the ATP/UTP receptor P2Y(2) via disulfide bridges. J Biochem 2018;163:475-80.
[161] Robaye B, Boeynaems JM, Communi D. Slow desensitization of the human P2Y(6) receptor. Eur J Pharmacol 1997;329:231-6.
[162] Brinson AE, Harden TK. Differential regulation of the uridine nucleotide-activated P2Y4 and P2Y6 receptors - Ser-333 and Ser-334 in the carboxyl terminus are involved in agonist-dependent phosphorylation desensitization and internalization of the P2Y4 receptor. J Biol Chem 2001;276:11939-48.
[163] Ecke D, Hanck T, Tulapurkar ME, Schafer R, Kassack M, Stricker R, et al. Hetero-oligomerization of the P2Y(11) receptor with the P2Y(1) receptor controls the internalization and ligand selectivity of the P2Y(11) receptor. Biochem J 2008;409:107-16.

Chapter 3:

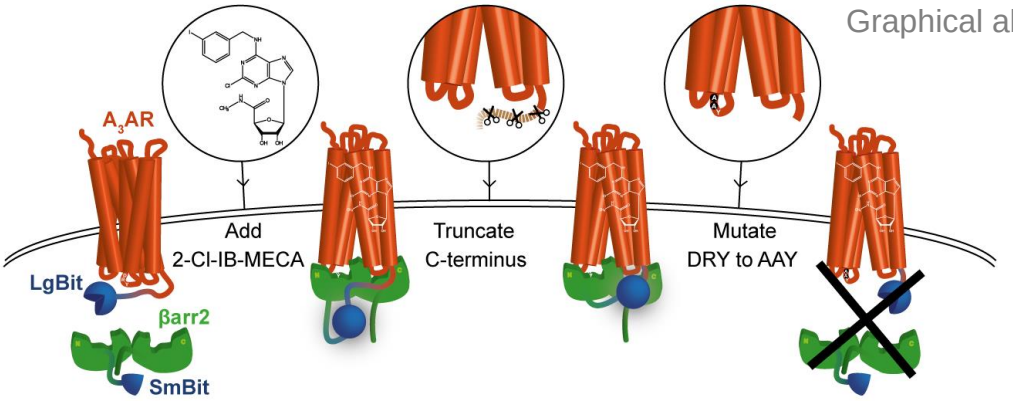
Molecular dissection of the human A_3 adenosine receptor coupling with β-arrestin2

Based on: Storme J, Cannaert A, Van Craenenbroeck K, Stove CP. Molecular dissection of the human A3 adenosine receptor coupling with beta-arrestin2. Biochemical Pharmacology 2018;148:298-307.

Abstract

Besides classical G protein coupling, G protein-coupled receptors (GPCRs) are nowadays well known to show significant signalling via other adaptor proteins, such as β-arrestin2 (βarr2). The elucidation of the molecular mechanism of the GPCR-βarr2 interaction is a prerequisite for the structure-activity based design of biased ligands, which introduces a new chapter in drug discovery. The general mechanism of the interaction is believed to rely on phosphorylation sites, exposed upon agonist binding. However, it is not known whether this mechanism is universal throughout the GPCR family or if GPCR-specific patterns are involved. In recent years, promising orally active agonists for the human A_3 adenosine receptor (A_3AR), a GPCR highly expressed in inflammatory and cancer cells, have been evaluated in clinical trials for the treatment of rheumatoid arthritis, psoriasis, and hepatocellular carcinoma. In this study, the effect of cytoplasmic modifications of the A_3AR on βarr2 recruitment was evaluated in transiently transfected HEK293T cells, using a live-cell split-reporter system (NanoBit®, Promega), based on the structural complementation of NanoLuc luciferase, allowing real-time βarr2 monitoring. The A_3AR-selective reference agonist 2-Cl-IB-MECA yielded a robust, concentration dependent (5 nM to 1 µM) recruitment of βarr2 (logEC50: -7.798 ± 0.076). The role of putative phosphorylation sites, located in the C-terminal part and cytoplasmic loops, and the role of the 'DRY' motif was evaluated. It was shown that the A_3AR C-terminus was dispensable for βarr2 recruitment. This contrasts with studies in the past for the rat A_3AR, which pointed at crucial C-terminal phosphorylation sites. When combining truncation of the A_3AR with modification of the 'DRY' motif to 'AAY', the βarr2 recruitment was drastically reduced. Recruitment could be partly rescued by back-mutation to 'NQY', or by extending the C-terminus again. In conclusion, other parts of the human A_3AR, either cytosolic or exposed upon receptor activation, rather than the C-terminus alone, are responsible for βarr2 recruitment in a complementary or synergistic way.

Graphical abstract



3.1 Introduction

In recent years, the class of purinergic adenosine receptors undoubtedly has earned its place amongst the group of most intensively studied druggable macromolecular targets, known as G protein-coupled receptors (GPCRs). Mammalian adenosine receptors (ARs) have been cloned and characterized for different species and include 4 subtypes; the A_1, A_{2A}, A_{2B} and A_3 receptors, of which A_{2A}AR and A_{2B}AR generally stimulate adenylate cyclase through coupling to the G_s family of G proteins, whereas A_1AR and A_3AR couple to the G_i family. Adenosine is the main endogenous agonist for all four adenosine receptors. It is present in the extracellular space at a basal level, but can increase substantially under conditions of stress or when cell damage occurs [1-5]. With its 318 amino acid length, the human A_3AR is the smallest AR subtype and is widely distributed in the human body with high expression levels in lung and liver, and moderate to low expression levels in heart, brain and eyes [6-9]. Remarkably, the A_3AR is highly expressed in a variety of inflammatory and cancer cells (human tumour cell lines and primary tissue), compared to a more basal expression in other cell types, which makes this AR subtype a potentially interesting therapeutic target [10-12]. In recent years, promising orally active A_3AR agonists, such as the prototypical compounds IB-MECA, *N*^{6}-(3-iodobenzyl)-5'-*N*-methyl-carboxamidoadenosine, and its 2-chloro analogue 2-Cl-IB-MECA, have been evaluated in clinical trials for the treatment of rheumatoid arthritis, psoriasis, and hepatocellular carcinoma, respectively [3, 13-16]. On the other hand, antagonists are being investigated for the treatment of asthma/COPD and glaucoma [17, 18]. Noteworthy, different studies have pointed at the rather enigmatic role of the A_3AR under different pathophysiologic conditions, displaying a twofold nature of effects, i.e. being protective/harmful under ischemic conditions, pro/anti-inflammatory, and pro/antitumoural, depending on the altered level of adenosine *in vivo*, or the organ or the cell type studied *in vitro* [12].

Agonist-induced signalling of the A_3AR is primarily G_i-mediated by inhibition of adenylate cyclase activity, causing a decrease in cAMP levels. The A_3AR also displays other signalling, such as the coupling with phospholipase C, which causes a Ca^{2+} increase in several cellular models, and with members of the Rho GTPase and mitogen-activated protein kinase (MAPK) family, as well as with ATP-sensitive

potassium ion channels [7, 9, 12]. In general, the classic view on GPCR signalling has been reoriented from a merely G protein mediated, linear concept of on/off receptor pharmacology, towards a complex, multidimensional phenomenon of coupling to a network of downstream signalling proteins. This finding is nowadays well-established as functional selectivity or biased agonism, which is defined as the ability of a ligand to selectively activate a particular (panel of) signalling pathway(s) with a certain efficacy, leading to a delineated physiological response or possibly favoured therapeutic effect [19-22]. The most extensively studied G protein-independent signal transduction pathway for which biased agonism has been thoroughly explored, is the coupling to the arrestin adaptor protein family [23-25]. Of the four arrestin isoforms that have been identified (arrestin 1 - 4), arrestin 1 and 4 are expressed in the visual system, whereas arrestin 2 (β-arrestin1) and arrestin 3 (β-arrestin2) are ubiquitously expressed. β-arrestins were originally discovered as inhibitory adaptor proteins that could “switch-off” GPCR signalling, a process well-described as GPCR desensitization. Their originally depicted function is thus considered to be protective, balancing the physiologic effect under sustained agonist stimulation. After short- or long-term agonist stimulation, β-arrestin is recruited to the GPCR, where it adapts its active conformation and sterically inhibits further interaction with the G protein [26, 27]. Once activated, β-arrestin takes up its second role as an adaptor for internalization proteins, directing the GPCR-arrestin complex towards clathrin-coated pits for endocytosis [28, 29]. Following internalization, GPCRs can either be recycled back to the plasma membrane, be targeted to larger endosomes and more slowly recycled, or even be degraded in lysosomes, the latter representing the onset for GPCR downregulation. Finally, one of the most striking features of activated arrestin is the ability to initiate its own distinct downstream G protein-independent signalling [30, 31], with β-arrestin2 (βarr2) serving a prominent role for the regulation of non-visual GPCRs [28, 32]. The exact molecular mechanism linking an agonist-induced GPCR conformation to the coupling and activation of βarr2 remains to be elucidated. Although to date, no consensus motif has been identified for βarr2 binding in the varying sequence of GPCRs, it is generally assumed that βarr2 recruitment is triggered by phosphorylation of serine/threonine residues by G protein-coupled (GRKs) or 2nd messenger regulated kinases after agonist binding. These putative phosphorylation sites are distributed throughout the cytoplasmic exposed parts of the GPCR, primarily considered to be the C-terminus and

intracellular loops [20, 33, 34]. Besides, the highly conserved 'DRY' motif is known to be involved in G protein interaction, possibly serving an additional role in arrestin binding [35, 36].

For the human A_3AR, the exact molecular features of βarr2 coupling have remained fully unexplored. Studies in the past have shown phosphorylation, desensitization and internalization for the rat A_3AR, pointing at crucial C-terminal phosphorylation sites [37-42]. However, the nature of βarr2 interaction with the human A_3AR has not been elucidated, leaving the role of the C-terminus and/or additional cytoplasmic sites undefined. Here, the effect of cytoplasmic modifications of the A_3AR on βarr2 recruitment was studied, using a live-cell split-reporter system (NanoBit®, Promega) based on the structural complementation of the NanoLuc luciferase, allowing real-time βarr2 monitoring. The role of putative phosphorylation sites, located in the C-terminal part and cytoplasmic loops, and the role of the 'DRY' motif was evaluated.

3.2 Materials & Methods

3.2.1 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 A_3AR construct (**NM_000677.2**, transcript variant 2 of the ADORA3 gene) and human βarr2 construct (**NM_004313**) were purchased from Origene Technologies (Rockville, MD, USA). The NanoBit® vectors were kindly provided by Promega (Madison, WI, USA). Self-designed primers were synthesized by Eurofins Genomics (Ebersberg, Germany). Restriction enzymes, Phusion High-Fidelity PCR Master Mix with HF buffer (containing a Phusion™ polymerase with low error rate for high-fidelity PCR), T4 DNA ligase, Phusion Site-Directed Mutagenesis Kit, and One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* were from Thermo Fisher Scientific (Pittsburg, PA, USA). Water for Molecular Biology (Sterile, RNase-free, DNase-free, Protease-free, Ca^{2+}-free, Mg^{2+}-free, RT-PCR tested) was from Merck-Millipore (Merck KGaA, Darmstadt Germany). E.Z.N.A.® MicroElute Cycle-Pure kit, E.Z.N.A.® MicroElute Gel Extraction kit and E.Z.N.A.® Plasmid DNA Mini/Midi kit were from

VWR International (Radnor, PA, USA). GelRed was from Biotium (Fremont, CA, USA). Luria Bertani Broth and Agar were from Lab M (Heywood, UK). GoTaq® DNA Polymerase, Thermosensitive Alkaline Phosphatase (TSAP), Fugene® HD transfection reagent and Nano-Glo® Live Cell reagent were purchased from Promega (Madison, WI, USA). Sanger DNA sequencing was performed by GATC Biotech SARL (Mulhouse, France). Dulbecco's Modified Eagle medium (DMEM), Opti-MEM® I Reduced Serum Medium, penicillin/streptomycin, Dulbecco's phosphate-buffered saline (DPBS) amphotericin B, glutamine and Trypsin-EDTA (0.05%), and 4',6-diamidino-2-phenylindole dihydrochloride were from Thermo Fisher Scientific (Pittsburg, PA, USA). Fetal bovine serum (FBS), poly-D-lysine, formaldehyde solution (36.5-38% in H_2O), ampicillin sodium salt, agarose High Resolution for molecular biology, and DMSO suitable for cell culture were purchased from Sigma Aldrich (Steinheim, Germany). Reference agonists 2-Cl-IB-MECA and NECA were from Tocris Bioscience (Bio-techne, Abingdon, UK).

3.2.2 Development of A_3AR- and βarr2 NanoBit® plasmid constructs

The NanoBit® system (Promega) was implemented for the generation of fusion proteins consisting of the human A_3AR or βarr2 connected via a peptide linker with the split subunits of NanoLuc luciferase. In these fusion proteins, the larger 18 kDa part of Nanoluc (Large Bit, LgBit) or the smaller 1 kDa part (Small Bit, SmBit) was C-terminally coupled to the A_3AR, or N- or C-terminally coupled to βarr2. Two full-length A_3AR-containing fusion constructs were developed: A_3AR-LgBit and A_3AR-SmBit. Four different βarr2-containing fusion constructs were developed: βarr2-LgBit, βarr2-SmBit, LgBit-βarr2 and SmBit-βarr2. The different inserts were constructed by performing a PCR reaction on the human A_3AR coding sequence, or βarr2 coding sequence (as described previously by our research group [43]), with primers containing a specific restriction site and a Kozak sequence. The reaction was performed with the Phusion High-Fidelity PCR Master Mix according to the manufacturer's instructions, using 200 pg plasmid DNA template and 0.5 µM final concentration of forward and reverse primers. A 3-step PCR cycling protocol was performed with a Mastercycler™ Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) at the following conditions: initial denaturation (98°C - 30s), denaturation (98°C - 10s), annealing (Tm – 30s), extension (72°C – time depending on the length

of the coding sequence), final extension (72°C - 5min), for 35 cycles. The PCR product was run on agarose gel and purified with a MicroElute Gel extraction kit, to ensure that no original template plasmid DNA could interfere with subsequent cloning steps. The amplified coding sequence and the vector of destination were both cut with the specific restriction enzyme, and purified with a MicroElute Cycle Pure Kit or Gel extraction kit , respectively. Finally, the resulting insert was ligated using T4 DNA Ligase into the digested vector, which was dephosphorylated at the 5' end in advance, to avoid re-ligation. All primers, PCR conditions, and restriction enzymes used are given in Table 3.1. Subsequently, the ligation product was transformed into One Shot Mach *E. coli*. After plating, ampicillin-resistant colonies were PCR-screened with Taq-polymerase for containing the insert of interest in the right direction. Colonies that contained the correct constructs were grown in LB with ampicillin (100 µg/mL) and plasmid DNA was isolated with a Plasmid DNA mini/midi kit. The length of the fusion constructs was evaluated by RE digest and coding sequences were sequence-verified by Sanger sequencing.

Table 3.1: PCR conditions (a-c) and restriction enzymes (RE) used for development of NanoBit® fusion constructs

Template Sequence		Primers (F: forward – R: reverse)[a]	Tm (°C)[b]	Ext. time (s)[c]	RE	Fusion construct
A_3AR- and βarr2 NanoBit® plasmid construct development						
A_3AR	F	ACTCAAGAGCTC***ACC***ATGCCCAACAACAGC	69.5	25	SacI	A_3AR-LgBit
	R	ACTCAAGAGCTCCCTCAGAATTCTTCTCAATGC				
	F	ACTCAACTCGAG***ACC***ATGCCCAACAACAGC	69		XhoI	A_3AR-SmBit
	R	ACTCAACTCGAGCCCTCAGAATTCTTCTCAATGC				
βarr2	F	ACTCAAGAATTC***ACC***ATGGGGGGAGAAACCCGGGACC	71.1	35	EcoRI	βarr2-LgBit & βarr2-SmBit
	R	ACTCAAGAATTCCCGCAGAGTTGATCATCATAGTCG				
	F	ACTCAAGAATTCAATGGGGGGAGAAACCCGGGACC	69.7			LgBit-βarr2 & SmBit- βarr2
	R	ACTCAAGAATTC**TCA**GCAGAGTTGATCATCATAGTCG				
Truncation of the A_3AR C-terminus						
A_3AR	F	ACTCAAGAGCTC***ACC***ATGCCCAACAACAGC	71	25	SacI	A_3ART^{309}-LgBit
	R	ACTCAAGAGCTCCCAAAGAATCAGAGGGATGGC				
	F	ACTCAAGAGCTC***ACC***ATGCCCAACAACAGC	72.5			A_3ART^{305}-LgBit
	R	ACTCAAGAGCTCCGGGATGGCAGACCACACAGG				
	F	ACTCAAGAGCTC***ACC***ATGCCCAACAACAGC	72			A_3ART^{302}-LgBit
	R	ACTCAAGAGCTCCGACCACACAGGCTTTGAGG				

a: Primers (5'->3') containing the specific restriction site (underlined), Kozak sequence (***bold italic***) and coding sequence of interest (*italic*).
b: Annealing temperature.
c: Extension time.
A stop codon (bold) or extra nucleotides (marked grey) to ensure a correct reading frame were added when necessary.

3.2.3 Modifications of the A_3AR: truncation of the C-terminus, mutation of phosphorylation sites in IL3, and mutation of the 'DRY' motif

The combination A_3AR-LgBit / SmBit-βarr2 was the set-up of choice for implementation of modifications in the A_3AR sequence. All developed fusion constructs described below were selected and sequence-verified as described above.

Three A_3AR C-terminal truncation mutants (Figure 3.1; A_3ART^{309}, A_3ART^{305}, A_3ART^{302}) were generated from the A_3AR-LgBit plasmid as described above, following the conditions described in Table 3.1.

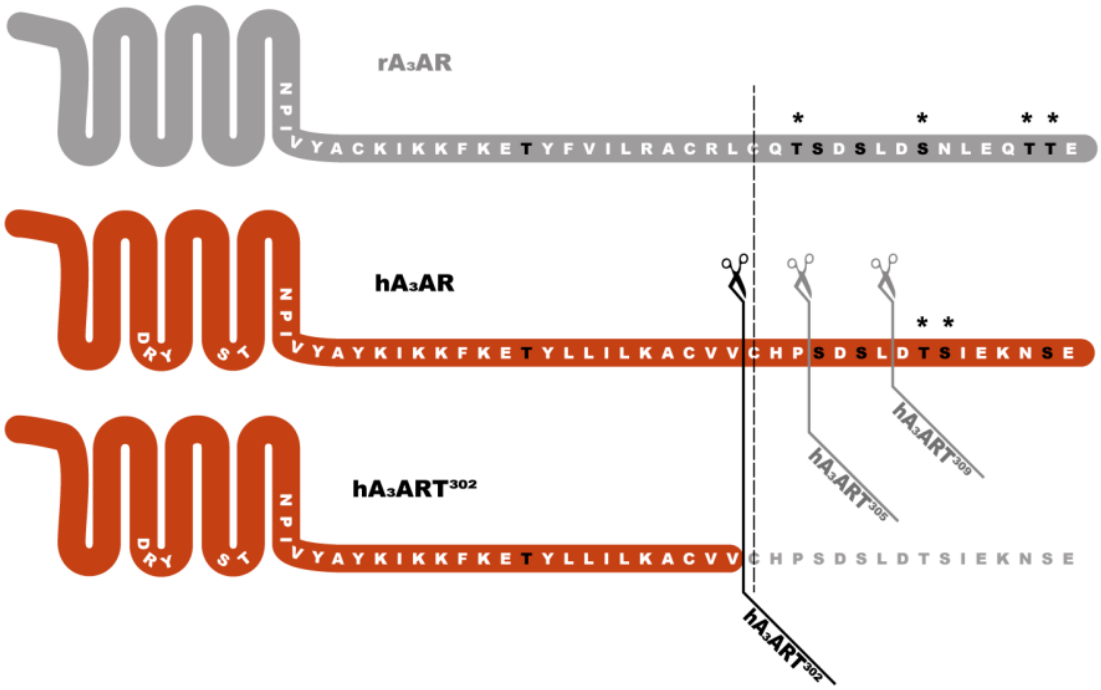


Figure 3.1: Alignment of the human and rat A_3AR C-terminal part: rA_3AR: rat A_3AR, hA_3AR: human A_3AR, hA_3ART309: truncated human A_3AR (residues 1-309), hA_3ART305: truncated human A_3AR (residues 1-305), hA_3ART302: truncated human A_3AR (residues 1-302). Serine and threonine residues are depicted in black, with potential phosphorylation sites predicted by NetPhos server (score > 0.5) marked by an asterisk. The predicted palmitoylation C^{303} is marked with a dotted line. Positions for truncation are indicated by scissors.

The original NanoBit® linker connecting the A_3AR and LgBit in the A_3AR-LgBit and A_3ART^{302}-LgBit fusion constructs was adjusted through deletion of a part of its sequence ('GAQGNSGSSGGGGSGGGGSSG' -> 'GAQGNGGGG'). A 3- or 2-step PCR cycling protocol with primers containing a 5' phosphate was performed on the A_3AR-LgBit fusion construct; phosphorylated primers and PCR conditions are given in Table 3.2. The PCR-amplified linear fusion construct was gel purified, and religated with T4 DNA Ligase in rapid ligation buffer, using a site-directed mutagenesis kit according to the manufacturer's instructions. The linker of the A_3ART^{302}-LgBit construct was adjusted by RE digest of A_3ART^{302}-LgBit and A_3ARL-LgBit, and recombination to A_3ARLT^{302}-LgBit.

Table 3.2: PCR conditions (a-c) used for adjustment of the NanoBit® linker sequence

Template sequence	Linker Sequence	Primers (F: forward – R: reverse)[a]		Tm (°C)[b]	Ext. time (min:sec)[c]	Fusion construct
Adjustment of the Linker sequence –GAQGNSGSSGGGGSGGGGSSG-						
A_3AR-LgBit	GAQGNGGGG	F	GGTGGAGGTGGTGTCTTCACAC	66.7	2:15	A_3ARL-LgBit
		R	ATTCCCCTGAGCTCCCTC			

a: 5' phosphorylated primers (5'P->3'), b: annealing temperature, c: extension time.

One serine and one threonine residue in the third intracellular loop (IL3) of A_3ART^{302} were mutated to alanine (S215A and T228A), to create the mutated, truncated A_3AR construct A_3ARLT^{302}M12-LgBit. The aspartic acid and arginine residue of the 'DRY' motif were modified step-wise in the full length A_3AR and A_3ART^{302}. Mutations to uncharged amino acids with similar polarity (D107N and R108Q) were introduced in a single or combined way (A_3ARLNRY-LgBit, A_3ARLDQY-LgBit, and A_3ARLT^{302}NQY-LgBit). Mutations to hydrophobic alanine residues were introduced as well (A_3ARLARY-LgBit, A_3ARLDAY-LgBit, A_3ARLAAY-LgBit, A_3ARLT^{302}AAY-LgBit). Phosphorylated primers and PCR conditions for site-directed mutagenesis to construct all mentioned modified fusion constructs are given in Table 3.3.

Table 3.3: PCR conditions (a-c) used for introduction of A_3AR mutations in IL3 and the 'DRY' motif

Template sequence		Primers (F: forward – R: reverse)[a]	Tm (°C)[b]	Ext. time (min:sec)[c]	Fusion construct
Mutation of phosphorylation sites in the A_3AR IL3					
A_3ARLT^{302}-LgBit	F	GGGAGTTCAAGGCAGCTAAGTCCTTG	68.4	2:05	A_3ARLT^{302}M2-LgBit
	R	GTCCATAAAATGCACCTGTCTCTTTGG			
A_3ARLT^{302}M2-LgBit	F	AACTTATCTAACGCTAAAGAGACAGGTGC	72	2:15	A_3ARLT^{302}M12-LgBit
	R	CAGACTGAGTTTGTTCCGAATGATG			
Mutation of the A_3AR DRY motif					
A_3ARL-LgBit	F	ATCGCTGTG**GACCAATAC**TTGCG	72	2:15	A_3ARLDQY-LgBit
	R	GGCCAGCAAGGACATGATGG			
	F	ATCGCTGTG**AACCGATAC**TTGC		1:40	A_3ARLNRY-LgBit
	R	CAGCAAGGACATGATGGAGGCGTGGG			
	F	GCCATCGCTGTG**GACGCATAC**TTGCGG			A_3ARLDAY-LgBit
	R	CAGCAAGGACATGATGGAGGCGTGGG			
	F	GCCATCGCTGTG**GCCCGATAC**TTGCG			A_3ARLARY-LgBit
	R	CAGCAAGGACATGATGGAGGCGTGGG			
	F	GCCATCGCTGTG**GCCGCATAC**TTGCG			A_3ARLAAY-LgBit
	R	CAGCAAGGACATGATGGAGGCGTGGG			
A_3ARLT^{302}-LgBit	F	ATCGCTGTG**GACGCATAC**TTGC		2:15	A_3ARLT^{302}DAY-LgBit
	R	GGCCAGCAAGGACATGATGG			
	F	GCCATCGCTGTG**GCCGCATAC**TTGCG			A_3ARLT^{302}AAY-LgBit
	R	CAGCAAGGACATGATGGAGGCGTGGG			
	F	GCCATCGCTGTG**AACCAATAC**TTGCGG			A_3ARLT^{302}NQY-LgBit
	R	CAGCAAGGACATGATGGAGGCGTGGG			
A_3ARLT^{302}M12-LgBit	F	ATCGCTGTG**GACGCATAC**TTGC			A_3ARLT^{302}M12DAY-LgBit
	R	GGCCAGCAAGGACATGATGG			

a: 5' phosphorylated primers (5'P->3') with mutated nucleotides (underlined), (mutated) DRY motif is shown in **bold**.
b: Annealing temperature.
c: Extension time.

3.2.4 A_3AR NanoBit® βarr2 reporter assay in HEK293T cells

Human embryonic kidney (HEK) 293T cells were cultured in full DMEM (DMEM supplemented with 10% heat-inactivated FBS, 2mM of glutamine, 100IU/ml of penicillin, 100µg/ml of streptomycin and 0.25µg/ml of amphotericin B) and maintained at 37°C, 5% CO2, under humidified atmosphere.

The day before transfection, cells were seeded on 6-well plates at a density of 5x10^5 cells/well in 2 mL full DMEM. The next day, cells were transiently transfected using FuGENE® HD reagent according to the manufacturer's instructions, with a ratio of FuGENE:DNA 3:1. Transfection mixes contained 1.65 µg of A_3AR and βarr2-construct each. On the third day, cells were treated with trypsin/EDTA and reseeded on precoated, white 96-well plates at 5x10^4 cells/well and incubated overnight (37°C, 5% CO_2). Plate coating was performed by incubation with 50 µL of a 0.1 mg/mL poly-D-lysine solution per well for 1h (37°C, 5% CO_2), subsequent removal of the solution and drying to air. This coating was indispensable to avoid cell loss in subsequent

washing steps, minimizing the well-to-well variation in luminescence signal during read-out caused by differences in cell number.

On day 4, i.e. 48 hours after transfection and after recovery from trypsin treatment, the read-out was performed at room temperature (RT): cells were washed twice with Opti-MEM® I, and 90 µL of this reduced serum medium was placed on the cells. The Nano-Glo Live Cell detection reagent was freshly prepared by dilution of the cell-permeable furimazine substrate (Nano-Glo Live Cell substrate) in aqueous buffer (Nano-Glo LCS Dilution buffer) that ensures similar tonicity to commonly used cell culture buffers. Twenty-five µL of this reagent was added to each well and luminescence was monitored in a GloMAX96 or Navigator luminometer (Promega) until the signal stabilized (equilibration period of 15-20 minutes). Once a stable signal was observed, monitoring was interrupted for addition of 20 µL 6.75× concentrated agonist solution in Opti-MEM® I and luminescence was monitored for at least 90 minutes. The final in-well concentration ranged from 0.1 nM up to 100 µM agonist (maximum 0.1% DMSO). A solvent control (blank sample) of 0.0001-0.1% DMSO in Opti-MEM® I was each time included.

3.2.5 Immunofluorescence microscopy

For immunofluorescence microscopy, a (2xHA)-tag was inserted N-terminally to the A_3AR, using phosphorylated primers and a 2-step PCR protocol (Table 3.4), for the generation of the following constructs: (2xHA)-A_3ARL-LgBit, (2xHA)-A_3ARLT^{302}-LgBit, and (2xHA)-A_3ARLT^{302}AAY-LgBit. The PCR-amplified linear tagged constructs were processed using the site-directed mutagenesis kit, as described above.

Table 3.4: PCR conditions (a-c) used for insertion of the (2xHA)-tag

Template sequence	Primers (F: forward – R: reverse)[a]		Tm (°C)[b]	Ext. time (min:sec)[c]	Fusion construct
A_3ARL-LgBit	F	TACCCATACGATGTTCCAGATTACGCTATGCCCAACAACAGC	72	1:50	**(2xHA)-A_3ARL-LgBit**
A_3ARLT^{302}-LgBit					**(2xHA)-A_3ARLT^{302}-LgBit**
A_3ARLT^{302}AAY-LgBit	R	AGCGTAATCTGGAACATCGTATGGGTACATGGTGAGCTCCCACTTAGG			**(2xHA)-A_3ARLT^{302}AAY-LgBit**

a: 5' phosphorylated primers (5'P->3'), b: annealing temperature, c: extension time.

The day before transfection, cells were seeded on poly-D-lysine-coated coverslips at a density of 1.25x10^5 cells in 2 mL full DMEM. The next day, cells were transiently transfected with 3.3 µg of one of the above mentioned A_3AR-constructs using FuGENE® HD reagent according to the manufacturer's instructions. On the third day, cells were washed once with Opti-MEM® I and incubated for 25 minutes (37°C, 5% CO_2) with primary purified anti-HA.11 Epitope Tag (Clone 16B12) mouse antibody (Biolegend, San Diego, CA), diluted 1/2000 in Opti-MEM® I. Following aspiration of the medium, the cells were fixated with 3.7% formaldehyde in PBS (15 minutes at RT). Subsequently, coverslips were washed 3 times with wash buffer (140 mM NaCl, 25 mM Tris, 2.7 mM KCl, 1 mM $CaCl_2$, adjusted to pH 7.4 with HCl) and incubated with 200 µL Blotto with Triton (30 mg/mL dry milk, 0.1% Triton X-100, 1 mM $CaCl_2$, 50 mM Tris HCl pH 7.5) for 20 minutes at RT. Following washing, coverslips were incubated with the secondary Alexa Fluor 594 goat anti-mouse IgG (H+L) antibody (Invitrogen™ Molecular Probes™, Thermo Fisher Scientific Pittsburg, PA, USA) 1/500 in Blotto for 20 minutes at RT in the dark. After washing, cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) 1/500 in Blotto for 5 minutes at RT in the dark. After a last washing cycle, coverslips were mounted using 10 µL of mounting medium and stored at 4°C in the dark until imaging. Conventional fluorescence microscopy was performed on a Zeiss Axiovert 200 M microscope with a 63x oil-immersion objective, images were captured using an AxioCam MRm camera, and processed on AxioVision Rel. 4.6 software.

3.2.6 Data analysis

Concentration-response (area under the curve; AUCs) were calculated, where the absolute signals were corrected for solvent control samples, and for inter-well variability. For calculation of logEC50 values, a sigmoidal curve was fitted by analysing concentration-response data using GraphPad Prism software (San Diego, CA, USA). A non-linear regression model (variable slope) was fitted for the normalized responses. Statistics were performed using analysis of variance (ANOVA), followed by post-hoc analysis (Tukey) to detect statistical difference among groups ($P < 0.05$).

3.3 Results

3.3.1 Development of the A_3AR NanoBit® reporter assay for real-time monitoring of β-arrestin2 recruitment in HEK293T cells

For the development of an A_3AR NanoBit® reporter assay for βarr2 recruitment, an optimal assay set-up was selected by evaluation of different combinations for A_3AR- and βarr2 fusion constructs in HEK293T cells. This cell line was chosen because of its rapid growth characteristics and high transient transfection efficiency. As the A_3AR and βarr2 are membrane-bound and cytosolic, respectively, the LgBit- or SmBit part of Nanoluc can be coupled C-terminally to the A_3AR, and N- or C-terminally to βarr2, resulting in four different set-ups of the assay (Figure 3.2). For the two set-ups in which LgBit was attached to the A_3AR, and SmBit was either N- or C-terminally attached to βarr2, a clear rise in signal was observed immediately after addition of the A_3AR selective reference agonist 2-Cl-IB-MECA at a concentration of 100 nM, which was sustained for at least 90 minutes. Set-up A in Figure 3.2, in which LgBit is attached to the C-terminus of the A_3AR, and SmBit is attached to the N-terminus of βarr2, was chosen as optimal configuration set-up for further experiments.

The chosen set-up yielded a concentration-dependent effect of 2-Cl-IB-MECA, starting at about 5 nM and reaching a maximum at about 1 µM (Figure 3.3). Higher concentrations caused a severe drop in signal – possibly owing to toxicity. Up to 0.1% of DMSO, which was used for preparation of stock solutions of the reference agonist, caused no discernible solvent effect. Analysis of the concentration-response curve with non-linear regression provided a logEC50 of -7.798 ± 0.076 (Figure 3.5).

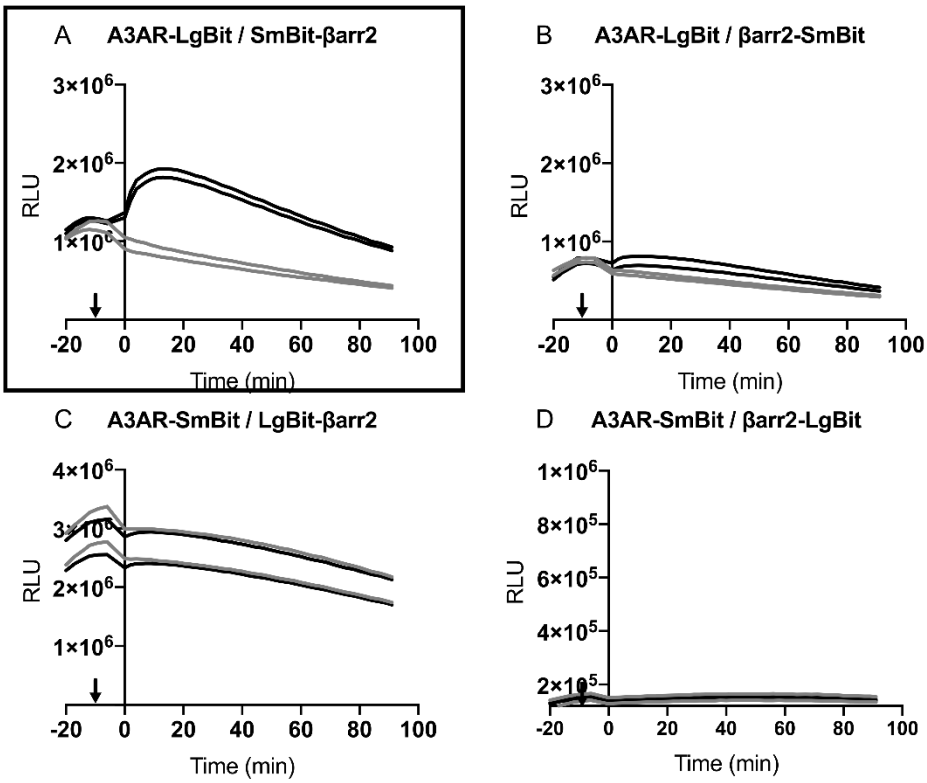


Figure 3.2: Selection of the optimal A_3AR NanoBit® reporter assay set-up: 4 combinations of A_3AR- and βarr2 fusion constructs were evaluated: A_3AR-LgBit / SmBit-βArr2, A_3AR-LgBit / βArr2-SmBit, A_3AR-SmBit / LgBit-βArr2 and A_3AR-SmBit / βArr2-LgBit. A concentration of 100 nM 2-Cl-IB-MECA was added (arrow) and luminescence was measured during ≥ 90 minutes (black lines). A solvent control of 0.0001% DMSO was included (grey lines). Measurements of duplicate wells in a 96-well plate are shown for one representative experiment (n=3).

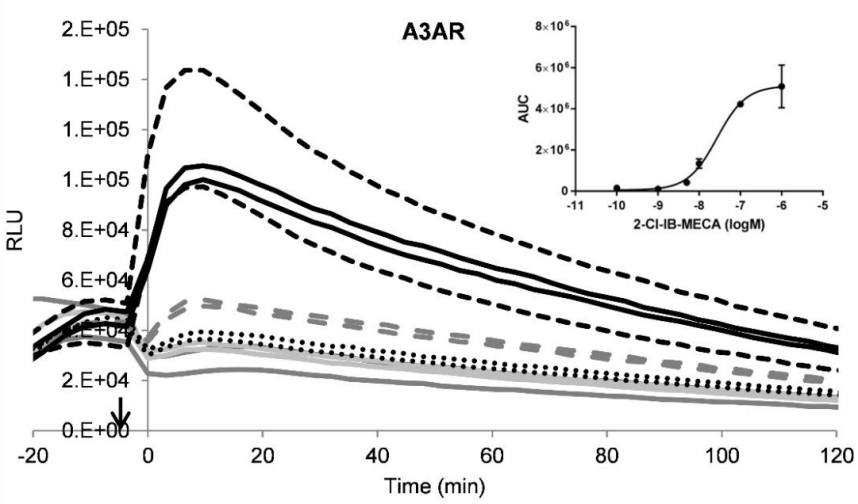


Figure 3.3: Concentration-dependence of βarr2 recruitment in the A_3AR NanoBit® reporter assay (A_3AR-LgBit/SmBit-βarr2): 1 nM - 5 nM - 10 nM - 100 nM - 1 µM 2-Cl-IB-MECA was added at the time point (indicated by the arrow) and luminescence was measured for > 90 minutes. A solvent control of 0.1% DMSO was included. – Inserted: Concentration-response (AUCs). Mean AUCs ± SEM of duplicate wells are shown for one representative experiment (n=3).

3.3.2 Truncation of the A_3AR C-terminus

The influence of C-terminal phosphorylation of the A_3AR on βarr2 recruitment was evaluated by truncation of the A_3AR C-terminus. Focusing on the short C-terminal part delineated by the palmitoylation C^{303}, three truncated A_3ARs (A_3ART) were evaluated in the assay (Figure 3.1). A_3ART^{309} is missing terminal residues 310-318 of the A_3AR-sequence, but two serine residues beyond C^{303} are still present. The shorter A_3ART^{305} still contains the predicted palmitoylation C^{303}, but is missing all five C-terminal serine/threonine residues. In the shortest A_3ART^{302}, C^{303} is removed as well. All three A_3ARTs, of which A_3ART^{302} is depicted in Figure 3.4, were still able to recruit βarr2 following stimulation with 100 nM 2-Cl-IB-MECA, giving a signal comparable with that of the full length A_3AR. Therefore, A_3ART^{302} was used for further elucidation of crucial contact points for βarr2 interaction.

To rule out a possible role of serine residues in the standard 21 amino acid linker with sequence 'GAQGNSGSSGGGGGSGGGGSSG', connecting A_3AR and LgBit in the A_3AR-LgBit and A_3ART^{302}-LgBit fusion constructs, this linker sequence was modified. Although only one serine residue (underlined) gave a score >0.5 using NetPhos server, the linker was shortened to 'GAQGNGGGG'. This did not influence the assay read-out; 2-Cl-IB-MECA as well as the non-selective agonist NECA triggered βarr2 recruitment, the latter showing an even faster onset (Figure 3.4). A concentration dependent effect was obtained with 2-Cl-IB-MECA (Figure 3.5; logEC50 of -7.657 ± 0.065). When the new linker was combined with A_3ART^{302}, the resulting A_3ARLT^{302}-LgBit fusion construct again gave very robust results in the assay, with a logEC50 of -7.884 ± 0.058 (Figure 3.4-3.5). All subsequent modifications of the A_3AR were based on constructs with the new shortened linker.

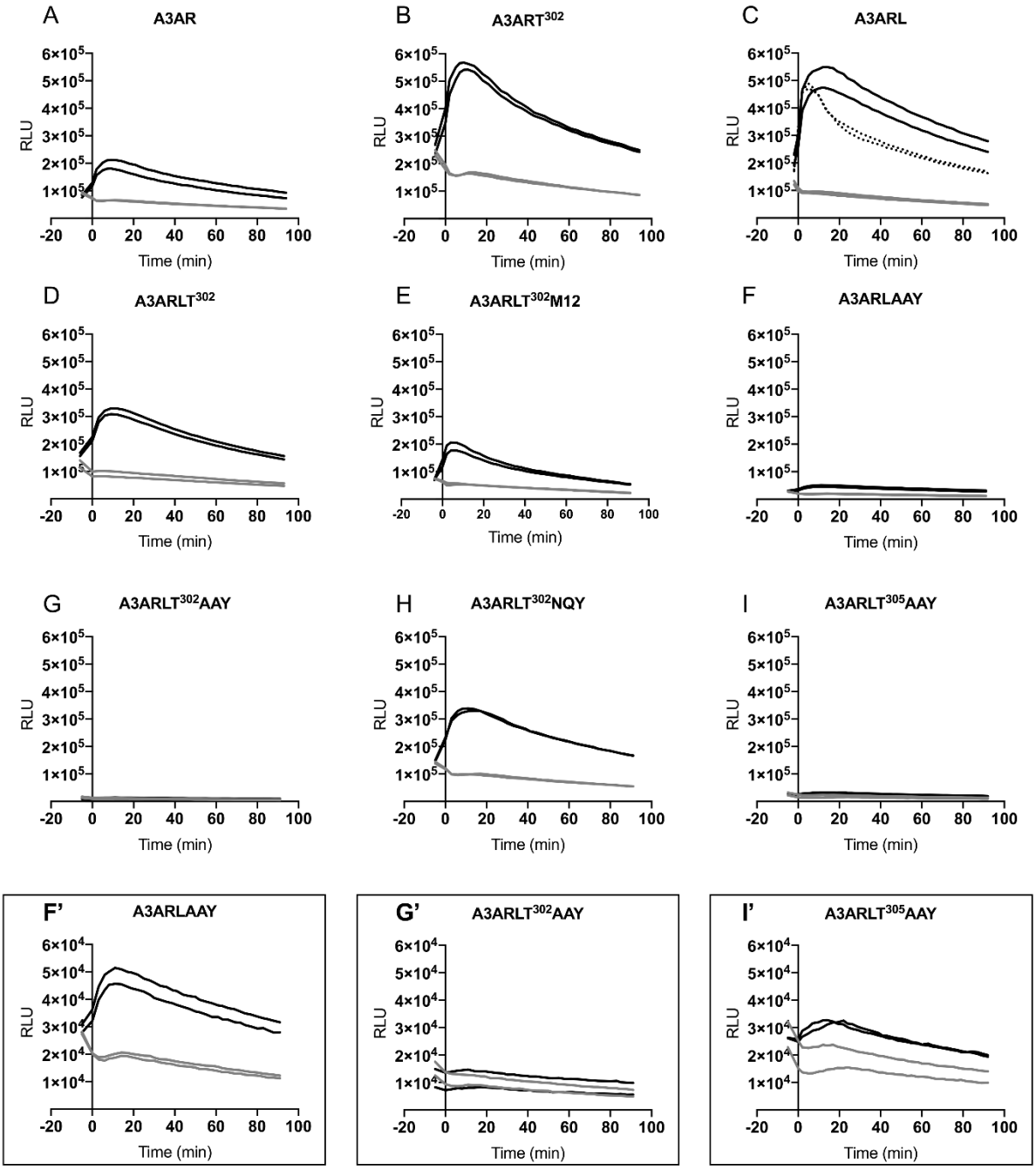


Figure 3.4: Evaluation of βarr2 recruitment in the A_3AR NanoBit® reporter assay. Panels A-D: Truncation of the human A_3AR C-terminus: A3AR: native A_3AR (original linker), A3ART^{302}: truncated A_3AR (original linker), A3ARL: native A_3AR (adjusted linker), A3ARLT^{302}: truncated A_3AR (adjusted linker). Panels E-G: Mutation of IL3 phosphorylation sites and the 'DRY' motif: A3ARLT^{302}M12: truncated A_3AR with IL3 mutations S215A & T228A (adjusted linker), A3ARLAAY: A_3AR with 'DRY' -> 'AAY' (adjusted linker), A3ARLT^{302}AAY: truncated A_3AR with 'DRY' -> 'AAY' (adjusted linker). Panels H-I: Restoration of response: A3ARLT^{302}NQY: truncated A_3AR with 'DRY' -> 'NQY' (adjusted linker), A3ARLT^{305}AAY: truncated A_3AR (C303 present) with 'DRY' -> 'AAY' (adjusted linker). Boxed panels F', G', I': zoomed graphs for clarity. A concentration of 100 nM 2-Cl-IB-MECA (black lines) or non-selective NECA (dotted lines) was added and luminescence was measured during ≥ 90 minutes. A solvent control of 0.1% DMSO was included (grey lines). Measurements of duplicate wells are shown for one representative experiment (n≥3).

3.3.3 Mutation of phosphorylation sites in intracellular loop 3 and mutation of the 'DRY' motif

Since the C-terminal serine/threonine residues were found to be dispensable for βarr2 recruitment, other putative phosphorylation sites in the A_3AR cytoplasmic loops were evaluated. Although no obvious serine/threonine clusters are present, two residues in intracellular loop 3 (IL3), S215 and T228, could serve as (part of) a pattern-wise phosphorylation motif (NetPhos >0.5). Mutating both residues to alanine in A_3ARLT^{302} gave the A_3ARLT^{302}M12-LgBit construct, which still provided a good βarr2 recruitment, with a logEC50 of -7.889 ± 0.023 upon stimulation with 2-Cl-IB-MECA (Figure 3.4-3.5).

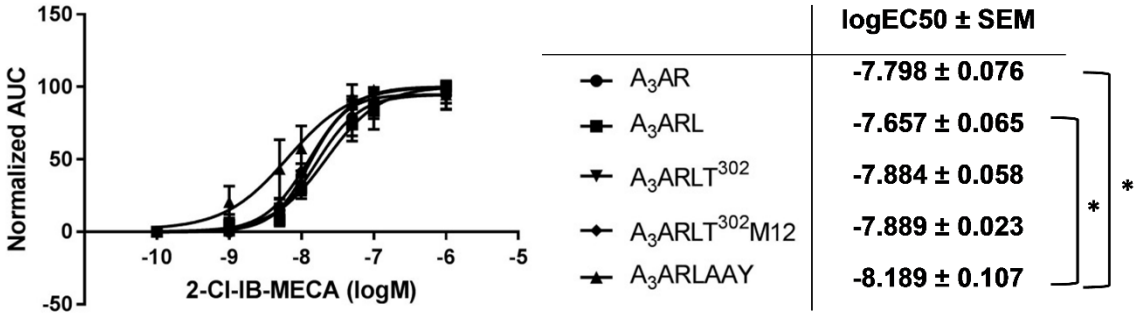


Figure 3.5: Analysis of the sigmoidal dose-response curves with non-linear regression: logEC50 ± SEM are given. Values are calculated based on ≥3 experiments performed in duplicate. Statistical significance is depicted, using analysis of variance (ANOVA), followed by post-hoc analysis (Tukey) (*P< 0.05).

The role of the A_3AR $D^{107}R^{108}Y^{109}$ motif was evaluated by site-directed mutagenesis of the D and R residue in a step-wise manner to uncharged amino acids with similar polarity, yielding D107N and R108Q mutants, respectively. These 'NRY'- and 'DQY'-mutants provided an unchanged response in the assay in comparison with the native DRY motif (data not shown). Subsequently, non-conservative mutations to hydrophobic alanine residues were introduced. Mutation of the 'DRY' motif to 'AAY' (Figure 3.4: A3ARLAAY) largely preserved concentration dependence of the βarr2 coupling, though was characterized by lower absolute values in the assay read-out, giving a logEC50 of -8.189 ± 0.107, which differed significantly ($P<0.05$) from that for full length A_3AR (Figure 3.5). Although significant, the difference is small and thus

possibly of limited scientific value. When combined with C-terminal truncation (A3ARLT^{302}AAY), this modification completely abolished the response to 2-Cl-IB-MECA: no rise in signal, nor a concentration-dependent effect could be observed upon agonist addition (Figure 3.4). Interestingly, the response of the A3ARLT^{302}AAY construct could be fully rescued by mutating the 1st two residues of the 'DRY' motif back to 'NQY' (A3ARLT^{302}NQY). When the 'AAY' modification was maintained, and the C-terminus was extended again beyond the palmitoylation C^{303} (A3ARLT^{305}AAY), a slight recovery of the signal was obtained as well (Figure 3.4).

3.3.4 Immunofluorescence microscopy

Immunofluorescence microscopy was used to confirm that the A_3ARLT^{302}AAY-mutant was still correctly expressed. To this end, HEK293T cells were transiently transfected with (2xHA)-tagged A_3AR-LgBit fusion constructs, followed by immunofluorescence microscopy with an anti-HA antibody (Figure 3.6). The images support the cell surface expression of the (2xHA)-A_3ARL-LgBit (A), (2xHA)-A_3ARLT^{302}-LgBit (B), and (2xHA)-A_3ARLT^{302}AAY-LgBit (C) fusion constructs.

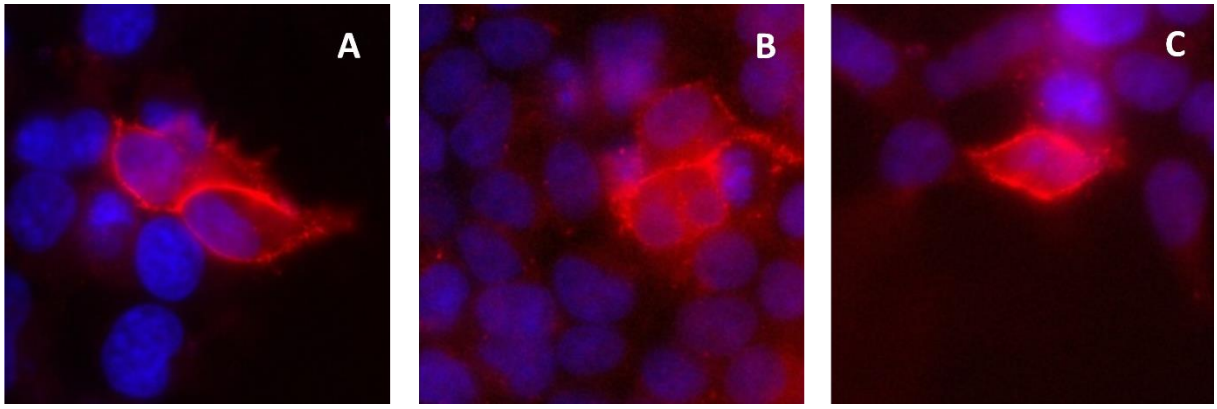


Figure 3.6: Cell surface expression of (2xHA)-tagged A_3AR-LgBit fusion constructs with immunofluorescence microscopy. HEK293T cells were transiently transfected with 3.3 µg of (2xHA)-A_3ARL-LgBit (panel A), (2xHA)-A_3ARLT^{302}-LgBit (panel B), or (2xHA)-A_3ARLT^{302}AAY-LgBit (panel C) and incubated with mouse anti-HA antibody. After fixation, cells were incubated with secondary Alexa Fluor 594 goat anti-mouse antibody and cell nuclei were stained with DAPI.

3.4 Discussion

In this study, we used a functional complementation approach to study the coupling between the human A_3 adenosine receptor (A_3AR) and the β-arrestin2 (βarr2) protein. Via step-wise modifications in the cytoplasmic portions of the A_3AR, we could delineate regions that were essential or dispensable for βarr2 recruitment. Originally discovered as an inhibitory adaptor protein mediating GPCR desensitization and endocytosis, βarr2 has now been established as a true signalling protein [26-31]. Biased agonists that preferentially signal via the G protein or βarr2 mediated pathway have been (re)discovered for different GPCR systems [19-22]. In the process of drug development, the oriented, structure-activity based design of biased ligands for GPCRs such as the A_3AR, could provide promising opportunities with respect to a favoured therapeutic profile that is devoid of side effects. However, the exact molecular aspects of the GPCR-βarr2 contact are only just emerging. Given the fact that a multitude of GPCRs all couple to only two βarrs, it has been hypothesized that certain cytoplasmic GPCR portions might be conserved to a certain extent amongst non-visual GPCRs [33, 34, 44]. The general image of GPCR-βarr interaction has been based on the rhodopsin-visual arrestin model, and has served as a template for all mammalian arrestins [45, 46]. β-arrestin recruitment to GPCRs would be induced by two driving forces: an agonist-induced conformational rearrangement of the receptor, exposing key cytoplasmic contact sites, and the phosphorylation of (exposed) clusters of serine/threonine residues located in the C-terminal tail and/or throughout cytoplasmic loops, by GRKs or 2^{nd} messenger regulated kinases. Complementary, arrestins contain an 'activation-sensor' and a 'phosphorylation-sensor', which should both be triggered in a sequential or synergistic way to allow βarr recruitment, high affinity binding and eventually full activation of the βarr molecule. Destabilisation of intramolecular βarr interactions by phosphorylated residues and other parts of the activated GPCR has been proposed as a driving force for a conformational rearrangement in the arrestin molecule, thereby liberating the arrestin C-terminal tail, which may serve as a docking site for internalization proteins [33, 34, 44]. Thus, part of the arrestin molecule engaged in GPCR binding is now well-defined, by resolution of the X-ray structures of all four arrestins and of GPCRs in complex with (ant)agonists, G proteins, or recently with arrestin [47-52]. However, the above mentioned binding site(s) in the GPCR remain ill-defined and have only

been specified for a few GPCRs [53, 54]. To date, there is no consensus on which GPCR residues are crucial for arrestin contact, in which cytoplasmic exposed parts they are located, and to which extent they are conserved throughout the heterogeneous GPCR family.

For the human A_3AR, the exact molecular features of βarr2 coupling have remained unexplored. In this study, the effect of cytoplasmic modifications of the A_3AR on βarr2 recruitment was evaluated. For live-cell monitoring of the A_3AR-βarr2 interaction, the NanoBit® technology from Promega was implemented [55]. This split-reporter based system depends on the structural complementation of the NanoLuc luciferase enzyme. The Nanoluc luciferase is a small (19.1 kDa) enzyme, producing high intensity, glow-type luminescence by conversion of the coelenterazine-derived cell-permeable substrate furimazine. The splitted Nanoluc parts, called Large Bit (LgBit; 18 kDa) and Small Bit (SmBit; 1 kDa), can be fused to two proteins of interest, making a reversible interaction possible. In our application, the interaction between the human A_3AR and βarr2 can be monitored in a kinetic way, under physiological conditions in living cells. This system was developed in parallel with a cannabinoid receptor system in our research group [43].

The A_3AR selective agonist 2-Cl-IB-MECA was used as a reference agonist in the assay. In the optimal assay set-up, A_3AR-LgBit / SmBit-βarr2, a clear rise in signal was observed immediately after the addition of 2-Cl-IB-MECA, which was detectable for at least 90 minutes. The response in the assay was concentration-dependent, starting at about 5 nM and reaching a maximum around 1 µM. Analysis of the sigmoidal concentration-response curve gave a logEC50 of -7.798 ± 0.076. This value is very similar to the one reported by Gao *et al.* (logEC50: -7.97 ± 0.14), who were the first to report on the interaction of βarr2 with the human A_3AR, using the PathHunter system (DiscoverX) in CHO cells**.** However, in this system the cells are incubated with the agonist for 60 minutes, not using real-time monitoring of βarr2 recruitment. The rapid onset of βarr2 recruitment they observed is consistent with the kinetics reported here with the NanoBit® assay. Interestingly, the logEC50 value of 2-Cl-IB-MECA for βarr2 recruitment is somewhat distinct from the logEC50 value reported for activation of the G protein pathway (-8.55) [56, 57].

At first sight, the cytoplasmic part of the human A_3AR does not reveal any obvious clusters of serine/threonine residues that are highly indicative for phosphorylation by GRKs or other kinases. A closer look at the C-terminal part shows six serine/threonine residues, of which one is located before and five are located beyond the predicted palmitoylation C^{303} (S^{306}, S^{308}, T^{311}, S^{312}, and S^{317}) (Figure 3.1). Of these five residues, only T^{311}/S^{312} were predicted as putative phosphorylation sites by NetPhos (score>0.5). The role of the A_3AR C-terminus in desensitization and internalization has been limited to studies performed on the rat A_3AR, but is unknown for the human A_3AR**.** Studies by Palmer *et al.* showed very rapid phosphorylation, desensitization and internalization for the rat A_3AR, in the order of minutes [37-42]. This is a unique feature in the AR field, as the A_{2A}AR and A_{2B}AR desensitize in <1h and the A_1AR only over a time-span of several hours. An explanation for these differences was sought in the C-terminus of the rat A_3AR, containing 6 serine/threonine residues, where the human A_1AR contains no potential phosphorylation sites in this corresponding region. Exchange of the C-terminal tails reversed the kinetics. The residues crucial for these observations were T^{307}, T^{318} and T^{319} for the rat A_3AR. Further, it has been suggested that C-terminal cysteine residues, present in the rat and human A_3AR, represent possible palmitoylation sites, and that these are relatively conserved among all GPCRs. These sites could regulate the accessibility of the C-terminal domain to activated GRKs [40]. As the sequence of the C-terminus of rat and human A_3AR is very alike, the C-terminal tail is considered a major trigger for β-arrestin recruitment. The rapid desensitization and internalization kinetics -in the order of minutes- were confirmed for the human A_3AR in a stable CHO cell line, and at the endogenous level in a human astrocytoma cell line [58, 59]. However, no mechanism with respect to βarr2 recruitment has been explored. Moreover, with respect to rodent modelling, possible differences between rat and human A_3AR cannot be ruled out, as there is only 72% shared identity between human and rat A_3AR sequence [4, 17, 60-62].

Therefore, the role of the C-terminal part was evaluated by truncating the human A_3AR. Surprisingly, full truncation of the C-terminal part, including removal of the predicted palmitoylation C^{303}, had no negative influence on the recruitment of βarr2 in our complementation assay (logEC50 of -7.884 ± 0.058). Therefore, in this context, the human A_3AR C-terminus can be regarded as dispensable for βarr2 recruitment.

Remarkably, this contrasts with studies in the past for the rat A_3AR, which pointed at crucial C-terminal phosphorylation sites with respect to receptor desensitization and internalization [37-42]. Although C-terminal phosphorylation has been reported as a major trigger for βarr interaction, there are examples of GPCRs that do not require phosphorylation [34]. In general, concerning the presence of C-terminal serine/threonine clusters, two classes of GPCRs have been identified with respect to βarr recruitment. Class A GPCRs, lacking obvious C-terminal phosphorylation clusters, show a transient interaction with βarr2, which leads to rapid GPCR recycling. Class B GPCRs do contain multiple C-terminal phosphorylation clusters, providing a stable GPCR-βarr complex that is mostly internalized and degraded [63]. From our study, it seems that the human A_3AR could be classified as class A GPCR with respect to βarr2 interaction. To further evaluate a role of possible putative phosphorylation sites, two residues were mutated in the third intracellular loop (S215A and T228A). Although highly predictive for phosphorylation (NetPhos >0.5), also these mutations did not hamper βarr2 recruitment (logEC50 of -7.889 ± 0.023). Thus, phosphorylation of cytoplasmic residues does not seem to play a crucial role in βarr2 recruitment for the human A_3AR.

Besides putative phosphorylation sites in the intracellular loops, other motifs crucial for arrestin binding can be exposed upon conformational rearrangement of the GPCR. Such a candidate motif is the ‘DRY’ motif, located at the boundary of TM helix 3 with IL2. The ‘DRY’ motif is highly conserved amongst class A GPCRs of the rhodopsin family, having a well-known role in G protein signalling, and has previously been reported to be involved in β-arrestin interaction [35, 54, 64]. In this study, mutation of the ‘DRY’ motif to ‘AAY’ clearly challenged, yet did not abolish the coupling of the A_3AR to βarr2. However, when introducing this non-conservative mutation in the truncated A_3AR, no increase in signal or concentration-dependent effect could be observed anymore. The fact that truncation of the A_3AR C-terminus alone did not influence βarr2 recruitment in the assay, but that the recruitment is drastically reduced by combining this truncation with mutation of the ‘DRY’ motif to ‘AAY’, could fit in the general image of β-arrestin ‘scanning’ the GPCR, based on multiple activation sensors in the arrestin molecule. Interestingly, βarr2 recruitment was fully rescued by back-mutating the ‘AAY’ residues to the conservatively mutated residues ‘NQY’, in the truncated A_3AR. To conclude, it can be said that other parts of

the human A_3AR, either cytosolic or exposed upon receptor activation, rather than the C-terminus alone, are responsible for βarr2 recruitment in a complementary or synergistic way.

Acknowledgments

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References

[1] Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W. Structure and function of adenosine receptors and their genes. N-S Arch Pharmacol 2000;362:364-74.

[2] Fredholm BB, Ijzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. Pharmacol Rev 2001;53:527-52.

[3] Fredholm BB, IJzerman AP, Jacobson KA, Linden J, Muller CE. International Union of Basic and Clinical Pharmacology. LXXXI. Nomenclature and Classification of Adenosine Receptors-An Update. Pharmacol Rev 2011;63:1-34.

[4] Jacobson KA, Gao ZG. Adenosine receptors as therapeutic targets. Nat Rev Drug Discov 2006;5:247-64.

[5] Jacobson KA. Introduction to adenosine receptors as therapeutic targets. Handb Exp Pharmacol 2009:1-24.

[6] Salvatore CA, Jacobson MA, Taylor HE, Linden J, Johnson RG. Molecular cloning and characterization of the human A3 adenosine receptor. Proc Natl Acad Sci U S A 1993;90:10365-9.

[7] Borea PA, Gessi S, Bar-Yehuda S, Fishman P. A3 adenosine receptor: pharmacology and role in disease. Handb Exp Pharmacol 2009:297-327.

[8] Moro S, Morizzo E, Jacobson KA. Molecular Modeling and Reengineering of A3 Adenosine Receptors. In: Borea PA, editor. A3 Adenosine Receptors from Cell Biology to Pharmacology and Therapeutics. Dordrecht: Springer Netherlands, 2010. p. 149-61.

[9] Cheong SL, Federico S, Venkatesan G, Mandel AL, Shao YM, Moro S, et al. The A3 adenosine receptor as multifaceted therapeutic target: pharmacology, medicinal chemistry, and in silico approaches. Med Res Rev 2013;33:235-335.

[10] Fishman P, Bar-Yehuda S, Synowitz M, Powell JD, Klotz KN, Gessi S, et al. Adenosine receptors and cancer. Handb Exp Pharmacol 2009:399-441.

[11] Di Virgilio F. Purines, purinergic receptors, and cancer. Cancer Res 2012;72:5441-7.

[12] Borea PA, Varani K, Vincenzi F, Baraldi PG, Tabrizi MA, Merighi S, et al. The A3 adenosine receptor: history and perspectives. Pharmacol Rev 2015;67:74-102.

[13] Ochaion A, Bar-Yehuda S, Cohen S, Barer F, Patoka R, Amital H, et al. The anti-inflammatory target A(3) adenosine receptor is over-expressed in rheumatoid arthritis, psoriasis and Crohn's disease. Cell Immunol 2009;258:115-22.

[14] Fishman P, Cohen S. The A3 adenosine receptor (A3AR): therapeutic target and predictive biological marker in rheumatoid arthritis. Clin Rheumatol 2016;35:2359-62.

[15] David M, Gospodinov DK, Gheorghe N, Mateev GS, Rusinova MV, Hristakieva E, et al. Treatment of Plaque-Type Psoriasis With Oral CF101: Data from a Phase II/III Multicenter, Randomized, Controlled Trial. J Drugs Dermatol 2016;15:931-8.

[16] Stemmer SM, Benjaminov O, Medalia G, Ciuraru NB, Silverman MH, Bar-Yehuda S, et al. CF102 for the treatment of hepatocellular carcinoma: a phase I/II, open-label, dose-escalation study. Oncologist 2013;18:25-6.

[17] Muller CE, Jacobson KA. Recent developments in adenosine receptor ligands and their potential as novel drugs. Biochim Biophys Acta 2011;1808:1290-308.
[18] Fishman P, Bar-Yehuda S, Liang BT, Jacobson KA. Pharmacological and therapeutic effects of A3 adenosine receptor agonists. Drug Discov Today 2012;17:359-66.
[19] Violin JD, Crombie AL, Soergel DG, Lark MW. Biased ligands at G-protein-coupled receptors: promise and progress. Trends Pharmacol Sci 2014;35:308-16.
[20] Bologna Z, Teoh JP, Bayoumi AS, Tang Y, Kim IM. Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology. Biomol Ther (Seoul) 2017;25:12-25.
[21] Rankovic Z, Brust TF, Bohn LM. Biased agonism: An emerging paradigm in GPCR drug discovery. Bioorg Med Chem Lett 2016;26:241-50.
[22] Shukla AK, Singh G, Ghosh E. Emerging structural insights into biased GPCR signaling. Trends Biochem Sci 2014;39:594-602.
[23] Violin JD, Lefkowitz RJ. Beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 2007;28:416-22.
[24] Rajagopal S, Rajagopal K, Lefkowitz RJ. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov 2010;9:373-86.
[25] Whalen EJ, Rajagopal S, Lefkowitz RJ. Therapeutic potential of beta-arrestin- and G protein-biased agonists. Trends Mol Med 2011;17:126-39.
[26] Ferguson SS. Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. Pharmacol Rev 2001;53:1-24.
[27] DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. Beta-arrestins and cell signaling. Annu Rev Physiol 2007;69:483-510.
[28] Shenoy SK, Lefkowitz RJ. beta-Arrestin-mediated receptor trafficking and signal transduction. Trends Pharmacol Sci 2011;32:521-33.
[29] Yoo SM, Kang DS, Benovic JL. Structural Basis for β-Arrestins in GPCR Trafficking. In: Gurevich VV, editor. The Structural Basis of Arrestin Functions. Cham: Springer International Publishing, 2017. p. 43-57.
[30] Gurevich EV, Gurevich VV. Arrestins: ubiquitous regulators of cellular signaling pathways. Genome Biol 2006;7:236.
[31] Zhuo Y, Klug CS. Overview of Arrestin Mediated Signaling with Receptors and Non-receptor Binding Partners. In: Gurevich VV, editor. The Structural Basis of Arrestin Functions. Cham: Springer International Publishing, 2017. p. 19-29.
[32] Spiller BW, Zhan X, Gurevich VV. Arrestin-3: The Structural Basis of Lower Receptor Selectivity. In: Gurevich VV, editor. The Structural Basis of Arrestin Functions. Cham: Springer International Publishing, 2017. p. 59-67.
[33] Gurevich VV, Gurevich EV. The structural basis of arrestin-mediated regulation of G-protein-coupled receptors. Pharmacol Ther 2006;110:465-502.
[34] Gurevich VV, Gurevich EV. Structural determinants of arrestin functions. Prog Mol Biol Transl Sci 2013;118:57-92.
[35] Rovati GE, Capra V, Neubig RR. The highly conserved DRY motif of class A G protein-coupled receptors: beyond the ground state. Mol Pharmacol 2007;71:959-64.
[36] Kim KM, Caron MG. Complementary roles of the DRY motif and C-terminus tail of GPCRS for G protein coupling and beta-arrestin interaction. Biochem Biophys Res Co 2008;366:42-7.

[37] Palmer TM, Benovic JL, Stiles GL. Agonist-dependent phosphorylation and desensitization of the rat A3 adenosine receptor. Evidence for a G-protein-coupled receptor kinase-mediated mechanism. J Biol Chem 1995;270:29607-13.
[38] Palmer TM, Benovic JL, Stiles GL. Molecular basis for subtype-specific desensitization of inhibitory adenosine receptors. Analysis of a chimeric A1-A3 adenosine receptor. J Biol Chem 1996;271:15272-8.
[39] Palmer TM, Stiles GL. Identification of threonine residues controlling the agonist-dependent phosphorylation and desensitization of the rat A(3) adenosine receptor. Mol Pharmacol 2000;57:539-45.
[40] Palmer TA, Ferguson G, Watterson KR. Dissecting the regulatory mechanisms controlling inhibitory adenosine receptor signaling. Drug Develop Res 2003;58:302-14.
[41] Ferguson G, Watterson KR, Palmer TM. Subtype-specific kinetics of inhibitory adenosine receptor internalization are determined by sensitivity to phosphorylation by G protein-coupled receptor kinases. Mol Pharmacol 2000;57:546-52.
[42] Ferguson G, Watterson KR, Palmer TM. Subtype-specific regulation of receptor internalization and recycling by the carboxyl-terminal domains of the human A1 and rat A3 adenosine receptors: consequences for agonist-stimulated translocation of arrestin3. Biochemistry 2002;41:14748-61.
[43] Cannaert A, Storme J, Franz F, Auwarter V, Stove CP. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. Anal Chem 2016;88:11476-85.
[44] Reiter E, Ahn S, Shukla AK, Lefkowitz RJ. Molecular mechanism of beta-arrestin-biased agonism at seven-transmembrane receptors. Annu Rev Pharmacol Toxicol 2012;52:179-97.
[45] Gurevich VV, Gurevich EV. The molecular acrobatics of arrestin activation. Trends Pharmacol Sci 2004;25:105-11.
[46] Park JY, Lee SY, Kim HR, Seo MD, Chung KY. Structural mechanism of GPCR-arrestin interaction: recent breakthroughs. Arch Pharm Res 2016;39:293-301.
[47] Han M, Gurevich VV, Vishnivetskiy SA, Sigler PB, Schubert C. Crystal structure of beta-arrestin at 1.9 A: possible mechanism of receptor binding and membrane Translocation. Structure 2001;9:869-80.
[48] Zhan X, Gimenez LE, Gurevich VV, Spiller BW. Crystal structure of arrestin-3 reveals the basis of the difference in receptor binding between two non-visual subtypes. J Mol Biol 2011;406:467-78.
[49] Palczewski K, Kumasaka T, Hori T, Behnke CA, Motoshima H, Fox BA, et al. Crystal structure of rhodopsin: A G protein-coupled receptor. Science 2000;289:739-45.
[50] Rasmussen SG, Choi HJ, Rosenbaum DM, Kobilka TS, Thian FS, Edwards PC, et al. Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. Nature 2007;450:383-7.
[51] Szczepek M, Beyriere F, Hofmann KP, Elgeti M, Kazmin R, Rose A, et al. Crystal structure of a common GPCR-binding interface for G protein and arrestin. Nat Commun 2014;5:4801.
[52] Kang Y, Zhou XE, Gao X, He Y, Liu W, Ishchenko A, et al. Crystal structure of rhodopsin bound to arrestin by femtosecond X-ray laser. Nature 2015;523:561-7.

[53] Oakley RH, Laporte SA, Holt JA, Barak LS, Caron MG. Molecular determinants underlying the formation of stable intracellular G protein-coupled receptor-beta-arrestin complexes after receptor endocytosis*. J Biol Chem 2001;276:19452-60.
[54] Marion S, Oakley RH, Kim KM, Caron MG, Barak LS. A beta-arrestin binding determinant common to the second intracellular loops of rhodopsin family G protein-coupled receptors. J Biol Chem 2006;281:2932-8.
[55] Dixon AS, Schwinn MK, Hall MP, Zimmerman K, Otto P, Lubben TH, et al. NanoLuc Complementation Reporter Optimized for Accurate Measurement of Protein Interactions in Cells. ACS chemical biology 2016;11:400-8.
[56] Gao ZG, Jacobson KA. Translocation of arrestin induced by human A(3) adenosine receptor ligands in an engineered cell line: comparison with G protein-dependent pathways. Pharmacol Res 2008;57:303-11.
[57] Gao ZG, Verzijl D, Zweemer A, Ye K, Goblyos A, Ijzerman AP, et al. Functionally biased modulation of A(3) adenosine receptor agonist efficacy and potency by imidazoquinolinamine allosteric enhancers. Biochem Pharmacol 2011;82:658-68.
[58] Trincavelli ML, Tuscano D, Cecchetti P, Falleni A, Benzi L, Klotz KN, et al. Agonist-induced internalization and recycling of the human A(3) adenosine receptors: Role in receptor desensitization and resensitization. J Neurochem 2000;75:1493-501.
[59] Trincavelli ML, Tuscano D, Marroni M, Falleni A, Gremigni V, Ceruti S, et al. A3 adenosine receptors in human astrocytoma cells: agonist-mediated desensitization, internalization, and down-regulation. Mol Pharmacol 2002;62:1373-84.
[60] Klaasse EC, Ijzerman AP, de Grip WJ, Beukers MW. Internalization and desensitization of adenosine receptors. Purinerg Signal 2008;4:21-37.
[61] Mundell S, Kelly E. Adenosine receptor desensitization and trafficking. Biochim Biophys Acta 2011;1808:1319-28.
[62] Verzijl D, Ijzerman AP. Functional selectivity of adenosine receptor ligands. Purinerg Signal 2011;7:171-92.
[63] Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled receptors delineate two major classes of receptors. J Biol Chem 2000;275:17201-10.
[64] Chen A, Gao ZG, Barak D, Liang BT, Jacobson KA. Constitutive activation of A(3) adenosine receptors by site-directed mutagenesis. Biochem Bioph Res Co 2001;284:596-601.

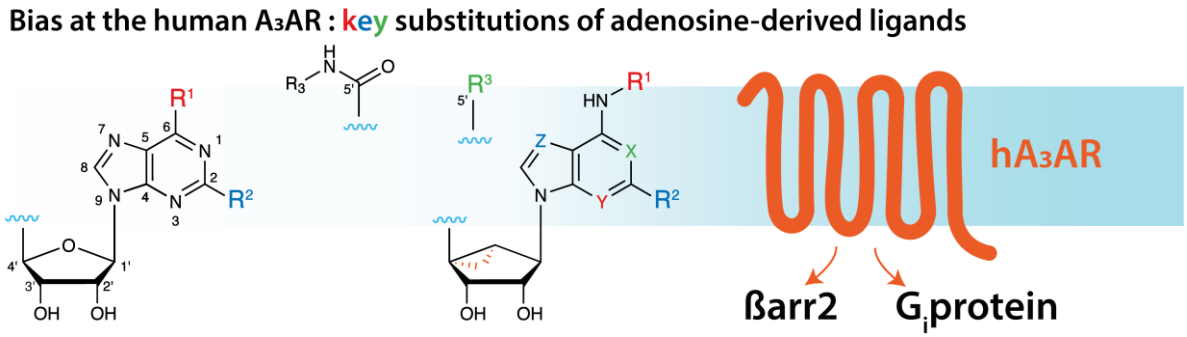
Chapter 4:
Probing Structure-Activity Relationship in β-Arrestin2 Recruitment of Diversely Substituted Adenosine Derivatives

Based on: Storme J, Tosh DK, Gao ZG, Jacobson KA, Stove CP. Probing Structure-Activity Relationship in β-Arrestin2 Recruitment of Diversely Substituted Adenosine Derivatives. Biochemical Pharmacology, 2018; 158: 103-113.

Abstract

In the adenosine receptor (AR) subfamily of G protein-coupled receptors (GPCRs), biased agonism has been described for the human A_1AR, A_{2B}AR and A_3AR. While diverse A_3AR agonists have been evaluated for receptor binding and G_i-mediated cAMP signalling, the β-arrestin2 (βarr2) pathway has been left largely unexplored. We screened nineteen diverse adenosine derivatives for βarr2 recruitment using a stable hA$_3$AR-NanoBit®-βarr2 HEK293T cell line. Their activity profiles were compared with a cAMP accumulation assay in stable hA$_3$AR CHO cells. Structural features linked to βarr2 activation were further investigated by the evaluation of an additional ten A_3AR ligands. The A_3AR-selective reference agonist 2-Cl-IB-MECA, which is a full agonist in terms of cAMP inhibition, only showed partial agonist behaviour in βarr2 recruitment. Highly A_3AR-selective (N)-methanocarba 5'-uronamide adenosine derivatives displayed higher potency in both cAMP signalling and βarr2 recruitment than reference agonists NECA and 2-Cl-IB-MECA. Their A_3AR-preferred conformation tolerates C2-position substitutions, for increased βarr2 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 A_3AR ligands with respect to G-protein and βarr2 signalling.

Graphical abstract



4.1 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 µM, may bind the four AR subtypes (A_1, A_{2A}, A_{2B} and A_3). 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 A_3AR (hA_3AR) is highly expressed in lung, liver and immune cells, with lower expression levels in the heart, brain and eye [4]. In recent years, the A_3AR 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]. A_3AR 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 A_3AR 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 A_3AR. Structure-based molecular modeling has led to the rational design of an impressive panel of potent, highly-selective A_3AR ligands [8]. For therapeutic application, these synthetic ligands must display good pharmacokinetic properties, as well as excellent A_3AR binding affinity, selectivity, efficacy and potency [14, 15]. Currently, functional evaluation of these A_3AR ligands relies mainly on the measurement of G_i protein-mediated signalling using cAMP accumulation assays. However, as with all GPCRs, the A_3AR 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 (βarr2) [16, 17]. βarr2 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 βarr2-dependent signalling pathways in A_3AR agonism has remained insufficiently explored. The application of functional assays to expand the structure-activity relationships of A_3AR 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 hA_3AR HEK293T cell assay system, which allows real-time monitoring of βarr2 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 hA_3AR-βarr2 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 A_3AR agonist (2-Cl-IB-MECA), we compared the βarr2 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 hA_3AR. Structural features linked to βarr2 activity were further investigated via the evaluation of an additional panel of ten A_3AR ligands, providing insight into the structure-activity relationship of A_3AR agonists with respect to βarr2 recruitment.

4.2 Materials & Methods

4.2.1 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 A_3AR construct (**NM_000677.2**, transcript variant 2 of the ADORA3 gene) and human βarr2 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-techne, 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].

4.2.2 Development of a stable A_3AR-NanoBit®-βarr2 HEK293T cell line by retroviral transduction

4.2.2.1 Production of retrovirus using the PhoenixA packaging cell line

To generate retroviral expression vectors, the coding sequences for the A_3AR-LgBit and SmBit-βarr2 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 4.1. The resulting retroviral expression vectors lead to co-expression of the fusion proteins A_3AR-LgBit and SmBit-βarr2 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 (ΦNX-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 µg/mL streptomycin and 0.25 µg/mL amphotericin B (full IMDM) under humidified atmosphere at 37°C, 5% CO_2. Twenty-four h before transfection, cells were seeded at a density of 10^6 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 µg 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 µM, 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×g, 4°C), aliquoted on ice without disturbing the pellet, and stored at -80°C. After the first harvest, puromycin selection (2 µg/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.

Table 4.1: PCR conditions (a-c) and restriction enzymes (RE; d) used to construct the retroviral expression vectors*

Coding Sequence		Primers (F: forward – R: reverse)[a]	Tm (°C)[b]	Ext. time (s)[c]	RE[d]	Retroviral expression vector*
A_3AR-LgBit	F	ACTCAAGGATCC***ACC***ATGCCCAACAACAGC	69.5	40	BamHI - SnaBI	pLZRS-A_3AR-LgBit-IRES-EGFP
	R	ACTCAATACGTA**TTA**GCTGTTGATGGTTACTCGG				
SmBit-βarr2	F	ACTCAAGGATCC***ACC***ATGGTGACCGGCTACCG	74	38	BamHI - NotI	pLZRS-SmBit-βarr2-IRES-dNGFR
	R	ACTCAAGCGGCCGC**TCA**GCAGA GTTGATCATCATAGTCG				

a: primers containing the specific restriction site (underlined), Kozak sequence (***bold italic***) and coding sequence of interest (*italic*).
b: Annealing temperature.
c: Extension time.
A stop codon (**bold**) to ensure a correct reading frame was added where necessary.

4.2.2.2 Retroviral transduction of HEK293T cells

HEK293T cells were seeded in a 96-well plate at 10^4 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 A_3AR- and βarr2-sequence containing retroviruses. Subsequently, the plate was centrifuged for 90 min at 950×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 A_3AR- and βarr2-fusion proteins, respectively.

4. 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 A_3AR- and βarr2 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.

4.2.3 Screening of synthetic A_3AR ligands

4.2.3.1 A_3AR NanoBit® βarr2 assay

In total, a panel of twenty-nine synthetic ligands was subjected to the stable A_3AR-NanoBit®-βarr2 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 $5x10^4$ cells/well and incubated overnight (37°C, 5% CO_2). 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.

4.2.3.2 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 A_3AR 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.

4.2.3.3 Binding assay

Binding experiments were performed as described previously [26, 28, 36], using [^{125}I]-*N*^{6}-(4-amino-3-iodobenzyl)adenosine-5'-*N*-methyluronamide ([^{125}I]I-AB-MECA) as a radioligand. Briefly, membranes were prepared from CHO cells stably expressing the human A_3AR. Cells were detached and resuspended in 50 mM Tris HCl buffer (pH 8.0) containing 10 mM $MgCl_2$, 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 [^{125}I]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 $MgCl_2$, 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. IC_{50} values were obtained from competition curves and converted to K_i values using the Cheng-Prusoff equation [37].

4.2.4 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 logEC_{50} values, a sigmoidal curve was fitted to the normalized responses by analysing concentration-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.

4.3 Results

4.3.1 Development of a stable A_3AR reporter assay for real-time monitoring of βarr2 recruitment

The originally-described reporter system for evaluation of βarr2 coupling to A_3AR [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 A_3AR-βarr2 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 A_3AR and βarr2 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 µM, was obtained with this cell line (Figure 4.1).

4.3.2 Screening of A_3AR ligands for βarr2 recruitment and cAMP signalling

A panel of nineteen synthetic A_3AR ligands was evaluated for βarr2 recruitment, using the stable hA_3AR-NanoBit®-βarr2 HEK293T cell line, and G-protein dependent cAMP signalling, using a cAMP accumulation assay in stable hA_3AR CHO cells. In Table 4.2, the maximal efficacy (E_{max}) and potency ($logEC_{50}$) of these compounds is shown for both signalling pathways, as well as their A_3AR binding K_i values and structures. To gain more insight into their βarr2-related activity profiles, an additional ten structural analogues were tested with the hA_3AR-βarr2 cell line (Table 4.3). The sigmoidal concentration-response curves depicted in Figure 4.2 show different

extents of maximal βarr2 recruitment for all compounds tested, relative to reference agonists NECA and 2-Cl-IB-MECA (black curves).

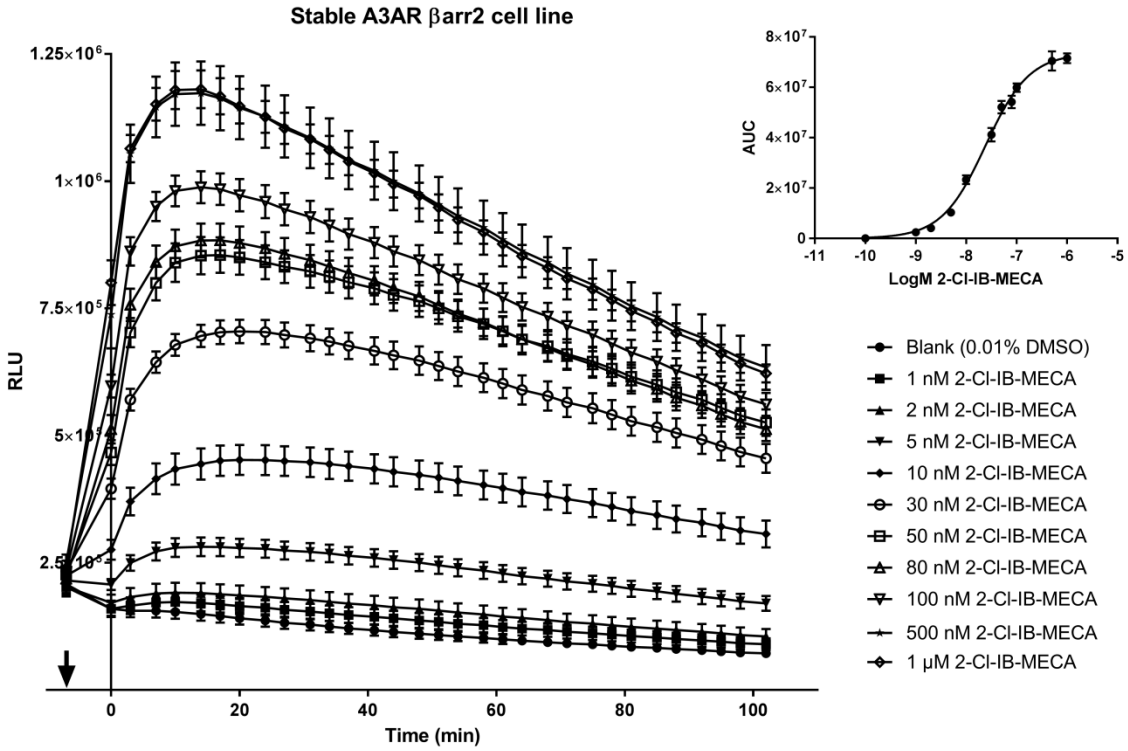
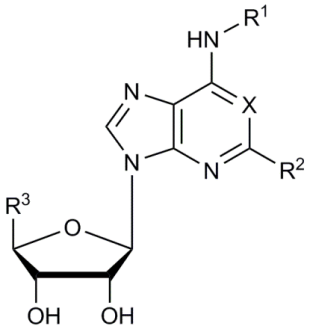
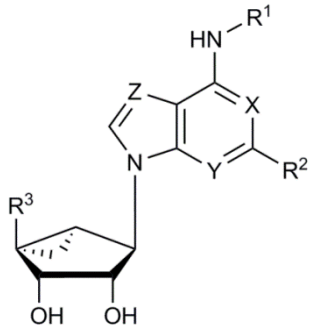


Figure 4.1: Concentration-dependent effect of reference A_3AR agonist 2-Cl-IB-MECA on βarr2 recruitment in a stable hA_3AR-NanoBit®-βarr2 HEK293T cell line. 1, 2, 5, 10, 30, 50, 80, 100, 500 nM and 1 µM 2-Cl-IB-MECA was added at the time point indicated by the arrow, and luminescence was measured for >90 min. A solvent control of 0.01% DMSO was included. Insert: Concentration-response (AUCs). AUCs (± SEM) of quadruplicate wells are shown for a representative experiment (n=3).

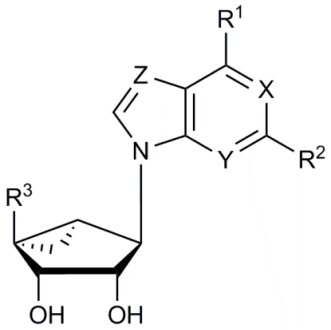
Table 4.2: Structures, efficacies (E_{max}), potencies ($logEC_{50}$), and hA_3AR binding affinities (K_i) of nineteen known A_3AR ligands. Data for βarr2 recruitment were obtained with the stable hA_3AR-NanoBit®-βarr2 HEK293T cell line, and those for cAMP inhibition were obtained with a stable hA_3AR CHO cell line.



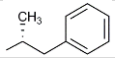
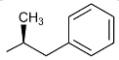
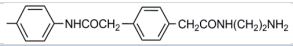
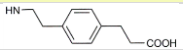
Compound series A and B



Compound series C, D and E

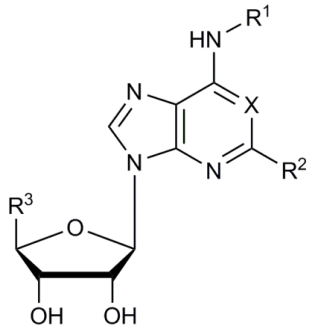


Compound series F

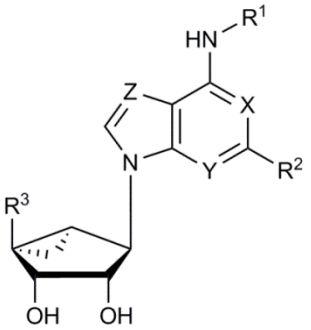
Compound	R^1	R^2	R^3	X	Y	Z	Stable A_3AR NanoBit® HEK293T cell line[A] Emax (%)	Stable A_3AR NanoBit® HEK293T cell line[A] $LogEC_{50}$ ± SEM	Stable A_3AR CHO cell line[B] Emax (%)	Stable A_3AR CHO cell line[B] $LogEC_{50}$	Ki (nM) ± SEM
A. Ribose 5'-OH analogues											
CPA	cyclopentyl	H	CH_2OH	N	-	-	56.9 ± 3.7	-5.82 ± 0.11	97 ± 4	-6.91	72 ± 12
CCPA	cyclopentyl	Cl	CH_2OH	N	-	-	33.5 ± 2.7	-6.08 ± 0.15	0	NA	38 ± 6
R-PIA		H	CH_2OH	N	-	-	56.6 ± 1.9	-6.35 ± 0.06	102 ± 6	-7.57	8.7 ± 0.9
S-PIA		H	CH_2OH	N	-	-	51.0 ± 1.4	-5.72 ± 0.04	97 ± 3	-6.68	68 ± 12
ADAC		H	CH_2OH	N	-	-	73.7 ± 3.1	-6.61 ± 0.12	103 ± 4	-7.00	13.3 ± 3.0
B. Ribose 5'-amide analogues											
NECA	H	H	$CONHC_2H_5$	N	-	-	100 ± 2.5	-6.99 ± 0.05	103	-7.42	35 ± 12
CGS21680	H		$CONHC_2H_5$	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
C. Methanocarba, C2-Cl											
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_2OH	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
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
D. Methanocarba, 5'-amides, C2-extended											
MRS5698	3-Cl-benzyl	(3,4-F_2-phenyl)ethynyl	$CONHCH_3$	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)ethynyl	$CONHCH_3$	N	N	N	91.6 ± 3.4	-8.51 ± 0.10	97.3 ± 2.9	-9.38	0.57 ± 0.10
E. Methanocarba, deaza-adenine											
MRS7144	CH_2CH_3	(5-Cl-thien-2-yl)ethynyl	$CONHCH_3$	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)ethynyl	$CO_2C_2H_5$	N	N	CH	NA	NA	1±7	NA	448 ± 13
F. Methanocarba, 5'-amides, lacking 6-NH											
MRS7195	CH_3	(5-Cl-thien-2-yl)ethynyl	$CONHCH_3$	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)ethynyl	$CONHCH_3$	N	N	N	68.4 ± 5.3	-5.52 ± 0.11	97.8 ± 0.1	-7.57	60 ± 19

A. E_{max} is relative to that of reference agonist NECA - B. E_{max} is relative to that of reference agonist NECA at 10 µM - NA, not significantly active at 10 µM

Table 4.3: Structures, efficacies (E_{max}), potencies ($logEC_{50}$), and hA_3AR binding affinities (K_i) of ten additional structural analogs tested in the stable hA_3AR-NanoBit®-βarr2 HEK293T cell line.



Compound series A



Compound series D and E

Compound	R^1	R^2	R^3	X	Y	Z	Stable A_3AR NanoBit® HEK293T cell line[A]		Ki (nM) ± SEM
							Emax (%)	$LogEC_{50}$ ± SEM	
A. Ribose 5'-OH analogues									
DCCA	cyclopentyl	Cl	CH_2OH	CH	-	-	5.8 ± 0.2	-5.83 ± 0.07	244 ± 37
N^6-benzyl-adenosine	benzyl	H	CH_2OH	N	-	-	34.9 ± 2.3	-6.07 ± 0.13	41.7 ± 5.3
N^6-phenyl-adenosine	phenyl	H	CH_2OH	N	-	-	75.8 ± 3.0	-6.23 ± 0.0	14.9 ± 3.1
D. Methanocarba, 5'-amides, C2-extended									
MRS5679	3-Cl-benzyl	(4-phenyl-phenyl)ethynyl	$CONHCH_3$	N	N	N	46.8 ± 3.3	-5.92 ± 0.11	3.06 ± 1.35
MRS5967	CH_3	(2-CH_3O-phenyl)ethynyl	$CONHCH_3$	N	N	N	92.5 ± 2.4	-8.33 ± 0.06	0.77 ± 0.17
MRS5663	CH_3	(2-Cl-phenyl)ethynyl	$CONHCH_3$	N	N	N	101.7 ± 3.6	-8.25 ± 0.08	0.58 ± 0.04
MRS5980	CH_3	(5-Cl-thien-2-yl)ethynyl	$CONHCH_3$	N	N	N	95.0 ± 4.0	-8.71 ± 0.11	0.7 ± 0.11
MRS7154	$(CH_2)_2CH_3$	(5-Cl-thien-2-yl)ethynyl	$CONHCH_3$	N	N	N	94.5 ± 4.8	-7.67 ± 0.11	1.1 ± 0.3
E. Methanocarba, deaza-adenine									
MRS7173	CH_3	(5-Cl-thien-2-yl)ethynyl	$CONHCH_3$	N	CH	N	78.2 ± 1.8	-7.26 ± 0.04	1.56 ± 0.2
MRS7232	CH_3	(5-Cl-thien-2-yl)ethynyl	$CO_2C_2H_5$	N	N	N	13.6 ± 1.6	-7.30 ± 0.17	5.38 ± 0.03

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

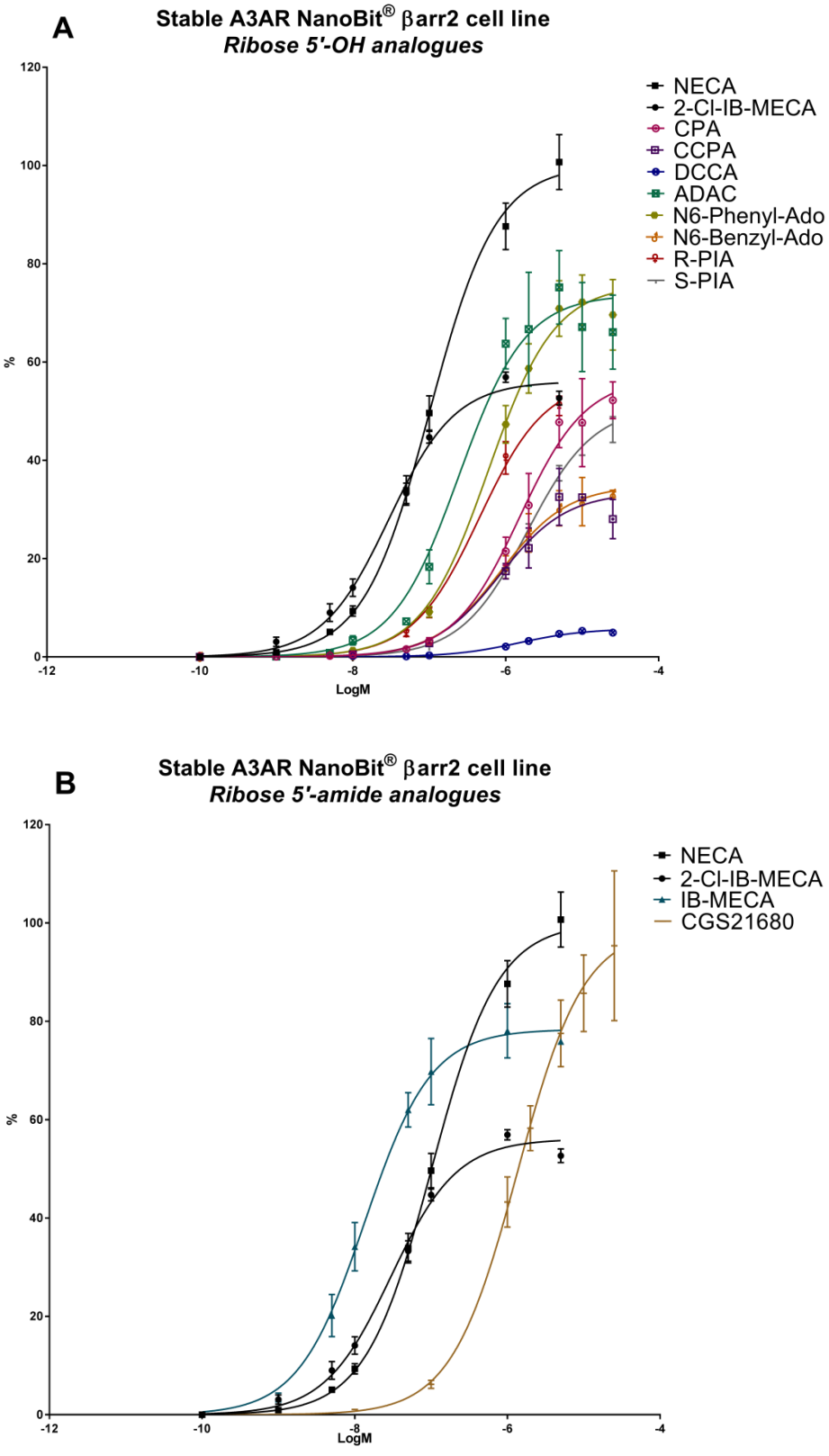


Figure 4.2, A-F: Non-linear regression analysis of the sigmoidal concentration-response curves for all twenty-nine synthetic A_3AR ligands tested in the stable hA_3AR-NanoBit®-βarr2 cell line. The curves were normalized to that of reference agonist NECA. AUCs (± SEM) were calculated based on at least 3 experiments performed in duplicate or triplicate.

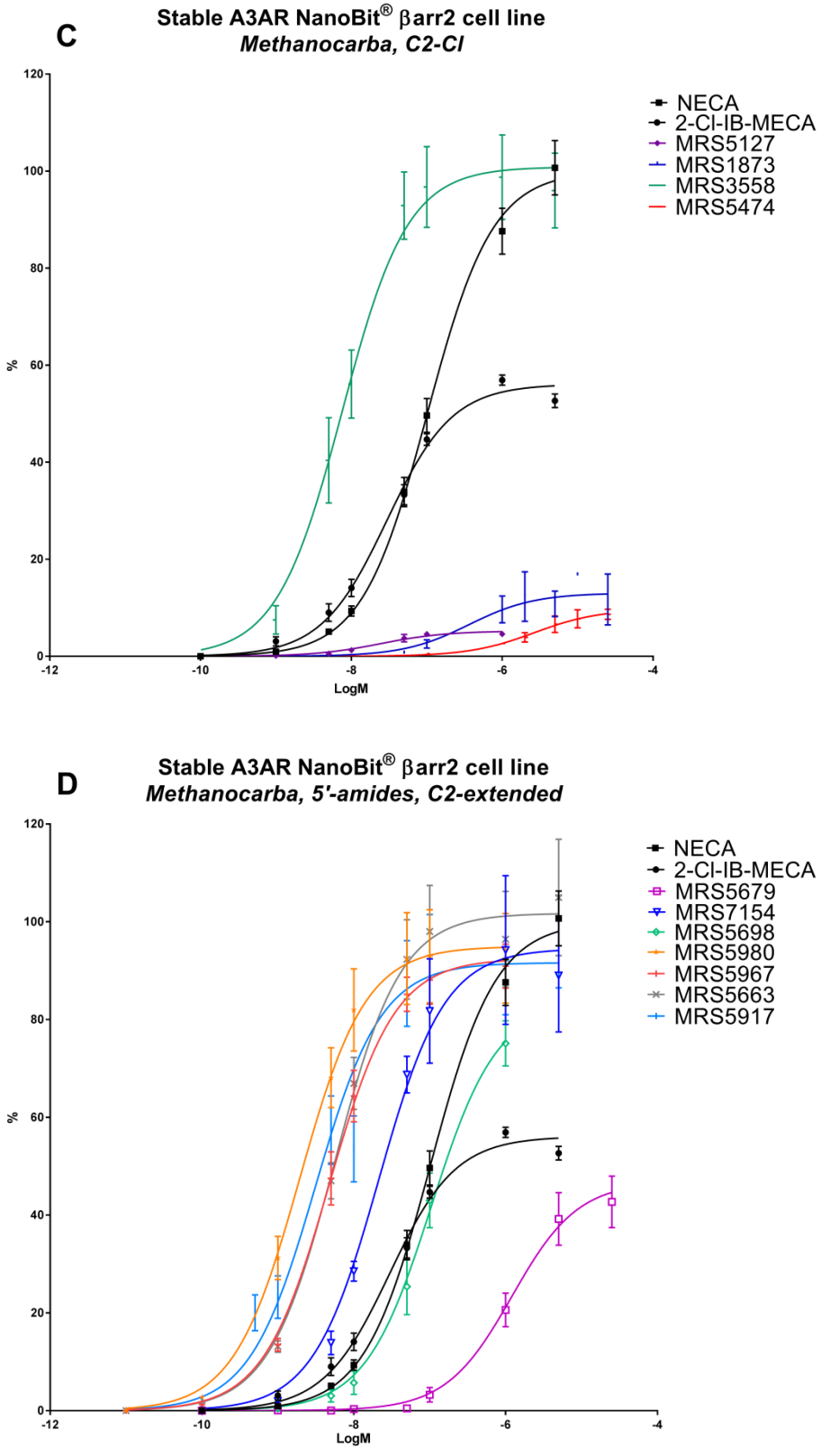


Figure 4.2, A-F (continued).

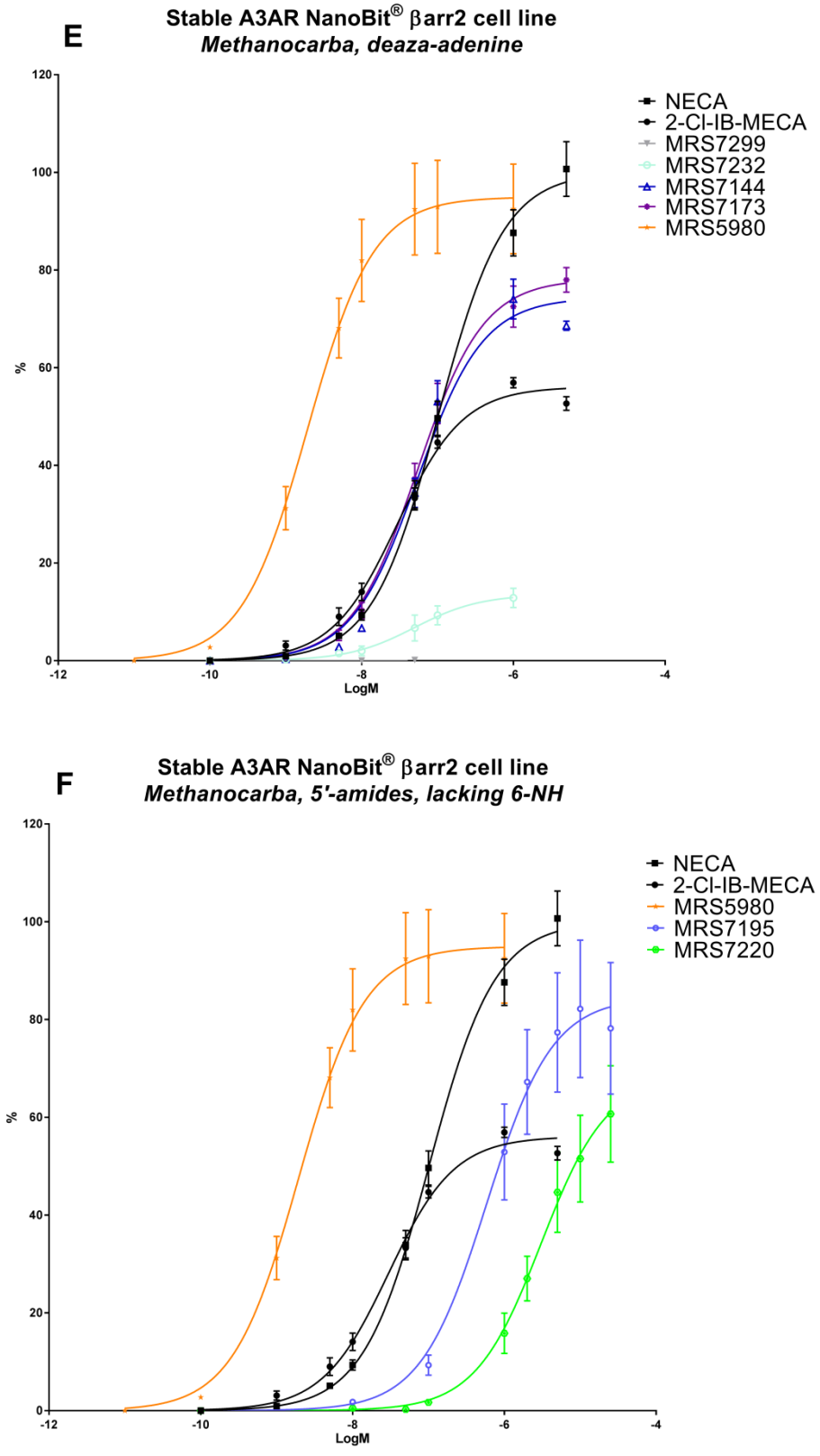


Figure 4.2, A-F (continued).

4.4 Discussion

The human A_3 adenosine receptor is gaining interest in the field of drug discovery as a therapeutic target for inflammatory diseases and cancer [4]. Two A_3AR 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 A_3AR'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 A_3AR 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 A_3AR homology model, which uses an agonist-bound A_{2A}AR X-ray structure [44], but not the recently solved antagonist-bound A_1AR [45], as a template, as well as opsin and the β_2AR receptor, has enabled additional prediction of nucleoside binding interactions at the A_3AR [46-48]. However, how this binding is translated into differential signalling has remained elusive.

Although the A_3AR generally couples to the G_i protein, inhibiting adenylate cyclase and leading to a decrease in cAMP levels, there is substantial coupling to $G_{\beta\gamma}$ 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 A_1AR, the A_{2B}AR, and A_3AR [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 A_3AR. First reports of the interaction of the human A_3AR 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 hA_3AR-NanoBit®-βarr2 HEK293T cell line that was used to screen a panel of nineteen synthetic ligands for βarr2 recruitment to the hA_3AR. The resulting βarr2-recruitment activity profiles and those obtained for G_i protein coupling (Table 4.2), using the cAMP accumulation assay in stable A_3AR CHO cells, were compared (Figure 4.3). 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 4.3, Figure 4.2). The efficacies reported are expressed relative to the non-selective AR reference agonist NECA, for consistency with respect to previous results reported for A_3AR signalling. NECA is considered to be a full agonist in multiple A_3AR signalling pathways — including the cAMP- and βarr2-pathways, as well as A_3AR mediated membrane hyperpolarization and intracellular Ca^{2+} mobilization [62]. The A_3AR-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 Ca^{2+} 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 A_3AR expression levels in the different assays may have varied and influenced the signal measured. However, with the NanoBit® system, we also evaluated βarr2 recruitment for 2-Cl-IB-MECA in CHO-K1 cells; potencies did not differ substantially between HEK293T cells ($logEC_{50}$ of -7.53 ± 0.04) and transiently transfected ($logEC_{50}$ of -7.26 ± 0.08) as well as stably-transduced ($logEC_{50}$ 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 βarr2 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 βarr2-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 $logEC_{50}$s for cAMP signalling and βarr2 recruitment (Figure 4.3B).

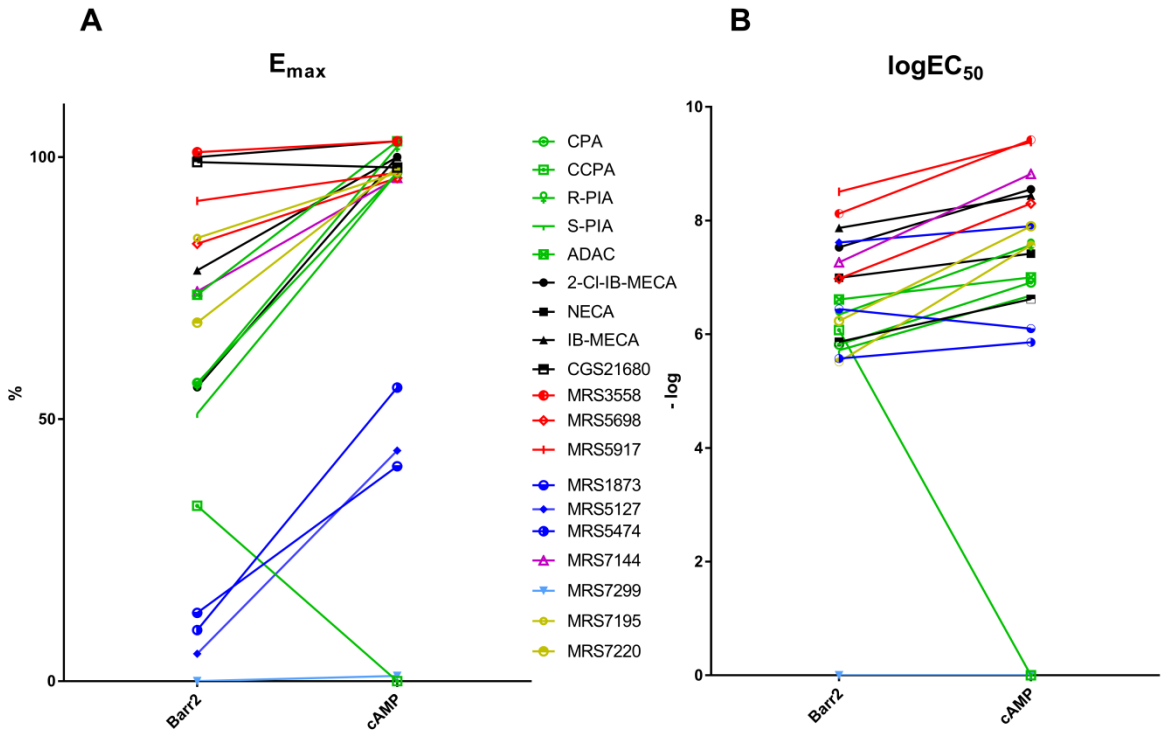


Figure 4.3: Comparison of the E_{max} relative to that of NECA (arbitrarily set at 100%) (A) and negative $logEC_{50}$ (B) of nineteen synthetic A_3AR ligands tested with the stable hA_3AR-NanoBit®-βarr2 HEK293T cell line (Barr2) versus the stable hA_3AR CHO cell line (cAMP).

Several *N*^{6} substituted adenosine derivatives were tested, as they are known to be quite potent A_3AR ligands from G-protein-dependent signalling experiments [61, 63]. *N*^{6}-cyclopentyladenosine (CPA), an A_1AR-selective agonist, and the *N*^{6}-phenylisopropyl R- and S-diastereomers R-PIA and S-PIA, which have demonstrated stereoselectivity in binding at the A_1AR, A_{2A}AR and A_3AR [19], show only partial agonist efficacy in βarr2 recruitment, in contrast to full agonism in cAMP signaling (Table 4.2; Figure 4.2A; Figure 4.3A). These compounds also have a low potency in βarr2 recruitment ($logEC_{50}$s of around -5.8), although R-PIA is more potent than S-

PIA (logEC_{50} of -6.35), as is also the case in cAMP signalling (Table 4.2; Figure 4.3B). The 2-chloro analogue of N^6-cyclopentyladenosine (CCPA) is a full antagonist in the cAMP assay, but a partial agonist in βarr2 recruitment (E_{max} of 34%; Table 4.2). The 1-deaza analogue of CCPA, DCCA, showed a further reduced 6% efficacy in βarr2 recruitment (Table 4.3), suggesting an importance of the 1-position ring nitrogen in βarr2-pathway activation. Furthermore**,** substitution of N^6 with a benzyl or phenyl group instead of a cyclopropyl group (as in CPA) reduces or stimulates βarr2 recruitment, respectively (Table 4.3). The N^6-chain elongated adenosine congener N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl]anilino]-carbonyl]methyl]phenyl]adenosine (ADAC), an agonist with selectivity for human A_1AR and A_3AR, has been studied for enhanced A_3AR-selectivity upon conjugation to a nanocarrier [64]. Amongst the ribose-5'-OH analogues, this compound performs substantially well in βarr2 recruitment (Table 4.2; Figure 4.3A). Overall, the N^6-substituted adenine 9-riboside (4'-CH_2OH) derivatives mentioned here all have intermediate potency for βarr2 recruitment (logEC_{50}s of around -6) compared to reference agonists NECA or 2-Cl-IB-MECA (logEC_{50}s of around -7).

A series of *5'*-alkyluronamide adenosine derivatives was developed as selective A_3AR agonists, and substitutions at the C2- and/or N^6- 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 (logEC_{50} from -7.42 to -6.62), as well as βarr2 recruitment (logEC_{50} from -6.99 to -5.87) (Table 4.2; Figure 4.2B). Introduction of a shorter methyluronamide group was reported to favour A_3AR binding compared to larger alkyl groups. An N^6-halobenzyl group maintains affinity at the A_3AR while reducing A_1AR and A_{2A}AR affinity, to increase A_3AR selectivity. Examples include the prototypical agonists IB-MECA [8] and 2-Cl-IB-MECA (Figure 4.2B; Figure 4.3, black curves) [21]. These agonists display high potency in both cAMP signalling and βarr2 recruitment (logEC_{50}s of around -8.5 and -7.5, respectively) (Table 4.2). 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 βarr2 recruitment (Table 4.2; Figure 4.2B). Thus, for (5'-uronamide) adenosine derivatives, the E_{max} of βarr2 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 βarr2 recruitment (logEC_{50}s of -9.42 and -8.12, respectively) (Table 4.2). Exchanging the 5’-uronamide group, as in the adenosine-like 4’-CH_2OH derivative MRS1873 [19] and the truncated 4’-H MRS5127, drastically lowered the E_{max} and potency towards both signalling pathways (Table 4.2; Figure 4.2C). 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 βarr2 recruitment as well as in cAMP signalling (Table 4.2; Figure 4.2C; Figure 4.3, 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 4.2; Figure 4.2D; Figure 4.3, red curves). To further evaluate C2-extension, the βarr2 activity profile of analogue MRS5679, containing a biphenyl substituent at the C2-position, was tested. Interestingly, this compound showed much lower potency (logEC_{50} of -5.92), and only half the E_{max} of MRS5698 (Table 4.3; Figure 4.2D). This might be explained by the characteristics of transmembrane helix 2 (TM2). When relying solely on the A_{2A}AR 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 βarr2 recruitment (Table 4.3; Figure 4.2D). 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 (logEC_{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 A_3AR selectivity. Thus, in contrast to ribose analogs, (N)-methanocarba 5'-uronamide adenosine derivatives seem to occupy the A_3AR binding site in a manner that maintains efficacy for βarr2 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 βarr2 recruitment (logEC_{50} of -9.38 and -8.5, respectively) (Table 4.2; Figure 4.2D). 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 βarr2 signalling (Table 4.3). MRS5917 and its arylethynyl congeners are highly promising orally active A_3AR 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 A_3AR is subject to desensitization and downregulation [66], which is known to be correlated with βarr2 recruitment. However, protection in animal models of pain results from prolonged A_3AR agonist action, which can be prevented by coadministration of an A_3AR antagonist [12]. Despite potential A_3AR downregulation, animal models have already revealed the persistent anti-inflammatory and anticancer effects of A_3AR agonists. Thus, the roles of G-protein versus βarr2-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 N^1-group is more dispensable for βarr2 recruitment, although it is characterized by a somewhat reduced but still substantially good $\log EC_{50}$ of approximately -7.3 (Table 4.2; Figure 2E; Figure 4.3, purple curve). Thus, the more stabilized A_3AR interaction of (N)-methanocarba 5′-methyluronamide adenosine derivatives tends to compensate for the loss of N1 in βarr2 recruitment, as well as cAMP signalling [31]. Also, the additionally tested 3-deaza analogue MRS7173 was fully efficacious in recruiting βarr2 (Table 4.3). 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 βarr2 recruitment (Table 4.2; Figure 4.2E; Figure 4.3, bright blue curve) [33]. When re-introducing the amino group (MRS7232), βarr2 recruitment was partially restored (Table 4.3; Figure 4.2E). The necessity of N^7 is a well-described feature in literature concerning A_3AR 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 A_3AR-selective ligands can partially compensate for the lack of an exocyclic amine, an otherwise important contributor to recognition in the A_3AR binding site [32]. This is demonstrated by the sustained A_3AR activity of MRS7195 and MRS7220 in cAMP signalling as well as in βarr2 recruitment (Table 4.2; Figure 4.2F; Figure 4.3, 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 A_3AR ligands using a βarr2-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 A_3AR 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 βarr2 signalling, as well as A_3AR desensitization, remains to be determined for the different therapeutic applications.

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References

[1] Borea PA, Gessi S, Merighi S, Varani K. Adenosine as a Multi-Signalling Guardian Angel in Human Diseases: When, Where and How Does it Exert its Protective Effects? Trends Pharmacol Sci 2016;37:419-34.

[2] Liu H, Xia Y. Beneficial and detrimental role of adenosine signaling in diseases and therapy. J Appl Physiol 2015;119:1173-82.

[3] Peleli M, Fredholm BB, Sobrevia L, Carlstrom M. Pharmacological targeting of adenosine receptor signaling. Mol Aspects Med 2017;55:4-8.

[4] Borea PA, Varani K, Vincenzi F, Baraldi PG, Tabrizi MA, Merighi S, et al. The A_3 adenosine receptor: history and perspectives. Pharmacol Rev 2015;67:74-102.

[5] Burnstock G. Purinergic Signalling: Therapeutic Developments. Front Pharmacol 2017;8:661.

[6] Tian YK, Marshall M, French BA, Linden J, Yang ZQ. The infarct-sparing effect of IB-MECA against myocardial ischemia/reperfusion injury in mice is mediated by sequential activation of adenosine A(3) and A(2A) receptors. Basic Res Cardiol 2015;110:16.

[7] Cronstein BN, Sitkovsky M. Adenosine and adenosine receptors in the pathogenesis and treatment of rheumatic diseases. Nat Rev Rheumatol 2017;13:41-51.

[8] Jacobson KA, Merighi S, Varani K, Borea PA, Baraldi S, Aghazadeh Tabrizi M, et al. A_3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy. Med Res Rev 2017.

[9] Gessi S, Merighi S, Sacchetto V, Simioni C, Borea PA. Adenosine receptors and cancer. Biochim Biophys Acta 2011;1808:1400-12.

[10] Di Virgilio F, Adinolfi E. Extracellular purines, purinergic receptors and tumor growth. Oncogene 2017;36:293-303.

[11] Chen Z, Janes K, Chen C, Doyle T, Bryant L, Tosh DK, et al. Controlling murine and rat chronic pain through A_3 adenosine receptor activation. FASEB J 2012;26:1855-65.

[12] Little JW, Ford A, Symons-Liguori AM, Chen Z, Janes K, Doyle T, et al. Endogenous adenosine A_3 receptor activation selectively alleviates persistent pain states. Brain 2015;138:28-35.

[13] Janes K, Symons-Liguori AM, Jacobson KA, Salvemini D. Identification of A_3 adenosine receptor agonists as novel non-narcotic analgesics. Br J Pharmacol 2016;173:1253-67.

[14] Chen JF, Eltzschig HK, Fredholm BB. Adenosine receptors as drug targets--what are the challenges? Nat Rev Drug Discov 2013;12:265-86.

[15] Schmidt J, Ferk P. Safety issues of compounds acting on adenosinergic signalling. J Pharm Pharmacol 2017;69:790-806.

[16] Rankovic Z, Brust TF, Bohn LM. Biased agonism: An emerging paradigm in GPCR drug discovery. Bioorg Med Chem Lett 2016;26:241-50.

[17] Bologna Z, Teoh JP, Bayoumi AS, Tang Y, Kim IM. Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology. Biomol Ther (Seoul) 2017;25:12-25.

[18] Storme J, Cannaert A, Van Craenenbroeck K, Stove CP. Molecular dissection of the human A_3 adenosine receptor coupling with β-arrestin2. Biochem Pharmacol 2018;148:298-307.

[19] Gao ZG, Kim SK, Biadatti T, Chen W, Lee K, Barak D, et al. Structural determinants of A(3) adenosine receptor activation: nucleoside ligands at the agonist/antagonist boundary. J Med Chem 2002;45:4471-84.
[20] Gallo-Rodriguez C, Ji XD, Melman N, Siegman BD, Sanders LH, Orlina J, et al. Structure-Activity-Relationships of N^6-Benzyladenosine-5'-Uronamides as A(3)-Selective Adenosine Agonists. J Med Chem 1994;37:636-46.
[21] Kim HO, Ji XD, Siddiqi SM, Olah ME, Stiles GL, Jacobson KA. 2-Substitution of N^6-Benzyladenosine-5'-Uronamides Enhances Selectivity for A_3 Adenosine Receptors. J Med Chem 1994;37:3614-21.
[22] Jacobson KA, Ji X, Li AH, Melman N, Siddiqui MA, Shin KJ, et al. Methanocarba analogues of purine nucleosides as potent and selective adenosine receptor agonists. J Med Chem 2000;43:2196-203.
[23] Tchilibon S, Joshi BV, Kim SK, Duong HT, Gao ZG, Jacobson KA. (N)-methanocarba 2,N^6-disubstituted adenine nucleosides as highly potent and selective A_3 adenosine receptor agonists. J Med Chem 2005;48:1745-58.
[24] Melman A, Gao ZG, Kumar D, Wan TC, Gizewski E, Auchampach JA, et al. Design of (N)-methanocarba adenosine 5'-uronamides as species-independent A_3 receptor-selective agonists. Bioorg Med Chem Lett 2008;18:2813-9.
[25] Melman A, Wang B, Joshi BV, Gao ZG, Castro S, Heller CL, et al. Selective A(3) adenosine receptor antagonists derived from nucleosides containing a bicyclo[3.1.0]hexane ring system. Bioorg Med Chem 2008;16:8546-56.
[26] Tosh DK, Chinn M, Ivanov AA, Klutz AM, Gao ZG, Jacobson KA. Functionalized congeners of A_3 adenosine receptor-selective nucleosides containing a bicyclo[3.1.0]hexane ring system. J Med Chem 2009;52:7580-92.
[27] Tosh DK, Paoletta S, Deflorian F, Phan K, Moss SM, Gao ZG, et al. Structural sweet spot for A_1 adenosine receptor activation by truncated (N)-methanocarba nucleosides: receptor docking and potent anticonvulsant activity. J Med Chem 2012;55:8075-90.
[28] Tosh DK, Deflorian F, Phan K, Gao ZG, Wan TC, Gizewski E, et al. Structure-guided design of A(3) adenosine receptor-selective nucleosides: combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. J Med Chem 2012;55:4847-60.
[29] Tosh DK, Padia J, Salvemini D, Jacobson KA. Efficient, large-scale synthesis and preclinical studies of MRS5698, a highly selective A_3 adenosine receptor agonist that protects against chronic neuropathic pain. Purinerg Signal 2015;11:371-87.
[30] Tosh DK, Finley A, Paoletta S, Moss SM, Gao ZG, Gizewski ET, et al. In vivo phenotypic screening for treating chronic neuropathic pain: modification of C2-arylethynyl group of conformationally constrained A_3 adenosine receptor agonists. J Med Chem 2014;57:9901-14.
[31] Tosh DK, Crane S, Chen Z, Paoletta S, Gao ZG, Gizewski E, et al. Rigidified A_3 Adenosine Receptor Agonists: 1-Deazaadenine Modification Maintains High in Vivo Efficacy. ACS Med Chem Lett 2015;6:804-8.
[32] Tosh DK, Ciancetta A, Warnick E, O'Connor R, Chen ZM, Gizewski E, et al. Purine (N)-Methanocarba Nucleoside Derivatives Lacking an Exocyclic Amine as Selective A(3) Adenosine Receptor Agonists. J Med Chem 2016;59:3249-63.
[33] Tosh DK, Ciancetta, A., Mannes, P., Warnick, E., Janowsky, A., Eshleman, A.J.; Gizewski, E., Brust, T.F., Bohn, L.M., Auchampach, J.A., Gao, Z.G.,

Jacobson, K.A. Repurposing of a nucleoside scaffold from adenosine receptor agonists to opioid receptor antagonists. ACS Omega 2018;3:12658-78.
[34] Nordstedt C, Fredholm BB. A modification of a protein-binding method for rapid quantification of cAMP in cell-culture supernatants and body fluid. Anal Biochem 1990;189:231-4.
[35] Post SR, Ostrom RS, Insel PA. Biochemical methods for detection and measurement of cyclic AMP and adenylyl cyclase activity. Methods Mol Biol 2000;126:363-74.
[36] Olah ME, Gallo-Rodriguez C, Jacobson KA, Stiles GL. ^{125}I-4-Aminobenzyl-5'-*N*-Methylcarboxamidoadenosine, a High-Affinity Radioligand for the Rat A(3) Adenosine Receptor. Mol Pharmacol 1994;45:978-82.
[37] Cheng Y, Prusoff WH. Relationship between Inhibition Constant (K1) and Concentration of Inhibitor Which Causes 50 Per Cent Inhibition (I50) of an Enzymatic-Reaction. Biochem Pharmacol 1973;22:3099-108.
[38] David M, Gospodinov DK, Gheorghe N, Mateev GS, Rusinova MV, Hristakieva E, et al. Treatment of Plaque-Type Psoriasis With Oral CF101: Data from a Phase II/III Multicenter, Randomized, Controlled Trial. J Drugs Dermatol 2016;15:931-8.
[39] Stemmer SM, Benjaminov O, Medalia G, Ciuraru NB, Silverman MH, Bar-Yehuda S, et al. CF102 for the treatment of hepatocellular carcinoma: a phase I/II, open-label, dose-escalation study. Oncologist 2013;18:25-6.
[40] Shen YC, Lin ZZ, Hsu CH, Hsu C, Shao YY, Cheng AL. Clinical trials in hepatocellular carcinoma: an update. Liver Cancer 2013;2:345-64.
[41] Muller CE. Medicinal chemistry of adenosine A3 receptor ligands. Curr Top Med Chem 2003;3:445-62.
[42] Jacobson KA, Klutz AM, Tosh DK, Ivanov AA, Preti D, Baraldi PG. Medicinal chemistry of the A_3 adenosine receptor: agonists, antagonists, and receptor engineering. Handb Exp Pharmacol 2009:123-59.
[43] Baraldi PG, Preti D, Borea PA, Varani K. Medicinal chemistry of A(3) adenosine receptor modulators: pharmacological activities and therapeutic implications. J Med Chem 2012;55:5676-703.
[44] Jaakola VP, Griffith MT, Hanson MA, Cherezov V, Chien EY, Lane JR, et al. The 2.6 angstrom crystal structure of a human A_{2A} adenosine receptor bound to an antagonist. Science 2008;322:1211-7.
[45] Glukhova A, Thal DM, Nguyen AT, Vecchio EA, Jorg M, Scammells PJ, et al. Structure of the Adenosine A_1 Receptor Reveals the Basis for Subtype Selectivity. Cell 2017;168:867-77.
[46] Cheong SL, Federico S, Venkatesan G, Mandel AL, Shao YM, Moro S, et al. The A_3 adenosine receptor as multifaceted therapeutic target: pharmacology, medicinal chemistry, and in silico approaches. Med Res Rev 2013;33:235-335.
[47] Ciancetta A, Jacobson KA. Structural Probing and Molecular Modeling of the A(3) Adenosine Receptor: A Focus on Agonist Binding. Molecules 2017;22:449.
[48] Ciancetta A, Jacobson KA. Breakthrough in GPCR Crystallography and Its Impact on Computer-Aided Drug Design. Methods Mol Biol 2018;1705:45-72.
[49] Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. μ-opioid receptor desensitization by β-arrestin-2 determines morphine tolerance but not dependence. Nature 2000;408:720-3.

[50] Groer CE, Tidgewell K, Moyer RA, Harding WW, Rothman RB, Prisinzano TE, et al. An opioid agonist that does not induce μ-opioid receptor--arrestin interactions or receptor internalization. Mol Pharmacol 2007;71:549-57.
[51] Siuda ER, Carr R, 3rd, Rominger DH, Violin JD. Biased μ-opioid receptor ligands: a promising new generation of pain therapeutics. Curr Opin Pharmacol 2017;32:77-84.
[52] Wei H, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc Natl Acad Sci USA 2003;100:10782-7.
[53] Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively engaging β-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. J Pharmacol Exp Ther 2010;335:572-9.
[54] Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, et al. A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci USA 2007;104:16657-62.
[55] Urs NM, Peterson SM, Caron MG. New Concepts in Dopamine D2 Receptor Biased Signaling and Implications for Schizophrenia Therapy. Biol Psychiatry 2017;81:78-85.
[56] Verzijl D, Ijzerman AP. Functional selectivity of adenosine receptor ligands. Purinerg Signal 2011;7:171-92.
[57] Gao ZG, Balasubramanian R, Kiselev E, Wei Q, Jacobson KA. Probing biased/partial agonism at the G protein-coupled A(2B) adenosine receptor. Biochem Pharmacol 2014;90:297-306.
[58] Baltos JA, Gregory KJ, White PJ, Sexton PM, Christopoulos A, May LT. Quantification of adenosine A(1) receptor biased agonism: Implications for drug discovery. Biochem Pharmacol 2016;99:101-12.
[59] Baltos JA, Paoletta S, Nguyen AT, Gregory KJ, Tosh DK, Christopoulos A, et al. Structure-Activity Analysis of Biased Agonism at the Human Adenosine A3 Receptor. Mol Pharmacol 2016;90:12-22.
[60] Baltos JA, Vecchio EA, Harris MA, Qin CX, Ritchie RH, Christopoulos A, et al. Capadenoson, a clinically trialed partial adenosine A_1 receptor agonist, can stimulate adenosine A_{2B} receptor biased agonism. Biochem Pharmacol 2017;135:79-89.
[61] Gao ZG, Jacobson KA. Translocation of arrestin induced by human A(3) adenosine receptor ligands in an engineered cell line: comparison with G protein-dependent pathways. Pharmacol Res 2008;57:303-11.
[62] Gao ZG, Verzijl D, Zweemer A, Ye K, Goblyos A, Ijzerman AP, et al. Functionally biased modulation of A(3) adenosine receptor agonist efficacy and potency by imidazoquinolinamine allosteric enhancers. Biochem Pharmacol 2011;82:658-68.
[63] Gao ZG, Blaustein JB, Gross AS, Melman N, Jacobson KA. N6-Substituted adenosine derivatives: selectivity, efficacy, and species differences at A_3 adenosine receptors. Biochem Pharmacol 2003;65:1675-84.
[64] Klutz AM, Gao ZG, Lloyd J, Shainberg A, Jacobson KA. Enhanced A_3 adenosine receptor selectivity of multivalent nucleoside-dendrimer conjugates. J Nanobiotechnology 2008;6:12.
[65] Auchampach JA, Gizewski ET, Wan TC, de Castro S, Brown GG, Jr., Jacobson KA. Synthesis and pharmacological characterization of

[(125)I]MRS5127, a high affinity, selective agonist radioligand for the A_3 adenosine receptor. Biochem Pharmacol 2010;79:967-73.

[66] Trincavelli ML, Tuscano D, Marroni M, Falleni A, Gremigni V, Ceruti S, et al. A(3) adenosine receptors in human astrocytoma cells: Agonist-mediated desensitization, internalization, and down-regulation. Mol Pharmacol 2002;62:1373-84.

Chapter 5:
The P2Y2 receptor C-terminal tail modulates but is dispensable for β-arrestin2 recruitment

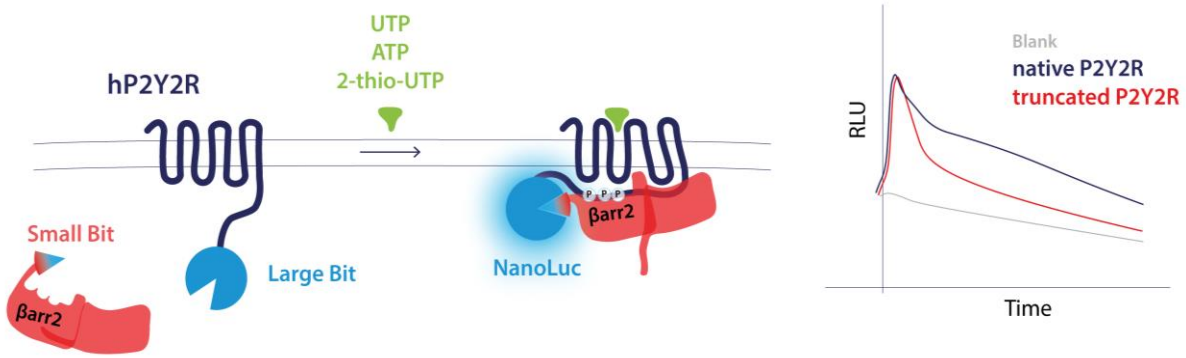
Abstract

This study evaluates the effect of cytoplasmic modifications of the human P2Y2 receptor (P2Y2R) on β-arrestin2 (βarr2) recruitment, using a live-cell split-reporter system (NanoBit®, Promega) in HEK293T cells, based on structural complementation of NanoLuc luciferase.

Upon stimulation with the nucleotides UTP, ATP and the P2Y2R-selective agonist 2-thio-UTP, a concentration dependent (100 nM to 100 µM) recruitment of βarr2 was observed. Interestingly, removal of the P2Y2R C-terminus did not hamper βarr2 recruitment upon stimulation with these agonists. Also the additional mutation of three serine/threonine residues in the third intracellular loop (T232, S233 and S243), representing potential phosphorylation sites, did not jeopardize βarr2 recruitment to the truncated P2Y2R upon stimulation with UTP. Remarkably, a clear difference in profile was observed for the time curve of βarr2 recruitment, which was more plateau-like for the full length P2Y2R, compared to more transient for the truncated P2Y2R. Phosphorylation of extracellular regulated kinase (ERK) showed that the truncated P2Y2R was still functional.

This study shows that the P2Y2R C-terminus is not crucial for βarr2 recruitment, although it does modulate βarr2 interaction in some kind of way. This P2Y2R-βarr2 NanoBit® reporter system can be used for the evaluation of βarr2 recruitment to the P2Y2R to gain insight into the coupling of βarr2 to the P2Y2R.

Graphical Abstract



5.1 Introduction

The human P2Y2R belongs to the P2Y family of G-protein coupled purinergic receptors and is activated in the human body by the endogenous nucleotides uridine-5'-triphosphate (UTP) and adenosine-5′-triphosphate (ATP), which show full agonist activity in the midnanomolar range. These nucleotides are released basally by cells, or by exocytosis of secretory vesicles from e.g. nerve terminals, immune cells or platelets, and their release is intensified under conditions of stress, infection or apoptosis. The P2Y2R is widely expressed, in heart and skeletal muscle, in epithelial and endothelial cells, immune cells, in the intestine, spleen, and at lower levels in the kidney, lung, and different regions of the brain [1-6]**.** Because of the presence and/or generation of purinergic nucleotides under various conditions, the P2Y2R is involved in a variety of physiological processes such as inhibition of bone formation and mineralization, immune cell recruitment and inflammatory processes, regulation of vascular tone, blood pressure and intraocular pressure, epithelial K^+/Cl^- secretion, pancreatic and renal functions, liver regeneration and more [3, 6, 7]. Correspondingly, the P2Y2R is involved in pathological processes, and there is therapeutic potential for P2Y2R agonists in a variety of diseases. The long acting P2Y2R agonist P^1,P^4-di(uridine-5′)-tetraphosphate (Up4U), known as diquafosol, is available on the Japanese market for the symptomatic treatment of dry eye disease [8]. Stimulation of the P2Y2R is speculated to compensate for the abnormal sodium, chloride and water transport across respiratory epithelium in cystic fibrosis, caused by a genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR) [9, 10]. P2Y2R agonists also have cardioprotective effects by reduction of postischemic myocardial damage [11], and have neuroprotective effects in neurodegenerative disorders such as Alzheimer [12]. P2Y2R antagonists can be useful as anti-inflammatory therapy in atherosclerosis, psoriasis and inflammatory airway diseases, and as anti-metastatic cancer therapy [13, 14].

Activation of the P2Y2R by endogenous ATP, UTP or synthetic (ant)agonists mainly occurs via coupling to G_q protein. This activates phospholipase C (PLC), which evokes a rise in inositol triphosphate (IP3) and diacylglycerol (DAG), leading to an increase in cytosolic Ca^{2+} and the activation of protein kinase C (PKC), respectively. Besides, coupling to other G protein subtypes as well as to other signalling proteins

has been described [15, 16]. Some of these pathways have shown their relevance towards aimed therapeutic effects evoked by synthetic P2Y2R drug candidates. However, little is known about the signalling repertoire underlying beneficial therapeutic effects or side effects. In this way, biased signalling might be a way of targeting specific signalling pathways, to ameliorate the therapeutic profile and the safety and/or potential of drugs and drug candidates. An interesting signalling partner is the desensitizing adaptor protein arrestin. It has been reported that the P2Y2R shows rapid desensitization [17] and internalization [18], which is regulated by β-arrestins (βarrs). The fast kinetics and extent of these processes might influence therapeutic outcomes of P2Y2R agonists in a positive or negative way; overstimulation by the agonist might be avoided, but on the other hand, the therapeutic effect might be diminished or tolerance might appear upon chronic drug treatment. Therefore, it might be of interest to prolong the therapeutic effect by specific design of P2Y2R selective ligands that preferentially activate the G-protein mediated- or other pathways, and leave the βarr pathway untouched, or vice versa.

In **Chapter 3**, we reported on the development of a bioassay based on functional complementation of the Nanoluc luciferase for evaluation of βarr2 recruitment to the human A_3 adenosine receptor [19]. In this study, we evaluated the role of the C-terminus and third intracellular loop (IL3) of the P2Y2R for coupling to βarr2, by truncation and mutation of the receptor, using the agonists UTP and ATP, as well as the more P2Y2R selective agonist 2-thio-UTP. A distinct kinetic pattern was observed with respect to βarr2 recruitment. Furthermore, phosphorylation of extracellular regulated kinase (ERK) as part of the mitogen-activated protein kinase (MAPK) signalling pathway was evaluated, to test if the modified P2Y2R was still functional. This pathway is known to be both G protein- and βarr-mediated [20, 21]. Hence, we provide insights in the coupling characteristics between the human P2Y2R and βarr2.

5.2 Materials & Methods

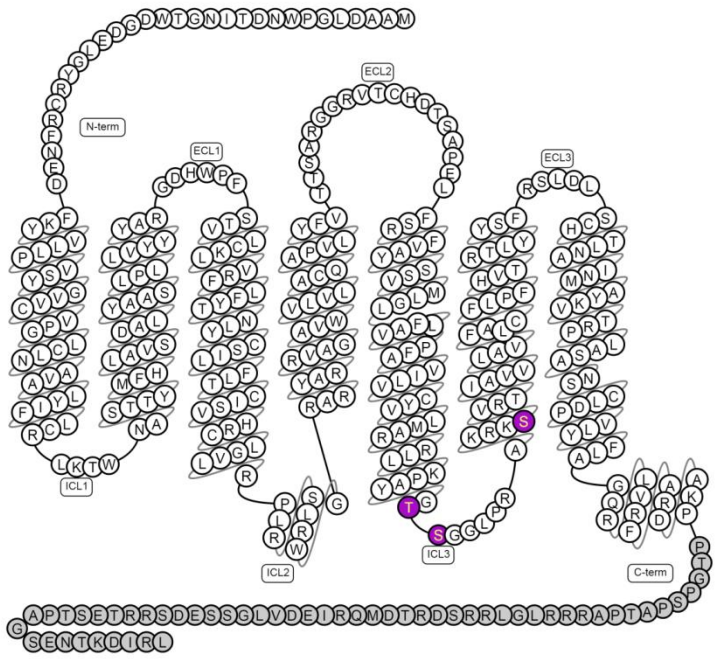
5.2.1 Chemicals and reagents

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

P2Y2R construct (**NM_002564.3**, transcript variant 2 of the P2RY2 gene) was a kind gift from Prof. L. Erb (Department of Biochemistry, Life Sciences Center, University of Missouri-Columbia, Columbia, USA). Hank's balanced salt solution (HBSS) was from Thermo Fisher Scientific (Pittsburg, PA, USA). Reference agonists UTP and ATP, and β-glycerol phosphate disodium salt pentahydrate were purchased from Sigma Aldrich (Steinheim, Germany). 2-thio-UTP was from Tocris Bioscience (Bio-techne, Abingdon, UK). Licor blocking agent, secondary goat anti-rabbit IRDye680RD (926-32221) and goat anti-mouse IRDye800CW (926-322210) were from LI-COR Biosciences (Lincoln, NE 68504 USA). Mouse anti-HA.11 Tag antibody (clone 16B12) was from BioLegend (San Diego, CA 92121). Rabbit anti-phospho-p44/42 MAPK antibody (T202/Y204) and mouse anti-p44/p42 MAPK antibody (L34F12) were from Cell Signalling Technology (Leiden, The Netherlands). All other chemicals and reagents used were purchased from the same suppliers as described previously in **Chapter 3** [19].

5.2.2 Development of P2Y2R NanoBit® plasmid constructs

The NanoBit® system (Promega) was implemented for the generation of fusion proteins consisting of the human P2Y2R connected via a peptide linker ('GAQGNSGSSGGGGSGGGGSSG') with one of the split subunits of NanoLuc luciferase (LgBit or SmBit), generating the two full length P2Y2R-containing constructs P2Y2R-LgBit and P2Y2R-SmBit. To exclude a role of the serine residues in the linker sequence, the linker was shortened to 'GGGG', giving the P2Y2R**L**-LgBit construct. Subsequently, the P2Y2R was truncated C-terminally after proline 322 (Figure 5.1), in both P2Y2R-LgBit as well as P2Y2R**L**-LgBit. In a next step, three serine/threonine residues – T232, S233 and S243 – in IL3 of P2Y2R**LT**-LgBit were mutated to alanine, to create the mutated, truncated P2Y2R construct P2Y2R**LT**-**AAA**-LgBit. All experimental conditions were similar as described previously in **Chapter 3** [19] and primers, PCR conditions, and restriction enzymes used are given in Table 5.1. All resulting fusion constructs were sequence-verified by Sanger sequencing.



◄ Figure 5.1: P2Y2R snake plot (constructed on gpcrdb.org): Deleted C-terminal part in truncation mutant P2Y2RT is shown in grey. Serine/threonine residues in IL3 that have been mutated to alanine (T232, S233, S243) are shown in purple.

Table 5.1: PCR conditions (a-c) and restriction enzymes (RE: d) used for development of NanoBit® fusion constructs*

Template Sequence		Primers (F: forward – R: reverse)[a]	Tm (°C)[b]	Ext. time (s)[c]	RE[d]	Fusion construct*
P2Y2R NanoBit® plasmid construct development						
P2Y2R	F	ACTCAACTCGAG***ACC****ATGGCAGCAGACC*	71.9	30	XhoI	P2Y2R-LgBit & P2Y2R-SmBit
	R	ACTCAACTCGAGCC*CAGCCGAATGTCC*				
Template Sequence		**Phosphorylated primers (F: forward – R: reverse)[a]**	**Tm (°C)[b]**	**Ext. time (min:sec)[c]**		**Fusion construct***
Insertion HA-tag - Adjustment of the Linker sequence – Truncation of the C-terminus – Mutation of phosphorylation sites in IL3						
P2Y2R-LgBit	F	GGTGGAGGTGGTGTCTTCACAC	67.6	1:40		P2Y2RL-LgBit
	R	CAGCCGAATGTCCTTAGTGTTCTCG				
P2Y2R-LgBit	F	GGCTCGAGCGGTGGTGGCG	67.5	1:35		P2Y2RT-LgBit
	R	TGGCTTGGCATCTCGGGCAAAGC				
P2Y2R-LgBit	F	GGTGGAGGTGGTGTCTTCACAC	68.3	1:35		P2Y2RLT-LgBit
	R	TGGCTTGGCATCTCGGGCAAAGC				
P2Y2RLT-LgBit	F	T AGG GCC AAG CGC AAG GCC GTG CGC	72 (2-step)	1:37		P2Y2RLT-T232A-S233A-S243A
	R	GG CAG GCC GCC CGC GGC CCC GTA GG				
P2Y2R-LgBit	F	TACCCATACGATGTTCCAGATTACGCT*ATGGCAGCAG ACCTGG*	72 (2-step)	1:50		(2xHA)-P2Y2R-LgBit
	R	AGCGTAATCTGGAACATCGTATGGGTACAT***GGT***CTCG AGCCAGAATTCC				
(2xHA)-P2Y2R-LgBit	F	GGTGGAGGTGGTGTCTTCACAC	67.6	1:40		(2xHA)-P2Y2RL-LgBit
	R	CAGCCGAATGTCCTTAGTGTTCTCG				
(2xHA)-P2Y2R-LgBit	F	GGCTCGAGCGGTGGTGGCG	67.5	1:35		(2xHA)-P2Y2RT-LgBit
	R	TGGCTTGGCATCTCGGGCAAAGC				
(2xHA)-P2Y2R-LgBit	F	GGTGGAGGTGGTGTCTTCACAC	68.3	1:35		(2xHA)-P2Y2RLT-LgBit
	R	TGGCTTGGCATCTCGGGCAAAGC				
(2xHA)-P2Y2RLT-LgBit	F	T AGG GCC AAG CGC AAG GCC GTG CGC	72 (2-step)	1:37		(2xHA)-P2Y2RLT-T232A-S233A-S243A
	R	GG CAG GCC GCC CGC GGC CCC GTA GG				

a: Primers (5'->3') containing the specific restriction site (underlined), Kozak sequence (***bold italic***) and coding sequence of interest (*italic*).
b: Annealing temperature.
c: Extension time.
Extra nucleotides (marked grey) to ensure a correct reading frame were added when necessary.

5.2.3 P2Y2R NanoBit® βarr2 reporter assay in HEK293T cells

The protocol of the reporter assay is similar as described in **Chapter 3** [19]. Human embryonic kidney (HEK) 293T cells were seeded in 6-well plates (5x10^5 cells/well) in full DMEM (DMEM supplemented with 10% heat-inactivated FBS, 2mM of glutamine, 100IU/ml of penicillin, 100µg/ml of streptomycin and 0.25µg/ml of amphotericin B) and transiently transfected the next day using FuGENE® HD reagent, with a ratio of FuGENE:DNA 3:1. Transfection mixes contained 1 µg of P2Y2R and βarr2-construct each, and 1.3 µg of pcDNA3.1. On the third day, cells were reseeded in poly-D-lysine coated, white 96-well plates at 5x10^4 cells/well and incubated overnight (37°C, 5% CO_2). On day 4, the read-out was performed: cells were washed twice with HBSS, and 90 µL of HBSS was placed on the cells. Twenty-five µL of Nano-Glo® Live Cell detection reagent, consisting of the cell-permeable furimazine substrate in aqueous buffer, was added to each well and luminescence was monitored in a Tristar Luminometer (Berthold) until the signal stabilized. Once a stable signal was observed, 20 µL 6.75× concentrated agonist solution in HBSS was added and luminescence was monitored for at least 90 minutes. As an alternative to furimazine, we also used a more stable substrate, in which a protecting group is added to furimazine to protect against (non-)enzymatic turnover, i.e. the Nano-Glo® Live Cell Vivazine™ substrate (Promega). A 1/1000 solution of this DMSO-based custom product in HBSS was placed upon the cells (100 µL/well) after the washing steps and luminescence was monitored until stabilization. Subsequently, 20 µL 6× concentrated agonist solution in HBSS was added. The final in-well concentration ranged from 100 nM agonist up to 100 µM or 500 µM agonist. A solvent control of H_2O in HBSS was each time included.

5.2.4 ERK phosphorylation

HEK293T cells were seeded at 1,2*10^6 cells/T25 falcon in full DMEM. The next day, the cells were transfected with the same ratios of DNA as described above, using HA-tagged P2Y2R constructs, with a total of 8.8 µg DNA. On the third day, cells were transferred to a 6-well plate and seeded at a density of 4.5*10^5 cells/well. The cells were serum-starved overnight using DMEM without FBS. On day 4, cells were stimulated with agonist UTP or ATP for the indicated times (2 min, 5 min, 10 min, 20

min). A solvent control was included, as well as untransfected cells, which were stimulated with the agonists for the time that provided maximum response for UTP or ATP (i.e. 10 min). Following stimulation, the reaction was stopped by washing the cells with 2 mL ice-cold PBS, and cells were lysed by scraping in 100 µL RIPA buffer (with freshly added phosphatase inhibitor β-glycerol phosphate). The cell lysate was transferred to an Eppendorf and was rotated for 2 hours at 4°C. After centrifugation at 8000 rpm (10 min, 4°C), 20 µl of supernatant was mixed with 5 µl of Laemmli 4x (pH 6.8, containing bromophenolblue and 50 µL/mL β-mercapto-ethanol) and analysed via Western Blot. Blots were blocked with PBS/Licor for 1h and incubated overnight with primary rabbit anti-phospho-p44/42 MAPK antibody (1/2000 in PBS) to detect phosphorylated MAPK. The next day, blots were washed and incubated with secondary goat anti-rabbit IRDye680 (1/10000) for 1h in the dark. Following visualisation, blots were incubated for 3h with mouse anti-p44/p42 MAPK antibody (1/2000 in PBS) to demonstrate equal protein loading, followed by secondary goat anti-mouse IRDye800 (1/10 000) for 1h in the dark, and visualised. Finally, blots were incubated with anti-HA antibody (1/2000). For quantification, the phospho-p44/42 MAPK signal was normalized against total p44/42 MAPK signal using ImageJ software.

5.2.5 Data analysis

Concentration-response cruves were generated, with calculation of the area under the curve (AUC), where the absolute signals were corrected for solvent control samples, and for inter-well variability. For calculation of $\log EC_{50}$ values, a sigmoidal curve was fitted by analysing concentration-response data using GraphPad Prism software (San Diego, CA, USA). A non-linear regression model (Hill Slope 1) was fitted for the normalized responses. Statistics were performed using analysis of variance (ANOVA), followed by post-hoc analysis (Tukey) to detect statistical difference among groups (**$P < 0.05$**).

5.3 Results

5.3.1 Development of the P2Y2R NanoBit® reporter assay for real-time monitoring of β-arrestin2 recruitment in HEK293T cells

For the development of a P2Y2R NanoBit® reporter assay for βarr2 recruitment, an optimal assay set-up was selected by evaluation of different combinations for P2Y2R- and βarr2 fusion constructs. For the two set-ups in which LgBit was attached to the P2Y2R, and SmBit was either N- or C-terminally attached to βarr2, a clear rise in signal was observed upon addition of reference agonist UTP at a concentration of 100 µM, which was sustained for more than 2 hours. Set-up A in Figure 5.2, in which LgBit is attached to the C-terminus of the P2Y2R, and SmBit is attached to the N-terminus of βarr2, was chosen as the optimal configuration for further experiments.

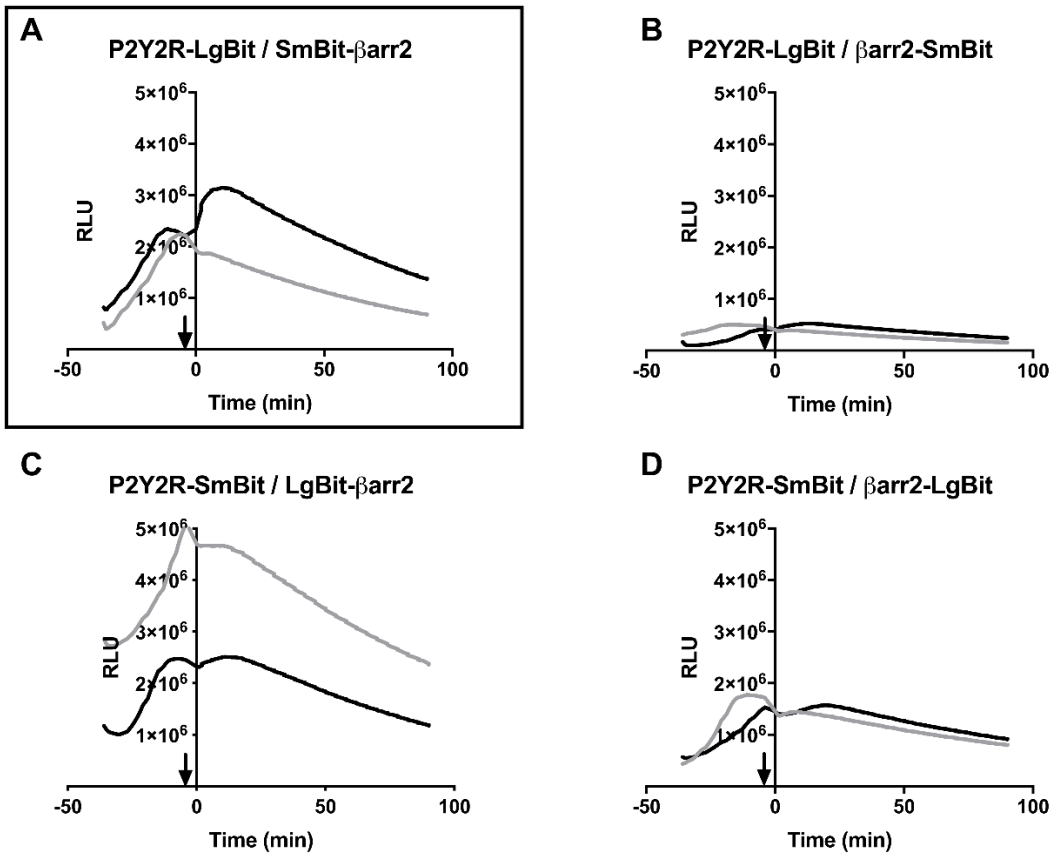


Figure 5.2: Selection of the optimal P2Y2R NanoBit® reporter assay set-up: 4 different combinations of P2Y2R- and βarr2 fusion constructs were evaluated: P2Y2R-LgBit/SmBit-βarr2, P2Y2R-LgBit/βarr2-SmBit, P2Y2R-SmBit/LgBit-βarr2 and P2Y2R-SmBit/βarr2-LgBit. 100 µM UTP was added (arrow) and luminescence was measured during at least 90 min (black lines). A solvent control was included (grey lines). Measurements are shown for one representative experiment (n = 3).

In this set-up, UTP caused a concentration-dependent βarr2 recruitment, starting at about 100 nM and reaching a maximum at 500 µM (Figure 5.3A). At very high agonist concentrations (500 µM), the curve showed a rather plateau-like course; this caused a deviation in the fitting of the sigmoidal curve when plotting the data points; hence, only data points from 0 up to 100 µM were included, and the 500 µM data point was left out in the sigmoidal curves that were fitted to obtain EC_{50} values for stimulation with UTP (Figure 5.4A) and ATP (Figure 5.4B).

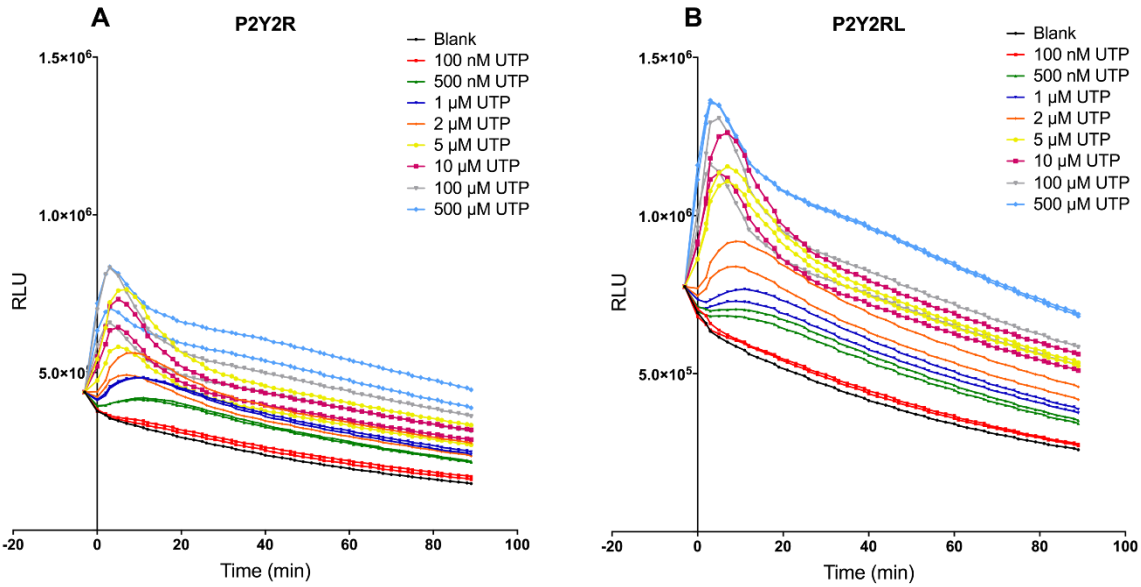


Figure 5.3: Concentration-dependence of βarr2 recruitment in the P2Y2R NanoBit® reporter assay: A: native P2Y2R (P2Y2R-LgBit/SmBit-βarr2) and B: native P2Y2R with adjusted linker (P2Y2RL-LgBit/SmBit-βarr2). 100 nM up to 500 µM UTP was tested and luminescence was measured for 90 minutes. A solvent control (blank) was included. Data are representative of one experiment performed in triplicate (n=3).

The concentration-response was independent of the presence of serine residues in the linker sequence between the P2Y2R and the LgBit protein (Figure 5.3B). This is also reflected by the similar $logEC_{50s}$ of P2Y2R and P2Y2R**L** for UTP (Figure 5.4C), ATP (Figure 5.4D) and 2-thio-UTP ($logEC_{50s}$ of -6.271 ± 0.036 and -6.020 ± 0.062, respectively; curve not shown).

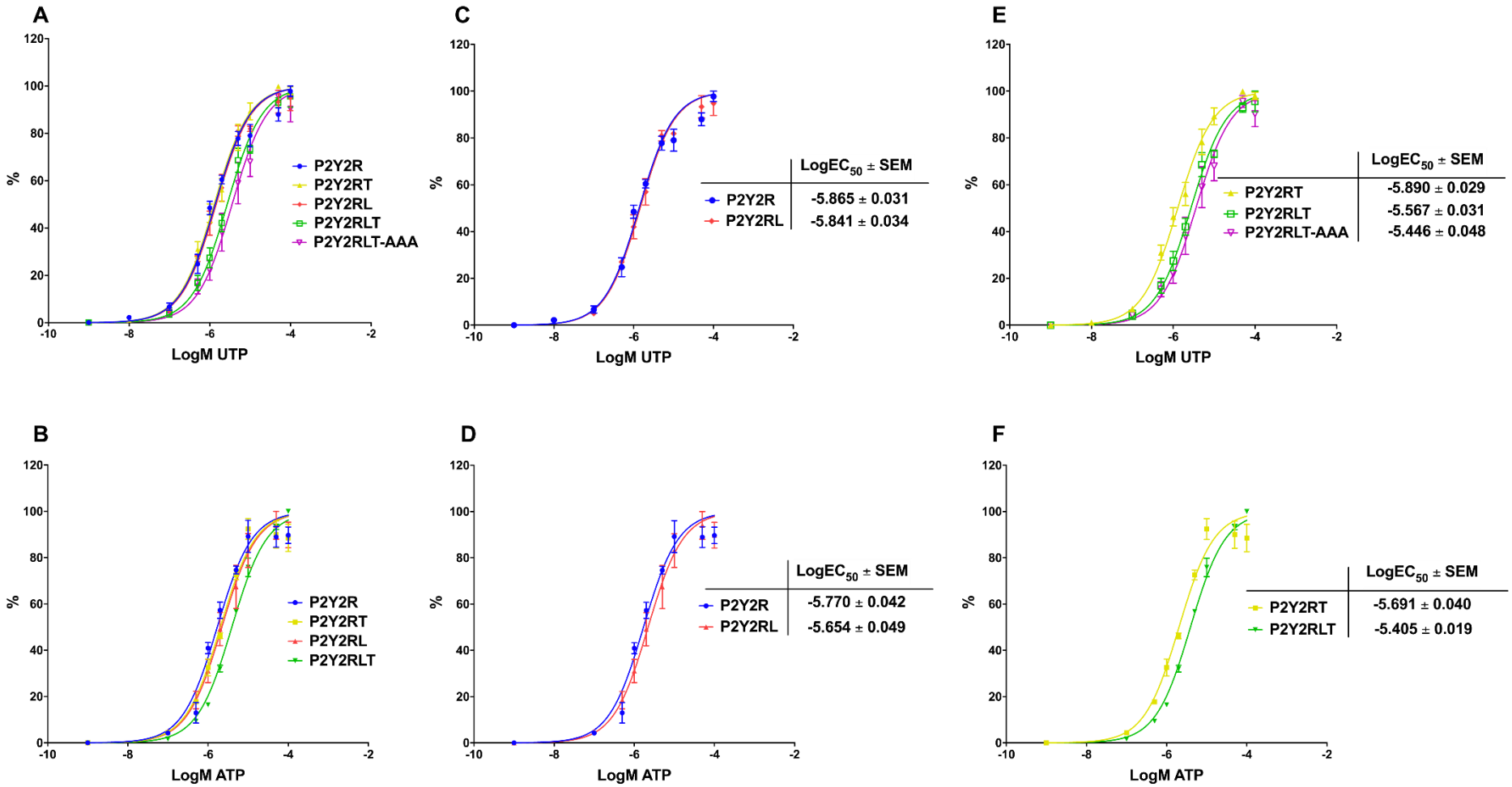


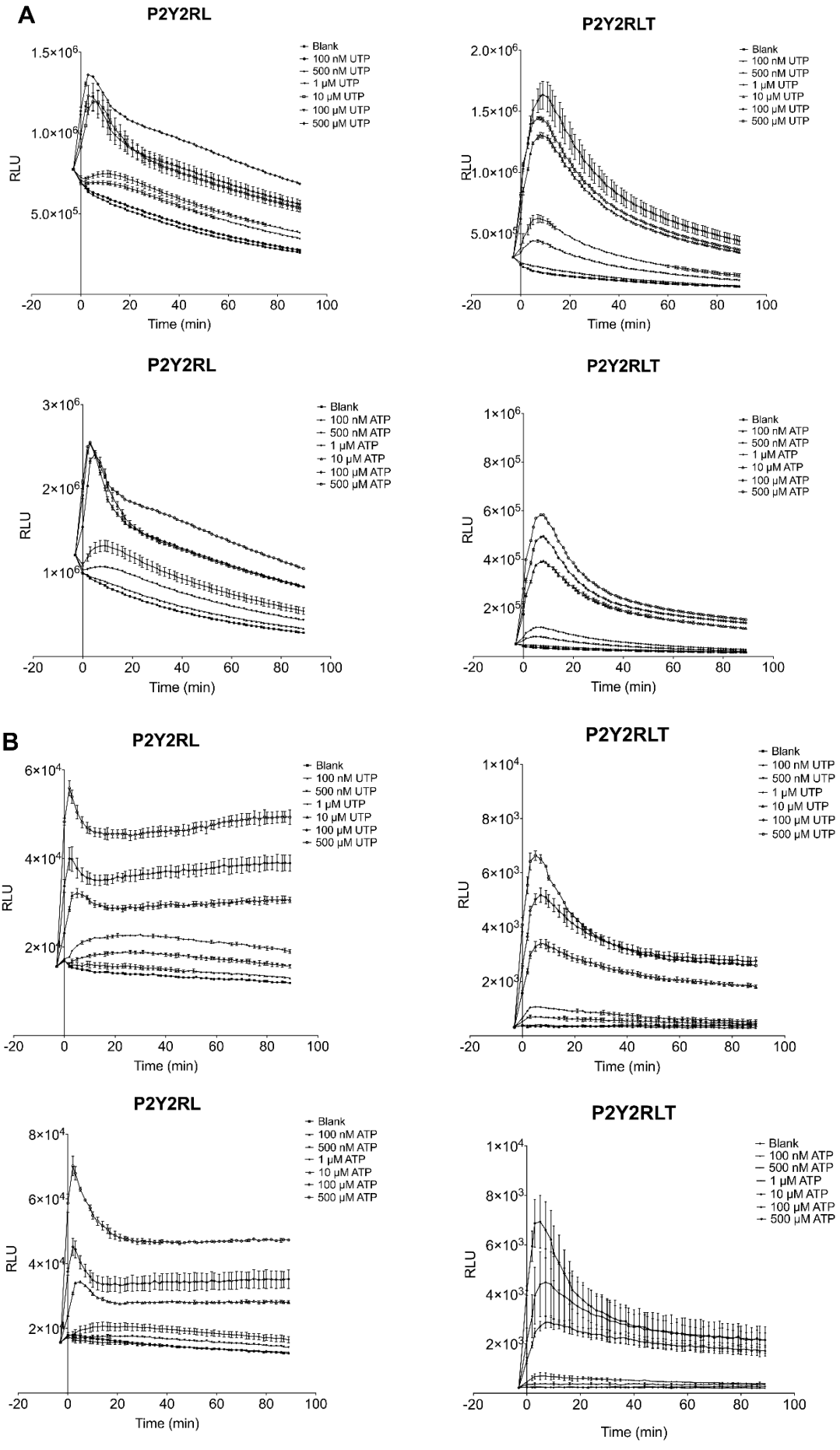
Figure 5.4: Sigmoidal concentration-response curves fitted with non-linear regression. Agonist concentrations up to 500 µM are included. Data points shown are mean AUCs ± SEM of duplicate wells of three independent experiments (n=3).

5.3.2 Truncation of the P2Y2R C-terminus and mutation of phosphorylation sites in intracellular loop 3 does not compromise βarr2 recruitment

The influence of C-terminal P2Y2R phosphorylation on βarr2 recruitment was evaluated by truncation of the P2Y2R C-terminus. Also the fusion construct with adjusted linker (P2Y2R**L**-LgBit) was tested to ensure that serine residues present in the original linker sequence would not function as a substitute for C-terminal serine/threonine residues. The truncated P2Y2Rs (both with original and shortened linker sequence) were still able to recruit βarr2, giving a signal comparable with that of the full length P2Y2R, upon stimulation with UTP (Figure 5.4E) and ATP (Figure 5.4F). For both UTP and ATP, the $logEC_{50s}$ did not differ significantly between P2Y2R, P2Y2R**L** and P2Y2R**T** **(P>0.05**). However, for the construct with the truncated P2Y2R fused to LgBit via the adjusted linker, which did not contain any serine residues, (P2Y2R**LT**), the $logEC_{50}$ was significantly different **(P ≤ 0.01)** from that of the other constructs (P2Y2R, P2Y2R**L**, P2Y2R**T**). In a next step, three additional residues in IL3 were mutated to alanine, creating the **P2Y2RLT-AAA**-LgBit construct, which still provided a good βarr2 recruitment (Figure 5.4E). The $logEC_{50}$ of **P2Y2RLT-AAA** for UTP was again significantly different **(P<0.001)** from the $logEC_{50s}$ of the other constructs (P2Y2R, P2Y2R**L**, P2Y2R**T**), but did not differ significantly from that of P2Y2R**LT (P = 0.1884)**.

Interestingly, a closer look at the time profiles of βarr2 recruitment revealed that the full length P2Y2R more has a plateau shape (especially at high concentrations) as opposed to a more transient profile for truncated P2Y2R (Figure 5.5A; P2Y2R**L** and P2Y2R**LT**). This difference was also observed with the original linker (data not shown) and when using a more stable furimazine variant (Figure 5.5B).

Figure 5.5 (next page): Concentration-dependence of βarr2 recruitment in the P2Y2R NanoBit® reporter assay with furimazine substrate (A) and with Nano-Glo® Live Cell Vivazine™ substrate (B): P2Y2RL-LgBit/SmBit-βarr2 and P2Y2RLT-LgBit/SmBit-βarr2. 100 nM up to 500 µM UTP (upper plots) or ATP (lower plots) was tested and luminescence was measured for 90 minutes. A solvent control (blank) was included. Data are representative of one experiment performed in triplicate (n=3).


A
P2Y2RL
P2Y2RLT
Blank
100 nM UTP
500 nM UTP
1 μM UTP
10 μM UTP
100 μM UTP
500 μM UTP
100 nM ATP
500 nM ATP
1 μM ATP
10 μM ATP
100 μM ATP
500 μM ATP
RLU
Time (min)
B

5.3.3 ERK phosphorylation

ERK phosphorylation was chosen as a means to evaluate general functionality of the receptor constructs. To this end, HA-tagged P2Y2R**L** and P2Y2R**LT** constructs were used to transiently transfect HEK293T cells prior to stimulation. For Western Blot analysis, lysates were loaded of transfected cells (P2Y2R**L(T)**-LgBit/SmBit-βarr2) that were left unstimulated or were stimulated for 2, 5, 10, or 20 minutes, as well as of untransfected cells that were stimulated for 10 minutes with 100 µM UTP or ATP (Figure 5.6A and B). Blots were analysed for phospho-p44/p42 ERK, followed by total p44/p42 ERK; the ratio is plotted in Figure 5.7. Both UTP and ATP caused a time dependent phosphorylation of ERK, irrespective of whether the receptor was truncated or not, suggesting that overall functionality of the truncated receptor was not impaired.

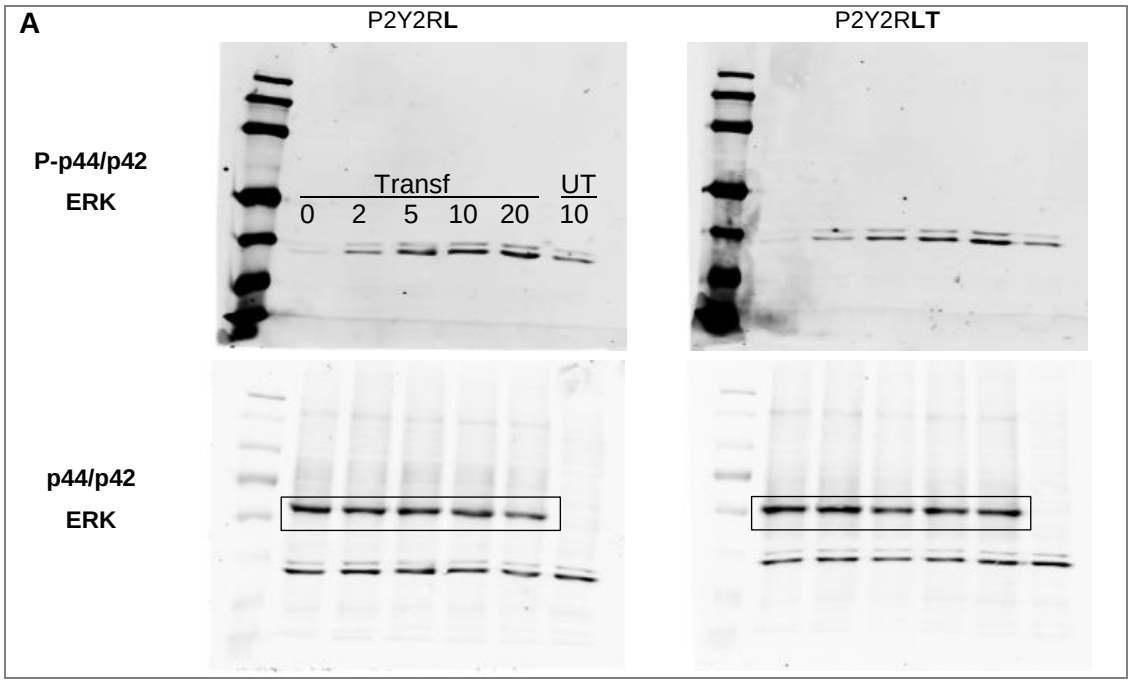


Figure 5.6: Western Blots of HEK293T cells transfected with P2Y2RL-LgBit/SmBit-βarr2 (P2Y2RL) or P2Y2RLT-LgBit/SmBit-βarr2 (P2Y2RLT), unstimulated (Transf 0) or stimulated for 2, 5, 10, or 20 minutes (Transf 2 – 5 – 10 – 20) with 100 µM UTP (A) or ATP (B). Untransfected HEK293T cells stimulated for 10 minutes (UT 10) with 100 µM UTP/ATP were included as well. Blots were developed with anti-phospho-p44/p42 ERK antibody, followed by anti-p44/p42 ERK antibody (for total ERK), and anti-HA antibody (boxed regions) to visualize HA-tagged P2Y2Rs.

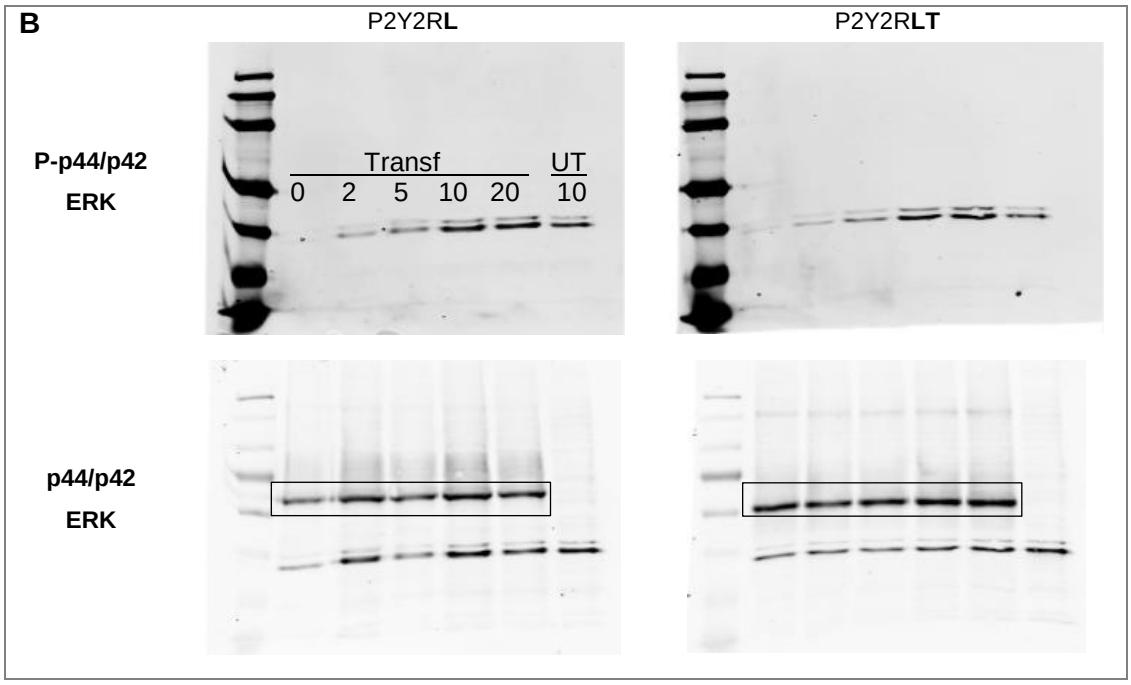


Figure 5.6 (continued).

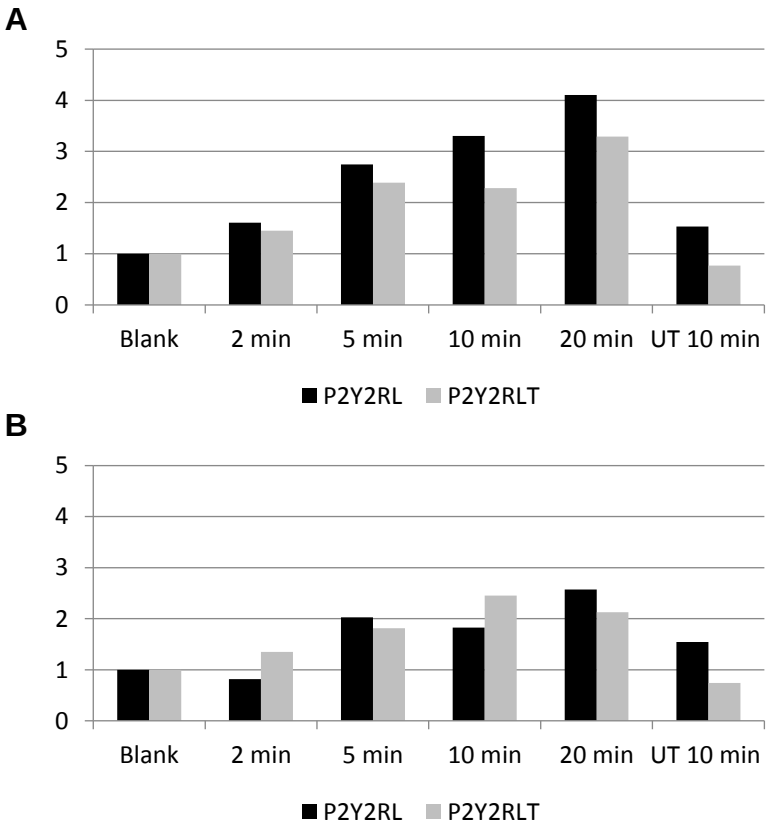


Figure 5.7: Ratios of phospho-p44/p42 ERK to total ERK, analysed via Western Blot. HEK293T cells transfected with P2Y2RL-LgBit/SmBit-βarr2 (P2Y2RL) or P2Y2RLT-LgBit/SmBit-βarr2 (P2Y2RLT) were left unstimulated (Blank; set to unity) or were stimulated for 2, 5, 10, or 20 minutes with 100 μM UTP (A) or ATP (B). Untransfected cells stimulated for 10 minutes with 100 μM UTP/ATP were included. Values are representative for one blot performed in triplicate.

5.4. Discussion

Although the structure of the P2Y2R is not available yet, structures of the P2Y1R and P2Y12R subtypes have led to P2Y2R homology models that can aid in the development of selective P2Y2R ligands [22, 23]. Compounds acting on P2Y2Rs have been proposed as interesting therapeutic agents for a range of inflammatory, cardiovascular and neurodegenerative diseases [13, 14]. Selective targeting of different downstream P2Y2R signalling pathways might offer opportunities to achieve distinct therapeutic outcomes, or to obtain a therapeutic profile without side effects. Therefore, exploring functional selectivity at the P2Y2R will be of importance in future (re)design of (existing or new) P2Y2R ligands. P2Y2R ligands are mostly derivatives of the endogenous nucleotide UTP, and are generally characterized by a low oral bioavailability and susceptibility to enzymatic degradation by ectonucleotidases. Some dinucleotide tetraphosphates have been developed that have an equal number of negative charges and are more resistant to enzymatic degradation. Hence, despite their extensive therapeutic application potential, only a discrete number of selective P2Y2R agonists and even fewer antagonists are available for clinical use today [24, 25]. To keep the number of ligands going into clinical trials growing, a better insight into the signalling downstream of the P2Y2R is warranted. Implementing functional assays may help to achieve this. The P2Y2R primarily couples to G_q protein, but also to G_o and G_{12} by interaction with $\alpha_v\beta_{3/5}$ integrins. Activation of G_o leads to activation of RhoA, whereas activation of G_{12} leads to activation of Rac and the Rac guanine nucleotide exchange factor (RacGEF), Vav2. These proteins are important in modulation of cytoskeletal rearrangement involved in cell migration and phagocytosis. Furthermore, P2Y2R coupling to ion channels, as well as crosstalk and dimerization with other GPCRs have been described [16, 26, 27].

The P2Y2R undergoes desensitization and internalization by contact with β-arrestins (βarrs) [16, 18, 28-30]. The effectiveness of P2Y2R signalling might be influenced by desensitization and/or downregulation of the receptor, which can have an impact on the therapeutic value of P2Y2R (ant)agonists. Different reports exist about the involvement of both βarr isoforms in regulation of the P2Y2R. In this study, the characteristics of the human P2Y2R-βarr2 coupling were explored in a P2Y2R NanoBit® βarr2 reporter assay in HEK293T cells. In this assay, βarr2 recruitment is

evaluated by functional complementation of two split parts of the NanoLuc luciferase enzyme, a Large Bit (LgBit) and a Small Bit (SmBit), fused via a linker to the C-terminus of P2Y2R and the N-terminus of βarr2, respectively. A solvent control was included in each read-out, as a correction for the influence of nucleotides spontaneously released by cells in response to stress such as mechanic stimulation [31].

Surprisingly, removal of the 55 amino acid long P2Y2R C-terminal tail did not hamper βarr2 recruitment upon stimulation with UTP, ATP, or the P2Y2R-selective agonist 2-thio-UTP. Therefore, we conclude that the P2Y2R C-terminus is not crucial for βarr2 recruitment. Also additional mutation of three serine/threonine residues in IL3 (T232, S233 and S243), representing potential phosphorylation sites, did not jeopardize βarr2 recruitment to the truncated P2Y2R upon stimulation with UTP. It was noted, however, that the $\log EC_{50s}$ for βarr2 recruitment upon stimulation with UTP/ATP differed significantly ($P \leq 0.01$) for the truncated P2Y2R, when fused via a short linker not containing any serine residues, compared to a linker that contained multiple serine clusters. We hypothesize that this may be related to a reduced flexibility or a less ideal orientation of LgBit to accommodate SmBit, in the context of the truncated receptor. Furthermore, a clear difference in profile was observed for the time curve of βarr2 recruitment, which was more plateau-like for the full length P2Y2R (especially at high agonist concentrations), compared to more transient for the truncated P2Y2R, and this independent of the linker or substrate used. Together, these observations indicate that, while the C-terminal tail is not required for βarr2 recruitment, it may still modulate βarr2 binding. Only little is known about the features of βarr coupling to P2YRs. However, there have been reports for the hP2Y1R, pointing at a differential role for potential phosphorylation sites in IL3 and the C-terminus. Fluorescently-tagged P2Y1R showed internalization, accompanied by translocation of fluorescently-tagged βarr2 to the plasma membrane in HEK293 cells, also in the presence of Ser/Ala, Thr/Ala (or Tyr/Ala) mutations in IL3 and the proximal C-terminus, but with crucial involvement of one serine and one threonine in the distal P2Y1R C-terminus [32, 33]. Although these specific Ser/Thr residues are highly conserved between species for the P2Y1R, they are not conserved in the P2Y2R. As the authors of this article also noted, it seems that for each GPCR, different regions can have a contribution in βarr interaction, as well as in βarr dependent

functionalities such as GPCR desensitization, internalization and βarr-dependent signalling.

Hoffmann *et al*. (2008) reported a different recruitment pattern of bovine βarr1 and βarr2 to the human P2Y2R upon stimulation with 100 µM UTP or ATP in HEK293 cells [18]. Both P2Y2R and βarrs were C-terminally tagged with fluorescent proteins, and their trafficking was evaluated using confocal microscopy and fluorescence resonance energy transfer (FRET). The assumed endogenous expression of P2Y2R in untransfected HEK293 cells did not seem to evoke βarr recruitment upon stimulation with UTP or ATP. In transfected cells, UTP recruited βarr1 and βarr2 to the same extent, which would classify the P2Y2R as a class B GPCR, while ATP showed less pronounced recruitment of βarr1 than βarr2, classifying the receptor as class A [34]. Furthermore, a differential behavior was observed in the ERK phosphorylation pathway; ATP-induced ERK phosphorylation was prolonged, while that of UTP was rather transient (showing a maximum at 10 minutes). The authors hypothesized that UTP and ATP induce different P2Y2R conformations, which may behave differently with respect to βarr recruitment and ERK phosphorylation. In our study, only βarr2 was evaluated, which gave a comparable recruitment in response to UTP and ATP. In contrast to Hoffmann *et al.* (2008), we did not observe a distinct time pattern for ERK phosphorylation upon stimulation with UTP versus ATP. Also truncation of the P2Y2R did not influence the kinetics of the ERK phosphorylation pattern. When stimulating untransfected HEK293 cells with agonists for 10 min, substantial ERK phosphorylation was observed as well, equivalent with the signal obtained after 2 min stimulation of transfected cells, but lower than stimulation for 5 min. This points at considerable activation of endogenous P2YRs in HEK293 cells by UTP, ATP, or their degradation products UDP and ADP. This is not surprising, as HEK293 cells have been reported to endogenously express different P2YR subtypes (P2Y1R, P2Y2R, P2Y4R, P2Y11R, and P2Y12R) [35-37]. Anyway, the time-dependent ERK stimulation in cells transfected with full length as well as truncated P2Y2R indicates that the receptor is still functional upon truncation.

5.5 Conclusions

The P2Y2R represents an interesting therapeutic target next to other members of the P2Y purinergic receptor family for the treatment of inflammatory, neurodegenerative, respiratory and (cardio)vascular disorders. Yet, (ant)agonists in clinical use have been limited due to inherently unfavorable features of highly polar nucleotide-derived P2Y2R ligands in the physiological setting. In addition, there is a need for fundamental knowledge on the role played by different signalling pathways in the different therapeutic goals of P2Y2R-based therapies. With this study, we present a human P2Y2R-βarr2 reporter system, which cannot only be used for the evaluation of βarr2 recruitment to the P2Y2R, to gain insight into the coupling of βarr2 to the P2Y2R, but which may also serve to screen for (biased) ligand activity. From our findings we conclude that neither the C-terminus nor potential phosphorylation sites in IL3 are crucial for βarr2 recruitment to the P2Y2R, although we did observe a difference in the profile of the time curve for the truncated P2Y2R.

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References

[1] Moore DJ, Chambers JK, Wahlin JP, Kong KB, Moore GB, Jenkins O, et al. Expression pattern of human P2Y receptor subtypes: a quantitative reverse transcription-polymerase chain reaction study. BBA-Gene Struct Expr 2001;1521:107-19.

[2] von Kugelgen I. Pharmacological profiles of cloned mammalian P2Y-receptor subtypes. Pharmacol Therapeut 2006;110:415-32.

[3] Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, et al. International union of pharmacology LVIII: Update on the P2Y G protein-coupled nucleotide receptors: From molecular mechanisms and pathophysiology to therapy. Pharmacol Rev 2006;58:281-341.

[4] Hillmann P, Ko GY, Spinrath A, Raulf A, von Kugelgen I, Wolff SC, et al. Key Determinants of Nucleotide-Activated G Protein-Coupled P2Y(2) Receptor Function Revealed by Chemical and Pharmacological Experiments, Mutagenesis and Homology Modeling. J Med Chem 2009;52:2762-75.

[5] Burnstock G, Verkhratsky A. Receptors for purines and pyrimidines. Purinergic Signalling and the Nervous System: Springer, 2012. p. 119-244.

[6] von Kugelgen I, Hoffmann K. Pharmacology and structure of P2Y receptors. Neuropharmacology 2016;104:50-61.

[7] Weisman GA, Ajit D, Garrad R, Peterson TS, Woods LT, Thebeau C, et al. Neuroprotective roles of the P2Y(2) receptor. Purinerg Signal 2012;8:559-78.

[8] Lau OC, Samarawickrama C, Skalicky SE. P2Y2 receptor agonists for the treatment of dry eye disease: a review. Clin Ophthalmol (Auckland, NZ) 2014;8:327.

[9] Kellerman D, Evans R, Mathews D, Shaffer C. Inhaled P2Y(2) receptor agonists as a treatment for patients with Cystic Fibrosis lung disease. Adv Drug Deliver Rev 2002;54:1463-74.

[10] Fajac I, De Boeck K. New horizons for cystic fibrosis treatment. Pharmacol Therapeut 2017;170:205-11.

[11] Djerada Z, Feliu C, Richard V, Millart H. Current knowledge on the role of P2Y receptors in cardioprotection against ischemia-reperfusion. Pharmacol Res 2017;118:5-18.

[12] de Diego-García L, Ramírez-Escudero M, Sebastián-Serrano Á, Diaz-Hernández JI, Pintor J, Lucas JJ, et al. Regulation of proteasome activity by P2Y2 receptor underlies the neuroprotective effects of extracellular nucleotides. Biochem Biophys Act Mol Basis Dis 2017;1863:43-51.

[13] Burnstock G. Purinergic Signalling: Therapeutic Developments. Front Pharmacol 2017;8:661.

[14] Burnstock G. The therapeutic potential of purinergic signalling. Biochem Pharmacol 2018;151:157-65.

[15] Erb L, Liao ZJ, Seye CI, Weisman GA. P2 receptors: intracellular signaling. Pflug Arch Eur J Phy 2006;452:552-62.

[16] Erb L, Weisman GA. Coupling of P2Y receptors to G proteins and other signaling pathways. Wiley Interdiscip Rev Membr Transp Signal 2012;1:789-803.

[17] Flores RV, Hernandez-Perez MG, Aquino E, Garrad RC, Weisman GA, Gonzalez FA. Agonist-induced phosphorylation and desensitization of the P2Y(2) nucleotide receptor. Mol Cell Biochem 2005;280:35-45.

[18] Hoffmann C, Ziegler N, Reiner S, Krasel C, Lohse MJ. Agonist-selective, Receptor-specific Interaction of Human P-2Y Receptors with beta-Arrestin-1 and-2. J Biol Chem 2008;283:30933-41.
[19] Storme J, Cannaert A, Van Craenenbroeck K, Stove CP. Molecular dissection of the human A(3) adenosine receptor coupling with beta-arrestin2. Biochem Pharmacol 2018;148:298-307.
[20] Gutkind JS. Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. Sci STKE 2000;2000:re1-re.
[21] Luttrell LM. Composition and function of G protein-coupled receptor signalsomes controlling mitogen-activated protein kinase activity. J Mol Neurosci 2005;26:253-63.
[22] Rafehi M, Neumann A, Baqi Y, Malik EM, Wiese M, Namasivayam V, et al. Molecular Recognition of Agonists and Antagonists by the Nucleotide-Activated G Protein-Coupled P2Y(2)Receptor. J Med Chem 2017;60:8425-40.
[23] Ciancetta A, Jacobson KA. Breakthrough in GPCR Crystallography and Its Impact on Computer-Aided Drug Design. Methods Mol Biol 2018;1705:45-72.
[24] Xu PF, Feng X, Luan HY, Wang JB, Ge RL, Li ZY, et al. Current knowledge on the nucleotide agonists for the P2Y2 receptor. Bioorgan Med Chem 2018;26:366-75.
[25] Rafehi M, Müller CE. Tools and drugs for uracil nucleotide-activated P2Y receptors. Pharmacol Therapeut 2018;190:24-80.
[26] Liu J, Liao ZJ, Camden J, Griffin KD, Garrad RC, Santiago-Perez LI, et al. Src homology 3 binding sites in the P2Y(2) nucleotide receptor interact with Src and regulate activities of Src, proline-rich tyrosine kinase 2, and growth factor receptors. J Biol Chem 2004;279:8212-8.
[27] Suzuki T, Namba K, Tsuga H, Nakata H. Regulation of pharmacology by hetero-oligomerization between A(1) adenosine receptor and P2Y(2) receptor. Biochem Biophys Res Co 2006;351:559-65.
[28] Morris GE, Nelson CP, Everitt D, Brighton PJ, Standen NB, Challiss RAJ, et al. G protein-coupled receptor kinase 2 and arrestin2 regulate arterial smooth muscle P2Y-purinoceptor signalling. Cardiovasc Res 2011;89:193-203.
[29] Morris GE, Nelson CP, Brighton PJ, Standen NB, Challiss RAJ, Willets JM. Arrestins 2 and 3 differentially regulate ETA and P2Y(2) receptor-mediated cell signaling and migration in arterial smooth muscle. Am J Physiol-Cell Ph 2012;302:C723-C34.
[30] Abe M, Watanabe K, Kuroda Y, Nakagawa T, Higashi H. Homodimer formation by the ATP/UTP receptor P2Y(2) via disulfide bridges. J Biochem 2018;163:475-80.
[31] Burnstock G, Knight GE. Cell culture: complications due to mechanical release of ATP and activation of purinoceptors. Cell Tissue Res 2017;370:1-11.
[32] Reiner S, Ziegler N, Leon C, Lorenz K, von Hayn K, Gachet C, et al. beta-Arrestin-2 interaction and internalization of the human P2Y1 receptor are dependent on C-terminal phosphorylation sites. Mol Pharmacol 2009;76:1162-71.
[33] Qi AD, Houston-Cohen D, Naruszewicz I, Harden TK, Nicholas RA. Ser352 and Ser354 in the carboxyl terminus of the human P2Y(1) receptor are required for agonist-promoted phosphorylation and internalization in MDCK cells. Br J Pharmacol 2011;162:1304-13.
[34] Oakley RH, Laporte SA, Holt JA, Caron MG, Barak LS. Differential affinities of visual arrestin, beta arrestin1, and beta arrestin2 for G protein-coupled

receptors delineate two major classes of receptors. J Biol Chem 2000;275:17201-10.
[35] Burnstock G, Knight GE. Cellular distribution and functions of P2 receptor subtypes in different systems. Int Rev Cytol 2004;240:31-304.
[36] Fischer W, Franke H, Groger-Arndt H, Illes P. Evidence for the existence of P2Y(12,4) receptor subtypes in HEK-293 cells: reactivation of P2Y(1) receptors after repetitive agonist application. N-S Arch Pharmacol 2005;371:466-72.
[37] Atwood BK, Lopez J, Wager-Miller J, Mackie K, Straiker A. Expression of G protein-coupled receptors and related proteins in HEK293, AtT20, BV2, and N18 cell lines as revealed by microarray analysis. BMC Genomics 2011;12:14.

Chapter 6:
Broader international context, relevance and future perspectives

G protein-coupled receptors (GPCRs) are amongst the most common targets of modern drug therapy, and tremendous research has been invested in the development of highly selective drug compounds with therapeutic potential for application in the clinical setting. It is now recognized that a compound not only has to display selectivity for the aimed GPCR subtype, but additionally has to reach a higher level of selectivity in the way it activates distinct GPCR signalling pathways. This phenomenon is known as 'functional selectivity' or 'ligand bias' and describes the extent to which a ligand selectively stimulates one or more signalling pathways downstream of the activated GPCR. Selective stimulation of a (subset of) signalling pathway(s) might provide a safer and better tolerated therapeutic profile with less adverse effects. Biased signalling has mostly been evaluated for the G protein- versus the arrestin pathway; the arrestin protein acts both as a regulator of GPCR signalling, controlling overstimulation of the receptor, and as a signalling protein as such [1, 2]. A growing body of evidence illustrates the promise of G protein- or arrestin-biased ligands as novel drug compounds at different GPCR systems [3, 4] (Figure 6.1); some examples of prototypical GPCR systems that are the targets of major classes of (non-)therapeutically used drugs are given below.

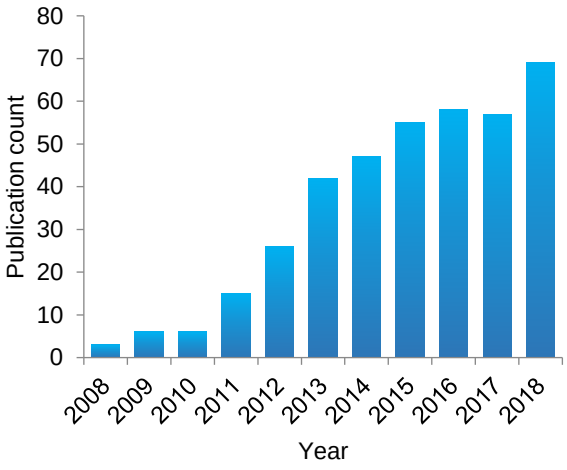

Figure 6.1: Number of publications that appeared during the last decade by using the key terms 'GPCR AND biased' in PubMED search engine.

Important to note is that functional selectivity and ligand bias in essence delineate different concepts. Functional selectivity refers to a differentiated pharmacological profile, which is very broad as it can be achieved through distinct mechanisms, at the level of pharmacokinetics, receptor subtype selectivity, receptor binding affinity, intrinsic efficacy, or intrinsic ligand bias. These mechanisms ultimately drive *in vivo* pharmacology. Intrinsic ligand bias is solely related to the stabilization of (subsets of) receptor conformation(s), and not to upstream or downstream factors. Hence, functional selectivity is influenced by cell- or tissue-related factors, while ligand bias is more system-independent. Receptor conformation(s) stabilized by ligand bias are evaluated in terms of ligand activity in a specific signalling pathway; bias occurs

when a ligand exhibits a difference in efficacy, potency or another parameter that quantifies the effective coupling with the receptor, relative to a reference ligand. By convention, the reference ligand, usually the endogenous agonist or a validated drug, is defined as unbiased, although this is not always truly the case. When measured appropriately, *in vitro* assays are able to identify intrinsic bias. This explains the relevance of translating data from overexpressed transfected receptor systems to *in vivo* differentiation of biased and unbiased ligands [1, 2, 5].

6.1 Examples of GPCR systems for which arrestin bias holds promise for therapeutic application

Angiotensin II type 1 receptor (AT1R)

The angiotensin II type 1 receptor (AT1R) is probably one of the best-studied examples for which biased ligands have been developed. Angiotensin II (AngII) is a vasoconstrictive peptide and agonist at the AT1R and signals through both the G_q-pathway and the βarr pathway. The AT1R regulates blood pressure and electrolyte homeostasis by vasoconstriction and fluid retention and is the target for the treatment of hypertension with angiotensin converting enzyme (ACE) inhibitors, or with angiotensin-receptor blockers (ARBs). However, the latter are known to reduce cardiac output as well [1, 6, 7]. Bias towards the βarr-pathway provides the desired reduction in blood pressure, while improving cardiac contractility and protecting against cardiac cell apoptosis [8]. This improved cardiac contractility is thus not driven by Ca^{2+} mobilization as such, but rather by a βarr-dependent enhanced sensitivity to Ca^{2+} by post-translational modification of cardiac myofilaments [2]. Such biased ligands have increasingly been reported for the development of better cardiovascular drugs, for example for the treatment of acute heart failure [9]. A Tyr4 and Phe8 isoleucine substituted AngII derivative [Sar^1,Ile^4,Ile^8]-AngII (SII) [10, 11], and a peptide analogue of angiotensin, Sar-Arg-Val-Tyr-Ile-His-Pro-D-Ala-OH (TRV027 or TRV120027) [6] both stimulate βarr-recruitment and βarr-mediated cytoprotective ERK activation, in the apparent absence of detectable G_q signaling. However, substantially weak coupling of the AT1R to G_q and/or coupling to other G proteins, e.g. for the biased agonist SII, has been reported for the AT1R (see **Chapter 1**) [12, 13]. TRV027 entered Phase IIb clinical studies to determine safety, efficacy and optimal dose for treatment of acute heart failure by IV infusion but failed

to show any benefit compared to placebo [14], in contrast to the benefit for treatment of chronic heart failure (Phase IIa). Therefore, more studies are required to decide if one should further invest in TRV027 or related biased AT1R agonists.

β-adrenergic receptors (βARs)

The G_s/G_i-coupled β1- and β2-adrenergic receptor (β1AR and β2AR) are known for the regulation of heart rate and contractility by endogenous catecholamines. βAR agonists can act as positive inotropes for the acute treatment of systolic ventricular dysfunction. βAR antagonists, referred to as β-blockers, are used for the treatment of hypertension, arrhythmia and heart failure [15]. The β2AR has been a model system for structural characterization of GPCR activation and (biased) signalling [4]. In an evaluation of clinically relevant β-blockers, only carvedilol selectively stimulated βarr-mediated signalling at the β2AR [16] and the β1AR [17], while not activating G_s-mediated signalling. However, a contributing role for $G_{\alpha i}$ signalling cannot be ruled out, again emphasizing more of a scaffolding function for βarr rather than stating clear signalling (see **Chapter 1**) [18]. Via the β1AR, carvedilol stimulates βarr1 to translocate to the nucleus and regulate miRNA processing, which is involved in cardioprotection and cardiomyocyte survival [19]. Remarkably, for the β1-selective β-blocker metoprolol, the βarr-pathway was associated with development of cardiac fibrosis [20]. This example underscores the importance of biased signalling in preclinical evaluation. All together, these studies show that β-blockers not simply provide a beneficial therapeutic profile by simply blocking all signalling pathways downstream of the βARs. β_2AR agonists are used as therapy for respiratory disease, particularly asthma, providing short- or long-term bronchodilation of airway smooth muscle, an effect achieved via the G_s-pathway. The rationale of long-acting bronchodilators (salmeterol) and short-acting bronchodilators (salbutamol) might be related to the βarr pathway [21]. Two racemic fenoterol stereoisomers are therapeutically used as G_s-biased β2AR agonists for the treatment of bronchial asthma and COPD [22].

Dopaminergic receptor D2 (D2R)

The $G_{i/o}$-coupled D2 dopamine receptor (D2R) represents one of the most validated drug targets for the treatment of neurologic and psychiatric disorders such as Parkinson's disease and schizophrenia. With the exception of aripiprazole, clinically

used antipsychotics for the treatment of schizophrenia traditionally are unbiased D2R antagonists; this antagonism underlies the therapeutic effect in the treatment of psychotic symptoms, but also evokes serious extrapyramidal side effects (dyskinesias) [5, 15]. Selective targeting of the βarr-pathway has appeared as a strategy to improve antipsychotic efficacy and uncouple this from the on-target motoric side effects [3]. Chen *et al.* ([23]) studied the SFSR, starting from aripiprazole, as a proof-of-concept for the discovery of βarr-biased D2R agonists. Their study was based on functional assays, i.e. ligand binding, cAMP signalling and βarr-recruitment, and on *in vivo* antipsychotic effects, evaluated in βarr2 knock-out mice. UNC9975 and UNC9994 were inactive towards $G_{i/o}$-regulated cAMP production but were partial agonists for βarr2 interaction, which was linked with their antipsychotic drug-like activity without motoric side effects. βarr contributed to the antipsychotic *in vivo* effect by evaluation of these ligands in βarr2-knock-out mice [24]. Interestingly, biased ligands with opposite pharmacology, i.e. biased to the G protein pathway, have been identified as well [25, 26]. As both G-protein-biased and βarr-biased D2R agonists have shown to be effective as antipsychotic agents, further studies are required to completely understand the relevant contributions of each of these signalling pathways to the antipsychotic efficacy and motoric side effects [4]. Functionally selective G protein-biased ligands may also result in improved therapies for neuropsychiatric disorders which require D2R stimulation, such as Parkinson's disease [3].

The μ-opioid receptor (μOR)

The μ-opioid receptor (μOR) is the target of the legal opiate analgesics morphine and codeine, the opioid analgesic fentanyl, the illegal opioid drug heroin and many other synthetic opioid drugs. In addition, μOR antagonists such as naloxone are used for the treatment of drug overdose. μOR agonists provide powerful analgesia via G_i-mediated inhibition of ion channels to hyperpolarize nociceptive fibers. However, typical opioid on-target adverse effects (nausea, vomiting, constipation, respiratory depression, sedation, dependence) may hamper sufficient dose-escalation to adequately relieve pain in the clinical setting [2]. The μOR was the first receptor shown to exhibit negative βarr-biased signalling; morphine stimulates the βarr pathway less than the G_i-mediated pathway. However, in βarr2-knock-out mice,

morphine-induced analgesia was amplified and prolonged, indicating that morphine still gives some βarr-mediated receptor desensitization [1, 27]. There has been growing interest in these G protein-biased - thus βarr negatively biased - opioid ligands for opioid drug discovery, as these appear to potentiate the opiates' analgesic effect and reduce some of the side effects (nausea, constipation, respiratory depression and tolerance) [28-30]. The influence of the βarr-pathway on more subjective opioid effects, i.e. dependence and withdrawal, is less clear, which complicates the prediction of the value of βarr-bias on opioid abuse [2]. In 2007, a novel non-nitrogen containing μOR agonist was discovered, named herkinorin, which was more negatively βarr2 biased than morphine [7, 15, 31]. In 2012, a completely different biased structure was developed, TRV130 or oliceridine [32], which has now finished Phase III clinical trials for the treatment of moderate to severe acute pain and is waiting for approval from the U.S. Food and Drug administration [4, 30, 33]. Therefore, this compound might represent an early clinical translation of ligand bias in a new chapter for GPCR drug discovery. Screening and structural studies are putting extensive effort in the discovery of biased μOR agonists [30, 34, 35].

Cannabinoid receptors (CBs)

The two major cannabinoid receptor subtypes, CB1 and CB2, are important drug targets for the regulation of neurotransmission, pain and inflammation. In the body, they are activated by the endocannabinoid lipids anandamide and 2-arachidonoylglycerol. Despite the long history of medical and recreational use of phyto-cannabinoids – the psychoactive constituent ∆9-tetrahydrocannabinol (THC) and the non-psychoactive constituent cannabidiol of marijuana – there are significant gaps in our understanding of the pharmacodynamic signalling of these ligands. CB1 is mainly expressed in the CNS and to a lesser extent in the periphery, whereas CB2 is expressed in the immune system and during inflammation injury in the CNS. CB1 selective ligands have been investigated as treatments for the management of pain, addiction, obesity, movement disorders including Huntington disease, Parkinson disease, multiple sclerosis, and other neurodegenerative and psychiatric disorders. Ligands for CB2 hold promise for treating neuro-inflammatory diseases (multiple sclerosis), cancer, and hypertension [36-38]. Nonetheless, CB(1) ligands have a propensity to induce on-target adverse psycho-behavioral effects. CB receptors

primarily couple to $G_{i/o}$ proteins, and additionally to G_s and G_q, and ligand bias has been reported mainly between these different G protein subtypes [36, 37]. Studies revealed βarr recruitment to CB1 and CB2 [38-40], and bias towards the βarr pathway has also been studied for CB1 [41, 42] and CB2 [43-46]. However, if or how the biased properties of CB ligands correlate to therapeutic effects or unwanted side effects remains to be determined [4, 37]. For now, reports are mainly from heterologous expressing CB systems, and there is a lack of data from *in vivo* experiments or systems that endogenously express CB receptors.

6.2 Contemporary relevance & future perspectives for this research project

6.2.1 Project overview and critical observations

The consideration of ligand bias will continue to change the way scientists approach GPCR-targeted drug discovery in the future. Knowledge on ligand features to selectively stimulate the G protein and/or arrestin pathway would be of tremendous value in this discovery process. However, this knowledge has been a subject of controversy in latest years. To date, we do not know if the coupling to arrestin is based on a consensus mechanism or not; it is likely that it includes a barcode system with at least some level of consensus in the form of a phosphorylation pattern in the C-terminus or intracellular loops, or a pattern in the GPCR core. An open view on this is given in **Chapter 1**.

In **Chapter 2**, we provide an overview of what is known on biased signalling for G protein-coupled purinergic receptors, i.e. adenosine (P1) receptors and P2Y receptors (P2YRs). The focus is on functional selectivity with therapeutic relevance, and the arrestin pathway is discussed more in detail. In general, this pathway has been insufficiently explored for purinergic receptors, in contrast to the aforementioned GPCR systems, in which bias of the arrestin pathway has proven to hold therapeutic promise/relevance. Exploring the fundamental features of arrestin coupling to a GPCR is interesting in a prospective way for future development of biased purinergic ligands. Once adverse or toxic effects would appear in animal models, it would be interesting to modulate ligand structure in a discrete way,

exploring bias to the G protein- or arrestin pathway, without losing therapeutic effect due to major structural changes.

For two subtypes of the purinergic GPCR subfamily, the A3 adenosine receptor (A_3AR) (a P1 receptor) and the P2Y2 receptor (P2Y2R), we evaluated the coupling to βarr2 in detail, as described in **Chapter 3 and Chapter 5** of this thesis. These subtypes were chosen because there is a high interest in clinically relevant ligands for both subtypes. The coupling to βarr2 was characterized for the C-terminally truncated and mutated receptors, by monitoring of βarr2 recruitment using a live-cell reporter assay system, based on the functional complementation of the bioluminescent NanoLuc (NL) luciferase enzyme; a NanoBit® technology provided by the Promega company. In **Chapter 4**, a stable A_3AR βarr2 NanoBit® HEK293T cell line was used for the screening of a panel of 19 synthetic adenosine derivatives, and comparison of their activity profiles with those for cAMP signalling to provide insight into a possible SFSR. Although no extreme patterns of ligand bias were observed, the highly A_3AR-selective (N)-methanocarba 5'-uronamide adenosine derivatives showed great tolerance for substitution at the C2-position due to their preferred conformation at the A_3AR, giving very high potency and efficacy in both the cAMP and βarr2 pathway.

The bioluminescent NL-based assay system used in this research project has several advantages; it provides a fast read-out with the possibility of downscaling and high-throughput purposes, the signal is quantifiable with high sensitivity, the system is very flexible to adjustment of the GPCR and/or arrestin, and this is possible within a live-cell set-up. However, there are some intrinsic limitations of this assay as well. First of all, both receptor and arrestin are modified by fusion to the split parts of NL. This gives a high chance of influencing the normal behavior of these proteins. Besides, it is important to realize that βarr2 recruitment as such is evaluated in this assay, rather than a βarr-related functional outcome, e.g. receptor desensitization, internalization or ERK signalling. Hence, it would be premature to draw conclusions concerning arrestin 'activation'. However, patterns of recruitment are likely to be reflected in arrestin activation, and thus may give a preliminary SAR. Furthermore, recent findings on GPCR-arrestin interaction have pointed at a catalytic behaviour of the GPCR towards trafficking (and possibly downstream signalling) of arrestin. Hence, partial contact (e.g. as shown for the biased agonist carvedilol [47]), or

transient contact with the GPCR might be of equal relevance (see **Chapter 1**). A transient GPCR-arrestin interaction can perfectly be monitored with the assay system used here.

The NL luciferase is a bright, glow-type luciferase and is smaller (19 kDa) than other luciferases, such as Firefly luciferase (FL; 61 kDa), Renilla luciferase (RL; 36 kDa), and Gaussia luciferase (GL; 20 kDa) [48, 49]. The size of the luciferase is of importance when it is used as a split reporter to evaluate βarr recruitment to a GPCR, as smaller luciferases give less sterical hindrance. In this thesis, we opted for a bioluminescent reporter technology. Other βarr proximity assays rely on fluorescent reporters. There are inherent advantages and disadvantages when comparing bioluminescence to fluorescence imaging for *in vitro* use. Bioluminescence generally has lower background signal as cells can display substantial autofluorescence. Furthermore, there is an enzymatic amplification of the imaging signal with bioluminescent imaging. These two aspects increase the sensitivity of the technique. On the other hand, fluorescence lends itself towards simultaneous detection of a wider array of spectrally distinct fluorescent proteins or dyes. Therefore, there is a need for different luciferases with distinct spectral properties or luciferase substrates that provide these distinct spectral properties to image multiple processes in the same cell [50, 51]. Proximity assays can use only fluorescence (such as fluorescence resonance energy transfer (FRET)) or can use bioluminescence to evoke the fluorescent signal (bioluminescence resonance energy transfer (BRET)). In general, FRET gives a stronger signal than BRET, but the excitation of the donor can give substantial acceptor excitation, and the technique is not suited for light-sensitive cells. BRET generally gives a weaker signal, that however can be detected with sensitive equipment or by integration of the signal over a longer period of time. The advantage is that it does not give photo-bleaching nor autofluorescence problems.

Proximity assays such as the bioluminescent protein complementation NanoBit® assay used in this thesis, as well as fluorescent BRET assays – for example NanoBRET™ [52] – also hold potential for *in vivo* use in animal models. Animal models are inevitable to see if the findings will match with the *in vivo* setting, and to provide a link with therapeutic effect. However, certain things should be taken into account. With respect to bioluminescence imaging, the spectral properties are of

importance; with furimazine as a substrate, NL has an emission peak at 460 nm and thus emits blue light, which is preferentially absorbed and scattered by tissues. This challenges the visualization of deeper tissues, though the visualization is still substantially good. Luciferases with more red-shifted emission, such as FL with emission >500-600 nm, will better serve *in vivo* imaging. The advantage is that the distinct spectral properties of FL and NL, or for any other pair of luciferases, make dual bioluminescence *in vivo* imaging possible [50]**.** Furthermore, it would be a possibility to use a more red-shifted furimazine substrate [53], or even a red-shifted furimazine-modified-NL pair [54].

In vivo imaging has also been described with BRET. Alcobia *et al.* ([55]) reported on bioluminescent as well as fluorescent imaging in mice, using NanoBRET™ (based on NL), making use of a N-terminal NL-tagged GPCR and a fluorescently-tagged GPCR ligand**.** It is by all means challenging - but not impossible - to say that the NanoBit® system used here could be transferred to the *in vivo* setting, and this in a couple of ways**.** The system could be stably transfected into cancer cells and injected into mice to evaluate the influence of *in vivo* parameters on βarr2 recruitment, as well as to evaluate the influence of modifications at the receptor on tumour evolution. As a furimazine substrate, a more red-shifted analogue should better be used then. However, if one would like to first monitor (tumour) tissue expression of the receptor, this one should be coupled to fully functional NL, rather than only to LgBit, and a NanoBRET™-approach could be used by linking arrestin with a suitable acceptor-molecule. In a next step, it would even be possible to include a G protein that is coupled with another acceptor molecule in this set-up. Obviously, evaluating native expression levels adds another level of complexity, as genome editing is required in this case. The use of different furimazine substrates that either give blue or red emission, could then evoke excitation of arrestin or G protein, if these are in close proximity with the receptor. However, one should take in mind that for both bioluminescence as well as for BRET-based systems *in vivo*, substrates need to be injected intravenously, and this might harbor substantial toxicity; while luciferin has shown to be non-toxic [56], furimazine has shown toxicity [57].

6.2.2 Seeking a link of purinergic (biased) signalling with the clinical setting

There are numerous examples of biased signalling and/or functional selectivity at different GPCR systems. However, it is not always known *a priori* which signalling pathway has therapeutic relevance in the pathophysiological setting; this has definitely been the case for purinergic GPCRs. For both the A_3AR and the P2Y2R, it is not fully known which signalling pathways are important to obtain the clinically relevant therapeutic effects, nor if the βarr pathway is involved in this to any extent.

The A_3AR is upregulated in almost all cells of the immune system and in cancer cells compared to normal cells due to the high levels of adenosine and cytokines in the tumor- and/or inflammatory microenvironment. Adenosine might regulate the expression of its own receptors via an autocrine pathway. The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) binds to its receptor, resulting in upregulation of protein kinase B (PKB)/Akt and the nuclear factor κ-light-chain-enhancer of activated B cell (NF-κB) pathway, providing transcription of ADORA3 by the cAMP response element binding protein (CREB) transcription factor [58, 59]. Both pro- and anti-inflammatory effects, as well as pro- and anti-proliferative effects have been described by modulation of the A_3AR, depending on the cell type involved and (ant)agonist concentrations used. The A_3AR contributes to neutrophil chemotaxis and migration by a polarized distribution on the neutrophil membrane [60, 61], while in breast cancer cells it arrests cell motility by stimulating migration in opposite directions [62]. Both A_3AR agonists and antagonists have been studied for a potential anticancer effect, often with contrasting results. However, only A_3AR agonists have shown therapeutic utility, supported by *in vivo* studies for colon, prostate, melanoma and hepatocellular carcinoma [59]. The anti-inflammatory and anticancer effect of A_3AR agonists has mainly been attributed to a modulation of the NF-κB pathway and the Wnt pathway, resulting in a decrease in levels of factors involved in cell cycle progression and cell growth (**Figure 6.2**) [58, 63, 64]. The prototypical agonist IB-MECA (CF101), piclidenoson, is entering phase III clinical trials for the treatment of inflammatory autoimmune disorders such as psoriasis and rheumathoid arthritis (RA) [63, 65]. The molecular mechanism involves the inhibition of the NF-κB pathway and consequent inhibition of release of pro-inflammatory cytokines, such as TNF-α, interleukin 1 (IL-1), and interleukin 6 (IL-6) [58, 66]. The A_3AR is upregulated in

peripheral blood mononuclear cells (PBMCs) obtained from patients with RA and psoriasis and can be used as a predictive biomarker. In lymphocytes of RA patients, the immunosuppressive A_3AR inhibits inflammatory cytokine production; receptor density is inversely correlated with indexes for disease activity, by which the response to anti-rheumatic drugs can be monitored [59]. The 2-chloro analogue 2-Cl-IB-MECA (CF102), namodenoson, is currently in phase II clinical trials for the treatment of hepatocellular carcinoma, nonalcoholic fatty liver disease and nonalcoholic steatohepatitis (NASH). The signalling is believed to rely on deregulation of the Wnt pathway and the increased expression of pro-apoptotic proteins [67]. The A_3AR is expressed in peripheral blood cells, reflecting receptor status in remote tumor tissue, and can be used as a possible marker for cancer [64, 67]. Although not expressed at high levels in the heart, the A_3AR has cardioprotective effects when large amounts of adenosine are released during cardiac ischemia. Signalling might happen through activation of PKC and subsequent phosphorylation and thus inactivation of glycogen synthase kinase-3β (GSK-3β), reducing cardiac myocyte death [58]. Furthermore, regulation of sarcolemmal or mitochondrial K_{ATP} channels via PKC might be involved [58]. More recently, the A_3AR has been involved in the treatment of chronic neuropathic pain. However, IV administration of adenosine is associated with serious cardiac side effects. Hence, separating antinociceptive from cardiovascular effects is important in

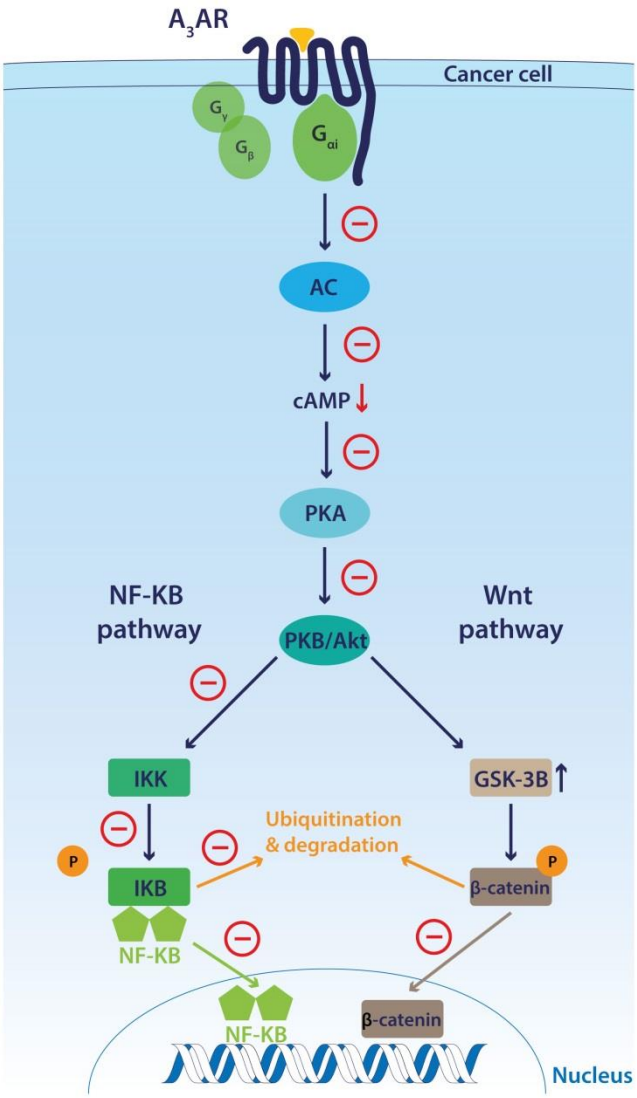

Figure 6.2: A_3AR stimulation leads to inhibition of PKB/Akt, evoking a deregulation of the NF-κB pathway (left) and the Wnt pathway (right). As a result, levels of cyclin D1 and c-Myc, which play a crucial role in cell cycle progression, are decreased. (IKK = IκB kinase)

developing adenosine-based therapeutics. Highly A_3AR selective (N)-methanocarba adenosine derivatives, such as MRS5698, have been developed for this purpose [68, 69]. The A_3AR acts by reducing downstream neuro-inflammatory events, leading to an overall reduction in pro-inflammatory cytokines (TNF and IL1β) [68]. Hence, A_3AR agonists might provide dual anticancer and anti-nociceptive benefits in the treatment of a variety of cancer-related pain states. Remarkably, the anti-nociceptive effects have been shown to result from prolonged A_3AR stimulation and A_3AR agonists are thus not subject to analgesic tolerance. For autoimmune disorders and for cancer as well, chronic administration of A_3AR agonists maintains anti-inflammatory/anticancer effects even during A_3AR downregulation. Hence, a possible role for G protein and/or βarr signalling in A_3AR therapeutic effects remains subject to debate.

Some P2Y2R signalling features have been linked to the therapeutic effect of P2Y2R agonists in clinical trials or already in use. The P2Y2R agonist P^1,P^4-di(uridine-5′)-tetraphosphate (Up4U), known as diquafosol (or INS365), is available on the Japanese and Korean market for the symptomatic treatment of dry eye disease and is also recommended for retinal detachment. The intracellular Ca^{2+} increase by P2Y2R stimulation is thought to open Cl^- channels in the apical membrane, followed by Cl^- flux and water transport [70]. A similar P2Y2R signalling mechanism might be of relevance in the treatment of cystic fibrosis (CF) [71]. In CF, a genetic defect in the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel results in abnormal sodium, chloride and water transport across the respiratory epithelium. Stimulation of the P2Y2R is speculated to compensate for the malfunctioning of the CFTR, by the coordinated inhibition of an epithelial Na^+ channel (ENaC) and stimulation of a Ca^{2+} dependent Cl^- channel (CaCC), resulting in increased Cl^- secretion [72]. However, the exact signalling mechanism needs further exploration. The P2Y2R agonist P^1-(Uridine-5')-P^4-(2'-deoxycytidine-5')-tetraphosphate (Up4dC), or denufosol (INS37217) [73], has made it far into clinical trials for CF [74, 75], but failed in phase III studies [76, 77]. Purinergic signalling also has a role in inflammatory airway diseases; increased amounts of extracellular ATP have been found in lungs of patients with asthma and COPD, leading to increased airway inflammation and bronchoconstriction, mediated via P2 receptors. There is clinical interest in the development of highly selective antagonists that inhibit specific signalling pathways of P2R subtypes [78]. Given the possible benefit of nucleotides

in the treatment of airway diseases both in an agonistic and antagonistic way, the development of P2Y subtype-selective ligands is needed. The P2Y2R also has a contributive neuroprotective role in the management of neurological disorders [79], next to other purinergic receptors, such as the P2X7R and the A_{2A}AR [80, 81]. Particularly in the early stages of disease where neuro-inflammatory responses may play a role in tissue repair, P2Y2R activation has been shown to promote neuroprotective responses [82]. Relevant in this context is a study by Camden *et al.* ([83]), who showed that P2Y2Rs enhanced α-secretase-dependent amyloid precursor protein processing to a non-amyloidogenic product in astrocytoma cells. This prevents the processing via β- and γ-secretase to amyloid-β, the main component of the plaques associated with Alzheimer's disease. On the other hand, P2Y2R antagonism might be useful as anti-metastatic cancer therapy and as anti-inflammatory therapy to treat psoriasis and atherosclerosis. By its expression on endothelial cells, P2Y2R activation opens the endothelial barrier, and the receptor is highly expressed in the tumor micro-environment by cancer cells, as well as by infiltrating immune cells [84-86].

6.2.3 Concluding aspects for this project in drug development and drug screening

- X-ray structures and X-ray derived homology models are routinely used for molecular docking of compound libraries to discover new lead compounds [87-89]. It is important to not only rely on crystal structures of receptors bound to (biased) (ant)agonists, but also in complex with their coupling effectors, i.e. G-proteins and arrestins [90]. As these structures represent a static snapshot and ligand bias may arise from an ensemble of receptor conformations, insights derived from these structures should be interpreted in conjunction with data on ligand efficacy and/or potency, originating from binding assays and functional assays [5, 91]. Furthermore, it is important that functional assays not only monitor one single time-point but also evaluate kinetic properties, as these can influence the bias profile. The assay applied in this thesis could definitely serve this purpose, as it evaluates kinetic properties as well; ideally, it is performed in parallel with G protein dependent functional assays.

- The assay we developed is highly flexible to elucidate structural features of βarr2 coupling and for screening purposes of ligand panels to deduct SAR or SFSR relationships. Interestingly, use of the assay presented here is not solely restricted to screening for drug development, but can also be used for drug detection. There is a substantial contribution of βarr2 to the psychomotor and rewarding effects of addictive drugs [92]. This was exploited by the development of a CB and a μOR NanoBit® system for the screening of drugs of abuse in the toxicological/forensic setting in our laboratory. These assays can be used for characterization of SAR of pure compounds as well as for the screening for the presence of drugs in biological samples [93-96]. By specifically modifying the βarr2 molecule, the sensitivity was substantially improved; two βarr2 truncation mutants, βarr2T382 AND βarr2T366, lacking the clathrin adaptor protein site and the restricting C-tail, respectively, gave an increased recruitment to the CB receptor and were implemented in these assays [97]. It was also tempting to increase the selectivity of the assay by mutating the 'DRY' motif to 'AAY', as this had been shown in literature to increase bias towards the βarr pathway and away from the G protein pathway [98]. However, this was without any result.

- While it is possible to evaluate known drug molecules used in the clinical setting for possible bias to associate pre-existing but unknown functional selectivity with a beneficial profile of activity in humans, this research project on purinergic GPCRs rather maps the βarr-coupling features and functional selectivity profile in a prospective way. In a next step, once a lead compound has been identified that displays a beneficial therapeutic profile in mice, minor SAR-adjustments can be introduced to guide lead optimization and to determine whether bias offers a therapeutic advantage or can be used to fine-tune the therapeutic profile.

Biased signalling and functional selectivity are two challenging concepts in an exciting, though complicated area of research that holds a lot of opportunities for present and future drug discovery. However, if ligands can be 'biased', ligand classification as agonists, antagonists or inverse agonists becomes assay dependent, which requires more nuanced screening approaches. This implies the

use of a panel of assays that evaluate ligand activity in each of the signalling pathways of interest. Examples are G protein dependent assays and βarr dependent assays, such as the cAMP assay and the NanoBit® assay, described in **Chapter 4**, respectively. Performing such assays in parallel by means of high throughput screening is not trivial, because it necessitates the generation of dose-response curves; hence, these assays have to be low-cost and easily upscalable. Current models for quantification of bias are available, based on the dose-response deducted system E_{max} and ligand EC_{50} (see **Chapter 1**), and practical examples of large scale screening studies are arising [99]. The quantified bias then represents a code for the complex phenotypic cellular response. However, at this moment, there is still a lack of information on the degree of bias that should be reached in each pathway to obtain a desired physiologic/therapeutic effect. Only for GPCR systems that have been studied for many decades, e.g. the μOR system, the target product profile is sufficiently clear to allow the development of clinically useful biased agonists [100, 101]. Hence, screening assays should be seen as a cost-effective way to identify compounds that can go from screening to more complex (*in vivo*) assays [102].

Because biased ligands exert a signalling profile different from that of endogenous ligand(s), unexpected effects might show up in the *in vivo* setting. Besides, once a biased ligand is introduced in the body, additional factors, such as pharmacokinetic bias (in drug absorption and metabolism) complicate the aimed effect at the targeted receptor. It is difficult if not impossible to predict how the biological effects of biased ligands might differ from those of unbiased ligands. Therefore, one must try to link results from *in vitro* screening (identifying intrinsic ligand bias) with *in vivo* biological response (which encompasses many confounding factors that influence the final functional selectivity of a biased compound) [100, 101]. The usual way to do this is by using mouse (or other animal) models. Both βarr knock-out mouse models as well as biased receptor mutants have been used to predict this. However, both approaches do not correctly mimic the action of biased ligands, as the observed effect can result from enhanced G protein activation or simply the loss of arr-dependent signalling. Hence, bias can best be evaluated *in vivo* in a wild-type animal setting [100]. Very recent is the use of genetically encoded biosensors for use as screening systems to measure spatiotemporal (biased) signalling at the whole organ level in the *in vivo* setting [103]. The NanoBit® assay might also have a place in this. To further

complicate matters, the measured/observed bias might not directly be translatable from one species to another. Hence, species bias can exist. Even stronger, as levels of signalling proteins differ between cell types, these can also change with disease states (giving rise to ‘dynamic bias’) and even between individuals [101]. Therefore, it will be of continuous importance to screen for candidate biased ligands using functional assays; these candidates then can be used in physiological systems to eventually obtain biased therapeutic lead compounds.

Taking all the above into account, it can be said that despite all the efforts, there is one question concerning the quest for biased ligands in drug discovery; is the chase worth the catch, or is it just too soon to choose a path? In the context of GPCR signalling, it is safe to say that the more we know, the more we realize there is so much we don’t know. Nevertheless, it seems that the quest for biased signaling is worth an effort but also requires increasing attention to the translation of *in vitro* bias to the *in vivo* setting (whole cell and body systems) [102, 104].

References

[1] Violin JD, Lefkowitz RJ. beta-arrestin-biased ligands at seven-transmembrane receptors. Trends Pharmacol Sci 2007;28:416-22.

[2] Violin JD, Crombie AL, Soergel DG, Lark MW. Biased ligands at G-protein-coupled receptors: promise and progress. Trend Pharmacol Sci 2014;35:308-16.

[3] Bologna Z, Teoh JP, Bayoumi AS, Tang Y, Kim IM. Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology. Biomol Ther 2017;25:12-25.

[4] Tan L, Yan W, McCorvy JD, Cheng J. Biased Ligands of G Protein-Coupled Receptors (GPCRs): Structure-Functional Selectivity Relationships (SFSRs) and Therapeutic Potential. J Med Chem 2018.

[5] Rankovic Z, Brust TF, Bohn LM. Biased agonism: An emerging paradigm in GPCR drug discovery. Bioorg Med Chem Lett 2016;26:241-50.

[6] Violin JD, DeWire SM, Yamashita D, Rominger DH, Nguyen L, Schiller K, et al. Selectively Engaging beta-Arrestins at the Angiotensin II Type 1 Receptor Reduces Blood Pressure and Increases Cardiac Performance. J Pharmacol Exp Ther 2010;335:572-9.

[7] Correll CC, McKittrick BA. Biased Ligand Modulation of Seven Transmembrane Receptors (7TMRs): Functional Implications for Drug Discovery. J Med Chem 2014;57:6887-96.

[8] Rajagopal K, Whalen EJ, Violin JD, Stiber JA, Rosenberg PB, Premont RT, et al. beta-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. Proc Natl Acad Sci USA 2006;103:16284-9.

[9] DeWire SM, Violin JD. Biased Ligands for Better Cardiovascular Drugs Dissecting G-Protein-Coupled Receptor Pharmacology. Circ Res 2011;109:205-16.

[10] Holloway AC, Qian HW, Pipolo L, Ziogas J, Miura SI, Karnik S, et al. Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors. Mol Pharmacol 2002;61:768-77.

[11] Wei HJ, Ahn S, Shenoy SK, Karnik SS, Hunyady L, Luttrell LM, et al. Independent beta-arrestin 2 and G protein-mediated pathways for angiotensin II activation of extracellular signal-regulated kinases 1 and 2. Proc Natl Acad Sci USA 2003;100:10782-7.

[12] Sauliere A, Bellot M, Paris H, Denis C, Finana F, Hansen JT, et al. Deciphering biased-agonism complexity reveals a new active AT1 receptor entity. Nat Chem Biol 2012;8:622-30.

[13] Wang J, Hanada K, Gareri C, Rockman HA. Mechanoactivation of the angiotensin II type 1 receptor induces beta-arrestin-biased signaling through Galphai coupling. J Cell Biochem 2018;119:3586-97.

[14] Pang PS, Butler J, Collins SP, Cotter G, Davison BA, Ezekowitz JA, et al. Biased ligand of the angiotensin II type 1 receptor in patients with acute heart failure: a randomized, double-blind, placebo-controlled, phase IIB, dose ranging trial (BLAST-AHF). Eur Heart J 2017;38:2364-73.

[15] Rajagopal S, Rajagopal K, Lefkowitz RJ. Teaching old receptors new tricks: biasing seven-transmembrane receptors. Nat Rev Drug Discov 2010;9:373-86.

[16] Wisler JW, DeWire SM, Whalen EJ, Violin JD, Drake MT, Ahn S, et al. A unique mechanism of beta-blocker action: Carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci USA 2007;104:16657-62.
[17] Kim IM, Tilley DG, Chen J, Salazar NC, Whalen EJ, Violin JD, et al. beta-Blockers alprenolol and carvedilol stimulate beta-arrestin-mediated EGFR transactivation. Proc Natl Acad Sci USA 2008;105:14555-60.
[18] Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, et al. Galphai is required for carvedilol-induced beta1 adrenergic receptor beta-arrestin biased signaling. Nat Commun 2017;8:1706.
[19] Kim IM, Wang YC, Park KM, Tang YP, Teoh JP, Vinson J, et al. Arrestin1-Biased beta 1-Adrenergic Receptor Signaling Regulates MicroRNA Processing. Circ Res 2014;114:833-44.
[20] Nakaya M, Chikura S, Watari K, Mizuno N, Mochinaga K, Mangmool S, et al. Induction of cardiac fibrosis by beta-blocker in G protein-independent and G protein-coupled receptor kinase 5/beta-arrestin2-dependent Signaling pathways. J Biol Chem 2012;287:35669-77.
[21] Carter AA, Hill SJ. Characterization of isoprenaline- and salmeterol-stimulated interactions between beta 2-adrenoceptors and beta-arrestin 2 using beta-galactosidase complementation in C2C12 cells. J Pharmacol Exp Ther 2005;315:839-48.
[22] Reinartz MT, Kalble S, Littmann T, Ozawa T, Dove S, Kaever V, et al. Structure-bias relationships for fenoterol stereoisomers in six molecular and cellular assays at the beta(2)-adrenoceptor. N-S Arch Pharmacol 2015;388:51-65.
[23] Chen X, Sassano MF, Zheng L, Setola V, Chen M, Bai X, et al. Structure-functional selectivity relationship studies of beta-arrestin-biased dopamine D(2) receptor agonists. J Med Chem 2012;55:7141-53.
[24] Allen JA, Yost JM, Setola V, Chen X, Sassano MF, Chen M, et al. Discovery of beta-Arrestin-Biased Dopamine D-2 Ligands for Probing Signal Transduction Pathways Essential for Antipsychotic Efficacy. Proc Natl Acad Sci USA 2011;108:18488-93.
[25] Chun LS, Vekariya RH, Free RB, Li Y, Lin DT, Su P, et al. Structure-Activity Investigation of a G Protein-Biased Agonist Reveals Molecular Determinants for Biased Signaling of the D2 Dopamine Receptor. Frontiers in synaptic neuroscience 2018;10:2.
[26] Weiwer M, Xu QH, Gale JP, Lewis M, Campbell AJ, Schroeder FA, et al. Functionally Biased D2R Antagonists: Targeting the beta-Arrestin Pathway to Improve Antipsychotic Treatment. ACS chemical biology 2018;13:1038-47.
[27] Bohn LM, Lefkowitz RJ, Gainetdinov RR, Peppel K, Caron MG, Lin FT. Enhanced morphine analgesia in mice lacking beta-arrestin 2. Science 1999;286:2495-8.
[28] Bohn LM, Gainetdinov RR, Lin FT, Lefkowitz RJ, Caron MG. mu-Opioid receptor desensitization by beta-arrestin-2 determines morphine tolerance but not dependence. Nature 2000;408:720-3.
[29] Allouche S, Noble F, Marie N. Opioid receptor desensitization: mechanisms and its link to tolerance. Front Pharmacol 2014;5:280.
[30] Siuda ER, Carr R, Rominger DH, Violin JD. Biased mu-opioid receptor ligands: a promising new generation of pain therapeutics. Curr Opin Pharmacol 2017;32:77-84.

[31] Groer CE, Tidgewell K, Moyer RA, Harding WW, Rothman RB, Prisinzano TE, et al. An opioid agonist that does not induce mu opioid receptor-arrestin interactions or receptor internalization. Mol Pharmacol 2006.
[32] Chen XT, Pitis P, Liu GD, Yuan C, Gotchev D, Cowan CL, et al. Structure Activity Relationships and Discovery of a G Protein Biased mu Opioid Receptor Ligand, [(3-Methoxythiophen-2-yl)methyl]a2[(9R)-9-(pyridin-2-y1)-6-oxaspiro-[4.5]clecan-9-yl]ethylpamine (TRV130), for the Treatment of Acute Severe Pain. J Med Chem 2013;56:8019-31.
[33] Fossler MJ, Sadler BM, Farrell C, Burt DA, Pitsiu M, Skobieranda F, et al. Oliceridine, a Novel G Protein-Biased Ligand at the mu-Opioid Receptor, Demonstrates a Predictable Relationship Between Plasma Concentrations and Pain Relief. II: Simulation of Potential Phase 3 Study Designs Using a Pharmacokinetic/Pharmacodynamic Model. J Clin Pharmacol 2018;58:762-70.
[34] Madariaga-Mazon A, Marmolejo-Valencia AF, Li YM, Toll L, Houghten RA, Martinez-Mayorga K. Mu-Opioid receptor biased ligands: A safer and painless discovery of analgesics? Drug Discovery Today 2017;22:1719-29.
[35] Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue ZZ, Morgenweck J, et al. Bias Factor and Therapeutic Window Correlate to Predict Safer Opioid Analgesics. Cell 2017;171:1165-75.
[36] Laprairie RB, Bagher AM, Denovan-Wright EM. Cannabinoid receptor ligand bias: implications in the central nervous system. Curr Opin Pharmacol 2017;32:32-43.
[37] Mallipeddi S, Janero DR, Zvonok N, Makriyannis A. Functional selectivity at G-protein coupled receptors: Advancing cannabinoid receptors as drug targets. Biochem Pharmacol 2017;128:1-11.
[38] Ibsen MS, Connor M, Glass M. Cannabinoid CB1 and CB2 Receptor Signaling and Bias. Cannabis and cannabinoid research 2017;2:48-60.
[39] van der Lee MM, Blomenröhr M, van der Doelen AA, Wat JW, Smits N, Hanson BJ, et al. Pharmacological characterization of receptor redistribution and β-arrestin recruitment assays for the cannabinoid receptor 1. J Biomol Screen 2009;14:811-23.
[40] Mcguinness D, Malikzay A, Visconti R, Lin K, Bayne M, Monsma F, et al. Characterizing Cannabinoid CB2 Receptor Ligands Using DiscoveRx PathHunter (TM) beta-Arrestin Assay. J Biomol Screen 2009;14:49-58.
[41] Laprairie RB, Bagher AM, Kelly MEM, Dupre DJ, Denovan-Wright EM. Type 1 Cannabinoid Receptor Ligands Display Functional Selectivity in a Cell Culture Model of Striatal Medium Spiny Projection Neurons. J Biol Chem 2014;289:24845-62.
[42] Delgado-Peraza F, Ahn KH, Nogueras-Ortiz C, Mungrue IN, Mackie K, Kendall DA, et al. Mechanisms of Biased beta-Arrestin-Mediated Signaling Downstream from the Cannabinoid 1 Receptor. Mol Pharmacol 2016;89:618-29.
[43] Atwood BK, Wager-Miller J, Haskins C, Straiker A, Mackie K. Functional Selectivity in CB2 Cannabinoid Receptor Signaling and Regulation: Implications for the Therapeutic Potential of CB2 Ligands. Mol Pharmacol 2012;81:250-63.
[44] Dhopeshwarkar A, Mackie K. Functional Selectivity of CB2 Cannabinoid Receptor Ligands at a Canonical and Noncanonical Pathway. J Pharmacol Exp Ther 2016;358:342-51.

[45] Soethoudt M, Grether U, Fingerle J, Grim TW, Fezza F, de Petrocellis L, et al. Cannabinoid CB2 receptor ligand profiling reveals biased signalling and off-target activity. Nat Commun 2017;8:13958.
[46] Morales P, Goya P, Jagerovic N. Emerging strategies targeting CB2 cannabinoid receptor: Biased agonism and allosterism. Biochem Pharmacol 2018.
[47] Kumari P, Srivastava A, Banerjee R, Ghosh E, Gupta P, Ranjan R, et al. Functional competence of a partially engaged GPCR-beta-arrestin complex. Nat Commun 2016;7:13416.
[48] Hall MP, Unch J, Binkowski BF, Valley MP, Butler BL, Wood MG, et al. Engineered Luciferase Reporter from a Deep Sea Shrimp Utilizing a Novel Imidazopyrazinone Substrate. ACS chemical biology 2012;7:1848-57.
[49] England CG, Ehlerding EB, Cai WB. NanoLuc: A Small Luciferase Is Brightening Up the Field of Bioluminescence. Bioconjugate Chem 2016;27:1175-87.
[50] Stacer AC, Nyati S, Moudgil P, Iyengar R, Luker KE, Rehemtulla A, et al. NanoLuc Reporter for Dual Luciferase Imaging in Living Animals. Mol Imaging 2013;12:1-13.
[51] Kaskova ZM, Tsarkova AS, Yampolsky IV. 1001 lights: luciferins, luciferases, their mechanisms of action and applications in chemical analysis, biology and medicine. Chem Soc Rev 2016;45:6048-77.
[52] Machleidt T, Woodroofe CC, Schwinn MK, Mendez J, Robers MB, Zirnmerman K, et al. NanoBRET-A Novel BRET Platform for the Analysis of Protein-Protein Interactions. ACS chemical biology 2015;10:1797-804.
[53] Shakhmin A, Hall MP, Machleidt T, Walker JR, Wood KV, Kirkland TA. Coelenterazine analogues emit red-shifted bioluminescence with NanoLuc. Organic & Biomolecular Chemistry 2017;15:8559-67.
[54] Yeh HW, Karmach O, Ji A, Carter D, Martins-Green MM, Ai HW. Red-shifted luciferase-luciferin pairs for enhanced bioluminescence imaging. Nat Methods 2017;14:971-74.
[55] Alcobia DC, Ziegler AI, Kondrashov A, Comeo E, Mistry S, Kellam B, et al. Visualizing Ligand Binding to a GPCR In Vivo Using NanoBRET. iScience 2018;6:280-8.
[56] Adams ST, Miller SC. Beyond D-luciferin: expanding the scope of bioluminescence imaging in vivo. Curr Opin Chem Biol 2014;21:112-20.
[57] Shipunova VO, Shilova ON, Shramova EI, Deyev SM, Proshkina GM. A Highly Specific Substrate for NanoLUC Luciferase Furimazine Is Toxic in vitro and in vivo. Russ J Bioorg Chem 2018;44:225-8.
[58] Fishman P, Bar-Yehuda S, Liang BT, Jacobson KA. Pharmacological and therapeutic effects of A(3) adenosine receptor agonists. Drug Discov Today 2012;17:359-66.
[59] Jacobson KA, Merighi S, Varani K, Borea PA, Baraldi S, Aghazadeh Tabrizi M, et al. A3 Adenosine Receptors as Modulators of Inflammation: From Medicinal Chemistry to Therapy. Med Res Rev 2018;38:1031-72.
[60] Chen Y, Corriden R, Inoue Y, Yip L, Hashiguchi N, Zinkernagel A, et al. ATP release guides neutrophil chemotaxis via P2Y2 and A3 receptors. Science 2006;314:1792-5.
[61] Butler M, Sanmugalingam D, Burton VJ, Wilson T, Pearson R, Watson RP, et al. Impairment of adenosine A3 receptor activity disrupts neutrophil migratory

capacity and impacts innate immune function in vivo. Eur J Immunol 2012;42:3358-68.
[62] Ledderose C, Hefti MM, Chen Y, Bao Y, Seier T, Li LL, et al. Adenosine arrests breast cancer cell motility by A3 receptor stimulation. Purinerg Signal 2016;12:673-85.
[63] Gessi S, Merighi S, Varani K. Adenosine Receptors: The Status of the Art. The Adenosine Receptors: Springer, 2018. p. 1-11.
[64] Gessi S, Merighi S, Borea PA, Cohen S, Fishman P. Adenosine Receptors and Current Opportunities to Treat Cancer. The Adenosine Receptors: Springer, 2018. p. 543-55.
[65] Fishman P, Cohen S. The A(3) adenosine receptor (A(3)AR): therapeutic target and predictive biological marker in rheumatoid arthritis. Clin Rheumatol 2016;35:2359-62.
[66] Ravani A, Vincenzi F, Bortoluzzi A, Padovan M, Pasquini S, Gessi S, et al. Role and Function of A(2A) and A(3) Adenosine Receptors in Patients with Ankylosing Spondylitis, Psoriatic Arthritis and Rheumatoid Arthritis. Int J Mol Sci 2017;18:697.
[67] Bar-Yehuda S, Stemmer SM, Madi L, Castel D, Ochaion A, Cohen S, et al. The A(3) adenosine receptor agonist CF102 induces apoptosis of hepatocellular carcinoma via de-regulation of the Wnt and NF-kappa B signal transduction pathways. Int J Oncol 2008;33:287-95.
[68] Tosh DK, Finley A, Paoletta S, Moss SM, Gao ZG, Gizewski ET, et al. In vivo phenotypic screening for treating chronic neuropathic pain: modification of C2-arylethynyl group of conformationally constrained A3 adenosine receptor agonists. J Med Chem 2014;57:9901-14.
[69] Tosh DK, Padia J, Salvemini D, Jacobson KA. Efficient, large-scale synthesis and preclinical studies of MRS5698, a highly selective A3 adenosine receptor agonist that protects against chronic neuropathic pain. Purinerg Signal 2015;11:371-87.
[70] Lau OC, Samarawickrama C, Skalicky SE. P2Y2 receptor agonists for the treatment of dry eye disease: a review. Clin Ophthalmol 2014;8:327.
[71] Fajac I, De Boeck K. New horizons for cystic fibrosis treatment. Pharmacol Therapeut 2017;170:205-11.
[72] Kellerman D, Evans R, Mathews D, Shaffer C. Inhaled P2Y(2) receptor agonists as a treatment for patients with Cystic Fibrosis lung disease. Adv Drug Deliver Rev 2002;54:1463-74.
[73] Yerxa BR, Sabater JR, Davis CW, Stutts MJ, Lang-Furr M, Picher M, et al. Pharmacology of INS37217[P-1-(uridine 5 ')-P-4-(2 '-deoxycytidine 5 ')tetraphosphate, tetrasodium salt], a next-generation P2Y(2) receptor agonist for the treatment of cystic fibrosis. J Pharmacol Exp Ther 2002;302:871-80.
[74] Deterding R, Retsch-Bogart G, Milgram L, Gibson R, Daines C, Zeitlin PL, et al. Safety and tolerability of denufosol tetrasodium inhalation solution, a novel P2Y(2) receptor agonist: Results of a phase 1/phase 2 multicenter study in mild to moderate cystic fibrosis. Pediatr Pulm 2005;39:339-48.
[75] Deterding RR, LaVange LM, Engels JM, Mathews DW, Coquillette SJ, Brody AS, et al. Phase 2 randomized safety and efficacy trial of nebulized denufosol tetrasodium in cystic fibrosis. Am J Resp Crit Care 2007;176:362-9.
[76] Moss RB. Pitfalls of Drug Development: Lessons Learned from Trials of Denufosol in Cystic Fibrosis. J Pediatr-Us 2013;162:676-80.

[77] Smith C, Strain WD. Case study in drug discovery and development: When it doesn't go to plan Denufosol for the treatment of cystic fibrosis. Int J Pharma Res Rev 2016;5:29-33.
[78] Pelleg A, Schulman ES, Barnes PJ. Extracellular Adenosine 5 '-Triphosphate in Obstructive Airway Diseases. Chest 2016;150:908-15.
[79] de Diego-Garcia L, Ramirez-Escudero M, Sebastian-Serrano A, Diaz-Hernandez JI, Pintor J, Lucas JJ, et al. Regulation of proteasome activity by P2Y2 receptor underlies the neuroprotective effects of extracellular nucleotides. Biochim Biophys Acta Mol Basis Dis 2017;1863:43-51.
[80] Burnstock G. Purinergic Signalling: Therapeutic Developments. Front Pharmacol 2017;8:661.
[81] Burnstock G. The therapeutic potential of purinergic signalling. Biochem Pharmacol 2018;151:157-65.
[82] Woods LT, Ajit D, Camden JM, Erb L, Weisman GA. Purinergic receptors as potential therapeutic targets in Alzheimer's disease. Neuropharmacology 2016;104:169-79.
[83] Camden JM, Schrader AM, Camden RE, Gonzalez FA, Erb L, Seye CI, et al. P2Y(2) nucleotide receptors enhance alpha-secretase-dependent amyloid precursor protein processing. J Biol Chem 2005;280:18696-702.
[84] Chadet S, Jelassi B, Wannous R, Angoulvant D, Chevalier S, Besson P, et al. The activation of P2Y(2) receptors increases MCF-7 breast cancer cells migration through the MEK-ERK1/2 signalling pathway. Carcinogenesis 2014;35:1238-47.
[85] Di Virgilio F. Purines, purinergic receptors, and cancer. Cancer Res 2012;72:5441-7.
[86] White N, Burnstock G. P2 receptors and cancer. Trends in Pharmacological Sciences 2006;27:211-7.
[87] Ciancetta A, Jacobson KA. Structural Probing and Molecular Modeling of the A(3) Adenosine Receptor: A Focus on Agonist Binding. Molecules 2017;22:449.
[88] Ciancetta A, Jacobson KA. Breakthrough in GPCR Crystallography and Its Impact on Computer-Aided Drug Design. Methods Mol Biol 2018;1705:45-72.
[89] Jacobson KA, Costanzi S. New Insights for Drug Design from the X-Ray Crystallographic Structures of G-Protein-Coupled Receptors. Mol Pharmacol 2012;82:361-71.
[90] Smith JS, Lefkowitz RJ, Rajagopal S. Biased signalling: from simple switches to allosteric microprocessors. Nat Rev Drug Discov 2018;17:243-60.
[91] Shukla AK, Singh G, Ghosh E. Emerging structural insights into biased GPCR signaling. Trends Biochem Sci 2014;39:594-602.
[92] Porter-Stransky KA, Weinshenker D. Arresting the Development of Addiction: The Role of beta-Arrestin 2 in Drug Abuse. J Pharmacol Exp Ther 2017;361:341-8.
[93] Cannaert A, Storme J, Franz F, Auwarter V, Stove CP. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. Anal Chem 2016;88:11476-85.
[94] Cannaert A, Franz F, Auwärter V, Stove CP. Activity-based detection of consumption of synthetic cannabinoids in authentic urine samples using a stable cannabinoid reporter system. Anal Chem 2017;89:9527-36.

[95] Cannaert A, Vasudevan L, Friscia M, Mohr ALA, Wille SMR, Stove CP. Activity-Based Concept to Screen Biological Matrices for Opiates and (Synthetic) Opioids. Clin Chem 2018;64:1221-9.
[96] Cannaert A, Ambach L, Blanckaert P, Stove CP. Activity-Based Detection and Bioanalytical Confirmation of a Fatal Carfentanil Intoxication. Front Pharmacol 2018;9:486.
[97] Cannaert A, Storme J, Hess C, Auwärter V, Wille SM, Stove CP. Activity-Based Detection of Cannabinoids in Serum and Plasma Samples. Clinical chemistry 2018:clinchem. 2017.285361.
[98] Gyombolai P, Toth AD, Timar D, Turu G, Hunyady L. Mutations in the 'DRY' motif of the CB1 cannabinoid receptor result in biased receptor variants. J Mol Endocrinol 2015;54:75-89.
[99] Winpenny D, Clark M, Cawkill D. Biased ligand quantification in drug discovery: from theory to high throughput screening to identify new biased opioid receptor agonists. Brit J Pharmacol 2016;173:1393-403.
[100] Luttrell LM, Maudsley S, Bohn LM. Fulfilling the Promise of "Biased" G Protein-Coupled Receptor Agonism. Mol Pharmacol 2015;88:579-88.
[101] Michel MC, Charlton SJ. Biased Agonism in Drug Discovery-Is It Too Soon to Choose a Path? Mol Pharmacol 2018;93:259-65.
[102] Kenakin T. Is the Quest for Signaling Bias Worth the Effort? Mol Pharmacol 2018;93:266-9.
[103] van Unen J, Woolard J, Rinken A, Hoffmann C, Hill S, Goedhart J, et al. A perspective on studying GPCR signaling with RET biosensors in living organisms. Mol Pharmacol 2015:mol. 115.098897.
[104] Berg KA, Clarke WP. Making Sense of Pharmacology: Inverse Agonism and Functional Selectivity. The international journal of neuropsychopharmacology 2018;21:962-77.

Conclusion & Summary of this thesis

G protein-coupled receptors (GPCRs) represent the largest class of membrane proteins in the human body and are the target of approximately one third of drugs available on the drug market today. For decades, GPCR activation has been looked at as simple as the on/off-switching of intracellular signalling via G proteins, driven by the binding of ligands to the GPCR, e.g. hormones, neurotransmitters or exogenous drug molecules. However, since the mid-nineties, this paradigm has totally been changed and evolved towards a complex, dynamic process in which signalling can occur towards multiple intracellular signalling pathways. A pathway that has achieved utmost attention, not only for its role in ending and thereby controlling GPCR signalling, but also as a signalling protein as such, is the one mediated by (β-) arrestin. An important finding regarding the multiple signalling pathways triggered by GPCRs is that they can be activated to a different extent by different GPCR ligands, a concept that is referred to as 'biased signalling' or 'functional selectivity'. By means of this biased signalling, a beneficial therapeutic profile might be obtained that is characterized by less or even no side effects. An open view on this is given in introductory **Chapter 1** of this thesis.

The nucleoside adenosine and nucleotides UTP, UDP, ATP, and ADP are the endogenous ligands of the P1 and P2Y receptor subclasses of purinergic GPCRs, respectively. In the human body, their levels are orchestrated by physiologic conditions (stress, inflammation) as well as pathologic disorders. Out of these two purinergic GPCR classes, two GPCR subtypes were chosen, the A3 adenosine receptor (A_3AR) belonging to the P1 receptor class, and the P2Y2 receptor (P2Y2R) belonging to the P2Y receptor class, for elucidation of their coupling properties with βarr2. These receptors represent interesting therapeutic targets for inflammatory, neurodegenerative as well as neoplastic pathological conditions, with several newly developed ligands now entering and/or progressing in clinical trials. Nevertheless, biased signalling for these receptors, and for the purinergic GPCR class in general, has long remained in the dark and is only now being explored; an overview can be found in **Chapter 2**.

The molecular characteristics of the A_3AR and P2Y2R coupling to βarr2 were explored in **Chapters 3 and 5**, respectively, using a live-cell, real-time reporter assay in HEK293T cells that is based on the functional complementation of the split

NanoLuc luciferase enzyme, developed using the NanoBit® technology (Promega). In this assay, a big part of NanoLuc is attached to the receptor and a complementary small peptide is attached to βarr2. Upon βarr2 recruitment to the GPCR, the two parts reconstitute the functional enzyme and bioluminescence can be observed by addition of a cell-permeable substrate. For both the A_3AR and P2Y2R, it was shown that the C-terminus is dispensable for βarr2 recruitment, although differences in kinetic profile were revealed upon truncation of the P2Y2R. For the A_3AR, the 'DRY' motif (located at the boundary of TM3 and IL2) seemed to have a role in the coupling, together with the C-terminus.

In **Chapter 4**, an A_3AR ligand panel of synthetic adenosine derivatives was tested in a stable form of the reporter assay (stable HEK293T cell line). Profiles of βarr2-activity were compared to profiles of G protein activity using results from a cAMP accumulation assay. Although there were ligands with a pronounced bias towards one of the examined signalling pathways, it was found that stable bicyclic adenosine derivatives represent a good starting point for further design of highly A_3AR-selective and potent compounds in the G protein as well as the βarr pathway, and a good back-bone for fine-tuning modifications towards G protein or βarr activity.

For many GPCR systems, there are high hopes for clinically promising biased ligands (see **Chapter 6**). As of today, no consensus has been found in the coupling characteristics of βarr to GPCRs, each GPCR system should be looked at and evaluated separately. Emphasis has mostly been on the role of the phosphorylated C-terminus for recruitment, contact and/or activation of arrestins. The question however is whether we should *go with the flow* or also investigate other contact sites at the (cytosolic) site of the GPCR. Molecular modelling techniques, relying on structural computer models, *in vivo* (knock-out) animal models, as well as fundamental functional GPCR studies should be combined, to clear the path towards the development of biased ligands with a hopefully beneficial therapeutic profile, devoid of or with reduced side effects. However, for many GPCR systems, as is the case for purinergic GPCRs, it is still not clear which turn to take to end up with a clinically relevant profile. The work within this thesis can be seen in this context, aiming to have contributed a piece to the large and complex puzzle of biased signalling and functional selectivity.

Samenvatting

G proteïne-gekoppelde receptoren (GPCRs) zijn de grootste klasse van membraaneiwitten aanwezig in het menselijk lichaam. Deze receptoren zijn een therapeutisch doelwit voor ruim één derde van de vandaag op de markt beschikbare geneesmiddelen. Sinds jaar en dag werd de signalisatie via deze receptoren gezien als een soort sleutel-slot mechanisme, waarbij de binding van liganden - zoals hormonen, neurotransmitters of exogene geneesmiddelmolecules - de koppeling met en signalisatie via het zogenaamde G proteïne initieert. Sinds de jaren ’90 echter, hebben nieuwe inzichten deze visie zodanig bijgestuurd dat GPCR signalisatie tegenwoordig aanzien wordt als een complexer, dynamischer gebeuren, waarbij naast koppeling met G proteïnes ook koppeling met andere cytosolische adaptor-proteïnes kan voorkomen. Tot de meest bestudeerde signalisatie-proteïnes behoren ongetwijfeld de (β-)arrestines - waarin niet toevallig de term ‘arrest’ onthuld wordt, gezien ze de GPCR signalisatie begrenzen. Elk van de verschillende types signalisatie-proteïnes (of dus signalisatie-wegen) kunnen in verschillende mate geactiveerd worden na binding van een ligand met de GPCR, zodat een differentieel signalisatieprofiel ontstaat dat gelinkt is aan deze specifieke ligand. Zo zal de signalisatie uitgaande van een bepaald (exogeen) ligand anders zijn - dus een bepaalde mate van ‘bias’ bezitten - dan deze veroorzaakt door een ander (bijvoorbeeld endogeen) ligand van de GPCR. Dit concept is een relatief iets en wordt aangeduid met de term ‘*biased* signalisatie’ of ‘functionele selectiviteit’. Onder invloed van deze differentiële (of ‘*biased*’) signalisatie ter hoogte van de GPCR, wordt gehoopt een farmacologisch-therapeutisch profiel te bekomen dat op selectieve wijze (het) gewenste therapeutisch(e) effect(en) verwezenlijkt, terwijl neveneffecten afgezwakt of in het beste geval vermeden worden. Hoe de koppeling van GPCRs met het G proteïne en/of arrestine kan bijdragen tot dit fenomeen en welk onderzoek hier reeds naar verricht werd, wordt in detail besproken in **Hoofdstuk 1**.

Dit fundamentele onderzoeksproject heeft zijn focus op de koppeling van purinerge GPCRs met β-arrestine 2 (βarr2). Het nucleoside adenosine en de nucleotides UTP, UDP, ATP, en ADP zijn de endogene liganden van de purinerge P1 (of adenosine) en P2Y receptoren. In het lichaam kunnen de concentraties van deze molecules sterk toenemen onder (patho)fysiologische condities zoals stress, inflammatie, ischemie en/of hypoxie. Uit deze twee purinerge receptor subklasses werden twee

receptoren gekozen, de A_3 adenosine receptor (A_3AR) en de P2Y2 receptor (P2Y2R), waarvoor de koppelingskarakteristieken met βarr2 onderzocht werden. Beide GPCRs zijn betrokken in de pathofysiologie van inflammatoire, neurodegeneratieve alsook neoplastische aandoeningen; de laatste tien jaar verschenen dan ook verschillende purinerge '*lead compounds*' die momenteel verder geëvalueerd worden in klinische studies of reeds hun intrede deden in de klinische setting. Het onderzoek naar de mogelijks differentiële signalisatie van deze en toekomstige purinerge liganden staat nog in de kinderschoenen maar lijkt een snelgroeiende, veelbelovende onderzoekstak naar een toekomstig therapeutisch, efficiënt en bovenal veilig purinerg geneesmiddelengebruik. Een overzicht wordt gegeven in **Hoofdstuk 2**.

Het doel van **Hoofdstuk 3** en **Hoofdstuk 5** was na te gaan welke cytoplasmatische delen van de A_3AR en de P2Y2R, respectievelijk, verantwoordelijk zijn voor koppeling van de receptor met βarr2. Hiertoe werd een reporter assay in HEK293T cellen ontwikkeld, gebaseerd op de functionele complementatie van het nanoluciferase enzym, gebruik makend van de NanoBit® technologie (Promega). In deze assay is een groot deel van het nanoluciferase gekoppeld aan de receptor en een complementair klein peptide aan βarr2. Bij rekrutering van βarr2 naar de GPCR, reconstitueren de twee delen opnieuw het functionele enzym en kan een bioluminescent signaal gemonitord worden na toevoeging van een celpermeabel substraat. Voor zowel de A_3AR als de P2Y2R werd aangetoond dat de C-terminus niet cruciaal is voor rekrutering van βarr2 naar de receptor, alhoewel het verwijderen van de C-terminus van de P2Y2R een verschillend kinetisch profiel onthulde. Voor de A_3AR leek het 'DRY' motief, in combinatie met de C-terminus, meer cruciaal voor koppeling met βarr2.

In **Hoofdstuk 4** werd een panel synthetische adenosine derivaten getest met dezelfde hierboven vermelde reporter assay, dit keer in een stabiele HEK293T cellijn. De profielen van βarr2-activatie werden vergeleken met deze van G proteïne-activatie, gebaseerd op de resultaten van een cAMP accumulatie assay. Hoewel geen grote verschillen werden opgemerkt in de activatie-profielen, bleek een subgroep van bicyclische adenosine derivaten een goed uitgangspunt te zijn voor toekomstig design van hoog-selectieve, potente liganden voor de A_3AR, welke dan

stapsgewijs gemodificeerd kunnen worden door het invoeren van specifieke functionele groepen, om zo een meer selectieve activatie van βarr- of G proteïne te verkrijgen.

Voor verschillende andere GPCRs, zoals de angiotensine II type 1 receptor en de μ-opioid receptor, werden reeds '*biased* liganden' ontwikkeld waarvoor een differentieel signalisatieprofiel aanleiding geeft tot klinisch gunstige, nauw therapeutisch afgelijnde effecten voor de behandeling van verschillende aandoeningen (zie **Hoofdstuk 6**). Gezien tot op heden geen consensus werd gevonden wat betreft GPCR-βarr koppelingskarakteristieken, dient elk GPCR systeem voorlopig zoveel mogelijk afzonderlijk benaderd te worden. Tot op heden werd de nadruk vooral gelegd op de rol van de gefosforyleerde C-terminus voor βarr rekrutering, -contact en/of -activatie. De vraag is echter of dit meer in detail moet bekeken worden, dan wel of er dient nagegaan te worden of er ook bijkomende (cytosolische) contactpunten met de GPCR van belang kunnen zijn. Modelleertechnieken die zich baseren op computer-gegenereerde GPCR structuren, *in vivo* (knock-out) diermodellen, alsook fundamentele, functionele GPCR platformen zoals de assay voorgesteld in deze thesis, kunnen hiertoe bijdragen. In deze context trachtte het werk voorgesteld in deze thesis een stukje bij te dragen aan de grote en complexe puzzel van functionele selectiviteit en '*biased'* (purinerge) liganden met een gunstig therapeutisch profiel.