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Targeting G protein-coupled receptor signalling by blocking G proteins

Adrian P. Campbell and **Alan V. Smrcka***

Department of Pharmacology, University of Michigan School of Medicine, Ann Arbor, MI, USA.

Abstract

G protein-coupled receptors (GPCRs) are the largest class of drug targets, largely owing to their druggability, diversity and physiological efficacy. Many drugs selectively target specific subtypes of GPCRs, but high specificity for individual GPCRs may not be desirable in complex multifactorial disease states in which multiple receptors may be involved. One approach is to target G protein subunits rather than the GPCRs directly. This approach has the potential to achieve broad efficacy by blocking pathways shared by multiple GPCRs. Additionally, because many GPCRs couple to multiple G protein signalling pathways, blocking specific G protein subunits can 'bias' GPCR signals by inhibiting only a subset of these signals. Molecules that target G protein α or βγ-subunits have been developed and show strong efficacy in multiple preclinical disease models and biased inhibition of G protein signalling. In this Review, we discuss the development and characterization of G protein α and βγ-subunit ligands and the preclinical evidence that this exciting new approach has potential for therapeutic efficacy in a number of indications, such as pain, thrombosis, asthma and heart failure.

G protein-coupled receptors (GPCRs) are important targets for current drugs and drug discovery largely owing to the wide range of physiologies and pathophysiologies in which GPCR targeting can have a major impact. GPCRs signal via direct interactions with heterotrimeric G proteins on the inner surface of the plasma membrane, where the GPCR acts as an exchange factor to enhance the release of GDP from the G protein, leading to the subsequent binding of GTP and conformational activation[1,2]. Heterotrimeric G proteins are composed of Gα, Gβ and Gγ subunits. The Gα subunit binds to either GTP or GDP; Gβ and Gγ subunits form a constitutive heterodimer that binds reversibly to the Gα subunit. GTP binding activates the Gα subunit, and the resulting conformational changes lead to dissociation from the receptor and from Gβγ subunits. These free subunits are now competent to interact with the downstream enzymes or channels to drive second messenger generation or changes in membrane potential that modulate cell physiology.

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* avsmrcka@umich.edu.

Competing interests

The authors declare no competing interests.

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Following activation, most GPCRs are phosphorylated by GPCR kinases (GRKs), then bind to arrestin and become internalized. There are seven isoforms of GRKs, GRK1–7, with GRK1 and GRK7 strictly found in the retina, where they function to desensitize rhodopsin[3,4]. Of the remaining GRKs, GRK2 and GRK3 are cytosolic and are recruited to the membrane by binding to the free Gβγ subunits that are released upon G protein activation and by coincident association with phosphatidylinositol- 4,5-bisphosphate (PtdIns(4,5)P_2, also known as PIP_2) in the plasma membrane. GRK4, GRK5 and GRK6 are constitutively membrane associated via carboxy-terminal polybasic regions that interact with negatively charged phospholipids and/or post-translational palmitoylation. β-Arrestins bind to phosphorylated GPCRs to mediate internalization of the receptor, which was originally thought to turn off and desensitize the receptor[5]. It has been proposed that β-arrestins directly transmit GPCR signals. However, recent data indicate that downstream signalling previously attributed to β-arrestins is in fact dependent on classical G protein signalling[6–9]. Indeed, internalized GPCRs activate G protein signalling on endosomes[10,11]. This activation results in a second wave of longer-term GPCR-dependent signalling that could partially explain the effects of β-arrestin depletion on downstream signalling.

GPCRs bind to many known drugs and are important potential targets for drug discovery[12]. Recently, there has been interest in targeting G proteins downstream of the receptors themselves. This approach has multiple advantages. Many complex diseases result from dysregulation of multiple GPCRs, such that targeting a single GPCR may not achieve the desired effects[13]. Primary examples are the chronic inflammatory diseases in which multiple chemokines (the receptors for which are GPCRs) are dysregulated. Additionally, it has become appreciated that dysregulation of the G protein systems themselves can drive disease. The involvement of activating mutations of protein $G\alpha_{q/11}$ subunits in driving uveal melanoma is discussed in detail below[14,15].

A current approach to identifying new GPCR therapeutics has been to identify ligands that interact with GPCRs in binding modes that favour specific conformations of the receptor that activate only select downstream pathways[16,17]. The emphasis has been on finding ligands that lead either to preferential activation of G proteins or to β-arrestin binding by GPCRs. One example is the discovery of μ-opioid receptor (MOR) agonists that bias MORs towards G protein activation over β-arrestin recruitment to improve the safety of opioid analgesics. The underlying basis for this idea comes from data from β-arrestin-knockout mice, which show enhanced G protein-dependent analgesia upon opioid treatment with fewer adverse effects such as respiratory depression and development of tolerance[18–20]. Recent clinical trial data indicate that a new G protein-biased MOR agonist, oliceridine, is effective at relieving postoperative pain, with significantly less nausea and respiratory depression[21,22].

Direct G protein targeting is an alternative approach to bias GPCRs by blocking selected post-receptor signalling pathways (FIG. 1). Targeting specific G protein sub units downstream of GPCRs can bias GPCR signals away from detrimental signalling pathways but leave pathways that are important for normal cell functioning intact. Targeting G protein subunits that are common to signalling downstream of receptor families may also improve therapeutic efficacy in complex disease such as heart failure, inflammation and asthma.

Finally, targeting G proteins themselves could have efficacy in treating diseases driven by G protein dysregulation. This Review discusses the strategy behind targeting G proteins and provides examples in which G protein inhibition has shown therapeutic efficacy in preclinical models, thereby demonstrating the potential power of this new approach to therapeutics.

Overview of G protein subunit families

The heterotrimeric G protein family consists of numerous, diverse individual subunit isoforms, with 20 different G protein α-subunits, 5 different β-subunits and 12 different γ-subunits that can associate combinatorially to produce a dizzying array of potential G protein heterotrimers[23–26]. GPCR coupling specificity and downstream target regulation are driven largely by the identity of the Gα subunit, and these can be classified into four different families, simplifying the problem to some degree[27]. For all of the G protein subunit classes discussed below, identification of direct regulatory interactions between specific G protein α-subunit family members and targets were defined through purified protein reconstitution experiments[28–31]. This approach has laid out a direct mechanistic biochemical basis for much, but not all, of the specificity observed in cellular physiology. Purified protein reconstitutions have also clearly defined direct interactions between Gβγ subunits and downstream effectors[32–34]. Gβ and Gγ diversity is important in physiology, but individual classification based on biochemical properties has not been successful[26,35]. For the purposes of pharmacological targeting, the Gβγ subunits will be discussed as a single class.

$G\alpha_s$

The Gα subunits of the $G\alpha_s$ family were the first G proteins discovered and were purified on the basis of their ability to stimulate adenylyl cyclase[28]. $G\alpha_s$–GTP directly binds to adenylyl cyclase, resulting in increased catalytic activity and cAMP production[36]. Although there are numerous modulators of the nine adenylyl cyclase isoforms, all are activatable by $G\alpha_s$. There are three $G\alpha_s$ isoforms: two splice variants — $G\alpha_{s\ short}$ and $G\alpha_{s\ long}$ — and the distinct gene product $G\alpha_{olf}$. Suramin and related small molecules inhibit activation of $G\alpha_s$ but have multiple targets and are not cell permeable; therefore, they are not useful for targeting $G\alpha_s$ in vivo[37]. There is potential interest in targeting members of the $G\alpha_s$ family because activating mutations in $G\alpha_s$ lead to numerous syndromes, including benign pituitary adenomas and pancreatic adenocarcinomas, and are found in 3.5% of all tumour sequences in the COSMIC database (see Related links).

$G\alpha_i$

$G\alpha_i$ subunits were discovered as inhibitors of adenylyl cyclase, and one of them, $G\alpha_o$, was found as a high-abundance Gα subunit in brain extracts[38–41]. The canonical function of $G\alpha_i$ family G proteins is to bind directly to and inhibit adenylyl cyclase isoforms, thereby leading to a decreased cAMP concentration in cells. The $G\alpha_i$ family consists of $G\alpha_o$, $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_z$ and $G\alpha_t$ (REFS[27,42]). All three $G\alpha_i$ isoforms (1, 2 and 3) inhibit adenylyl cyclase in biochemical experiments with no clearly distinguishable isoform-specific functions[36,43]. $G\alpha_o$ also weakly inhibits adenylyl cyclase and has no other clearly defined function, although recent work suggests a role for $G\alpha_o$ in the Golgi apparatus regulating

neurite outgrowth[44]. All of the $G\alpha_i$ family members, except for $G\alpha_z$ (REF.[45]), are inhibited by pertussis toxin (PTX) through ADP-ribose modification of a unique cysteine at the carboxyl terminus of $G\alpha_i$ subunits, which inhibits the interaction of $G\alpha_i$ with receptors, presumably by steric occlusion[41,46]. No specific small-molecule inhibitors of $G\alpha_i$ family subunits have been identified.

$G\alpha_q$ and $G\alpha_{11}$

$G\alpha_q$ and its closely related homologue $G\alpha_{11}$ (which are 90% identical) are responsible for activation of phospholipase Cβ (PLCβ) downstream of GPCR activation[29,47,48]. PLC hydrolyses the membrane lipid PtdIns(4,5)P_2 to inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3, also known as IP_3) and diacylglycerol (DAG), each of which initiates a signal transduction cascade. Ins(1,4,5)P_3 causes the release of calcium into the cytoplasm and DAG activates protein kinase C (PKC); both of these pathways are ubiquitous regulators of cell physiology[49–52]. $G\alpha_q$ or $G\alpha_{11}$ regulation of PLCβ and subsequent Ca^{2+} release are major drivers of cell function throughout the body. These include platelet aggregation, smooth muscle contraction and exocytosis, as well as many others[53–57]. $G\alpha_q$ family members in humans are $G\alpha_q$, $G\alpha_{11}$, $G\alpha_{14}$ and $G\alpha_{15}$, all of which can activate PLCβ[58]. These subunits have distinct tissue distributions, but no biochemical specificity for target interactions has yet been defined and most physiological data suggest that $G\alpha_q$ and $G\alpha_{11}$ have overlapping functions[47,53,59,60]. However, one study has shown that $G\alpha_q$ and $G\alpha_{11}$ have different roles in GPCR-mediated sensitization of mechanical and thermal nociception in mice that were often distinct and non-compensatory[61].

Although PLCβ is recognized as the canonical target for $G\alpha_{q/11}$, other direct targets of $G\alpha_{q/11}$ have been identified. These include p63RhoGEF, which binds to $G\alpha_{q/11}$ to initiate the activation of Rho by converting Rho–GDP to Rho–GTP. The canonical mechanism for GPCR-mediated activation of Rho downstream of GPCRs is through activation of the $G\alpha_{12}$ or $G\alpha_{13}$ family and direct activation of p115RhoGEF; however, depending on the cell type and the receptor, $G\alpha_{q/11}$-dependent Rho activation may predominate. Examples of RhoGEFs that are activated by $G\alpha_{q/11}$ include p63RhoGEF and TRIO[62], which have been implicated in the regulation of vascular tone[63,64] and uveal melanoma[65], respectively. Another alternative $G\alpha_{q/11}$-regulated pathway involves a direct interaction between $G\alpha_q$ and PKCζ[66]. The Ribas group has demonstrated a direct interaction between $G\alpha_q$ and PKCζ[67] and showed that this interaction is important for extracellular- signal-regulated kinase 5 (ERK5) activation in cardiac myocytes and fibroblasts in response to angiotensin II (ATII)[68].

Specific bioavailable inhibitors for $G\alpha_q$ family members have been identified and are currently the only Gα subunit inhibitors with therapeutic potential[69,70]. These will be discussed below and are a major subject of this Review.

$G\alpha_{12}$ and $G\alpha_{13}$

$G\alpha_{12}$ and $G\alpha_{13}$ were originally identified in a homology screen for novel G protein subunits[23,71]. Later, these subunits were found to directly interact with p115RhoGEF and increase its ability to activate Rho[30,72]. The $G\alpha_{12}$ or $G\alpha_{13}$ pathway is the major pathway through which GPCRs activate Rho.

Gβγ subunits

Early studies indicated that the Gα subunits were the only transducers of G protein signals, but seminal observations demonstrating regulation of inwardly rectifying K^+ channels revealed that Gβγ subunits are bona fide signal transducers[32]. It is now well accepted that the Gβγ subunit heterodimer can activate a wide range of effector targets, the majority of which interact with Gβγ via a single hotspot interface on Gβ[35,73–76]. There are 5 different Gβ isoforms and 12 different Gγ isoforms in humans. $Gβ_{1–4}$ are highly homologous, sharing ~80–90% sequence identity, and $Gβ_5$ shares about 50% identity with those four. $Gβ_5$ is unusual in that it is not thought to interact with Gγ subunits in vivo but rather forms dimers with regulator of G protein signalling (RGS) proteins of the R7 family[77–79]. No clear specific differences have been noted between $Gβ_{1–4}$ in biochemical reconstitution or cell transfection experiments, although some subtle preferences have been noted[80–84]. Co-transfected $Gβ_5γ$ is often less effective at activating downstream targets, but because $Gβ_5$ subunits are not thought to interact with Gγ or Gα subunits in the traditional sense, this apparent biochemical selectivity does not likely reflect physiological specificity[85].

Gγ subunits are more diverse, with intrafamily identities as low as 25%. Similar to Gβ subunits, activation of most effectors by $Gβγ_x$ combinations does not reveal any particular specificity either with purified proteins or upon transfection. $Gγ_1$-containing Gβγ complexes are consistently less potent that other Gβγ subunits with respect to effector activation, but because $Gγ_1$ is almost exclusively expressed in the retina (see Bgee database), this cannot be taken as evidence for physiological Gγ subunit target specificity[80,81].

Most effectors bind to a highly conserved surface on Gβ subunits that roughly corresponds to the Gα–Gβγ interface. In GPCR–G protein crystal structures, interactions between receptors and Gγ subunits have not been observed. In two recent cryo-electron microscopy structures of calcitonin and glucagon-like peptide 1 (GLP1) receptors in complex with G proteins, some contacts between the receptor and Gβ were observed, but the importance of these interactions is not clear[86–88]. Gγ subunits are not directly involved in target interactions in cases for which this has been defined. These data likely explain why no clear, specific biochemical roles have been attributed to individual isoforms of Gβγ. On the other hand, a number of Gγ and Gβ subunit knockout or knockdown studies indicate exquisite specificity in vivo[89–93]. This finding strongly suggests specific roles for Gβ and Gγ subunits in physiological signalling, but the mechanistic nature of this specificity is not understood. An emerging concept is that different Gγ subunits may impart specific Gβγ plasma membrane affinities, but the physiological relevance of this is not yet clear[94–96].

Because Gβγ subunits do not undergo major conformational changes upon G protein activation, their activities are regulated by the activity of the Gα subunits[2]. Activation of Gα leads to dissociation from Gβγ, which exposes a protein–protein interaction surface on Gβ that can bind to downstream targets[35,74,97–99]. Interestingly, PTX inhibits the activation of many and/or most Gβγ subunit signalling pathways in cells, although PTX does not have any direct actions on Gβγ[35]. PTX inhibits Gβγ signalling because it modifies $Gα_i$ in the $Gα_iβγ$ heterotrimer, preventing $Gα_i$ activation, thereby inhibiting release of Gβγ. This observation indicates that despite the requirement for Gβγ assembly with all Gα subunits

for efficient GPCR coupling, and the presumed Gβγ release upon activation of all GPCRs, Gβγ signalling downstream seems to preferentially involve $G\alpha_i$-coupled receptors, although some exceptions have been noted. One of the targets of downstream Gβγ signalling is GRK2, which functions in a negative feedback loop[100]. Cellular expression of the carboxy-terminal, Gβγ binding fragment from GRK2 selectively inhibits Gβγ signalling in cells in vivo and has been used to test for the specific involvement of Gβγ signalling in particular signalling pathways and pathologies[101–104]. Subsequently, prototypical small molecules that bind directly to Gβγ and inhibit Gβγ signalling were developed and used to validate Gβγ as a potential therapeutic target in a number of conditions[75,105]. The use of Gβγ as a potential therapeutic target will be discussed in detail later in this Review.

Pharmacological targeting of Gα

$G\alpha_q$ family inhibitors

To date, the only specific bioavailable inhibitors of Gα subunits that have a good level of validation are inhibitors of $G\alpha_q$ family members. YM-254890 is a cyclic peptide that was isolated from *Chromobacterium* spp. QS3666 as an inhibitor of ADP-dependent platelet aggregation[70,106–108] (FIG. 2a). These and subsequent studies demonstrated that YM-254890 inhibits $G\alpha_{q/11}$ signalling downstream of multiple $G\alpha_q$-linked GPCRs in platelets and other native and heterologous systems without affecting other GPCR signalling pathways[70]. A highly related compound, FR900359 (FIG. 2a), was isolated from the ornamental primrose plant, *Ardisia crenata*, and also has potent and specific effects on $G\alpha_{q/11}$ signalling[69]

YM-254890 was co-crystallized with $G\alpha_q$, which gave insight into its mechanism of action and suggests paths to the development of Gα subunit-specific inhibitors[109]. YM-254890 binds to a hinge between two independent domains of the Gα subunit, the α-helical domain and the Ras-like domain, and GDP and GTP bind at the interface between the domains[110,111] (FIG. 3). GDP release and subsequent GTP binding require the interface between these domains to open and allow the nucleotide to diffuse into, and out of, the nucleotide binding site[112,113]. Binding of YM-254890 at the hinge between these domains is predicted to prevent domain opening and thereby prevent GDP release and GTP binding, leading to G protein inhibition. FR900359 is structurally very similar to YM-254890 and likely binds in a very similar mode. Indeed, FR900359 was demonstrated to be a potent inhibitor of GDP release in biochemical assays[69].

FR900359 has been extensively characterized in vitro to assess its specificity and efficacy for inhibition of $G\alpha_{q/11}$ -mediated signal transduction[69]. These studies used cell-based assays to examine $G\alpha_{q/11}$-stimulated PLC activity by monitoring inositol phosphate (IP) production, bioluminescence resonance energy transform (BRET) to monitor G protein subunit dissociation and dynamic mass redistribution (DMR) to measure cellular responses to GPCR activation. These data all showed strong specificity for inhibition of $G\alpha_{q/11}$ but not for other G protein-mediated responses. In this same study, in a more physiologically relevant system, FR900359 relaxed phenylephrine/α_1-adrenergic receptor (α_1-AR)-dependent constriction of tail vein arteries. Interestingly, these effects were not diminished with FR900359 washout, suggesting a slowly reversible mechanism of action that is likely

non-covalent. This property may improve the therapeutic utility of this compound in vivo and may partially explain its long duration of action in vivo as described below.

Specific inhibition of $G\alpha_q$-mediated activation of downstream effectors has also been demonstrated using a 27mer peptide derived from a helix–turn–helix region of PLCβ3, which corresponds to a region critical for interaction with $G\alpha_q$. This peptide inhibited PLC activation by $G\alpha_q$ in reconstituted lipid vesicles and inhibited $G\alpha_q$ signalling in cells when transfected as a fusion construct with attached fluorescent proteins[114]. Although it was effective, the peptide has not demonstrated bio-availability when applied exogenously.

Preclinical studies with $G\alpha_{q/11}$ inhibitors in thrombosis.

YM-254890 was originally developed by Astellas Pharma as an antithrombotic agent. It was dropped as a therapeutic programme likely owing to concerns with systemic blood pressure effects that result from inhibiting $G\alpha_q$ in the vasculature. In humans, the receptors for thrombin — proteinase-activated receptors 1 (PAR1) and 4 (PAR4) — mediate platelet aggregation in part through $G\alpha_{q/11}$-induced Ca^{2+} release from endoplasmic reticulum stores[115]. The purinergic receptors $P2Y_1$ and $P2Y_{12}$ are other important pharmacological targets in platelets. The $P2Y_1$ receptor couples to $G\alpha_{q/11}$ whereas $P2Y_{12}$ is coupled to $G\alpha_i$, with stimulation of either receptor resulting in activation of platelet aggregation[116,117]. YM-254890 was originally isolated in a screen to identify inhibitors of platelet aggregation in response to ADP detection by $P2Y_1$ (REF.[106]). YM-254890 effectively and potently inhibited thrombus formation in a vascular carotid injury model in mice but also significantly increased bleeding time using the $FeCl_3$ assay[107,108]. A substantial decrease in blood pressure was also observed with bolus injections in mice and rats. As a result of these side effects, it was suggested that YM-254890 and similar compounds are best utilized as locally delivered agents.

Preclinical studies with $G\alpha_{q/11}$ inhibitors in asthma.

Because of the widely known role of $G\alpha_q$-coupled receptors, such as M_3 muscarinic receptors, ATII receptors and endothelin receptors, in mediating smooth muscle contraction, conditions involving excessive smooth muscle tone could be ameliorated by inhibiting $G\alpha_{q/11}$ pathways. It is through these types of pathway that $G\alpha_{q/11}$ inhibitors have hypotensive effects in vascular smooth muscle. More recent studies have focused on potential uses in the treatment of asthma. The standard of care for acute asthma is bronchodilators, such as β_2-AR agonists, which produce cAMP and cause smooth muscle relaxation, or antagonists of GPCRs coupled to $G\alpha_q$ pathways, such as antagonists of the M_3 muscarinic receptor or the leukotriene receptor, which may chronically mediate elevated bronchial tone.

The Benovic group first investigated the possibility of inhibiting $G\alpha_{q/11}$ directly as a way to ameliorate bronchoconstriction with the idea that multiple $G\alpha_q$-coupled receptors could be contributing to elevated tone and that targeting a single receptor may not achieve optimal efficacy[118]. In this study, FR900359 inhibited airway smooth muscle growth in vitro, which could prevent airway occlusion during airway remodelling in asthma. FR900359 also blocked constriction of lung slices stimulated with a muscarinic agonist, carbachol, or with

histamine. These data suggested that targeting $G\alpha_{q/11}$ could alleviate airway occlusion by blocking both smooth muscle remodelling and smooth muscle constriction mechanisms.

In a more recent study, a comprehensive analysis of inhibition of $G\alpha_{q/11}$ signalling in vitro, ex vivo and in vivo was performed[119]. As suggested from previous work, $G\alpha_{q/11}$ inhibition with FR900359 completely reversed methacholine-induced murine tracheal and lung tissue constriction. Methacholine is often used in humans to test for airway hyperresponsiveness. The same results were observed with histamine-induced porcine and human airway constriction ex vivo, with 80% relaxation observed after treatment with 1 μM FR900359.

Because of the blood pressure side effects with systemic administration of YM-254890 or FR900359, it was postulated that local delivery in the airways may be an ideal strategy to produce therapeutic efficacy. FR900359 was therefore delivered to mice via inhalation as an aerosol and airway resistance was measured. A single dose of FR900359 blocked acute methacholine-induced increases in airway resistance without affecting basal tone. This effect persisted for up to 24 hours, suggesting that FR900359 is stable in vivo and/or only slowly dissociates from $G\alpha_{q/11}$ after binding. As postulated, aerosol delivery of FR900359 did not affect blood pressure or heart rate at doses that strongly inhibited airway constriction.

To further explore the utility of FR900359, it was tested in a murine allergic airway sensitization model that uses ovalbumin. This model results in airway inflammatory cell infiltration and hyperresponsiveness to the muscarinic agonist methacholine. FR900359 treatment completely blocked sensitized respiratory system resistance in response to methacholine. As a more physiologically relevant model, FR900359 application was tested in mice sensitized with an intratracheal house dust mite challenge. This model also results in inflammatory cell infiltration and mucin production, which was not inhibited by FR900359, but, again, FR900359 was very effective at inhibition of hyperresponsiveness to methacholine.

These studies are the first to demonstrate a potential therapeutic use for an inhibitor of a G protein α-subunit. Targeting G protein α-subunits has the potential for pleiotropic effects and could result in multiple side effects. These studies indicate that local application of Gα protein subunit inhibitors, in this case a $G\alpha_{q/11}$ inhibitor, may circumvent these issues. Additionally, because multiple GPCRs that signal through $G\alpha_{q/11}$ mediate airway hyperresponsiveness, a strategy that targets $G\alpha_{q/11}$ has the potential to be more efficacious than a single selective GPCR antagonist.

Preclinical studies with $G\alpha_{q/11}$ inhibitors in melanoma.

$G\alpha_{q/11}$ signalling pathways have been implicated in oncogenic signalling in certain types of cancer. In melanoma, for example, metabotropic glutamate receptor 1 (mGluR1), which is a $G\alpha_q$-coupled receptor, is highly elevated, and as such, FR900359 was tested in a variety of melanoma cell lines for inhibition of ERK signalling and cell proliferation[69]. In cell lines for which growth was sensitive to FR900359, a high basal level of inositol phosphates (IPs) was detected that was suppressed by FR900359, indicating high basal $G\alpha_{q/11}$ activation. FR900359 caused the sensitive cells to change from a proliferative migrating phenotype to a

differentiated, non-dividing and non-migratory state. This finding suggests that $G\alpha_{q/11}$ inhibition is useful for treating melanoma and inhibiting its metastatic progression.

Constitutively active $G\alpha_{q/11}$ mutations $G\alpha_{q/11}$(Q209L) and $G\alpha_{q/11}$(R183C) are prevalent in patients with uveal melanoma[14,15]. These mutations prevent hydrolysis of GTP, maintaining $G\alpha_{q/11}$ in the GTP-bound state, and therefore do not require GDP–GTP exchange to become active. Thus, FR900359 or YM-254890 would not be predicted to inhibit $G\alpha_{q/11}$(Q209L) activity in cells. However, FR900359 has been shown to suppress IP production and proliferation in a human melanoma cell line that carries a $G\alpha_{q/11}$(Q209L) mutation[69]. One possibility is that in a cellular context some low level of nucleotide exchange is required to maintain $G\alpha_{q/11}$(Q209L) in the active state. Indeed a recent study demonstrated that FR900359 drives constitutively active $G\alpha_q$ (Q209L) into the inactive, GDP-bound state by suppressing nucleotide exchange, which results in inhibition of downstream signalling pathways[190]. Additionally, treatment with FR900359 inhibited the proliferation and dedifferentiation of $G\alpha_q$ (Q209L)-driven uveal melanoma cell lines[190], suggesting that FR900359 could be considered as a treatment for uveal melanoma.

For patients with activating $G\alpha_{q/11}$ mutations in uveal melanoma, local application of the compound could bypass systemic effects of FR900359 administration. If topical application was possible, this could provide a useful route for delivery.

Other considerations for $G\alpha_{q/11}$ inhibitors.

As discussed above, both YM-254890 and FR900359 are GDP dissociation inhibitors and would be predicted to inhibit GTP binding and activation of $G\alpha_q$. Thus, this strategy would be expected to be effective under conditions in which the upstream GPCR is overexpressed but not under conditions in which the G protein is constitutively active; $G\alpha_{q/11}$(Q209L) is predicted to have a negligible rate of GTP hydrolysis in vitro and is locked in the GTP-bound state. Thus, a mechanism that inhibits nucleotide exchange would not be expected to regulate these proteins. Nevertheless, the Kostenis group showed that FR900359 is able to inhibit IP accumulation driven by $G\alpha_{q/11}$(Q209L) and $G\alpha_{q/11}$(R183C) in HEK293 cells, although fairly high concentrations of FR900359 were used in these experiments[69]. A possible explanation for this observation is that although the GTP hydrolysis rate by $G\alpha_{q/11}$(Q209L) is negligible in a purified system, in cells there may be factors such as RGS proteins that increase the hydrolysis rate enough that nucleotide exchange becomes a factor in its activation.

Although the detailed characterization of FR900359 by the Kostenis group provided strong evidence for the specificity of FR900359 for $G\alpha_{q/11}$, subsequent analysis has revealed unexpected effects of FR900359 on $G\alpha_i$-mediated signalling[120]. In some cases, GPCR-mediated PLC signalling proceeds through a $G\alpha_i$ -mediated pathway that is inhibited by PTX[121–123]. These PTX-sensitive responses are mediated by $G\beta\gamma$-dependent PLC activation rather than $G\alpha_{q/11}$ activity[33,124,125]. In these studies, FR900359 inhibited PTX-sensitive IP mobilization and ERK1–ERK2 activation stimulated by adenosine A_1, M_2 muscarinic and $P2Y_{12}$ purinergic receptors expressed in CHO cells. FR900359 did not inhibit $G\alpha_i$-mediated inhibition of cAMP production. One possible explanation is that FR900359 can bind directly to $G\beta\gamma$ and inhibit downstream signalling to PLC. Given the very high specificity of this

compound for $G\alpha_q$ versus $G\alpha_s$ and $G\alpha_i$ in other respects, this seems unlikely but remains possible. Another potential explanation may reside in the cooperative nature of PLCβ3 activation by G protein subunits. PLCβ3 is prominently expressed in various cell lines and is likely the relevant PLC isoform in CHO cells[124]. PLCβ3 is synergistically activated by a combination of $G\alpha_{q/11}$ and $G\beta\gamma$[126,127]. It is possible that the $G\beta\gamma$ released upon activation of $G\alpha_i$-coupled receptors in CHO cells is insufficient to activate PLC without a concomitant low-level activation by $G\alpha_{q/11}$. Perhaps a low basal level of $G\alpha_{q/11}$ signalling in these cells is required to observe $G\beta\gamma$-dependent stimulation and FR900359 inhibits this low-level $G\alpha_{q/11}$ basal activity.

A related issue is that FR900359 and YM-254890 stabilize the $G\alpha_q$–GDP state, which has a high affinity for $G\beta\gamma$. Thus, FR900359 and YM-254890 would inhibit signalling by both $G\alpha_q$ and $G\beta\gamma$ released from $G\alpha_q$ heterotrimers. As discussed above, $G\beta\gamma$ signalling is primarily associated with $G\alpha_i$ signalling, but some examples of $G\beta\gamma$ signalling are associated with release from $G\alpha_q$ heterotrimers[128,129].

Prospects for Gα inhibitor development

FR900359 is a very powerful tool for dissecting $G\alpha_q$-mediated signal transduction pathways, and the data discussed above suggest that this approach, and perhaps this compound, is an effective clinical lead. Total synthesis of FR900359 and YM-254890 analogues has been successfully achieved[130,131], and some of the derivatives have half-maximal inhibitory concentration (IC_{50}) values for $G\alpha_{q/11}$ inhibition approaching that of FR900359 (REF.[132]). Synthetic production has allowed diversification of the YM-254890 scaffold, which could yield new Gα subunit subtype-specific inhibitors. Despite substantial divergence in sequence between Gα subunits in the YM-254890 and/or FR900359 binding site, all the YM-254890 derivatives synthesized thus far retain high selectivity for $G\alpha_{q/11}$ inhibition[132]. Nevertheless, the structure–activity relationship of YM-254890 derivatives has only very recently begun to be explored, therefore the potential for development of G protein-specific inhibitors remains.

Pharmacological targeting of Gβγ

Rationale for targeting Gβγ

Initial data suggesting that $G\beta\gamma$ targeting is of clinical utility are based on in vivo expression of a protein-based inhibitor of $G\beta\gamma$, the carboxy-terminal fragment of GRK2 (GRK2ct)[102,103,133–135]. This protein fragment contains the pleckstrin homology (PH) domain region of GRK2, which directly binds to $G\beta\gamma$. Numerous cellular expression studies indicate that this domain can specifically inhibit signalling downstream of $G\beta\gamma$ without affecting other GPCR-initiated signalling pathways[101].

The first utilization of this inhibitor by the Koch group showed that cardiomyocyte-specific expression of GRK2ct in mice improved cardiac function in an animal model of heart failure[103]. GRK2ct inhibits the recruitment of GRK2 to the β-AR, thereby preventing receptor desensitization, which is associated with heart failure[103]. This group and others have used expression of GRK2ct in various preclinical models that together provide

evidence that targeting Gβγ could have therapeutic utility[102–104,136,137]. Other ideas for clinical utility come from the roles for Gβγ signalling in specific physiologies that will be discussed below.

Prototype small-molecule inhibitors of Gβγ subunit signalling have been identified that block a subset of Gβγ-dependent signals downstream of GPCR activation[105]. For example, some molecules block potentially detrimental Gβγ signals without affecting Gα signals, allowing potentially beneficial Gα signals and some Gβγ signals to proceed. As discussed earlier, oliceridine is a biased ligand for the MOR that preferentially activates G protein pathways over β-arrestin pathways[138]. As discussed in more detail below, preclinical data indicate that it is also possible to bias MOR signals with Gβγ inhibitors to favour pain relief while avoiding the detrimental effects of MOR activation. Developing biased ligands for each individual receptor involves painstaking screening and analysis to find appropriate ligands. Molecules that target Gβγ have the potential to bias any receptor that signals through Gβγ. A Gβγ-targeted drug could potentially be coadministered with a GPCR-targeted drug to improve the side-effect profile. This strategy would obviate the need for discovery and approval of biased ligands for every receptor. It is also likely that Gβγ-targeted compounds can bias GPCR signals in ways that cannot be achieved by GPCR-directed biased ligands, thus providing an alternative approach to altering pharmacological efficacy.

It is possible that virus-based strategies for delivery of protein inhibitors of Gβγ could have direct clinical application[135]. In addition to the potential of GRK2ct, the Palczewski laboratory has recently developed Gβγ-directed nanobodies that inhibit Gβγ signalling without interfering with Gα signalling[139]. Viral delivery of either of these proteins has potential for selective delivery to target tissues, but issues with developing therapeutics based on viral therapies must be overcome, and protein-based Gβγ inhibitors will not be discussed further.

Small-molecule Gβγ inhibitors

Prototypical Gβγ inhibitors were discovered in a competition screen with the peptide SIGK for binding to Gβγ[5,140]. The SIGK peptide has been co-crystallized with Gβγ and defines an interaction surface on Gβ that coordinates direct binding to downstream targets and Gα subunits[73]. Thus, compounds that inhibit binding of this peptide likely either bind directly to this surface or alter the surface in a way that interferes with peptide binding and potentially with Gβγ effectors. Gβ subunits are prototypical members of the WD40 repeat protein family and mediate downstream effector activation through direct protein–protein interactions[141]. Traditionally, protein–protein interactions are difficult to disrupt with small molecules, in part because protein interaction surfaces are often flat, making it difficult for small molecules to bind with sufficient energy to disrupt high-affinity interactions[142] (BOX 1). The surface to which SIGK binds is not flat but is rather a concave surface present on the top face of all WD40 repeat proteins, and results from the toroid structure formed as a consequence of the circular folding of the seven blades of the WD40 repeat propeller. This motif is common for protein–protein interactions in biology and has been targeted

successfully by small molecules, leading to multiple potentially clinically important drugs[143–145].

Two prototypical Gβγ-binding molecules have been extensively characterized in vitro and in vivo. These molecules — gallein and M119 — are structurally similar and are related to fluorescein (FIG. 2b). They bind to Gβγ with an apparent affinity of approximately 1 μM; fluorescein does not bind to Gβγ[140,146]. A potentially important property of these molecules is that the binding is very slowly reversible, which could explain their efficacy despite low apparent potency (k_{off} = 0.0003 s^{-1})[146,147] (BOX 2). These compounds are commercially available, are well tolerated in mice and have been widely used by the scientific community[140,148–152]. This profile has allowed for the characterization of the effects of inhibiting Gβγ subunits on GPCR signalling, the specificity of the approach and the evaluation of Gβγ as a potential therapeutic target in a number of animal models of disease.

Another approach to identify small-molecule inhibitors of Gβγ was developed by the Hamm laboratory[153]. This laboratory identified an interaction between Gβγ and components of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex, which is involved in regulating synaptic vesicle fusion. A surface plasmon resonance-based screen was used to identify molecules that would inhibit Gβγ–SNARE interactions. Several molecules were identified in this screen but have not yet been tested in physiological systems.

Downstream signalling specificity

One concern with targeting Gβγ is that Gβγ is a participant in every G protein signalling pathway because it is required for G protein activation by GPCRs, likely because Gβγ stabilizes the conformation of Gα that is required for GPCR interactions[1,112]. As such, completely blocking Gβγ subunit function would completely decouple the G protein system. Thus, the pharmacological approach to blocking Gβγ function must prevent downstream signalling without interfering with G protein activation in general. Proof of principle that this is achievable comes from the capacity of GRK2ct, M119 or gallein to block Gβγ signalling without affecting GPCR-dependent Gα activation[101].

Another potential concern is the ubiquitous expression of Gβγ subunits in every cell downstream of every GPCR; consequently, inhibition of downstream signalling by Gβγ could have wide-ranging effects. In reality, Gβγ subunit signalling is highly restricted to cell types in which Gβγ-responsive effectors are expressed. An example of this is regulation of phosphoinositide 3-kinase (PI3K) signalling by Gβγ in immune cells in response to chemokines. Chemokine receptors couple primarily to $G\alpha_i$ and drive a number of immune cell responses by pathways that are Gβγ-dependent, including PI3K activation and subsequent phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3, also known as PIP_3) production, both of which are blocked by treatment with PTX. Chemokine-dependent PtdIns(3,4,5)P_3 responses are very robust in immune cells such as neutrophils and macrophages[121]. The strength of this response is likely because they express high levels of the Gβγ-responsive PI3Kγ isoform, which consists of P110γ and P101 subunits[34,154]. By contrast, GPCR-dependent PTX-sensitive PtdIns(3,4,5)P_3 responses are not readily observable in other cell types, likely because these cells do not express PI3Kγ. Thus, two

levels of potential selectivity are present in this strategy. One level of selectivity is for $G\alpha_i$-coupled receptors because $G\beta\gamma$-dependent signalling responses arise primarily from $G\alpha_i$, and the second level is due to the restricted expression of $G\beta\gamma$-regulated downstream targets such as PI3Kγ.

As another source of specificity, small-molecule inhibitors of $G\beta\gamma$ that have been identified thus far inhibit a subset of protein–protein interactions without affecting interactions with $G\alpha$ subunits[105,140,146]. $G\beta\gamma$ inter actions with downstream targets have been examined by both X-ray crystallography and mutagenesis[74,155–157]. One key feature of these interactions is that the targets share a common binding surface on $G\beta$ but the details of the interactions are unique for each target[75]. This observation suggests that it is possible for small molecules to selectively interfere with downstream targets by binding to the subset of amino acids on $G\beta$ that is required for binding to a subset of targets (FIG. 4). This contrasts with protein-based approaches with GRK2ct and $G\beta\gamma$-directed nanobodies that occupy a large area on the $G\beta$ protein interaction surface[139]. As yet, no X-ray crystal structure exists for a small molecule bound to $G\beta\gamma$, but the empirical data in which small molecules selectivity block a subset of $G\beta\gamma$ targets support this idea[105,158].

Still, some issues remain because even with restricted expression, inhibition of some targets would have major side effects. Particular targets of concern include ion channels such as the G protein-activated inward rectifier K^+ channel (GIRK) and the N-type voltage-gated calcium channels[32,159,160]. $G\beta\gamma$ activates GIRK channels in neurons and in atria, leading to a hyperpolarization-induced decrease in action potential firing. Therefore, when considering the use of $G\beta\gamma$ inhibitors in cardiac or immune therapy, interfering with the regulation of action potentials would have highly undesirable side effects, such as arrhythmias. However, empirical data using prototypical $G\beta\gamma$ blockers indicate that these pathways are unaffected by $G\beta\gamma$ inhibitors, and animals treated with gallein show no signs of arrhythmias or alterations in heart rate[161].

Another approach to generating specificity for $G\beta\gamma$ signalling would be to target specific $G\beta\gamma$ subtypes. As discussed, there are multiple subtypes of $G\beta$ and $G\gamma$ subunits that have differential tissue distributions and signalling functions in cells and in vivo. Most of the diversity resides in the $G\gamma$ subunits, which have not been shown to interact with effectors, and the surface that has been targeted on $G\beta$ is highly conserved among $G\beta$ subunits (excep t $G\beta_5$). These data suggest that direct, selective, small-molecule targeting of specific $G\beta\gamma$ subunit sub-types will be difficult.

Preclinical models of $G\beta\gamma$ targeting

Opioid analgesia.—Activation of MOR produces analgesia by virtue of its ability to inhibit the release of neurotransmitters in peripheral nociceptors as well as in the descending circuit in the periaqueductal grey of the brain. MOR is $G\alpha_i$-coupled and inhibits neurotransmitter release by regulating a variety of systems in neurons. $G\beta\gamma$ activates GIRK channels to hyperpolarize presynaptic neurons and thus reduce excitability, and $G\beta\gamma$ inhibits the N-type calcium channels that are responsible for Ca^{2+} influx upon neuronal depolarization; both of these mechanisms inhibit neurotransmitter release. $G\beta\gamma$ also interacts with synaptosomal-associated protein 25 (SNAP25) to directly inhibit synaptic

vesicle fusion[162]. Thus, complete inhibition of Gβγ signalling would be expected to eliminate opioid efficacy.

Chronic MOR activation and very-low-dose morphine have also been shown to promote hyperalgesia[163,164]. Several lines of evidence suggest that this phenomenon involves activation of PLCβ3 and subsequent downstream PKC activation[163–165]. Mice with global deletion of PLCβ3 show enhanced sensitivity to MOR activation, which results in a tenfold greater sensitivity to morphine in a 55°C tail flick assay of antinociception[166]. This finding indicates that PLCβ3 activation downstream of MOR inhibits MOR-dependent analgesia. PLCβ3 is responsive to both $G\alpha_q$ and Gβγ, and because MOR is coupled to $G\alpha_i$, any mechanism for PLCβ3 activation must involve Gβγ subunits[124,126,167]. M119 inhibited Gβγ-dependent PLCβ2 and PLCβ3 activation without affecting K^+ channel and Ca^{2+} channel inhibition by Gβ, which suggested that M119 or gallein could increase the analgesic potency of morphine[105].

In initial experiments, M119 was administered through intracerebroventricular injection into mice and morphine dose-dependent analgesia was assessed in the tail flick antinociception test[105]. M119 injection indeed increased the potency of morphine by tenfold. M119 was ineffective when injected into mice lacking PLCβ3, supporting the idea that the effect of M119 is through inhibition of Gβγ regulation of PLCβ3. The observation that M119 injection into the periaqueductal grey inhibited the formation of Gβγ–PLCβ3 complexes in this tissue further supports this mechanism of action[164].

Follow-up experiments showed that M119 was also effective when injected intraperitoneally, was specific for MOR activation and had no effect on δ-opioid or κ-opioid receptor-mediated antinociception[158]. M119 also blocked development of acute tolerance — co-injection of M119 and morphine prevented the rightward shift in dose dependence for antinociception that accompanies repeated morphine injection. In the same study, intracerebroventricular injection of M119 before intraperitoneal administration of high-dose morphine prevented the development of withdrawal symptoms upon naloxone injection. These experiments indicate that M119 acts on central MORs but when injected intraperitoneally it is possible that its actions may be mediated peripherally as well as centrally.

The ability of M119 to shift the dose–response curve for morphine suggests that, if administered in conjunction with morphine, considerably less opioid will be required to achieve the same level of pain relief. These studies indicate that M119 or other Gβγ inhibitors coadministered with μ-opioids would inhibit the development of tolerance and ameliorate withdrawal symptoms. On the other hand, if M119 potentiated all effects of morphine, including the side effects, such an approach would have limited utility. A subsequent study confirmed that gallein potentiated morphine-dependent antinociception when administered intraperitoneally. Gallein did not potentiate morphine-dependent respiratory depression, inhibition of gastrointestinal transit or conditioned place preference[168]. Together, these data indicate that gallein coadministration with opioids increases the therapeutic window for opioid use, which would increase the safety profile of well-characterized opioids and reduce their addictive potential.

New treatments for chronic pain are a major unmet need. Opioids are highly effective at treating acute pain such as postoperative pain, but prolonged usage results not only in tolerance but also in hyperalgesic sensitization of pain responses. This finding has also been shown to be dependent on PLCβ3 signalling, suggesting that M119 or gallein could prevent the hyper sensitization with chronic opioid use[165]. A recent study indeed showed that gallein inhibited hyperalgesic priming in response to repeated opioid administration in mice[163]. Thus, Gβγ inhibitors might be developed that would allow for safe longer-term use of opioids for chronic pain.

Chronic inflammatory disease.—As discussed above, Gβγ signalling is a major driver of signal transduction in immune cells because chemokine receptors signal through $G\alpha_i$. The majority of the downstream processes driven by chemokine receptors are regulated by Gβγ, although some roles specific to $G\alpha_i$ have recently been identified[169,170]. Chemokine receptors are highly sought- after targets for the development of anti-inflammatory treatments for diseases such as rheumatoid arthritis and lupus. These chronic inflammatory diseases involve dysregulation of multiple chemokines and their receptors, so targeting individual GPCRs may have limited efficacy. Blockade of a common target such as Gβγ downstream of multiple chemokine receptors could have greater efficacy in these conditions.

Gallein delivered either intraperitoneally or orally was able to block acute inflammation in a carrageenan foot pad injection model[140]. More recently, gallein was tested in a mouse model of lupus nephritis. In those experiments, gallein was administered intraperitoneally three times a week during disease progression for 20 weeks, or after the animals developed active lupus[171]. Gallein prevented the clinically relevant end point of proteinuria, as well as glomerular inflammatory infiltration, either when given prophylactically or after disease development. Interestingly, gallein treatment also inhibited germinal centre formation in the spleen by reducing both their size and number. These data validate the idea that Gβγ inhibition may have therapeutic utility in chronic inflammatory diseases.

Heart failure.—Cardiac myocyte-specific expression of the protein-based Gβγ inhibitor GRK2ct increased cardiac performance in heart failure[102,103]. This observation suggests that gallein or M119 could be used to inhibit the development of heart failure. Initial studies demonstrated that intraperitoneal administration of gallein improved cardiac function and inhibited the development of cardiac hypertrophy and fibrosis stimulated by chronic β-AR stimulation with isoprenaline administration (for 1 week)[172]. Gallein also and blocked hypertrophy progression in a cardiac-specific calsequestrin transgenic mouse model of cardiac failure[172].

Follow-up studies examined gallein in a pressure overload model of heart failure after establishment of hypertrophy[161]. Gallein administration at 10 mg/kg intraperitoneally preserved cardiac function and halted hypertrophic growth. One of the hallmarks of heart failure is increased plasma concentration of neurohumoral factors such as adrenaline and noradrenaline. Adrenaline release is inhibited by presynaptic $G\alpha_i$-coupled α_2-ARs that are in turn inhibited by GRK2 (REF.[137]) (FIG. 5). GRK2 is upregulated in heart failure and leads to desensitization of α_2-ARs, reducing feedback inhibition of noradrenaline release. In a recent study, inhibition of Gβγ-regulated GRK2 activation, using adenoviruses to express

GRK2ct, prevented α_2-AR desensitization and reduced plasma adrenaline levels in a heart failure model. This finding suggested that systemic administration of a small molecule such as gallein achieves high therapeutic efficacy through a dual mechanism of action: through direct effects in cardiac myocytes and by lowering circulating catecholamine levels. Indeed, treatment with gallein normalized plasma adrenaline and noradrenaline levels and restored α_2-AR-mediated feedback inhibition of catecholamine release[161].

Chronic heart failure can lead to complications, such as the development of secondary renal disease. This disease results in part from activation of the endothelin system, and renal damage is characterized by renal fibrosis and inflammation. A recent study examined the effects of gallein on renal damage caused indirectly by pressure overload-induced cardiac hypertrophy or directly by bilateral ischaemia–reperfusion in a mouse model of acute kidney injury (AKI)[173]. Gallein administration at 10 mg/kg per day prevented elevation of plasma creatinine and renal fibrosis in the transverse aortic constriction model and inhibited renal damage, apoptosis and endothelin elevation in the AKI model.

It has been proposed that Gβγ inhibition in cardiac myocytes inhibits progression of heart failure by preventing Gβγ-dependent recruitment of GRK2, thereby preventing desensitization of the β-AR (FIG. 5). However, treatment with Gβγ inhibitors likely blocks other pathways associated with Gβγ in the heart. For example, it was recently shown that Gβγ can directly bind to and activate ERK, leading to its phosphorylation and trans-location to the nucleus in cardiac myocytes; ERK trans-location is associated with the development of cardiac hypertrophy[174]. Another study identified Gβγ as a regulator of PLCε activation at the Golgi apparatus, which is also critical for endothelin 1 (ET1)-driven hypertrophy[175,176]. Gallein inhibits Gβγ-dependent regulation of PLCε in cardiac myocytes, and Gβγ inhibition has the potential to inhibit ERK activation. Thus, Gβγ inhibition has the potential to be highly efficacious by virtue of its ability to block activation of multiple prohypertrophic targets by Gβγ.

Fibrosis.—In both the heart failure model and the kidney damage model, Gβγ inhibition with gallein prevented the development of fibrosis[173,177]. It is possible that this is a direct cellular effect on fibrosis. Indeed, in mouse embryo fibroblasts, gallein at 10 μM inhibited fibroblast activation in vitro[177].

Other effects of Gβγ blockade.—M119 and gallein have been widely used in cellular systems to assess the potential for blockade of Gβγ in particular disease-related systems. These include proliferation, migration and invasion of cancer cells, T cell regulation, Alzheimer disease models and other biological pathways. Some examples of these studies are listed in TABLE 1.

Prospects for Gβγ inhibitor development.—Gallein and M119 have been powerful tools to dissect Gβγ subunit signalling pathways and are widely available to the scientific community. These compounds are related and are flat, aromatic xanthene dye derivatives, and there are concerns with development of such compounds as human therapeutics owing to potential off-target effects. M119 and gallein seem to be fairly specific in vivo as no major side effects or toxic effects of these molecules were observed with chronic administration.

This observation demonstrates that on-target-based toxicity is not likely to be a major problem. Nevertheless, new small-molecule screens should be pursued to identify novel Gβγ binding scaffolds that are more tractable for pharmaceutical development. Protein–protein interactions are notoriously difficult drug targets but, as has been discussed, WD40 proteins are unique in this regard[145]. Recent discoveries and observations have demonstrated that inhibition of WD40 proteins by small, drug-like molecules is possible and has also elucidated some basic themes about these drugs. Indeed, these WD40 protein–protein interaction inhibitors have been developed to the point of entering clinical trials, which suggests that further screening and the use of structure–activity relationship modelling to refine chemical leads has a reasonable chance of success. Furthermore, these results suggest that Gβγ is indeed a viable pharmacological target.

Outlook

Targeting G protein subunits has emerged as a viable approach to achieve pharmacological efficacy for a number of indications that perhaps would not be achieved by targeting individual GPCRs. Currently, there are specific inhibitors only for $G\alpha_q$ or general inhibitors of Gβγ subunits. Future work is likely to identify novel specific inhibitors for other classes of Gα subunits, and higher potency, selective inhibitors for Gβγ subunits. G proteins are involved in a wide range of physiologies and it is likely that they will emerge as targets for other unexplored therapeutic applications.

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Glossary

Periaqueductal grey	An anatomical region in the brainstem that surrounds the cerebral aqueduct. This region is enriched in opioid receptors that are thought to mediate the central analgesic actions of opioid analgesics
Hyperalgesia	Increased sensitivity to pain. Repeated opioid treatment for chronic pain can paradoxically increase sensitivity to pain stimuli over time

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Box 1 | Development of PPI inhibitors targeting WD40 repeat proteins

In general, protein–protein interaction (PPI) inhibitors share features that seem logical or intuitive. PPI inhibitors tend to be larger than typical drugs (500–800 Da), which is unsurprising because PPIs are often expected to inhibit interactions that typically involve several-hundred to low-thousand $Å^2$ (REF.[2]) interfaces of each protein. The size of these interfaces was perhaps the greatest barrier when envisioning efficacious and potent PPI inhibitors in the first place; however, one of the identified themes for PPI mechanisms is that blocking a few critical residues in protein hot spots is often sufficient to effectively inhibit the PPI. These regions often have unique flexibility, which allows for adaptability to structurally distinct partner proteins but also gives rise to the existence of small, transient pockets where PPI inhibitors can bind[178]. Gβγ has a hot spot that has been implicated in effector protein binding. Thus, Gβγ may have a PPI interface that is amenable to small-molecule binding.

A pattern noted for PPI inhibitors of WD40 repeat (WDR) proteins is a three-pronged or triradiate structure[145]. They often have substituents around a central ring that extend in three directions, one of which extends into the central core of the WDR protein. A rudimentary hypothesis is that such a structure is necessary to both imbue sufficient contact for high-affinity binding and cover sufficient surface area to disrupt the PPI. This observation should be interpreted with caution as the sample size for WDR PPI inhibitors is quite small; however, it is an interesting approach to keep in mind when developing Gβγ inhibitors. It could be argued that gallein has such a structure, with three rings extending from the central oxygen-containing heterocycle. This general structure can also be seen in other less well-characterized Gβγ binders[105]. Although no structural data have been generated for these compounds bound to Gβγ, they all displace binding of the SIGK peptide, which has been crystalized bound to the hot spot near the top of the central pore.

Box 2 | Target residence time in therapeutic efficacy and specificity

An emerging idea in drug discovery is the concept that drug residence time — that is, the rate of dissociation of the drug from its target (k_{off}) — can be an important determinant of therapeutic specificity and efficacy[147]. If a drug has a relatively short plasma half-life, its therapeutic utility could be limited unless target engagement is sustained after the compound is cleared from the plasma. Additionally, if a compound engages multiple targets but dissociates particularly slowly from the desired target, a long residence time could increase specificity. The effects of FR900359 in cells and in ex vivo systems were sustained after compound washout[69] and airway relaxation was sustained in vivo for at least 24 hours after a single dose[119]. Binding of both gallein and M119 to Gβγ is slowly reversible, as measured by surface plasmon resonance[146]. The plasma half-life of gallein is 1–2 hours (A.S. unpublished data), and its affinity for Gβγ is in the high-nanomolar range, yet a dosing regimen of three times weekly was sufficient to ameliorate lupus symptoms over the course of the 20 weeks of disease development[171]. Thus, it appears likely that these compounds achieve therapeutic efficacy at least in part owing to their long residence times.

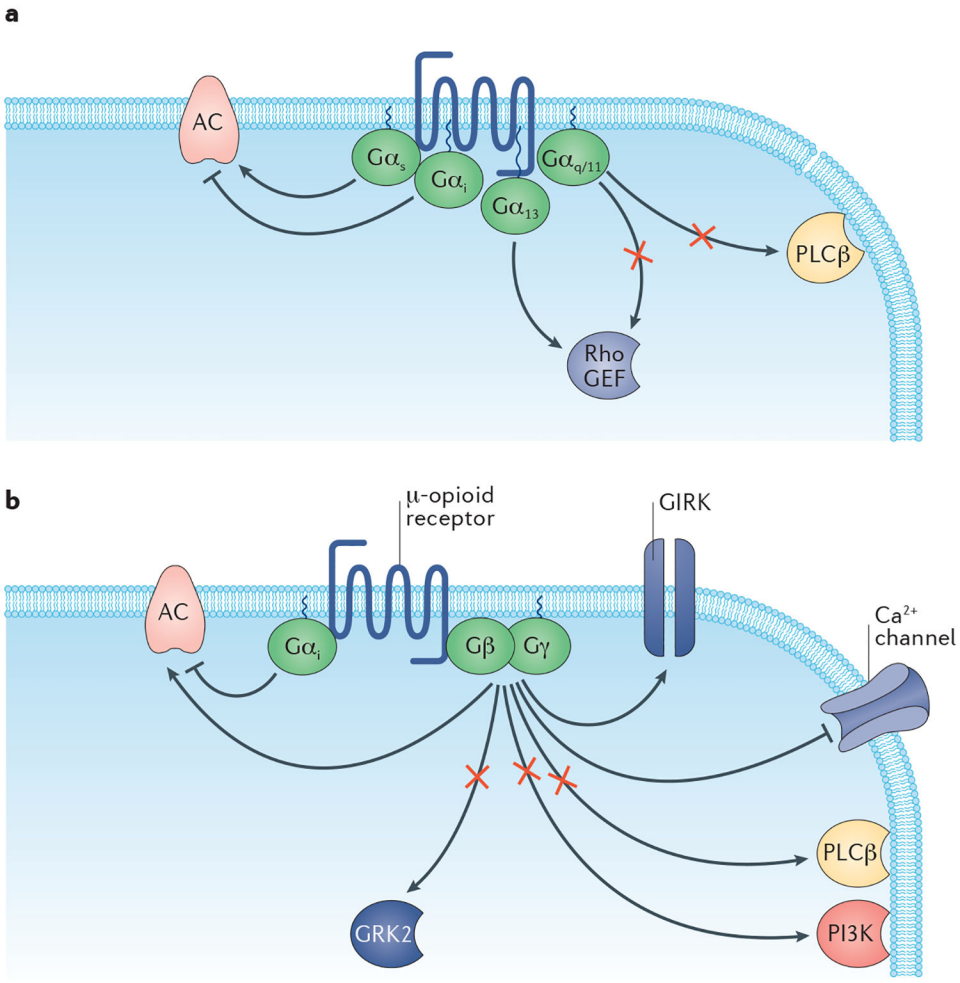

Figure 1. Diagrammatic representation of how G protein inhibition can bias GPCR signalling. **a**| For G protein-coupled receptors (GPCRs) that couple to multiple G protein signalling pathways, inhibition of a specific G protein (inhibition of $G\alpha_q$ is shown as an example) will block some, but not all, pathways. **b** | Another way to bias GPCR signalling is to block of a subset of Gβγ targets. In this example, three downstream targets — phosphoinositide 3-kinase (PI3K), phospholipase Cβ (PLCβ) and GPCR kinase 2 (GRK2) — are selectively blocked. This strategy alters some Gβγ signals downstream of GPCRs but leaves other signals associated with these receptors intact. AC, adenylyl cyclase; GIRK, G protein-activated inward rectifier K^+ channel.

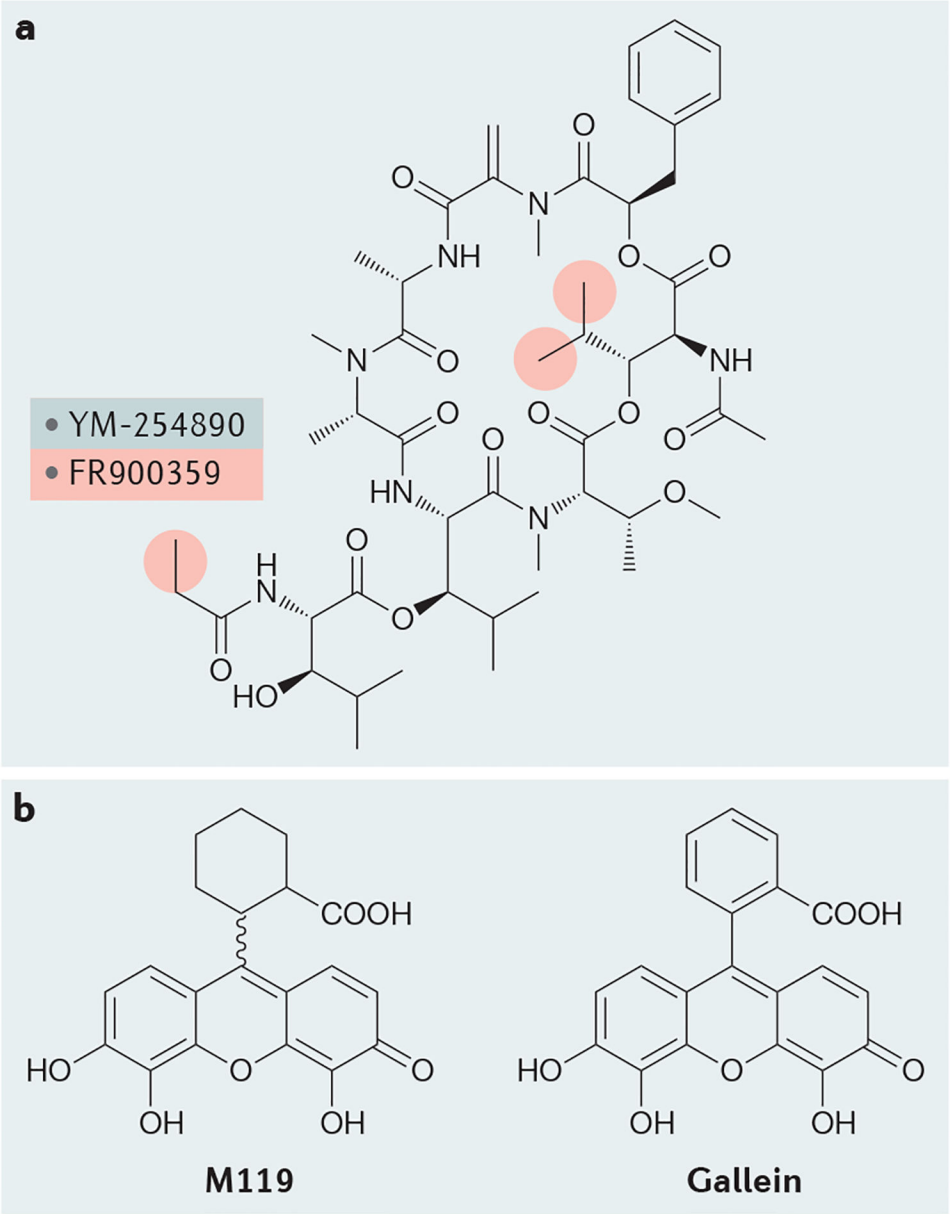

Figure 2 |. Structures of inhibitors of G protein signalling.
a | The $G\alpha_q$ inhibitors YM-254890 (black only) and FR900359 (with red modifications) are shown. **b** | The $G\beta\gamma$ inhibitors, M119 and gallein, differ only in the saturation of the carboxyphenyl ring.

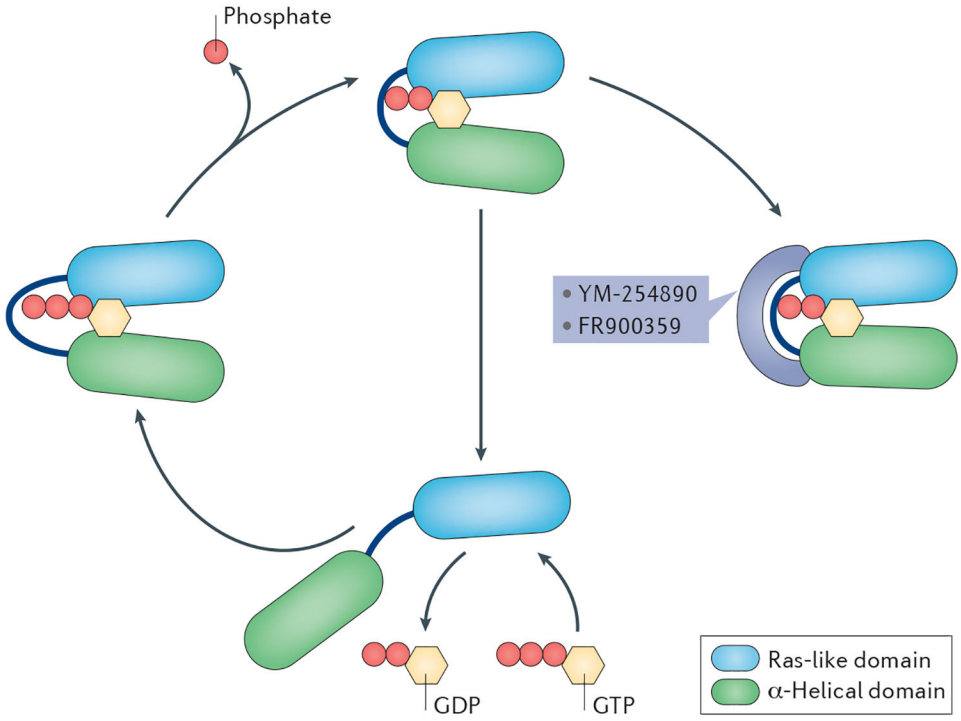

Figure 3 |. Schematic of Gα subunit inhibition by YM−254890 and FR900359.
Canonical G protein α-subunit signalling is initiated when GDP is released from the nucleotide-binding pocket, thereby allowing GTP to bind. The guanine nucleotide-free state is associated with separation of the α-helical domain (green) from the Ras-like domain (blue), which allows GDP to exit the nucleotide binding site. Gα returns to a closed state once GTP is bound, with minor structural perturbations in the hinge or switch region and around the nucleotide binding site. Signalling is terminated when the intrinsic GTPase activity of Gα hydrolyses GTP to GDP, returning Gα to its quiescent state. YM-254890 or FR900359 (purple) binds to the hinge region of $G\alpha_q$, preventing the separation of the domains necessary for GDP release and Gα activation.

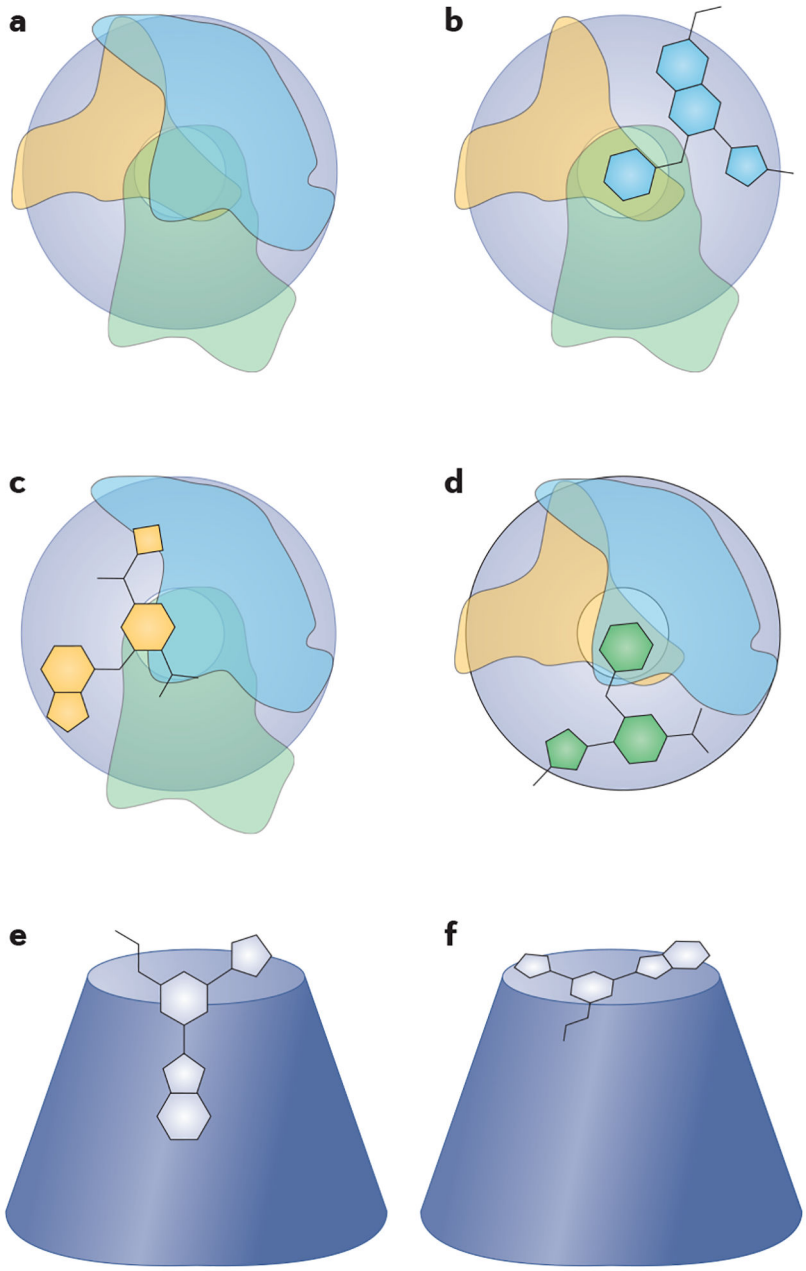

Figure 4 |. Mechanisms for selectively inhibiting signalling downstream of Gβγ.
Most downstream effector proteins (hypothetical footprints represented by orange, green and light blue shapes) interact with the hotspot surface on one face of the toroid structure of Gβ (dark blue). **a** | Downstream signalling proteins interact with Gβγ via distinct but overlapping binding sites. **b–d** | Compounds may anchor in the core but project into different interaction sites, thereby creating selective inhibition of specific binding partners. **e**,**f** | There are numerous potential ways in which small molecules could binding to the Gβγ hot spot. One possibility is a message–address-type pose (part **e**). One branch of the inhibitor would bind within the core of the toroid, mimicking a high-affinity binding pocket (address) and other branches would extend across the hotspot surface to disrupt protein binding (message).

Alternatively, flat molecules may bind across the surface of the hot spot, disrupting large areas of protein binding (part **f**).

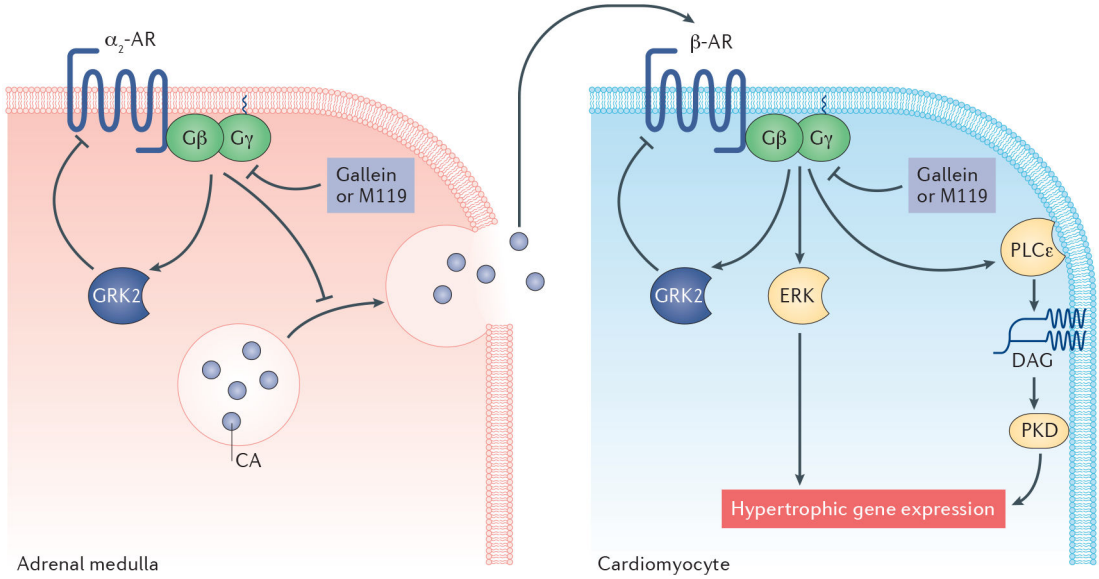

Figure 5 |. Possible targets of Gβγ inhibitors in heart failure.

Hypertrophic pathways in the myocardium and adrenal gland contribute to pathological hypertrophy and heart failure. Gallein or M119 is able to inhibit a number of Gβγ interactions in cardiomyocytes but also alters signalling in the adrenal medulla and thus demonstrates a multifactorial approach to the pharmacological treatment of cardiac hypertrophy and heart failure. In the adrenal medulla, inhibition of Gβγ increases the release of catecholamines (CAs), such as dopamine, adrenaline and noradrenaline. Adrenaline stimulates the β-adrenergic receptor (β-AR) on cardiomyocytes; therefore, inhibition of Gβγ could have numerous therapeutic effects in heart failure. DAG, diacylglycerol; ERK, extracellular-signal-regulated kinase; GRK2, G protein-coupled receptor kinase 2; PKD, protein kinase D; PLCε, phospholipase Cε.

Table 1 |

Selected gallein or M119 effects in ex vivo systems

Ex vivo cellular or organ system	Signalling pathway	Indication	
Hippocampal neurons	Amyloid precursor protein-Gao	Blocks amyloid-p-dependent cell death. Reverses memory impairment in 3xTgAD mice	148
LNCaP prostate cancer cell line	Olfactory receptor 51E2	Inhibits cell invasiveness in vitro and metastasis spread in a xenograft model	149
HEK293 cell line	DAT	Inhibits dopamine efflux through DAT	179
HEK293 and KNRK cell lines	PAR2-PKD	Trafficks PAR2 to the plasma membrane	150
T cells	TCR	Enhances TCR-dependent IL-2 production	180
MCF7 breast cancer cell line	PKC and PI3K	Decreases invasiveness but not proliferation	181
Rat mesenteric resistance arteries	CGRP-cAMP	Increases relaxation	182
HeLa cell line	PLC-PKD	Inhibits cargo transport from the Golgi to the plasma membrane	183
HeLa cell line	S1P-RAC-CDC42	Alters cargo sorting into exosomal vesicles of multivesicular endosomes	152
MDA-MB-231 breast cancer cell line	CXCR4-RAC1	Inhibits migration and invasion	184
MDA-MB-231 and MCF10A breast cancer cell lines	SDF1, LPA, PAR1 and PAR2	Inhibits proliferation and migration	185
PC3 prostate cancer cell line	LPA, PAR1 and SDF1-AKT	Inhibits proliferation and tumour sphere formation, triggers apoptosis and enhances sensitivity to paclitaxel	186
HEK293 cell line	ADGRB2 and a disease-associated mutation	Inhibits downstream signalling to NFAT	187
NIH 3T3 cell line	SHH -SMO-PLA2	Inhibits GLI activation	188
Primary human glioblastoma cells	PREX1	Inhibits motility and invasion	189

ADGRB2, adhesion G protein-coupled receptor B2; CGRP, calcitonin gene-related peptide; CXCR4, CXC-chemokine receptor 4; DAT, dopamine transporter; IL-2, interleukin 2; LPA, lysophosphatidic acid; NFAT, nuclear factor of activated T cells; PAR2, proteinase- activated receptor 2; PI3K, phosphoinositide 3-kinase; PKD, protein kinase D; PLA2, secretory phospholipase A2 receptor; PLC, phospholipase C; PREX1, phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein; RAC1, Ras-related C3 botulinum toxin substrate 1; S1P, sphingosine 1-phosphate; SDF1, stromal cell-derived factor 1; SHH, sonic hedgehog protein; SMO, smoothened homologue; TCR, T cell receptor.