BIOMOLECULES & THERAPEUTICS

Invited Review
Biomol Ther 25(1), 12-25 (2017)

Biased G Protein-Coupled Receptor Signaling: New Player in Modulating Physiology and Pathology

Zuzana Bologna[1], Jian-peng Teoh[1], Ahmed S. Bayoumi[1], Yaoliang Tang[1] and Il-man Kim[1,2,*]

[1]Vascular Biology Center, Medical College of Georgia, [2]Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta University, GA 30912, USA

Abstract

G protein-coupled receptors (GPCRs) are a family of cell-surface proteins that play critical roles in regulating a variety of pathophysiological processes and thus are targeted by almost a third of currently available therapeutics. It was originally thought that GPCRs convert extracellular stimuli into intracellular signals through activating G proteins, whereas β-arrestins have important roles in internalization and desensitization of the receptor. Over the past decade, several novel functional aspects of β-arrestins in regulating GPCR signaling have been discovered. These previously unanticipated roles of β-arrestins to act as signal transducers and mediators of G protein-independent signaling have led to the concept of biased agonism. Biased GPCR ligands are able to engage with their target receptors in a manner that preferentially activates only G protein- or β-arrestin-mediated downstream signaling. This offers the potential for next generation drugs with high selectivity to therapeutically relevant GPCR signaling pathways. In this review, we provide a summary of the recent studies highlighting G protein- or β-arrestin-biased GPCR signaling and the effects of biased ligands on disease pathogenesis and regulation.

Key Words: β-arrestin, biased signaling, G protein-coupled receptor, G protein

INTRODUCTION

G protein-coupled receptors (GPCRs) represent the largest family of cell surface molecules involved in signal transduction. More than 1000 receptors for sensory (e.g., odor and light) and chemical stimuli (e.g., catecholamines, amino acids, peptides, and ions) have been identified based on their common structural and biochemical properties (Muller, 2000). Because GPCRs represent 1-5% of the total cell surface proteins in mammals, it is not surprising that nearly 30% of United States Food and Drug Administration (FDA)-approved drugs target GPCRs (Overington *et al*., 2006).

Historically, GPCRs were assumed to exist in equilibrium between active and inactive states, and thus activation of GPCRs would equally affect all downstream signaling pathways. However, accumulating evidences indicate that GPCRs exist in multiple conformational states where each conformation confers different downstream effects. In this context, some ligands are able to induce a differential receptor conformation which activates a different subset of signaling events, causing bias receptor signaling (Liu *et al*., 2012). Biased GPCR signaling has been mainly studied on adrenergic and angiotensin receptors, but accumulating evidences indicate that this phenomenon may be extended to a wide variety of GPCRs currently targeted by pharmacological agents. This notion further complicates drug discovery efforts, but also holds the promise to design specific biased ligands that antagonize detrimental signaling pathways while stimulating beneficial downstream processes. Here, we seek to summarize the current knowledge of biased signaling on GPCRs and to discuss how identified biased ligands of selected receptors modulate disease outcomes.

ACTIVATION OF GPCRs

GPCRs are often referred to as seven-transmembrane receptors (7TMRs) because their structures are characterized by the presence of seven α-helices crossing the plasma membrane. GPCRs are consisted of intracellular and extracellular loops. The NH_2 terminus is exposed to the extracellular environment and the COOH terminus is located in the

Open Access **https://doi.org/10.4062/biomolther.2016.165**

Received Jul 30, 2016 **Revised** Aug 19, 2016 **Accepted** Aug 23, 2016
Published Online Jan 1, 2017

***Corresponding Author**
E-mail: ILKIM@augusta.edu
Tel: +1-706-721-9414, Fax: +1-706-721-9799

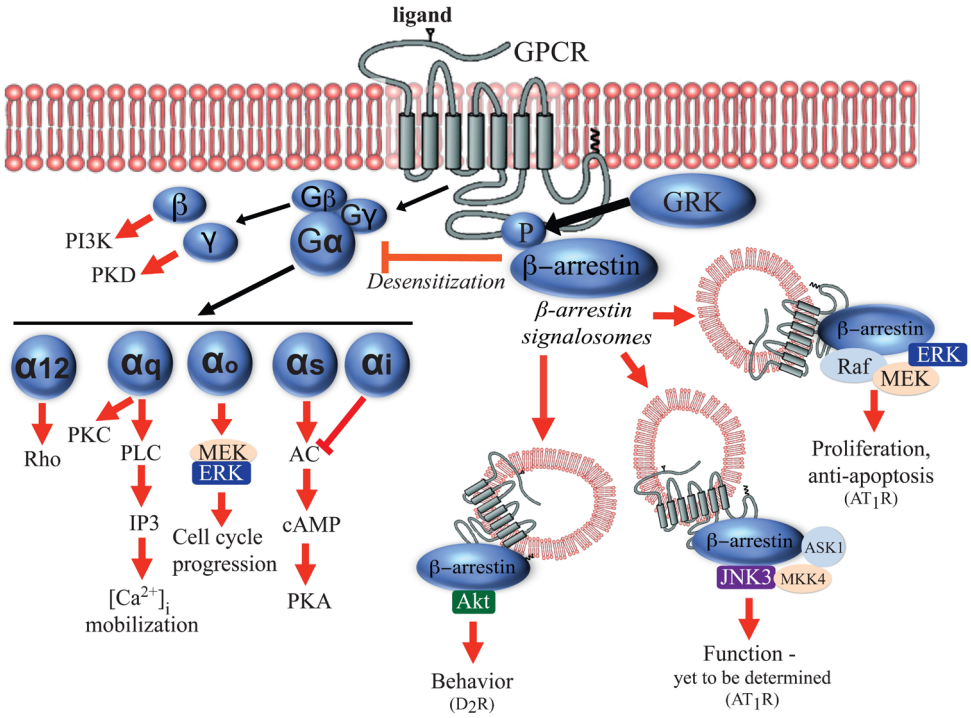

Fig. 1. Examples of G protein- and β-arrestin-mediated downstream signaling pathways on GPCRs. Upon agonist binding to GPCRs, both G proteins ($G\alpha_{12}$, $G\alpha_{q/11}$, $G\alpha_{i/o}$, $G\alpha_s$, Gβ and Gγ subunits) and β-arrestin are activated to mediate a variety of distinct downstream signaling pathways. Stimulation of Gβ subunit can activate PI3Kγ and Gγ subunit can activate PKD. $G\alpha_{12}$ can activate Rho kinase signaling pathways and $G\alpha_q$ can induce the mobilization of calcium from intracellular stores through activation of PLC/IP3. $G\alpha_o$ signaling activates MEK/ERK pathway to mediate cell cycle progression. $G\alpha_s$ proteins promote AC-induced PKA activation. Phosphorylation of GPCRs by GRK results in the recruitment of β-arrestin, which in turn desensitizes G protein signaling, mediates receptor trafficking to endosomes, and activates β-arrestin-dependent signaling.

intracellular part. The intracellular domains and loops mediate the interaction between the receptor and intracellular signaling partners such as G proteins (Gether, 2000; Hermans, 2003). The binding of exogenous ligands alters the conformation of critical domains of the seven-transmembrane helix pocket, which in turn causes the conformation changes of intracellular domains of the receptor. These changes promote the association of the receptor with a variety of heterotrimeric G proteins. They are composed of an α-subunit interacting with a βγ complex. Activation of the receptor promotes the exchange of a molecule of GDP by a molecule of GTP within the active site of the α-subunit. The binding of GTP to α-subunit causes the dissociation of the heterotrimeric complex, and both the GTP-bound α-subunit and the released βγ complex are then able to interact with intracellular or membrane effectors (e.g., enzymes or ion channels). The intrinsic GTPase activity of the α-subunit hydrolyses GTP into GDP, restoring its initial inactive conformation and its affinity for the βγ complex [for detailed reviews, see (Wess, 1997; Bockaert and Pin, 1999; Gether, 2000; Hermans, 2003)]. Up to now, at least 23 α-subunits derived from 17 different genes have been identified and are classified into four families ($G\alpha_{i/o}$, $G\alpha_s$, $G\alpha_{q/11}$, and $G\alpha_{12}$). At least 6 different β-subunits and 12 γ-subunits have been also discovered (Gautam *et al*., 1998; Vanderbeld and Kelly, 2000). Upon dissociation from the heterotrimeric complex, the various Gα subunits interact with the well-studied and classical effector enzymes in a highly specific manner. For instance, $G\alpha_s$ activates (and $G\alpha_i$ inhibits) adenylyl cyclase (AC), $G\alpha_t$ activates photoreceptor cGMP phosphodiesterase (PDE), and $G\alpha_q$ activates phospholipase C (PLC)-β (Skiba *et al*., 1996; Hamm, 1998). On the other hand, various Gβ subunits can activate or deactivate AC, activate PLCs or phosphatidylinositol 3-kinase (PI3K). Gγ subunits can activate various kinases including protein kinase D (PKD) (Morris and Malbon, 1999; Vanderbeld and Kelly, 2000) (Fig. 1).

In addition to signaling through G proteins, GPCRs can also activate G protein-independent signaling pathways mainly through multi-functional adaptor proteins called arrestins. The arrestins are a small family of proteins originally discovered in the visual system. Arrestins, which include arrestin-1 and -4 (expressed in retinal rods and cones) and ubiquitously expressed arrestin-2 (β-arrestin1) and arrestin-3 (β-arrestin2), were initially characterized for their roles in GPCR desensitization (uncoupling of the G protein from the cognate receptor) (Shukla *et al*., 2011; Lefkowitz, 2013). Homologous desensitization is initiated by stimulation of the receptor with high concentrations of its agonist, resulting in a change in the receptor conformation to its active state. G protein-coupled receptor kinases (GRKs) can then phosphorylate the specific serine/threonine residues at C-terminus or intracellular loops of the activated receptor, which increases the affinity of β-arrestin for the receptor, thus resulting in the uncoupling of the Gα subunit from the receptor. By interacting with components of the endocytic machinery such as clathrin and the adaptor pro-

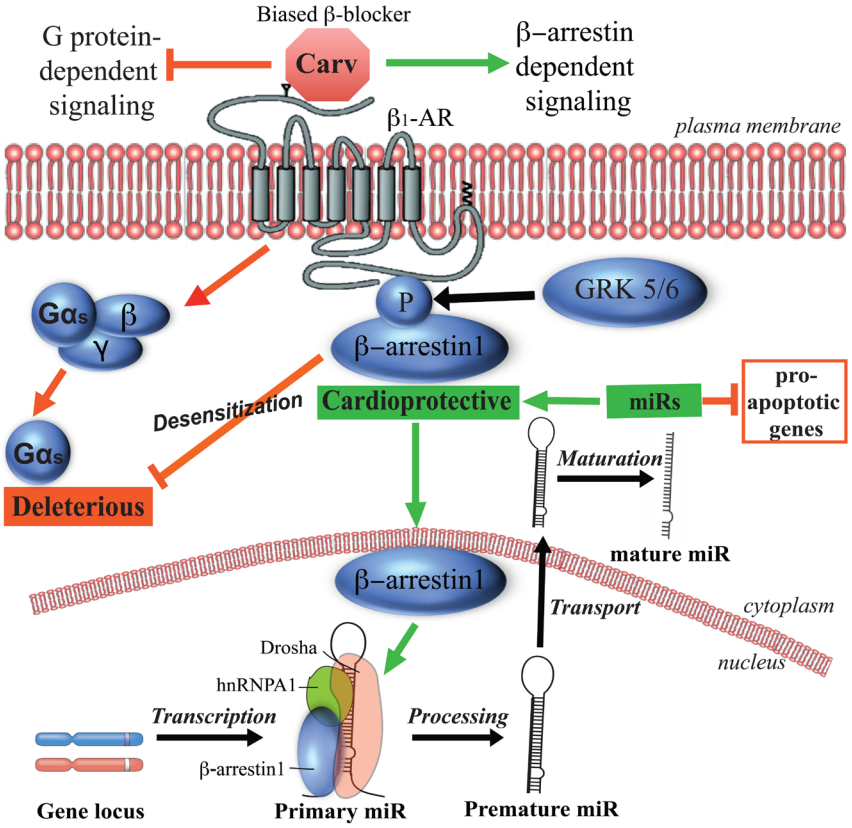

Fig. 2. Carvedilol-mediated β-arrestin biased signaling on β_1-adrenergic receptors in cardiomyocytes and hearts. Carvedilol selectively stimulates GRK5/6- and β-arrestin-dependent cardioprotective signaling without activating deleterious G protein signaling. Carvedilol-mediated GRK5/6 phosphorylation of β_1-adrenergic receptors leads to β-arrestin1's translocation into nucleus where β-arrestin1 interacts with a subset of primary miRs and components of the Drosha microprocessor complex. This results in an increased level of a subset of miRs, which act as cardioprotective miRs by repressing pro-apoptotic genes in cardiomyocytes and hearts.

tein 2 (AP2) complex, β-arrestins also target the GPCRs for clathrin-mediated endocytosis and internalization (Lefkowitz, 1998; Ferguson, 2001). In addition to receptor endocytosis, β-arrestins were found to play prominent roles in signaling, trafficking, and ubiquitination of receptors (Shenoy *et al*., 2001). It has been shown that internalized receptor-β-arrestin complexes can form a scaffold for mitogen-activated protein kinases (MAPKs) including ERK1/2, p38 and c-Jun N terminal kinase-3 (JNK3) to generate signalosomes, which may mediate long-lasting cell signaling in the cytosol (Luttrell *et al*., 1999; McDonald *et al*., 2000; Gong *et al*., 2008; Song *et al*., 2009). β-Arrestins have been also reported to scaffold AKT, PI3K and PDE4 in the context of various specific receptors both *in vitro* and *in vivo* [reviewed in (DeWire *et al*., 2007)]. Lastly, β-arrestin1 has been recently proposed to mediate nuclear signaling such as microRNA (miR) processing after activation of β_1-adrenergic receptors (β_1-ARs) by biased ligands [(Kim *et al*., 2014), see chapter 3.1]. The examples of G protein- and β-arrestin-mediated downstream signaling pathways on GPCRs are summarized in Fig. 1 and 2.

MOLECULAR BASIS OF BIASED GPCR SIGNALING

As our understanding on *in vivo* effects of GPCR-targeting drugs becomes more profound, it has become clear that therapeutic strategies often require the modulation of a single specific downstream signaling pathway (i.e. G protein- v.s. β-arrestin-mediated signaling) in a given cell type or tissue. Most drugs targeting GPCRs often lack the specificity in this regard, and as such produce undesirable side effects. In recent years, there have been significant efforts to develop ligands that preferentially activate one beneficial GPCR signaling pathway, not another detrimental pathway. These functionally selective biased ligands have demonstrated the potential to use as novel therapies and have opened new possibilities in GPCR drug discovery (Rankovic *et al*., 2016).

Although the detailed molecular mechanism of biased signaling is not yet well understood, it has been reported that biased GPCR ligands induce a unique receptor conformation, activating a particular signaling pathway. It is understood that the GPCR conformation stabilized by a G protein-biased ligand is distinct from the conformation stabilized by a β-arrestin-biased ligand. For example, a fluorescence-based study on activation of the arginine-vasopressin type 2 receptor by biased and unbiased ligands provided an interesting experimental notion, suggesting that the transmembrane helix 6 (TM6) and third intracellular loop at the receptor are associated with selective G protein signaling, whereas the TM7 and helix 8 (H8) regions at the receptor are required for selective

Table 1. Overview of biased ligands on selected GPCRs

Receptor	Biased ligand	Pathway	Therapeutic area	References
Angiotensin I receptor	SII TRV120027	β-arrestin	Cardiovascular & Renal	Ahn *et al*., 2009; Boerrigter *et al*., 2011; Violin *et al*., 2010
Apelin receptor	MM07	$G\alpha_i$	Cardiovascular	Brame *et al*., 2015
Arginine- vasopressin V2 receptor	MCF14 MCF18 MCF57	$G\alpha_s$	Renal	Jean-Alphonse *et al*., 2009
β_1-adrenergic receptor	Carvedilol Alprenolol	β-arrestin	Cardiovascular	Kim *et al*., 2008, 2014
β_2-adrenergic receptor	Fenoterol Carvedilol	$G\alpha_s$ β-arrestin	Pulmonary Cardiovascular	Woo *et al*., 2009; Xiao *et al*., 2003 Wisler *et al*., 2007
Dopamine D1 receptor	SKF83959 SKF38393 SKF82957 SKF75670	$G\alpha_q$	Neurology & Behavior	Conroy *et al*., 2015; Rashid *et al*., 2007
Dopamine D2 receptor	UNC9975 UNC9994	β-arrestin2	Neurology & Behavior	Chen *et al*., 2012; Park *et al*., 2016
	MLS1547	$G\alpha_{i/o}$	Neurology & Behavior	Free *et al*., 2014
Histamine H_2 receptor	Famotidine	$G\alpha_s$	Gastrointestinal	Alonso *et al*., 2015
Histamine H_4 receptor	JNJ7777120	β-arrestin2	Inflammatory	Rosethorne and Charlton, 2011
κ-Opioid receptor (KOR)	Isoquinoline 2.1 6'-GNTI RB-64	$G\alpha_{i/o}$	Neurology & Behavior	Zhou *et al*., 2013 Rives *et al*., 2012 White *et al*., 2015
μ-Opioid receptor (MOR)	TRV130 NAP	$G\alpha_{i/o}$ $G\alpha_{i/o}$	Neurology & Behavior Neurology & Behavior	Chen *et al*., 2013; DeWire *et al*., 2013 Zhang *et al*., 2016
Serotonin 5-HT_{2B} receptor	Ergotamin	β-arrestin	Neurology & Behavior	Wacker *et al*., 2013

β-arrestin recruitment (Rahmeh *et al*., 2012).

Another recent study used site-specific fluorine-19 nuclear magnetic resonance (19F-NMR) labels in the β_2-AR to unveil conformational changes of the receptor upon activation by biased and unbiased ligands. It was shown that unbiased ligand's binding to the receptor primarily shifts the equilibrium toward the G protein-specific active state of helix 6, while β-arrestin-biased ligands predominantly regulate the conformational states of helix 7 (Liu *et al*., 2012). Also, Woo *et al*. (2014) showed that phosphorylation of the receptor itself does not necessarily lead to a switching of the receptor coupling to different G proteins as once proposed (Daaka *et al*., 1997). It was also shown that the biased ligand-mediated signaling depends on the specific interaction between the ligand and the β_2-AR's tyrosine 308 residue positioned on transmembrane helix 7 (Woo *et al*., 2014). Despite these advances in biased signaling field, further structural studies are needed to unravel how the interaction between GPCR and ligand translates into the receptor conformation for selective coupling to different G proteins and β-arrestins. Such progress would move us closer to structure-based design of drugs specific to a particular signaling pathway.

It is well appreciated that the G protein-independent effector on GPCRs, β-arrestin, is recruited upon phosphorylation of GPCRs by GRKs specifically on their C terminal and intracellular loops. An interesting "barcode" hypothesis suggests that different GRKs phosphorylate distinct sites on the C terminus and internal loops of the receptor, thereby establishing a "barcode" that would instruct or determine the conformation for different β-arrestin functions. This would in turn determine the differential roles of GRKs and β-arrestins (Butcher *et al*., 2011). A detailed mapping of the β_2AR phosphorylation by GRKs supported this hypothesis by demonstrating that the β-arrestin-biased ligand carvedilol recruited different GRKs and induced a different phosphorylation pattern from that of a full agonist isoproterenol (Nobles *et al*., 2011). The experimental evidence also indicates that the biased ligands can stabilize both the receptor and β-arrestin in conformations that are distinct from those associated with unbiased ligands. For example, Shukla *et al*. (2008) used an intramolecular bioluminescence resonance energy transfer (BRET)-based biosensor of β-arrestin2 and a combination of biased ligands and/or biased mutants of three different GPCRs to show that β-arrestin can adopt multiple "active" conformations. These findings suggest the possibility that multiple distinct β-arrestin conformations can form various complexes with different binding partners, and thereby engage in different downstream signaling pathways.

Another interesting phenomenon in biased signaling observed for multiple GPCRs *in vitro* is the ability of biased ligands to recruit different subtypes of β-arrestins within a single receptor. Such differential involvement of β-arrestins can determine ligands' downstream effects on receptor such as trafficking, ubiquitination, or signaling. For example, it is known that the recruitment of β-arrestin1 and 2 can mediate internalization of mu-opioid receptor (MOR). Interestingly, morphine, an agonist with the low rate of MOR internalization primarily recruits β-arrestin2, whereas another agonist DAMGO can recruit either β-arrestin1 or 2, which leads to the

high level of receptor internalization. DAMGO was also shown to induce receptor ubiquitination in a β-arrestin1-dependent manner (Groer *et al*., 2011). Similarly, in delta-opioid receptor (DOR), an agonist SNC80, which causes the high internalization of the receptor, was shown to preferentially recruit β-arrestin1 over β-arrestin2. In contrast, agonists ARM390 and JNJ20788560 preferentially engage with β-arrestin2, resulting in the low rate of receptor internalization (Pradhan *et al*., 2016). Endogenous ligands for the C-C chemokine receptor 7 also showed differential internalizing properties. The ligand CCL19 with high-internalizing capacity preferentially recruits β-arrestin2 over 1, whereas CCL21 with low-internalizing capacity engages with neither (Byers *et al*., 2008). In addition, binding of ATP induces the greater interaction between P2Y2 receptor and β-arrestin1, whereas UTP nonselectively recruits both β-arrestin1 and 2 (Hoffmann *et al*., 2008). These studies support a novel interesting notion that different ligands for the same receptor can form distinct receptor-arrestin complexes.

Altogether, recent advances on GPCR biased signaling field suggest that biased GPCR ligands may have an important therapeutic potential in various diseases including cardiovascular diseases, neurological diseases, and cancers. As summarized in the following chapter and Table 1, we seek to provide recent scientific progress on identifying novel biased ligands on selected highly-profiled GPCRs.

BIASED SIGNALING ON SELECTED GPCRS

β-adrenergic receptors and their biased ligands

β-adrenergic receptors (β-ARs), prototypical members of GPCR superfamily, are known for their regulation of contractile function in the heart. The stimulation of cardiac β_1- and β_2-AR by catecholamines such as adrenaline and noradrenaline activates the canonical G_s-AC-cAMP-PKA signaling cascade, which increases calcium mobilization across different cellular compartments and sensitizes contractile proteins to cytosolic calcium. The overall physiological effect of cardiac β-AR stimulation is an increase in heart contractility (inotropic effect) and heart rate (chronotropic effect) (Rodefeld *et al*., 1996). The major subtype, β_1-AR couples to the $G\alpha_s$ protein, whereas β_2-AR is able to couple to both $G\alpha_s$ and $G\alpha_i$ proteins (Kilts *et al*., 2000; Xiang and Kobilka, 2003; Perrino and Rockman, 2007). Physiologically, the inotropic response to catecholamine stimulation is mediated mainly by β_1-AR because the β_2-AR-$G\alpha_s$-mediated AC-cAMP-PKA response is inhibited by the co-activated β_2-AR-$G\alpha_i$ signaling (Xiao *et al*., 1995). However, β_2-AR can regulate the effect of β_1-AR on excitation-contraction coupling by activating $G\alpha_i$ signaling. It is also known that activation of the β_2-AR-$G\alpha_i$ signaling protects the cardiomyocytes from the pro-apoptotic stimuli of excessive β_1-AR stimulation and activates a pro-survival PI3K-Akt signaling cascade (Chesley *et al*., 2000; Zhu *et al*., 2001). However, prolonged activation of $G\alpha_i$ through a synthetic receptor construct has been shown to lead to a depressed cardiac function and eventually the development of dilated cardiomyopathy in mice (McCloskey *et al*., 2008). Switching of β_2-AR coupling from $G\alpha_s$ to $G\alpha_i$ was found to play an important role in ischemic preconditioning-induced cardioprotection in the mouse heart (Tong *et al*., 2005). In addition, the enhanced β_2-AR-$G\alpha_i$ signaling contributes to the dysfunction of both β_1-AR and β_2-AR in the failing heart (Xiao *et al*., 2003; Xiao and Balke, 2004). In 2007, Noma *et al*. showed that β_1-AR-mediated β-arrestin signaling confers cardioprotection independent on G protein-mediated second messenger signaling (Noma *et al*., 2007), bringing up a concept of biased signaling. Here, we summarize recent advances on β-AR biased signaling and highlight some of biased ligands which are used in clinic for a therapy.

Fenoterol: Racemic fenoterol is unique among the β_2-AR agonists because it has been identified as a biased β_2-AR ligand selectively coupling β_2-AR to $G\alpha_s$ protein. In the study by Xiao *et al*., (2003) dysfunction of β_2-AR but not β_1-AR in the model of failing spontaneous hypertensive rats was induced by enhanced $G\alpha_i$ signaling. Disruption of $G\alpha_i$ signaling by pertussin toxin restored the blunted β_2-AR contractile response in the failing heart. Interestingly, β_2-AR agonist fenoterol had similar beneficial effects, which were due to selective activation of β_2-AR-$G\alpha_s$ signaling (Xiao *et al*., 2003). Functional selectivity of fenoterol was shown to depend on the stoichiometry at its two chirality centers (Woo *et al*., 2009). Based on these observations, at least two other fenoterol derivatives (S, R')-4'-methoxy-fenoterol and (S, R')-4'-methoxy-1-naphthylfenoterol have been identified to selectively activate only $G\alpha_s$ signaling pathways on β_2-AR. These biased β_2-AR ligands are therapeutically used for bronchial asthma and chronic obstructive lung disease (Reinartz *et al*., 2015). These studies suggest that identifying novel $G\alpha_s$-biased β_2-AR ligands, which display a better efficacy, may provide a new therapy with the sustained efficacy by switching off β-arrestin-dependent receptor desensitization and down-regulation.

Carvedilol: Carvedilol is a well-known neurohormonal antagonist with multiple activities efficiently used in patients with congestive heart failure, stable angina pectoris (Dunn *et al*., 1997), myocardial infarction (Doughty *et al*., 2001), and myocardial ischemia-reperfusion injury (Brunvand *et al*., 1998). It blocks both the β_1- and β_2-AR, resulting in the improved myocardial function and attenuation (or reversal) of adverse myocardial remodeling in heart failure. It also reduces the peripheral vascular resistance via vasodilation caused by antagonism of α_1-AR. In addition to these well-known properties, carvedilol has a number of ancillary activities including antioxidant, anti-inflammatory, and antiapoptotic actions (Ohtsuka *et al*., 2001; Dulin and Abraham, 2004; Mochizuki *et al*., 2007). Carvedilol was shown to confer a range of cardioprotective effects, which were hypothesized to stem from its antioxidant effects and/or from direct inhibition of proapoptotic pathways (Schwarz *et al*., 2003).

More importantly, carvedilol has been identified as a biased ligand on β_1-AR and β_2-AR, which selectively stimulates GRK5/6- and β-arrestin-dependent cardioprotective signaling without activating G proteins (Wisler *et al*., 2007; Kim *et al*., 2008). Our group also showed that after stimulation of β_1-AR by carvedilol, β-arrestin1 promotes the processing of five miRs (miR-125a-5p, miR-125b-5p, miR-150, miR-199a-3p and miR-214) in murine hearts and human cells (Kim *et al*., 2014). MiRs, a class of ~22 nucleotide small noncoding RNAs governing post-transcriptional repression of target mRNAs, were found to play important roles in normal cardiac physiology including the control of myocyte growth, contractility, and maintenance of cardiac rhythm as well as the pathogenesis of various heart diseases [reviewed in (Quiat and Olson, 2013)]. The hypothesis for the mechanism by which carvedilol promotes miR processing is that carvedilol-induced GRK5/6 phosphorylation of β_1AR mediates the recruitment of β-arrestin1 to the

ligand-occupied receptor, resulting in the translocation of β-arrestin1 to the nucleus where it interacts with a subset of primary miRs and components of the Drosha microprocessor complex. Formation of a nuclear complex of β-arrestin1 with the heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) and Drosha, which are crucial nuclear RNA-binding proteins involved in miR processing (Lee *et al*., 2003; Guil and Caceres, 2007), leads to activation of RNA helicase-independent miR processing (Kim *et al*., 2014). In the mouse hearts, 7-day carvedilol infusion induced the upregulation of miR-150, miR-214, miR-125b-5p and miR-199a-3p, which were shown to be cardioprotective in the mouse models of heart failure (Salloum *et al*., 2010; Aurora *et al*., 2012; Wang *et al*., 2014; Tang *et al*., 2015). Moreover, our group also showed that 7-day infusion of carvedilol induced a unique gene signature on multiple genes related to cardiac disease. Genes upregulated by carvedilol included those encoding proteins in the tight junctions, malaria and viral myocarditis pathway, while downregulated genes were those encoding proteins in the glycosaminoglycan biosynthesis and arrhythmogenic right ventricular cardiomyopathy (Teoh *et al*., 2015). These findings make us speculate that carvedilol-responsive miRs can regulate the expression of various detrimental genes to confer cardioprotection (Fig. 2). We postulate that β_1AR-mediated β-arrestin1 biased signaling provides an additional mechanism for the clinical efficacy of this β-blocker (Table 1).

Insulin signaling: Interestingly, a recent study suggests that insulin signaling can also mimic the effects of a biased β_2AR ligand and selectively activate a $G\alpha_i$-biased signaling pathway (Fu *et al*., 2014). Insulin and adrenergic stimulation represent two divergent regulatory systems that interact with overlapping downstream signaling pathways in adipocytes, liver, and skeletal and cardiac muscle. Stimulation of insulin receptor (IR) as well as βARs increases the glucose uptake in cardiac and skeletal muscle cells (Nevzorova *et al*., 2006; Ciccarelli *et al*., 2011). In the heart, IR and β_2AR form a complex and the stimulation of both receptors shares common downstream signaling components including GRK2 (Cipolletta *et al*., 2009; Ciccarelli *et al*., 2011), $G\alpha_i$ (Song *et al*., 2001) and β-arrestin (Luan *et al*., 2009). Stimulation with either insulin or adrenergic receptors antagonizes the ability of the other to activate glucose transport (Morisco *et al*., 2006) and to modulate myocyte survival (Rane *et al*., 2010).

In both diabetes and heart failure, circulating insulin levels are chronically elevated, leading to persistent stimulation of IRs. Despite the insulin resistance of adipocytes and skeletal muscle cells, the heart retains its insulin sensitivity to activate IR signaling cascades in type 2 diabetes (Wright *et al*., 2009; Cook *et al*., 2010). Hyperactive insulin signaling was shown to significantly accelerate adverse left ventricular remodeling in pressure overload-induced hypertrophy in rodents (Shimizu *et al*., 2010). In the animal model of ischemia/reperfusion, insulin inhibited β-AR action in the hearts (Yu *et al*., 2008). It was shown that in the animal hearts, insulin could directly impair adrenergic signaling pathways for contractile function via an IR-β_2AR signaling complex. Insulin stimulation promotes crosstalk with β_2AR pathways via insulin receptor substrate (IRS) and GRK2-mediated phosphorylation of the β_2AR, which selectively activates a $G\alpha_i$-biased β_2AR signaling cascade to inhibit cAMP/PKA activities. Consequently, this IR-β_2AR crosstalk leads to impaired β-AR-induced contractile function in cardiomyocytes and perfused mouse hearts (Fu *et al*., 2014). However, these findings still need to be confirmed in humans, as it is known that diabetes is one of the risk factors for heart failure (Nichols *et al*., 2001), and heart failure is an insulin-resistant state (Cook *et al*., 2010). If the hyperinsulinemia can indeed inhibit β_1AR signaling via $G\alpha_i$-biased β_2AR signaling in humans, it is possible that hyperinsulinemic subjects with type 2 diabetes and heart failure might have increased sensitivity to the cardio-depressive effects of nonselective or β_1-blockade, which is the standard care for managing patients with heart failure (Fu *et al*., 2014).

Biased ligands on angiotensin receptors

Angiotensin II (AngII) type I receptor (AT_1R), a primary regulator of blood pressure, is a prototype GPCR in the study of biased agonism. Upon binding of its natural ligand AngII to the receptor, $G\alpha_q$ proteins are activated, resulting in intracellular inositol triphosphate (IP3) production, calcium mobilization, protein kinase C (PKC) activation, which altogether mediate the physiological effects of AT_1R such as vasoconstriction and fluid retention. Moreover, the conformational rearrangement of 7 transmembrane α helices of the receptor also leads to the recruitment of β-arrestins, which mediates G protein-independent signaling, leading to overall positive inotropic and cardioprotective effects (Ikeda *et al*., 2015). Angiotensin receptor blockers (ARBs) are clinically used for their anti-hypertensive activity, and some ARBs also show variable efficacies toward the protection against organ damage in diabetic nephropathy, cardiac hypertrophy, arrhythmia, and renal failure (Burnier and Brunner, 2000). Multiple studies suggest that such additional tissue-protective benefits of ARBs may be mediated by β-arrestin signaling (Kim *et al*., 2005; Miura *et al*., 2013), thus the development of biased ligands has resulted in a promising therapy.

SII: One of the first β-arrestin-biased ligands to be described was the peptide [Sar^1, Ile^4, Ile^8]-Ang (SII). This peptide was reported to exert anti-apoptotic cytoprotective effects in rat vascular smooth muscle cells (Ahn *et al*., 2009) and induce positive inotropic and lusitropic effects in rat primary cardiomyocytes by stimulating endogenous AT_1R-β-arrestin signaling (Rajagopal *et al*., 2006). SII was also able to activate MAPK signaling in perfused rodent hearts (Aplin *et al*., 2007).

TRV120027: Utilizing SII as a pharmacological probe *in vitro* and *ex vivo* was helpful in elucidating downstream AT_1R-mediated β-arrestin signaling. However, due to its low-affinity for the receptor, it has been difficult to study SII's potential pharmacological benefit in *in vivo* models. To overcome this limitation, custom-synthetic peptides have been developed based on SII sequence. Among the identified ligands, TRV120027 (TRV027) exhibited an improved potency compared to AngII in stimulating β-arrestin signaling, but no detectable G protein activation. TRV027 was shown to stimulate β-arrestin recruitment with subsequent activation of several kinase pathways such as p42/44 mitogen-activated protein kinase, Src, and Akt-endothelial nitric-oxide synthase pathways. TRV027 was also shown to increase cardiomyocyte contractility *in vitro* and decrease mean arterial pressure in rats *in vivo*, similar as unbiased ARBs such as losartan. However, unlike the unbiased ARBs, which decrease cardiac performance, TRV027 increased cardiac performance and preserved cardiac stroke volume (Violin *et al*., 2010). Moreover, in healthy and heart failure canines, TRV027 also reduced pulmonary capillary wedge pressure, systemic and renal vascular resistance while

preserving renal functions (Boerrigter *et al*., 2011). Because of this unique pharmacological profile, TRV027 has already entered phase II clinical trial as a novel therapeutic agent for acute heart failure (Ikeda *et al*., 2015) (Table 1).

Biased signaling in apelin receptor

The apelin receptor (also known as APJ, APLNR, AGTRL1) is a class A GPCR discovered in 1993 based on its sequence similarity with the AT_1R (O'Dowd *et al*., 1993). APJ is widely expressed in brain and peripheral organs including heart, lung, kidney, or placenta (Hosoya *et al*., 2000; Pope *et al*., 2012). Since its discovery, a number of physiological and pathophysiological roles for the receptor have been identified, including regulation of cardiovascular function, fluid homeostasis, and the adipoinsular axis [reviewed in (Pitkin *et al*., 2010)]. APJ does not bind AngII but its natural ligand, apelin is a potent inotropic and vasodilatory agent. Apelin induces coupling of APJ to $G\alpha_i$ (Habata *et al*., 1999) with additional evidence for the involvement of $G\alpha_q$-coupled activation of PLC and PKC (Japp and Newby, 2008). Interestingly, the apelin receptor in the heart may act as a mechanosensor for stretch in an apelin-independent/G protein-independent manner through recruitment of β-arrestin (Scimia *et al*., 2012).

Apelin receptor system represents an attractive target in pathologies such as pulmonary hypertension and heart failure (Chong *et al*., 2006; Chandra *et al*., 2011), which encourages to the development of its synthetic ligands. However, the limitation in translation to clinic is that chronic administration of an agonist would likely cause receptor desensitization with subsequent β-arrestin-mediated downregulation and loss of therapeutic efficacy. Thus, the development of agonists biased toward G protein signaling is essential. MM07, a cyclic apelin peptide, was shown to preferentially activate G protein responses with the low potency toward β-arrestin and receptor internalization. In rats, systemic infusions of this peptide caused a dose-dependent increase in cardiac output greater than apelin. Moreover, MM07 was an effective vasodilator in human forearm without loss of effects on repeat dosing, providing proof-of-concept of a clinical potential for biased ligands (Brame *et al*., 2015). Another APJ ligand, K17P was also reported to be G protein-biased and displayed the strong impairment of β-arrestin-dependent signaling. This molecule lacks the vasodilator capacity of its mother molecule, which is due to the deletion of single C-terminal phenylalanine (Ceraudo *et al*., 2014). This reflects the impact of the specific structure of the ligand in determining the signaling pathway activated in the receptor (Table 1).

Biased signaling in histamine receptors

The human histamine H_4 receptor (H_4R) belongs to the GPCR family and is considered as an important receptor in immune and inflammatory processes (Leurs *et al*., 2009). H_4R was originally thought to signal only through $G\alpha_i$ proteins and recently shown to also recruit and signal via β-arrestin2. This discovery made by its antagonist JNJ7777120, which was identified as a biased ligand in a β-arrestin2 recruitment assay (Rosethorne and Charlton, 2011). The therapeutic potential of JNJ7777120 has been successfully studied in the mouse model of chronic dermatitis, where it inhibited pruritus and skin inflammation when used in combination with H1R antagonist (Ohsawa and Hirasawa, 2012). Based on its indolecarboxamide structure, various JNJ7777120 analogues have been recently developed with the similar biased affinity toward β-arrestin2 (Nijmeijer *et al*., 2013). The potentially improved therapeutic efficacy of these substances is yet to be evaluated.

Currently, one of the most clinically relevant therapies for histamine receptors is achieved through the regulation of H_2R, which is pathologically implicated in gastric acid-related diseases but widely expressed in most tissues. The H_2R stimulation increases adenylate cyclase activity and induces cAMP accumulation. The most commonly used H_2R blocker, famotidine was shown to act as an "inverse agonist" by diminishing G protein-mediated increase of cAMP. Interestingly, famotidine also mimicked the effect of histamine, and induced receptor desensitization and internalization along with increased ERK phosphorylation in gastric epithelial cells (Alonso *et al*., 2015) (Table 1).

Biased signaling in dopamine receptors

Dopamine receptors are another well-studied family of GPCRs largely because dopamine neurotransmission is important in multiple neuropsychiatric disorders. Among the dopamine receptors, dopamine receptor D2 (D2R) is one of the most validated drug targets in neurology and psychiatry. However, most drugs targeting the D2R are problematic, either being less efficacious than desired or possessing adverse side effects due to the activation or blockade of a subset of downstream signaling pathways.

D2R couples $G\alpha_{i/o}$ to negatively regulate cAMP-PKA pathways and modulate intracellular Ca^{2+} levels by acting on ion channels or by triggering the release of Ca^{2+} from intracellular stores. In addition, more recent discoveries showed that dopamine receptors exert their *in vivo* effects through β-arrestin2-mediated protein kinase B (Akt)- glycogen synthase kinase 3 (GSK3) signaling cascades (Beaulieu *et al*., 2005). In humans, the Akt1/GSK3β signaling pathways are implicated in schizophrenia as evidenced by the low levels of Akt1 protein and reduced phosphorylation of GSK3β in the brain and lymphocytes of schizophrenic patients (Emamian *et al*., 2004). As Akt and GSK3 responses are mediated through ligands that are biased for β-arrestin signaling (Beaulieu *et al*., 2005), this may represent a novel approach to develop drugs with fewer side effects, greater therapeutic selectivity, and enhanced efficacy for treating schizophrenia. Indeed, two newly synthetized β-arrestin-biased ligands for D2R, UNC9975 and UNC9994 have already shown some promise by displaying robust antipsychotic drug-like activities in wild-type mice, which were abolished in β-arrestin2 knockout mice (Chen *et al*., 2012). These two biased ligands also reduced schizophrenia-like behaviors in phencyclidine-treated or NR1-knockdown hypoglutamatergic mice, where they increased performance in various neurobehavioral tests, and elicited a lower level of catalepsy than standard antipsychotic drug and D2R antagonist haloperidol (Park *et al*., 2016).

Interestingly, biased ligands with the opposing pharmacology for the D2R, that is, the stimulation of G protein signaling pathways without activation of β-arrestin recruitment have been recently identified. The first example is MLS1547, which was shown to robustly activate G proteins while antagonizing of β-arrestin recruitment to the D2R (Free *et al*., 2014). Identification of such functionally selective ligands should help to dissect the roles of both signaling arms of the D2R in physiology and pathology. Moreover, functionally selective G protein-

biased ligands may also result in improved therapies for certain neuropsychiatric disorders such as Parkinson's disease, in which D2R stimulation is desired (Table 1). Several recent advances on D1R-biased signaling are also summarized in Table 1.

Biased signaling in opioid receptors

Opioid receptors are GPCRs, which are widely studied due to their crucial roles in pain management, drug abuse/addiction, and mood disorders. There are three major subtypes of opioid receptors: δ-receptor (DOR), κ-receptor (KOR), and μ-receptor (MOR). Majority of opioids exert their analgesic activities primarily via activating MOR. Upon activation, MOR predominately couples to $G\alpha_{i/o}$, which orchestrates downstream signaling cascades including those contributing to antinociception. On the other hand, activation of β-arrestins, especially β-arrestin2 induces receptor internalization and desensitization, diminishing G protein-mediated signaling. Recent studies have shown that some MOR agonists such as fentanyl and [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) have the high efficacy to recruit β-arrestin, whereas other opioids such as morphine are biased toward G protein signaling (McPherson *et al*., 2010; Molinari *et al*., 2010). MOR-mediated β-arrestin activation has been associated with the adverse effects of opioids such as dependence, and gastrointestinal and respiratory dysfunction. Indeed, in the absence of β-arrestin2, morphine produced less tolerance, dependence, constipation, and respiratory suppressive side effects. Moreover, a significantly enhanced and prolonged analgesic effect of morphine was observed in loss-of-function of β-arrestin2 (Thompson *et al*., 2015). Therefore, the development of biased G protein ligands for MOR holds a greater therapeutic potential compared to an unbiased one. In fact, a G protein-biased MOR ligand TRV130 (made by Trevena Inc., King of Prussia, PA, USA) with little β-arrestin recruitment activity has recently been reported to show potent analgesic effects with reduced respiratory depression and constipation compared to morphine (DeWire *et al*., 2013). TRV130 was delivered intravenously and has successfully completed phase I trials for safety in healthy volunteers (Soergel *et al*., 2014). The first encouraging results from phase II trials were reported in a randomized and double-blinded study in patients experiencing moderate-to-severe post-operative pain after bunionectomy. The results showed that TRV130 rapidly produces profound analgesia with no serious adverse effects, suggesting that G protein-biased MOR activation is a promising target for novel analgesics (Viscusi *et al*., 2016).

Interestingly, biased ligands that are selective agonists at one desired pathway could also act as biased competitive antagonists for the undesired pathway. In general, MOR ligands with low efficacy for G protein activation also have low efficacy for β-arrestin2 recruitment, and in fact partial agonists such as buprenorphine do not significantly recruit β-arrestin2 in cell models (McPherson *et al*., 2010). In clinic, partial MOR agonists with bias toward antagonism of the β-arrestin2 would be beneficial for the treatment of opioid-induced constipation, which often complicates an analgesic therapy in patients. The pursuit of highly selective and potent non-peptide MOR ligands has yielded several more promising compounds including NAP, which acts as a peripherally selective MOR partial agonist. Being a P-glycoprotein substrate, NAP has limited access to the central nervous system. Moreover, it has no apparent analgesic effect due to its low efficacy in activating G protein-mediated signaling with no apparent effect on β-arrestin2 recruitment. However, its therapeutic potential lies in its ability to antagonize MOR full agonist-induced intracellular calcium flux and β-arrestin2 recruitment (Zhang *et al*., 2016). NAP dose-dependently restored the morphine-impaired intestinal motility without precipitating significant withdrawal of symptoms and thus held great promise in the treatment of opioid-induced constipation (Yuan *et al*., 2012) (Table 1). In addition to multiple GPCRs as aforementioned, we summarize recent advances on serotonin 5-HT_{2B} receptor- and arginine-vasopressin V2 receptor-mediated biased signaling and highlight some biased ligands in Table 1.

Biased GPCR signaling in cancer

In contrast to the successful implementation of GPCR biased signaling concept for clinical benefit in the cardiovascular, neurological, behavior fields, there have been no reports demonstrating the utility of GPCR biased signaling for the treatment of cancer. However, a few recent studies reported the scientific progress in the potential use of biased signaling on endothelin receptors in cancer treatment.

Endothelin-1 (ET-1) is a peptide belonging to a family of the most potent vasoconstrictors. In addition to this function in the circulation, it has been implicated in various physiological and pathological conditions such as development, cell proliferation, differentiation, cardiac function and cancer (Schorlemmer *et al*., 2008; Rosano *et al*., 2013b). ET-1 is a well-recognized growth factor that is present in plasma and is produced by stromal and tumor cells. The ET-1 receptors, endothelin type A receptor (ET_AR) and endothelin type B receptor (ET_BR) are members of the GPCR family. ET_BR is coupled to $G\alpha_q$ and $G\alpha_i$, and expressed mainly in endothelial cells, while ET_AR, which is coupled to $G\alpha_q$, $G\alpha_s$ and $G\alpha_{12/13}$, is expressed in vascular smooth muscle cells and cardiomyocytes as well as solid tumors (Sakurai *et al*., 1990; Williams *et al*., 1991; Bagnato *et al*., 1999). In ovarian cancer, ET_AR/ET-1 axis has been shown to promote tumorigenesis by promoting anti-apoptosis, invasion, and neoangiogenesis (Spinella *et al*., 2004; Rosano *et al*., 2005). Indeed, ET_AR overexpression is associated with poor survival in patients with ovarian carcinoma (Teoh *et al*., 2014). However, a recent clinical study demonstrated that specific ET_AR antagonists are ineffective as auxiliary anti-cancer treatment (Cognetti *et al*., 2013). This might be explained by signaling bias of ET_AR, which mediates both oncogenic and tumor suppressive properties. The known oncogenic downstream effects of ET_AR are mediated by $G\alpha_q$-coupled or β-arrestin-dependent signaling pathways (Spinella *et al*., 2004; Rosano *et al*., 2013a). It was shown that GRK5/6-mediated phosphorylation of the receptor leads to the recruitment and nuclear translocation of β-arrestin, which in turn functions as an epigenetic regulator of several angiogenic/metastatic genes including β-catenin, thus promoting cell invasion (Rosano *et al*., 2013a; Teoh *et al*., 2014). On the other hand, ET_AR-mediated $G\alpha_s$ activation induces AC/cAMP/PKA signaling, which can confer tumor suppressive effects as reported in several carcinoma-derived cell lines (Takahashi *et al*., 2009; Follin-Arbelet *et al*., 2013; Teoh *et al*., 2014). Given that stimulation of the ET-1/ET_AR axis can activate both tumor suppressive and oncogenic properties in cancer cells, ligands biased toward $G\alpha_s$/cAMP/PKA signaling might represent a novel potential therapy of various malignancies. Unfortunately,

such therapeutic agents are yet to be developed.

Other GPCRs, which are involved in the progression of cancer and have been suggested as potential targets for yet-to-be-identified biased ligands, are CXC chemokine receptor 4 (CXCR4) and protease activated receptor 2 (PAR2). The enhanced expression of CXCR4 and aberrant downstream signaling are implicated in several cancers, where it is involved in tumor growth, vascularization, and metastasis (Guleng *et al*., 2005; Rubin, 2009). Also, PAR2, a GPCR with distinct biased signaling, has emerged as one of the promising therapeutic targets to inhibit rapidly metastasizing breast cancer cells (Morris *et al*., 2006). Therefore, the development of novel biased ligands for CXCR4 and PAR2 may open new opportunities for cancer treatment.

DISCOVERING BIASED SIGNALING ON GPCR

Over the past decade, there has been a surge in publications to describe the identification of biased ligands at a wide variety of GPCRs. Accordingly, quantifying ligand bias has been an active area of research. One way to quantify ligand bias is to plot β-arrestin activity against G protein activity. For biased ligands, there would be different levels of β-arrestin- and G protein-mediated efficacies. Such data can also be represented as a matrix that incorporates data from multiple assays, or ligand bias factors that compare β-arrestin activity against G protein activity in different assays (Rajagopal *et al*., 2010). The quantification of the relative levels of bias is important in the identification of lead compounds and in the optimization of drug screening for biased ligands. Despite this effort, there are still considerable gaps in our understanding of bias. The conventional high throughput screening methodology used in the pharmaceutical industry is inadequate for the needs of novel biased ligand discovery. The drug discovery and experimental studies of biased signaling mechanisms require the use of highly sensitive assays and a standardized methodology to quantify responses related to the signaling pathways. For example, assays and analyses must be configured to remove any apparent bias resulting from the biological assay system and thus correctly identify true ligand bias. In addition, at least one of the endpoints requires the accurate quantification of a poor response. Lastly, the analysis must be compatible for use with a large number of compounds, and the data must be presented to enable medicinal chemistry to derive the relationship between structure and activity (Winpenny *et al*., 2016).

In the past years, most groups have relied on comparing the maximal effects (E_{max}) and potencies (EC_{50}) of ligands for different signaling pathways. However, they are prone to errors in the interpretation in the setting of receptor reserve. For example, these parameters failed to account for the differences in the receptor reserve and amplification of different assays (Rajagopal *et al*., 2010). In assays with significant amplification such as second-messenger assays (e.g., cAMP formation), both full and partial agonists can reach the same maximal response, whereas in assays with little amplification such as assays that monitor the recruitment of β-arrestin to a receptor by enzyme complementation (Eglen *et al*., 2007), partial agonists have significantly lower maximal responses than full agonists (Rajagopal *et al*., 2010). Therefore, a partial agonist that reaches the maximal effect in one assay and half-maximal effect in another assay would be incorrectly identified as being biased compared with a full agonist, which reaches the maximal response in both assays. Also, the difference in potencies between the full agonist and partial agonist may be smaller in assays with less receptor reserve (Rajagopal *et al*., 2010, 2011). The current best practice in the identification and quantification of biased agonism is thus complex and an ongoing topic of debate.

The key requirements for measuring bias signaling are a common reference compound to overcome observational and systemic bias as well as a scale, which accounts for both potency and maximal response of ligands. They allow the relative activity of ligands to be compared across assays. The current 'gold standard' method is the operational model of agonism that allows for the systematically independent quantification of agonist activity via the relative transduction ratio coefficient $\Delta\log(\tau/K_A)$. The term τ incorporates agonist efficacy, receptor density and coupling within the system. The dissociation constant (K_A) is the reciprocal of the conditional affinity of the agonist in the functional system [for detailed reviews, see (Kenakin and Christopoulos, 2013)]. The alternative to this method is to use the bias factor β_{lig}, which is calculated by the ratios of the efficacy of agonists for a given signaling pathway in a cell. This method was first used to quantify ligand bias and to identify weak biased compounds in β_2-AR and AT_1R. This method differs from the previous one by assuming that a single estimate of KA for the receptor (obtained from biochemical binding studies) should be used to fit the data with the operational model [for detailed reviews, see (Rajagopal *et al*., 2011)]. However, the model to use the bias factor has limitations such as a possible error in the calculation of bias if the affinity of the agonist is changed when different signaling proteins are coupled to the receptor (Kenakin and Christopoulos, 2013). Another method for quantifying agonist bias offers the comparison of ratio of the maximal response (E_{max}) to the EC_{50} value for an agonist [$\Delta\log(E_{max}/EC_{50})$]. The equation has been employed to identify new biased MOR ligands. In this method, the relative signaling bias was quantified for each compound by calculating the difference in activity between β-arrestin recruitment assays (e.g., fluorescent labeling of β-arrestins and GPCRs) and G protein activation assays (e.g., cAMP assay). In such quantification, 0 represented no bias and +1 or -1 represented 10-fold bias for one signaling pathway [for detailed reviews, see (Winpenny *et al*., 2016)]. These methods for the identification and quantification of bias across large compound numbers have been essential for biased drug discovery.

One of increasingly recognized techniques for identification of GPCR signaling bias is BRET, which is a sensitive and non-destructive method commonly used in live cells to investigate protein-protein interactions or changes. BRET is a naturally occurring phenomenon resulting from the nonradioactive transfer of energy between luminescent donor and fluorescent acceptor proteins. In the sea pansy *Renilla reniformis*, the luminescence resulting from the catalytic degradation of coelenterazine by luciferase (*R*luc, donor) is transferred to the green fluorescent protein (GFP, acceptor), which in turn emits fluorescence upon dimerization of the two proteins. The BRET response depends on the distance and the relative orientation of the donor and acceptor. In many studies, donor and acceptor are tagged individually on two proteins that potentially assemble in signaling complexes. In other cases, subtle changes in the protein conformation upon complex assembly

or disassembly can be studied using modified proteins that are labeled with both BRET donor and acceptor moieties engineered into the same protein in an intramolecular setting. Because of its fast reaction kinetics, this method allows for the real time detection of complexes or conformational changes that may be transient (Angers *et al*., 2000; Milligan, 2004). BRET has been successfully applied to study GPCR dimerization (Angers *et al*., 2000), and more recently to study proximal interactions of GPCRs with different signaling effectors including G proteins and β-arrestins (Shukla *et al*., 2008; Molinari *et al*., 2010; Ceraudo *et al*., 2014). Recent new advances in BRET technology include the discovery of biosensors, which do not involve the labeling of receptor as either a BRET donor or acceptor, and allow the identification of biased signaling from unknown compounds for any GPCR of interest (Namkung *et al*., 2016).

In addition to BRET, fluorescence resonance energy transfer (FRET) proximity and conformation assays as well as signaling assays such as MAPK activation have been widely used for the discovery of biased ligands (Rajagopal *et al*., 2010).

CONCLUSIONS

The drug discovery strategy targeting only the primary ligand binding sites of GPCRs is becoming more and more difficult. As our understanding on *in vivo* effects of GPCR-targeting drugs becomes more profound, therapeutic strategies have often required the modulation of only a part of downstream effector pathways of a GPCR in a specific cell or tissue. Biased ligands with functional selectivity in specific GPCRs thus represent a new generation of drugs with increased specificity and fewer adverse effects. Much has been speculated regarding the potential advantages of the pharmacological feature of biased signaling and there are several biased ligands entering clinical studies. However, many gaps still exist. For example, few large animal and human studies have been performed. To completely translate existing basic research outcomes into clinical therapeutic options, further collaborative research should be pursued. Moreover, it is important for physicians and pharmacists to be continuously informed about which currently used drugs belong to the category of biased GPCR ligands and in which cases their use is beneficial or should be avoided due to the known adverse/unwanted effects.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank the editors for inviting us to write this review. Due to space restrictions, the authors cannot cite many important literatures on this field. The authors apologize to all colleagues whose work contributed significantly. This work was supported by the American Heart Association Postdoctoral Fellowship 16POST26990020 to Zuzana Bologna, American Heart Association Predoctoral Fellowship 16PRE30210016 to Jianpeng Teoh, and American Physiological Society Shih-Chun Wang Young Investigator Award, American Heart Association Grant-in-Aid 12GRNT12100048 and Scientist Development Grant 14SDG18970040, and National Institutes of Health R01 HL124251 to Il-man Kim.

AUTHOR CONTRIBUTIONS

All authors were involved either in the primary writing process or substantial revision of the manuscript. Il-mam Kim gave the final approval of the version to be published. All authors read and approved the final manuscript.

REFERENCES

Ahn, S., Kim, J., Hara, M. R., Ren, X. R. and Lefkowitz, R. J. (2009) β-arrestin-2 mediates anti-apoptotic signaling through regulation of BAD phosphorylation. *J. Biol. Chem.* **284**, 8855-8856.

Alonso, N., Zappia, C. D., Cabrera, M., Davio, C. A., Shayo, C., Monczor, F. and Fernandez, N. C. (2015) Physiological implications of biased signaling at histamine H2 receptors. *Front. Pharmacol.* **6**, 45.

Angers, S., Salahpour, A., Joly, E., Hilairet, S., Chelsky, D., Dennis, M. and Bouvier, M. (2000) Detection of β_2-adrenergic receptor dimerization in living cells using bioluminescence resonance energy transfer (BRET). *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3684-3689.

Aplin, M., Christensen, G. L., Schneider, M., Heydorn, A., Gammeltoft, S., Kjolbye, A. L., Sheikh, S. P. and Hansen, J. L. (2007) The angiotensin type 1 receptor activates extracellular signal-regulated kinases 1 and 2 by G protein-dependent and -independent pathways in cardiac myocytes and Langendorff-perfused hearts. *Basic Clin. Pharmacol. Toxicol.* **100**, 289-295.

Aurora, A. B., Mahmoud, A. I., Luo, X., Johnson, B. A., van Rooij, E., Matsuzaki, S., Humphries, K. M., Hill, J. A., Bassel-Duby, R., Sadek, H. A. and Olson, E. N. (2012) MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca^{2+} overload and cell death. *J. Clin. Invest.* **122**, 1222-1232.

Bagnato, A., Salani, D., Di Castro, V., Wu-Wong, J. R., Tecce, R., Nicotra, M. R., Venuti, A. and Natali, P. G. (1999) Expression of endothelin 1 and endothelin A receptor in ovarian carcinoma: Evidence for an autocrine role in tumor growth. *Cancer Res.* **59**, 720-727.

Beaulieu, J. M., Sotnikova, T. D., Marion, S., Lefkowitz, R. J., Gainetdinov, R. R. and Caron, M. G. (2005) An Akt/β-arrestin 2/PP2A signaling complex mediates dopaminergic neurotransmission and behavior. *Cell* **122**, 261-273.

Bockaert, J. and Pin, J. P. (1999) Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.* **18**, 1723-1729.

Boerrigter, G., Lark, M. W., Whalen, E. J., Soergel, D. G., Violin, J. D. and Burnett, J. C., Jr. (2011) Cardiorenal actions of TRV120027, a novel β-arrestin-biased ligand at the angiotensin II ype I receptor, in healthy and heart failure canines: a novel therapeutic strategy for acute heart failure. *Circ. Heart Fail.* **4**, 770-778.

Brame, A. L., Maguire, J. J., Yang, P. R., Dyson, A., Torella, R., Cheriyan, J., Singer, M., Glen, R. C., Wilkinson, I. B. and Davenport, A. P. (2015) Design, characterization and first-in-human study of the vascular actions of a novel biased apelin receptor agonist. *Hypertension* **65**, 834-840.

Brunvand, H., Liu, G. L., Ma, X. L., Yue, T. L., Ruffolo, R. R., Jr. and Feuerstein, G. Z. (1998) SB 211475, a metabolite of carvedilol, reduces infarct size after myocardial ischemic and reperfusion injury in rabbits. *Eur. J. Pharmacol.* **356**, 193-198.

Burnier, M. and Brunner, H. R. (2000) Angiotensin II receptor antagonists. *Lancet* **355**, 637-645.

Butcher, A. J., Prihandoko, R., Kong, K. C., McWilliams, P., Edwards, J. M., Bottrill, A., Mistry, S. and Tobin, A. B. (2011) Differential G-protein-coupled receptor phosphorylation provides evidence for a signaling bar code. *J. Biol. Chem.* **286**, 11506-11518.

Byers, M. A., Calloway, P. A., Shannon, L., Cunningham, H. D., Smith, S., Li, F., Fassold, B. C. and Vines, C. M. (2008) Arrestin 3 me-

diates endocytosis of CCR7 following ligation of CCL19 but not CCL21. *J. Immunol.* **181**, 4723-4732.
Ceraudo, E., Galanth, C., Carpentier, E., Banegas-Font, I., Schonegge, A. M., Alvear-Perez, R., Iturrioz, X., Bouvier, M. and Llorens-Cortes, C. (2014) Biased signaling favoring G_i over β-arrestin promoted by an apelin fragment lacking the C-terminal phenylalanine. *J. Biol. Chem.* **289**, 24599-24610.
Chandra, S. M., Razavi, H., Kim, J., Agrawal, R., Kundu, R. K., Perez, V. D., Zamanian, R. T., Quertermous, T. and Chun, H. J. (2011) Disruption of the apelin-APJ system worsens hypoxia-induced pulmonary hypertension. *Arterioscler. Thromb. Vasc. Biol.* **31**, 814-820.
Chen, X., Sassano, M. F., Zheng, L. Y., Setola, V., Chen, M., Bai, X., Frye, S. V., Wetsel, W. C., Roth, B. L. and Jin, J. (2012) Structure-functional selectivity relationship studies of β-arrestin-biased dopamine D-2 receptor agonists. *J. Med. Chem.* **55**, 7141-7153.
Chen, X. T., Pitis, P., Liu, G. D., Yuan, C., Gotchev, D., Cowan, C. L., Rominger, D. H., Koblish, M., DeWire, S. M., Crombie, A. L., Violin, J. D. and Yamashita, D. S. (2013) Structure-activity relationships and discovery of a G protein biased μ opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro-[4.5]decan-9-yl]ethyl})amine (TRV130), for the treatment of acute severe pain. *J. Med. Chem.* **56**, 8019-8031.
Chesley, A., Lundberg, M. S., Asai, T., Xiao, R. P., Ohtani, S., Lakatta, E. G. and Crow, M. T. (2000) The β_2-adrenergic receptor delivers an antiapoptotic signal to cardiac myocytes through G_i-dependent coupling to phosphatidylinositol 3'-kinase. *Circ. Res.* **87**, 1172-1179.
Chong, K. S., Gardner, R. S., Morton, J. J., Ashley, E. A. and McDonagh, T. A. (2006) Plasma concentrations of the novel peptide apelin are decreased in patients with chronic heart failure. *Eur. J. Heart Fail.* **8**, 355-360.
Ciccarelli, M., Chuprun, J. K., Rengo, G., Gao, E., Wei, Z. Y., Peroutka, R. J., Gold, J. I., Gumpert, A., Chen, M., Otis, N. J., Dorn, G. W., Trimarco, B., Iaccarino, G. and Koch, W. J. (2011) G protein-coupled receptor kinase 2 activity impairs cardiac glucose uptake and promotes insulin resistance after myocardial ischemia. *Circulation* **123**, 1953-1962.
Cipolletta, E., Campanile, A., Santulli, G., Sanzari, E., Leosco, D., Campiglia, P., Trimarco, B. and Iaccarino, G. (2009) The G protein coupled receptor kinase 2 plays an essential role in β-adrenergic receptor-induced insulin resistance. *Cardiovasc. Res.* **84**, 407-415.
Cognetti, F., Bagnato, A., Colombo, N., Savarese, A., Scambia, G., Sehouli, J., Wimberger, P., Sorio, R., Harter, P., Mari, E., McIntosh, S., Nathan, F., Pemberton, K. and Baumann, K. (2013) A Phase II, randomized, double-blind study of zibotentan (ZD4054) in combination with carboplatin/paclitaxel versus placebo in combination with carboplatin/paclitaxel in patients with advanced ovarian cancer sensitive to platinum-based chemotherapy (AGO-OVAR 2.14) *Gynecol. Oncol.* **130**, 31-37.
Conroy, J. L., Free, R. B. and Sibley, D. R. (2015) Identification of G protein-biased agonists that fail to recruit β-arrestin or promote internalization of the D1 dopamine receptor. *ACS Chem. Neurosci.* **6**, 681-692.
Cook, S. A., Varela-Carver, A., Mongillo, M., Kleinert, C., Khan, M. T., Leccisotti, L., Strickland, N., Matsui, T., Das, S., Rosenzweig, A., Punjabi, P. and Camici, P. G. (2010) Abnormal myocardial insulin signalling in type 2 diabetes and left-ventricular dysfunction. *Eur. Heart J.* **31**, 100-111.
Daaka, Y., Luttrell, L. M. and Lefkowitz, R. J. (1997) Switching of the coupling of the β2-adrenergic receptor to different G proteins by protein kinase A. *Nature* **390**, 88-91.
DeWire, S. M., Ahn, S., Lefkowitz, R. J. and Shenoy, S. K. (2007) β-arrestins and cell signaling. *Annu. Rev. Physiol.* **69**, 483-510.
DeWire, S. M., Yamashita, D. S., Rominger, D. H., Liu, G. D., Cowan, C. L., Graczyk, T. M., Chen, X. T., Pitis, P. M., Gotchev, D., Yuan, C., Koblish, M., Lark, M. W. and Violin, J. D. (2013) A G protein-biased ligand at the μ-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine. *J. Pharmacol. Exp. Ther.* **344**, 708-717.
Doughty, R. N., Whalley, G. A., Walsh, H., Gamble, G., Sharpe, N. and Investigat, C. E. S. (2001) Effects of carvedilol on left ventricular remodelling in patients following acute myocardial infarction: The CAPRICORN echo substudy. *Circulation* **104**, 517.
Dulin, B. and Abraham, W. T. (2004) Pharmacology of carvedilol. *Am. J. Cardiol.* **93**, 3B-6B.
Dunn, C. J., Lea, A. P. and Wagstaff, A. J. (1997) Carvedilol. A reappraisal of its pharmacological properties and therapeutic use in cardiovascular disorders. *Drugs* **54**, 161-185.
Eglen, R. M., Bosse, R. and Reisine, T. (2007) Emerging concepts of guanine nucleotide-binding protein-coupled receptor (GPCR) function and implications for high throughput screening. *Assay Drug Dev. Technol.* **5**, 425-451.
Emamian, E. S., Hall, D., Birnbaum, M. J., Karayiorgou, M. and Gogos, J. A. (2004) Convergent evidence for impaired AKT1-GSK3β signaling in schizophrenia. *Nat. Genet.* **36**, 131-137.
Ferguson, S. S. (2001) Evolving concepts in G protein-coupled receptor endocytosis: The role in receptor desensitization and signaling. *Pharmacol. Rev.* **53**, 1-24.
Follin-Arbelet, V., Torgersen, M. L., Naderi, E. H., Misund, K., Sundan, A. and Blomhoff, H. K. (2013) Death of multiple myeloma cells induced by cAMP-signaling involves downregulation of Mcl-1 via the JAK/STAT pathway. *Cancer Lett.* **335**, 323-331.
Free, R. B., Chun, L. S., Moritz, A. E., Miller, B. N., Doyle, T. B., Conroy, J. L., Padron, A., Meade, J. A., Xiao, J. B., Hu, X., Dulcey, A. E., Han, Y., Duan, L. H., Titus, S., Bryant-Genevier, M., Barnaeva, E., Ferrer, M., Javitch, J. A., Beuming, T., Shi, L., Southall, N. T., Marugan, J. J. and Sibley, D. R. (2014) Discovery and characterization of a G protein-biased agonist that inhibits β-arrestin recruitment to the D_2 dopamine receptor. *Mol. Pharmacol.* **86**, 96-105.
Fu, Q., Xu, B., Liu, Y. M., Parikh, D., Li, J., Li, Y., Zhang, Y., Riehle, C., Zhu, Y., Rawlings, T., Shi, Q., Clark, R. B., Chen, X. W., Abel, E. D. and Xiang, Y. K. (2014) Insulin inhibits cardiac contractility by inducing a G_i-biased β_2-adrenergic signaling in hearts. *Diabetes* **63**, 2676-2689.
Gautam, N., Downes, G. B., Yan, K. and Kisselev, O. (1998) The G-protein β gamma complex. *Cell. Signal.* **10**, 447-455.
Gether, U. (2000) Uncovering molecular mechanisms involved in activation of G protein-coupled receptors. *Endocr. Rev.* **21**, 90-113.
Gong, K. Z., Li, Z. J., Xu, M., Du, J. H., Lv, Z. Z. and Zhang, Y. Y. (2008) A novel protein kinase A-independent, β-arrestin-1-dependent signaling pathway for p38 mitogen-activated protein kinase activation by β_2-adrenergic receptors. *J. Biol. Chem.* **283**, 29028-29036.
Groer, C. E., Schmid, C. L., Jaeger, A. M. and Bohn, L. M. (2011) Agonist-directed interactions with specific β-arrestins determine mu-opioid receptor trafficking, ubiquitination and dephosphorylation. *J. Biol. Chem.* **286**, 31731-31741.
Guil, S. and Caceres, J. F. (2007) The multifunctional RNA-binding protein hnRNP A1 is required for processing of miR-18a. *Nat. Struct. Mol. Biol.* **14**, 591-596.
Guleng, B., Tateishi, K., Ohta, M., Kanai, F., Jazag, A., Ijichi, F., Tanaka, Y., Washida, M., Morikane, K., Fukushima, Y., Yamori, T., Tsuruo, T., Kawabe, T., Miyagishi, M., Taira, K., Sata, M. and Omata, M. (2005) Blockade of the stromal cell-derived factor-1/CXCR4 axis attenuates *in vivo* tumor growth by inhibiting angiogenesis in a vascular endothelial growth factor-independent manner. *Cancer Res.* **65**, 5864-5871.
Habata, Y., Fujii, R., Hosoya, M., Fukusumi, S., Kawamata, Y., Hinuma, S., Kitada, C., Nishizawa, N., Murosaki, S., Kurokawa, T., Onda, H., Tatemoto, K. and Fujino, M. (1999) Apelin, the natural ligand of the orphan receptor APJ, is abundantly secreted in the colostrum. *Biochim. Biophys. Acta* **1452**, 25-35.
Hamm, H. E. (1998) The many faces of G protein signaling. *J. Biol. Chem.* **273**, 669-672.
Hermans, E. (2003) Biochemical and pharmacological control of the multiplicity of coupling at G-protein-coupled receptors. *Pharmacol. Ther.* **99**, 25-44.
Hoffmann, C., Ziegler, N., Reiner, S., Krasel, C. and Lohse, M. J. (2008) Agonist-selective, receptor-specific interaction of human P2Y receptors with β-arrestin-1 and -2. *J. Biol. Chem.* **283**, 30933-30941.
Hosoya, M., Kawamata, Y., Fukusumi, S., Fujii, R., Habata, Y., Hinuma, S., Kitada, C., Honda, S., Kurokawa, T., Onda, H., Nishimura, O. and Fujino, M. (2000) Molecular and functional characteristics of APJ - Tissue distribution of mRNA and interaction with the endog-

enous ligand apelin. *J. Biol. Chem.* **275**, 21061-21067.
Ikeda, Y., Kumagai, H., Motozawa, Y., Suzuki, J. and Komuro, I. (2015) Biased agonism of the angiotensin II type I receptor a potential strategy for the rreatment of acute heart failure. *Int. Heart J.* **56**, 485-488.
Japp, A. G. and Newby, D. E. (2008) The apelin-APJ system in heart failure pathophysiologic relevance and therapeutic potential. *Biochem. Pharmacol.* **75**, 1882-1892.
Jean-Alphonse, F., Perkovska, S., Frantz, M. C., Durroux, T., Mejean, C., Morin, D., Loison, S., Bonnet, D., Hibert, M., Mouillac, B. and Mendre, C. (2009) Biased agonist pharmacochaperones of the AVP V2 receptor may treat congenital nephrogenic diabetes insipidus. *J. Am. Soc. Nephrol.* **20**, 2190-2203.
Kenakin, T. and Christopoulos, A. (2013) Signalling bias in new drug discovery: detection, quantification and therapeutic impact. *Nat. Rev. Drug Discov.* **12**, 205-216.
Kilts, J. D., Gerhardt, M. A., Richardson, M. D., Sreeram, G., Mackensen, G. B., Grocott, H. P., White, W. D., Davis, R. D., Newman, M. F., Reves, J. G., Schwinn, D. A. and Kwatra, M. M. (2000) β2-adrenergic and several other G protein-coupled receptors in human atrial membranes activate both G_s and G_i. *Circ. Res.* **87**, 705-709.
Kim, I. M., Tilley, D. G., Chen, J., Salazar, N. C., Whalen, E. J., Violin, J. D. and Rockman, H. A. (2008) β-blockers alprenolol and carvedilol stimulate β-arrestin-mediated EGFR transactivation. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 14555-14560.
Kim, I. M., Wang, Y. C., Park, K. M., Tang, Y. P., Teoh, J. P., Vinson, J., Traynham, C. J., Pironti, G., Mao, L., Su, H. B., Johnson, J. A., Koch, W. J. and Rockman, H. A. (2014) β-arrestin1-biased β_1-adrenergic receptor signaling regulates microRNA processing. *Circ. Res.* **114**, 833-844.
Kim, J., Ahn, S., Ren, X. R., Whalen, E. J., Reiter, E., Wei, H. J. and Lefkowitz, R. J. (2005) Functional antagonism of different G protein-coupled receptor kinases for β-arrestin-mediated angiotensin II receptor signaling. *Proc. Natl. Acad. Sci. U.S.A.* **102**, 1442-1447.
Lee, Y., Ahn, C., Han, J. J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. and Kim, V. N. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-419.
Lefkowitz, R. J. (1998) G protein-coupled receptors III. New roles for receptor kinases and β-arrestins in receptor signaling and desensitization. *J. Biol. Chem.* **273**, 18677-18680.
Lefkowitz, R. J. (2013) Arrestins come of age: a personal historical perspective. *Prog. Mol. Biol. Transl. Sci.* **118**, 3-18.
Leurs, R., Chazot, P. L., Shenton, F. C., Lim, H. D. and de Esch, I. J. (2009) Molecular and biochemical pharmacology of the histamine H4 receptor. *Br. J. Pharmacol.* **157**, 14-23.
Liu, J. J., Horst, R., Katritch, V., Stevens, R. C. and Wuthrich, K. (2012) Biased signaling pathways in β_2-adrenergic receptor characterized by ^{19}F-NMR. *Science* **335**, 1106-1110.
Luan, B., Zhao, J., Wu, H. Y., Duan, B. Y., Shu, G. W., Wang, X. Y., Li, D. S., Jia, W. P., Kang, J. H. and Pei, G. (2009) Deficiency of a β-arrestin-2 signal complex contributes to insulin resistance. *Nature* **457**, 1146-1149.
Luttrell, L. M., Ferguson, S. S., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F. T., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G. and Lefkowitz, R. J. (1999) β-arrestin-dependent formation of β(2) adrenergic receptor-Src protein kinase complexes. *Science* **283**, 655-661.
McCloskey, D. T., Turcato, S., Wang, G. Y., Turnbull, L., Zhu, B. Q., Bambino, T., Nguyen, A. P., Lovett, D. H., Nissenson, R. A., Karliner, J. S. and Baker, A. J. (2008) Expression of a G_i-coupled receptor in the heart causes impaired Ca^{2+} handling, myofilament injury and dilated cardiomyopathy. *Am. J. Physiol. Heart Circ. Physiol.* **294**, H205-H212.
McDonald, P. H., Chow, C. W., Miller, W. E., Laporte, S. A., Field, M. E., Lin, F. T., Davis, R. J. and Lefkowitz, R. J. (2000) β-arrestin 2: a receptor-regulated MAPK scaffold for the activation of JNK3. *Science* **290**, 1574-1577.
McPherson, J., Rivero, G., Baptist, M., Llorente, J., Al-Sabah, S., Krasel, C., Dewey, W. L., Bailey, C. P., Rosethorne, E. M., Charlton, S. J., Henderson, G. and Kelly, E. (2010) μ-opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol. Pharmacol.* **78**, 756-766.
Milligan, G. (2004) Applications of bioluminescence- and fluorescence resonance energy transfer to drug discovery at G protein-coupled receptors. *Eur. J. Pharm. Sci.* **21**, 397-405.
Miura, S., Okabe, A., Matsuo, Y., Karnik, S. S. and Saku, K. (2013) Unique binding behavior of the recently approved angiotensin II receptor blocker azilsartan compared with that of candesartan. *Hypertens. Res.* **36**, 134-139.
Mochizuki, M., Yano, M., Oda, T., Tateishi, H., Kobayashi, S., Yamamoto, T., Ikeda, Y., Ohkusa, T., Ikemoto, N. and Matsuzaki, M. (2007) Scavenging free radicals by low-dose carvedilol prevents redox-dependent Ca^{2+} leak via stabilization of ryanodine receptor in heart failure. *J. Am. Coll. Cardiol.* **49**, 1722-1732.
Molinari, P., Vezzi, V., Sbraccia, M., Gro, C., Riitano, D., Ambrosio, C., Casella, I. and Costa, T. (2010) Morphine-like opiates selectively antagonize receptor-arrestin interactions. *J. Biol. Chem.* **285**, 12522-12535.
Morisco, C., Lembo, G. and Trimarco, B. (2006) Insulin resistance and cardiovascular risk: new insights from molecular and cellular biology. *Trends Cardiovasc. Med.* **16**, 183-188.
Morris, A. J. and Malbon, C. C. (1999) Physiological regulation of G protein-linked signaling. *Physiol. Rev.* **79**, 1373-1430.
Morris, D. R., Ding, Y., Ricks, T. K., Gullapalli, A., Wolfe, B. L. and Trejo, J. (2006) Protease-activated receptor-2 is essential for factor VIIa and Xa-induced signaling, migration and invasion of breast cancer cells. *Cancer Res.* **66**, 307-314.
Muller, G. (2000) Towards 3D structures of G protein-coupled receptors: a multidisciplinary approach. *Curr. Med. Chem.* **7**, 861-888.
Namkung, Y., Radresa, O., Armando, S., Devost, D., Beautrait, A., Le Gouill, C. and Laporte, S. A. (2016) Quantifying biased signaling in GPCRs using BRET-based biosensors. *Methods* **92**, 5-10.
Nevzorova, J., Evans, B. A., Bengtsson, T. and Summers, R. J. (2006) Multiple signalling pathways involved in β(2)-adrenoceptor-mediated glucose uptake in rat skeletal muscle cells. *Br. J. Pharmacol.* **147**, 446-454.
Nichols, G. A., Hillier, T. A., Erbey, J. R. and Brown, J. B. (2001) Congestive heart failure in type 2 diabetes: prevalence, incidence and risk factors. *Diabetes Care* **24**, 1614-1619.
Nijmeijer, S., Vischer, H. F., Sirci, F., Schultes, S., Engelhardt, H., de Graaf, C., Rosethorne, E. M., Charlton, S. J. and Leurs, R. (2013) Detailed analysis of biased histamine H_4 receptor signalling by JNJ 7777120 analogues. *Br. J. Pharmacol.* **170**, 78-88.
Nobles, K. N., Xiao, K. H., Ahn, S., Shukla, A. K., Lam, C. M., Rajagopal, S., Strachan, R. T., Huang, T. Y., Bressler, E. A., Hara, M. R., Shenoy, S. K., Gygi, S. P. and Lefkowitz, R. J. (2011) Distinct phosphorylation sites on the β_2-adrenergic receptor establish a barcode that encodes differential functions of β-arrestin. *Sci. Signal.* **4**, ra51.
Noma, T., Lemaire, A., Naga Prasad, S. V., Barki-Harrington, L., Tilley, D. G., Chen, J., Le Corvoisier, P., Violin, J. D., Wei, H., Lefkowitz, R. J. and Rockman, H. A. (2007) B-arrestin-mediated β_1-adrenergic receptor transactivation of the EGFR confers cardioprotection. *J. Clin. Invest.* **117**, 2445-2458.
O'Dowd, B. F., Heiber, M., Chan, A., Heng, H. H., Tsui, L. C., Kennedy, J. L., Shi, X. M., Petronis, A., George, S. R. and Nguyen, T. (1993) A human gene that shows identity with the gene encoding the angiotensin receptor is located on chromosome 11. *Gene* **136**, 355-360.
Ohsawa, Y. and Hirasawa, N. (2012) The antagonism of histamine H1 and H4 receptors ameliorates chronic allergic dermatitis via antipruritic and anti-inflammatory effects in NC/Nga mice. *Allergy* **67**, 1014-1022.
Ohtsuka, T., Hamada, M., Hiasa, G., Sasaki, O., Suzuki, M., Hara, Y., Shigematsu, Y. and Hiwada, K. (2001) Effect of β-blockers on circulating levels of inflammatory and anti-inflammatory cytokines in patients with dilated cardiomyopathy. *J. Am. Coll. Cardiol.* **37**, 412-417.
Overington, J. P., Al-Lazikani, B. and Hopkins, A. L. (2006) How many drug targets are there? *Nat. Rev. Drug Discov.* **5**, 993-996.
Park, S. M., Chen, M., Schmerberg, C. M., Dulman, R. S., Rodriguiz, R. M., Caron, M. G., Jin, J. and Wetsel, W. C. (2016) Effects of β-arrestin-biased dopamine D2 receptor ligands on schizophrenia-like behavior in hypoglutamatergic mice. *Neuropsychopharmacology* **41**, 704-715.

Perrino, C. and Rockman, H. A. (2007) Reversal of cardiac remodeling by modulation of adrenergic receptors: a new frontier in heart failure. *Curr. Opin. Cardiol.* **22**, 443-449.

Pitkin, S. L., Maguire, J. J., Bonner, T. I. and Davenport, A. P. (2010) International union of basic and clinical pharmacology. LXXIV. Apelin receptor nomenclature, distribution, pharmacology and function. *Pharmacol. Rev.* **62**, 331-342.

Pope, G. R., Roberts, E. M., Lolait, S. J. and O'Carroll, A. M. (2012) Central and peripheral apelin receptor distribution in the mouse: species differences with rat. *Peptides* **33**, 139-148.

Pradhan, A. A., Perroy, J., Walwyn, W. M., Smith, M. L., Vicente-Sanchez, A., Segura, L., Bana, A., Kieffer, B. L. and Evans, C. J. (2016) Agonist-specific recruitment of arrestin isoforms differentially modify delta opioid receptor function. *J. Neurosci.* **36**, 3541-3551.

Quiat, D. and Olson, E. N. (2013) MicroRNAs in cardiovascular disease: from pathogenesis to prevention and treatment. *J. Clin. Invest.* **123**, 11-18.

Rahmeh, R., Damian, M., Cottet, M., Orcel, H., Mendre, C., Durroux, T., Sharma, K. S., Durand, G., Pucci, B., Trinquet, E., Zwier, J. M., Deupi, X., Bron, P., Baneres, J. L., Mouillac, B. and Granier, S. (2012) Structural insights into biased G protein-coupled receptor signaling revealed by fluorescence spectroscopy. *Proc. Natl. Acad. Sci. U.S.A.* **109**, 6733-6738.

Rajagopal, K., Whalen, E. J., Violin, J. D., Stiber, J. A., Rosenberg, P. B., Premont, R. T., Coffman, T. M., Rockman, H. A. and Lefkowitz, R. J. (2006) β-arrestin2-mediated inotropic effects of the angiotensin II type 1A receptor in isolated cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 16284-16289.

Rajagopal, S., Ahn, S., Rominger, D. H., Gowen-MacDonald, W., Lam, C. M., DeWire, S. M., Violin, J. D. and Lefkowitz, R. J. (2011) Quantifying ligand bias at seven-transmembrane receptors. *Mol. Pharmacol.* **80**, 367-377.

Rajagopal, S., Rajagopal, K. and Lefkowitz, R. J. (2010) Teaching old receptors new tricks: biasing seven-transmembrane receptors. *Nat. Rev. Drug Discov.* **9**, 373-386.

Rane, S., He, M. Z., Sayed, D., Yan, L., Vatner, D. and Abdellatif, M. (2010) An antagonism between the AKT and β-adrenergic signaling pathways mediated through their reciprocal effects on miR-199a-5p. *Cell. Signal.* **22**, 1054-1062.

Rankovic, Z., Brust, T. F. and Bohn, L. M. (2016) Biased agonism: An emerging paradigm in GPCR drug discovery. *Bioorg. Med. Chem. Lett.* **26**, 241-250.

Rashid, A. J., So, C. H., Kong, M. M. C., Furtak, T., El-Ghundi, M., Cheng, R., O'Dowd, B. F. and George, S. R. (2007) D1-D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of G_q/11 in the striatum. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 654-659.

Reinartz, M. T., Kalble, S., Littmann, T., Ozawa, T., Dove, S., Kaever, V., Wainer, I. W. and Seifert, R. (2015) Structure-bias relationships for fenoterol stereoisomers in six molecular and cellular assays at the β_2-adrenoceptor. *Naunyn Schmiedebergs Arch. Pharmacol.* **388**, 51-65.

Rives, M. L., Rossillo, M., Liu-Chen, L. Y. and Javitch, J. A. (2012) 6'-Guanidinonaltrindole (6'-GNTI) is a G protein-biased κ-opioid receptor agonist that inhibits arrestin recruitment. *J. Biol. Chem.* **287**, 27050-27054.

Rodefeld, M. D., Beau, S. L., Schuessler, R. B., Boineau, J. P. and Saffitz, J. E. (1996) β-adrenergic and muscarinic cholinergic receptor densities in the human sinoatrial node: identification of a high β_2-adrenergic receptor density. *J. Cardiovasc. Electrophysiol.* **7**, 1039-1049.

Rosano, L., Cianfrocca, R., Tocci, P., Spinella, F., Di Castro, V., Spadaro, F., Salvati, E., Biroccio, A. M., Natali, P. G. and Bagnato, A. (2013a) β-arrestin-1 is a nuclear transcriptional regulator of endothelin-1-induced β-catenin signaling. *Oncogene* **32**, 5066-5077.

Rosano, L., Spinella, F. and Bagnato, A. (2013b) Endothelin 1 in cancer: biological implications and therapeutic opportunities. *Nat. Rev. Cancer* **13**, 637-651.

Rosano, L., Spinella, F., Di Castro, V., Nicotra, M. R., Dedhar, S., de Herreros, A. G., Natali, P. G. and Bagnato, A. (2005) Endothelin-1 promotes epithelial-to-mesenchymal transition in human ovarian cancer cells. *Cancer Res.* **65**, 11649-11657.

Rosethorne, E. M. and Charlton, S. J. (2011) Agonist-biased signaling at the histamine H4 receptor: JNJ7777120 recruits β-arrestin without activating G proteins. *Mol. Pharmacol.* **79**, 749-757.

Rubin, J. B. (2009) Chemokine signaling in cancer: One hump or two? *Semin. Cancer Biol.* **19**, 116-122.

Sakurai, T., Yanagisawa, M., Takuwa, Y., Miyazaki, H., Kimura, S., Goto, K. and Masaki, T. (1990) Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* **348**, 732-735.

Salloum, F. N., Yin, C. and Kukreja, R. C. (2010) Role of microRNAs in cardiac preconditioning. *J. Cardiovasc. Pharmacol.* **56**, 581-588.

Schorlemmer, A., Matter, M. L. and Shohet, R. V. (2008) Cardioprotective signaling by endothelin. *Trends Cardiovasc. Med.* **18**, 233-239.

Schwarz, E. R., Kersting, P. H., Reffelmann, T., Meven, D. A., Al-Dashti, R., Skobel, E. C., Klosterhalfen, B. and Hanrath, P. (2003) Cardioprotection by Carvedilol: antiapoptosis is independent of β-adrenoceptor blockage in the rat heart. *J. Cardiovasc. Pharmacol. Ther.* **8**, 207-215.

Scimia, M. C., Hurtado, C., Ray, S., Metzler, S., Wei, K., Wang, J. M., Woods, C. E., Purcell, N. H., Catalucci, D., Akasaka, T., Bueno, O. F., Vlasuk, G. P., Kaliman, P., Bodmer, R., Smith, L. H., Ashley, E., Mercola, M., Brown, J. H. and Ruiz-Lozano, P. (2012) APJ acts as a dual receptor in cardiac hypertrophy. *Nature* **488**, 394-398.

Shenoy, S. K., McDonald, P. H., Kohout, T. A. and Lefkowitz, R. J. (2001) Regulation of receptor fate by ubiquitination of activated β2-adrenergic receptor and β-arrestin. *Science* **294**, 1307-1313.

Shimizu, I., Minamino, T., Toko, H., Okada, S., Ikeda, H., Yasuda, N., Tateno, K., Moriya, J., Yokoyama, M., Nojima, A., Koh, G. Y., Akazawa, H., Shiojima, I., Kahn, C. R., Abel, E. D. and Komuro, I. (2010) Excessive cardiac insulin signaling exacerbates systolic dysfunction induced by pressure overload in rodents. *J. Clin. Invest.* **120**, 1506-1514.

Shukla, A. K., Violin, J. D., Whalen, E. J., Gesty-Palmer, D., Shenoy, S. K. and Lefkowitz, R. J. (2008) Distinct conformational changes in β-arrestin report biased agonism at seven-transmembrane receptors. *Proc. Natl. Acad. Sci. U.S.A.* **105**, 9988-9993.

Shukla, A. K., Xiao, K. H. and Lefkowitz, R. J. (2011) Emerging paradigms of β-arrestin-dependent seven transmembrane receptor signaling. *Trends Biochem. Sci.* **36**, 457-469.

Skiba, N. P., Bae, H. and Hamm, H. E. (1996) Mapping of effector binding sites of transducin α-subunit using $G\alpha_t$/$G\alpha_{i1}$ chimeras. *J. Biol. Chem.* **271**, 413-424.

Soergel, D. G., Subach, R. A., Sadler, B., Connell, J., Marion, A. S., Cowan, C. L., Violin, J. D. and Lark, M. W. (2014) First clinical experience with TRV130: pharmacokinetics and pharmacodynamics in healthy volunteers. *J. Clin. Pharmacol.* **54**, 351-357.

Song, X. F., Coffa, S., Fu, H. A. and Gurevich, V. V. (2009) How does arrestin assemble MAPKs into a signaling complex? *J. Biol. Chem.* **284**, 685-695.

Song, X. S., Zheng, X. L., Malbon, C. C. and Wang, H. Y. (2001) $G\alpha_{i2}$ enhances *in vivo* activation of and insulin signaling to GLUT4. *J. Biol. Chem.* **276**, 34651-34658.

Spinella, F., Rosano, L., Di Castro, V., Nicotra, M. R., Natali, P. G. and Bagnato, A. (2004) Inhibition of cyclooxygenase-1 and-2 expression by targeting the endothelin a receptor in human ovarian carcinoma cells. *Clin. Cancer Res.* **10**, 4670-4679.

Takahashi, A., Kato, K., Kuboyama, A., Inoue, T., Tanaka, Y., Kuhara, A., Kinoshita, K., Takeda, S. and Wake, N. (2009) Induction of senescence by progesterone receptor-B activation in response to cAMP in ovarian cancer cells. *Gynecol. Oncol.* **113**, 270-276.

Tang, Y. P., Wang, Y. C., Park, K. M., Hu, Q. P., Teoh, J. P., Broskova, Z., Ranganathan, P., Jayakumar, C., Li, J., Su, H. B., Tang, Y. L., Ramesh, G. and Kim, I. M. (2015) MicroRNA-150 protects the mouse heart from ischaemic injury by regulating cell death. *Cardiovasc. Res.* **106**, 387-397.

Teoh, J. P., Park, K. M., Broskova, Z., Jimenez, F. R., Bayoumi, A. S., Archer, K., Su, H. B., Johnson, J., Weintraub, N. L., Tang, Y. and Kim, I. M. (2015) Identification of gene signatures regulated by carvedilol in mouse heart. *Physiol. Genomics* **47**, 376-385.

Teoh, J. P., Park, K. M., Wang, Y. C., Hu, Q. P., Kim, S., Wu, G. Y., Huang, S., Maihle, N. and Kim, I. M. (2014) Endothelin-1/Endothelin A receptor-mediated biased signaling is a new player in modu-

lating human ovarian cancer cell tumorigenesis. *Cell. Signal.* **26**, 2885-2895.

Thompson, G. L., Kelly, E., Christopoulos, A. and Canals, M. (2015) Novel GPCR paradigms at the μ-opioid receptor. *Br. J. Pharmacol.* **172**, 287-296.

Tong, H., Bernstein, D., Murphy, E. and Steenbergen, C. (2005) The role of β-adrenergic receptor signaling in cardioprotection. *FASEB J.* **19**, 983-985.

Vanderbeld, B. and Kelly, G. M. (2000) New thoughts on the role of the β gamma subunit in G protein signal transduction. *Biochem. Cell Biol.* **78**, 537-550.

Violin, J. D., DeWire, S. M., Yamashita, D., Rominger, D. H., Nguyen, L., Schiller, K., Whalen, E. J., Gowen, M. and Lark, M. W. (2010) Selectively engaging β-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance. *J. Pharmacol. Exp. Ther.* **335**, 572-579.

Viscusi, E., Minkowitz, H., Webster, L., Soergel, D., Burt, D., Subach, R. and Skobieranda, F. (2016) Rapid reduction in pain intensity with oliceridine (TRV130), a novel μ receptor G protein Pathway Selective modulator (μ-GPS), vs. Morphine: an analysis of two phase 2 randomized clinical trials. *J. Pain* **17**, S82-S83.

Wacker, D., Wang, C., Katritch, V., Han, G. W., Huang, X. P., Vardy, E., McCorvy, J. D., Jiang, Y., Chu, M. H., Siu, F. Y., Liu, W., Xu, H. E., Cherezov, V., Roth, B. L. and Stevens, R. C. (2013) Structural features for functional selectivity at serotonin receptors. *Science* **340**, 615-619.

Wang, X. H., Ha, T. Z., Zou, J. H., Ren, D. Y., Liu, L., Zhang, X., Kalbfleisch, J., Gao, X., Williams, D. and Li, C. F. (2014) MicroRNA-125b protects against myocardial ischaemia/reperfusion injury via targeting p53-mediated apoptotic signalling and TRAF6. *Cardiovasc. Res.* **102**, 385-395.

Wess, J. (1997) G-protein-coupled receptors: molecular mechanisms involved in receptor activation and selectivity of G-protein recognition. *FASEB J.* **11**, 346-354.

White, K. L., Robinson, J. E., Zhu, H., DiBerto, J. F., Polepally, P. R., Zjawiony, J. K., Nichols, D. E., Malanga, C. J. and Roth, B. L. (2015) The G protein-biased κ-opioid receptor agonist RB-64 is analgesic with a unique spectrum of activities *in vivo*. *J. Pharmacol. Exp. Ther.* **352**, 98-109.

Williams, D. L., Jr., Jones, K. L., Colton, C. D. and Nutt, R. F. (1991) Identification of high affinity endothelin-1 receptor subtypes in human tissues. *Biochem. Biophys. Res. Commun.* **180**, 475-480.

Winpenny, D., Clark, M. and Cawkill, D. (2016) Biased ligand quantification in drug discovery: from theory to high throughput screening to identify new biased opioid receptor agonists. *Br. J. Pharmacol.* **173**, 1393-1403.

Wisler, J. W., DeWire, S. M., Whalen, E. J., Violin, J. D., Drake, M. T., Ahn, S., Shenoy, S. K. and Lefkowitz, R. J. (2007) A unique mechanism of β-blocker action: carvedilol stimulates β-arrestin signaling. *Proc. Natl. Acad. Sci. U.S.A.* **104**, 16657-16662.

Woo, A. Y., Jozwiak, K., Toll, L., Tanga, M. J., Kozocas, J. A., Jimenez, L., Huang, Y., Song, Y., Plazinska, A., Pajak, K., Paul, R. K., Bernier, M., Wainer, I. W. and Xiao, R. P. (2014) Tyrosine 308 is necessary for ligand-directed Gs protein-biased signaling of β_2-adrenoceptor. *J. Biol. Chem.* **289**, 19351-19363.

Woo, A. Y., Wang, T. B., Zeng, X. K., Zhu, W. Z., Abernethy, D. R., Wainer, I. W. and Xiao, R. P. (2009) Stereochemistry of an agonist determines coupling preference of β_2-adrenoceptor to different G proteins in cardiomyocytes. *Mol. Pharmacol.* **75**, 158-165.

Wright, J. J., Kim, J., Buchanan, J., Boudina, S., Sena, S., Bakirtzi, K., Ilkun, O., Theobald, H. A., Cooksey, R. C., Kandror, K. V. and Abel, E. D. (2009) Mechanisms for increased myocardial fatty acid utilization following short-term high-fat feeding. *Cardiovasc. Res.* **82**, 351-360.

Xiang, Y. and Kobilka, B. K. (2003) Myocyte adrenoceptor signaling pathways. *Science* **300**, 1530-1532.

Xiao, R. P. and Balke, C. W. (2004) Na^+/Ca^{2+} exchange linking β_2-adrenergic Gi signaling to heart failure: associated defect of adrenergic contractile support. *J. Mol. Cell. Cardiol.* **36**, 7-11.

Xiao, R. P., Ji, X. W. and Lakatta, E. G. (1995) Functional coupling of the β2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes. *Mol. Pharmacol.* **47**, 322-329.

Xiao, R. P., Zhang, S. J., Chakir, K., Avdonin, P., Zhu, W. Z., Bond, R. A., Balke, C. W., Lakatta, E. G. and Cheng, H. P. (2003) Enhanced G_i signaling selectively negates β_2-adrenergic receptor (AR)- but not β_1-AR-mediated positive inotropic effect in myocytes from failing rat hearts. *Circulation* **108**, 1633-1639.

Yu, Q. J., Si, R., Zhou, N., Zhang, H. F., Guo, W. Y., Wang, H. C. and Gao, F. (2008) Insulin inhibits β-adrenergic action in ischemic/reperfused heart: a novel mechanism of insulin in cardioprotection. *Apoptosis* **13**, 305-317.

Yuan, Y. Y., Stevens, D. L., Braithwaite, A., Scoggins, K. L., Bilsky, E. J., Akbarali, H. I., Dewey, W. L. and Zhang, Y. (2012) 6β-N-heterocyclic substituted naltrexamine derivative NAP as a potential lead to develop peripheral mu opioid receptor selective antagonists. *Bioorg. Med. Chem. Lett.* **22**, 4731-4734.

Zhang, Y., Williams, D. A., Zaidi, S. A., Yuan, Y. Y., Braithwaite, A., Bilsky, E. J., Dewey, W. L., Akbarali, H. I., Streicher, J. M. and Selley, D. E. (2016) 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-(4'-pyridylcarboxamido)morphinan (NAP) modulating the mu opioid receptor in a biased fashion. *ACS Chem. Neurosci.* **7**, 297-304.

Zhou, L., Lovell, K. M., Frankowski, K. J., Slauson, S. R., Phillips, A. M., Streicher, J. M., Stahl, E., Schmid, C. L., Hodder, P., Madoux, F., Cameron, M. D., Prisinzano, T. E., Aube, J. and Bohn, L. M. (2013) Development of functionally selective, small molecule agonists at kappa opioid receptors. *J. Biol. Chem.* **288**, 36703-36716.

Zhu, W. Z., Zheng, M., Koch, W. J., Lefkowitz, R. J., Kobilka, B. K. and Xiao, R. P. (2001) Dual modulation of cell survival and cell death by β_2-adrenergic signaling in adult mouse cardiac myocytes. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 1607-1612.