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Taking the heart failure battle inside the cell: small molecule targeting of Gβγ subunits

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Heart failure (HF) is devastating disease with poor prognosis, and remains a leading cause of death worldwide. In the U.S. alone, ~5.8 million people suffer from HF, with ~670,000 new cases per year and an estimated 2010 cost of \$39.2 billion [1]. Given the steady growth of diabetic and aging populations, new therapeutic approaches are desperately needed. Considering elevated sympathetic tone in progressive HF that ultimately leads to desensitization of the sympathetic response, normalizing cardiac sympathetic input has been an intense area of investigation. Although early attempts at modulating sympathetic tone initially failed with drugs such as phosphodiesterase inhibitors and chronic inotropes [2,3], recent advances in modulating pathologic sympathetic receptor response and desensitization have shown promising results in animal studies [4,5]. Such approaches form the basis for the current review.

β-Adrenergic Receptor signaling in the heart

An important component of HF is sympathetic stimulation that intensifies with the progression of HF. G-protein coupled receptors (GPCRs) play an important role in both local and systemic regulation of heart function. In particular, β-adrenergic receptors (β-AR) are critical regulators of cardiac contractility, including both chronotropy and inotropy. Elevated sympathetic nervous system activity and outflow is a salient characteristic of HF, reflected by an increase in both synaptic and circulating plasma catecholamines (CAs) epinephrine (adrenaline) and norepinephrine (noradrenaline), initiated as an adaptive process to compensate for decreased cardiac contractility. However, the positive inotropic effect of this sympathetic activation is far outweighed by its chronic, maladaptive effects that contribute significantly to disease progression, including: myocardial ischemia, pathologic hypertrophy, arrhythmogenicity, myocardial necrosis and apoptosis [6–8]. This maladaptive response results in part from chronic CA stimulation, which leads to chronic down-regulation and desensitization of cardiac β-ARs [9]. Attenuation and desensitization of β-AR signaling and responsiveness are mediated in part via Gβγ subunit interactions with several

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molecules associated with receptor desensitization, including β -AR kinase (β ARK1) [4] and phosphoinositide 3-kinase (PI3K) [10,11].

G $\beta\gamma$ and cardiac function

β ARK1 is a member of the GPCR kinase (GRK) family, and is also known as GRK2. GRK2 is a cytosolic enzyme that targets and phosphorylates agonist-occupied GPCRs, including myocardial β -ARs, via recruitment by and binding to the $\beta\gamma$ -subunits of heterotrimeric G-proteins (G $\beta\gamma$) following GPCR agonist stimulation [4]. Agonist-stimulated G $\beta\gamma$ -GRK2 interaction is a prerequisite for GRK2-mediated GPCR (including β -AR) phosphorylation, which initiates a cascade of events resulting in homologous receptor desensitization, internalization, degradation and down-regulation [12]. Interestingly, G $\beta\gamma$ -mediated recruitment of cytosolic PI3K in complex with cytosolic GRK2 is also directly implicated in receptor desensitization [10,11] and cardiac dysfunction [13–17]. Elevated expression and activity of GRK2 is a hallmark of human and experimental animal HF [4]. Furthermore, enhancing G $\beta\gamma$ -GRK2 (and PI3K) interaction by cardiac targeted overexpression of GRK2 (s) can directly cause HF in experimental animal models [18], and cardiac ablation of GRK2 either before or after myocardial injury is generally cardioprotective [19–21]. We and others have recently demonstrated that levels of GRK2 expression and activity from cardiac tissue and circulating lymphocytes correlate directly with the severity of human HF [22,23]. Taken together, these data indicate a pathologic role for multiple aspects of G $\beta\gamma$ signaling in cardiac dysfunction.

β ARKct and G $\beta\gamma$ signaling inhibition

Since G $\beta\gamma$ binding is a critical prerequisite for G $\beta\gamma$ -GRK2-PI3K-mediated GPCR desensitization, several approaches have been explored to interdict pathologic G $\beta\gamma$ interactions, including G $\beta\gamma$ -GRK2-PI3K interaction. The first reported approach exploited GRK2, which possesses three general domains, including an N-terminal RGS and protein recognition domain, a central kinase domain, and a C-terminal region encoding the G $\beta\gamma$ binding domain. To study the role of G $\beta\gamma$ signaling and interactions, the C-terminal 194 amino acids encoding the GRK2 G $\beta\gamma$ binding domain (β ARKct) was expressed in cells as a G $\beta\gamma$ peptide inhibitor, where it attenuated homologous β -AR desensitization in a GPCR-specific manner [24]. β ARKct expression attenuated β -AR desensitization without disrupting normal signaling. Subsequently, transgenic mice were created with myocardial-targeted expression of β ARKct, which demonstrated enhanced basal cardiac function and response to isoproterenol [18]. Mating of the cardiac-targeted β ARKct mice with the cardiac-targeted GRK2 overexpressing mice normalized cardiac function. Subsequent data also demonstrated that β ARKct disrupts recruitment not only of GRK2, but also the GRK2-PI3K complex [13–17]. These data provided direct evidence that the mechanism responsible for the phenotype in these mice was β ARKct inhibition of GPCR-initiated, G $\beta\gamma$ -mediated signaling [18,25].

To determine the role of G $\beta\gamma$ -mediated signaling and protein-protein interactions in the pathogenesis of HF, the cardioprotective potential of β ARKct has been assessed in numerous animal models of HF. The data repeatedly demonstrate a salutary, cardioprotective effect of β ARKct both by cardiac-restricted transgenesis, as well as through adenoviral delivery, in various models of both ischemic and non-ischemic HF [20,26,27]. Notably, β ARKct has not only normalized cardiac function, but has also normalized several aspects of β -AR signaling [22,27,28]. Furthermore, β ARKct was shown to be synergistic with β -AR blockers (standard medical therapy for HF) in the cardiac calsequestrin overexpressor (CSQ) mouse model of HF [28]. We have previously demonstrated that β ARKct-mediated normalization of cardiac function in two genetically engineered animal

models of HF is accompanied by normalization of cardiac gene expression in a large scale gene expression profiling study [26]. β ARKct has also been shown to normalize contractile function of failing human cardiac myocytes [29]. Importantly, β ARKct can also ameliorate enhancement of ischemia-reperfusion injury in cardiac GRK2 transgenic mice [74]. Interestingly, β ARKct has demonstrated efficacy in other cardiovascular diseases, including vascular restenosis and hypertension [30–33]. Thus, inhibition of $G\beta\gamma$ signaling and protein-protein interactions represents a promising therapeutic target in the treatment of HF.

The therapeutic potential of targeting $G\beta\gamma$ signaling in HF pathogenesis has been subsequently validated by directly comparing β ARKct to truncated phosducin [34], a $G\beta\gamma$ binding protein discovered in retinal cell membranes following activation of the GPCR transducin. Like GRK2 and PI3K, cytosolic phosducin is recruited to membrane $G\beta\gamma$ subunits upon GPCR activation, and inhibits subsequent $G\beta\gamma$ signaling, including GRK2 recruitment [35–38]. Viral gene delivery of β ARKct or a ~200 amino acid N-terminally truncated phosducin to the pacing-induced HF rabbit heart equally normalized cardiac function. Both peptides normalized isolated failing cardiomyocyte contractility, with mildly differential effects on β -AR signaling [34]. Table 1 provides a partial list of the effects of gain and loss of function of $G\beta\gamma$ -mediated signaling effectors in the heart.

Small molecules bind to the $G\beta\gamma$ “hot spot”

Following GPCR activation, GTP binding results in activation of the heterotrimeric G protein and conformational “release” of the $G\alpha$ subunit from the $G\beta\gamma$ subunits. Once dissociated, the $G\beta\gamma$ subunits are known to interact with multiple effector molecules to activate numerous downstream signaling cascades, including phospholipases, protein kinases, lipid kinases, (mitogen activated protein) MAP kinase pathways and K^+ and Ca^{2+} channels [39,40].

Evidence from a variety of laboratories supports the broad view that different effectors share an interaction surface on $G\beta\gamma$ subunits at a binding site also associated with the $G\alpha$ subunit, but despite sharing a binding site, individual target proteins utilize distinct binding modes. For example, a series of alanine substitution mutants at the $G\alpha/\beta\gamma$ interaction surface differentially affected $G\beta\gamma$ -dependent regulation of GRK2, PLC (phospholipase C) β 2, PLC β 3, adenylyl cyclase (AC) type I and type II [41,42]. To probe selectivity at this interaction interface in more depth, a random peptide phage display screen was conducted using $G\beta\gamma$ as the target. Four distinct families of peptides that bind a single site on $G\beta\gamma$ (specifically, on $G\beta$) have been identified suggesting a $G\beta\gamma$ binding “hot spot” [43]. Despite their shared binding site, the peptides appear to be selective blockers of $G\beta\gamma$ effector regulation, as they differentially blocked $G\beta\gamma$ -mediated activation of PLC β , PI3K γ and $G\beta\gamma$ -mediated inhibition of voltage-gated Ca^{2+} channels or AC I [44].

To identify small molecules that could potentially bind in the $G\beta\gamma$ hot spot and interdict specific aspects of downstream signaling, we screened an NCI chemical library for its ability to compete with phage-displayed peptide binding to $G\beta\gamma$. The novelty of this approach was utilization of the $G\beta\gamma$ -phage bound crystal structure to first perform an in silico structural modeling screen, followed by verification with an ELISA-based assay. We identified several compounds [45] that differentially blocked $G\beta\gamma$ protein-protein interactions, as well as $G\beta\gamma$ signaling upon GPCR agonist stimulation, in vitro. Importantly, one compound, M119, dose-dependently reduced $G\beta\gamma$ -GRK2 interactions in vitro and in an HL60 neutrophil-like cell line. Further, M119 potentiated opioid receptor dependent analgesia in vivo through a $G\beta\gamma$ -PLC β 3 mechanism. Importantly, and similar to β ARKct, M119 inhibited targeted aspects of $G\beta\gamma$ signaling and interactions without disrupting general GPCR function, activation of non-targeted $G\beta\gamma$ -mediated signaling pathways, or $G\beta\gamma$ -mediated regulation of

N-type Ca^{2+} channel or G-protein coupled inward rectifying K ACh (GIRK) channels [5,45,46]. These data indicate that these compounds selectively interfere with a selective subset of $\text{G}\beta\gamma$ interactions.

$\text{G}\beta\gamma$ inhibitory compounds in isolated cardiomyocytes and in acute heart failure models

Considering the important role of $\text{G}\beta\gamma$ -mediated signaling in β -AR-mediated cardiac contractility, and the proven efficacy of $\text{G}\beta\gamma$ inhibitory peptides in cardiac dysfunction as outlined above, we sought to assess the potential of M119 in myocardial cells. In isolated adult mouse cardiomyocytes, M119 and its highly homologous compound gallein reduced β -AR-mediated membrane recruitment of GRK2, with a mild reduction of membrane associated GRK2 at baseline and enhanced cAMP generation, particularly in response to the β -AR agonist isoproterenol, due to β -AR-mediated activation of AC. M119 and gallein enhanced cardiomyocyte contractility both at baseline and in response to β -AR stimulation. This effect was completely inhibited by propranolol, suggesting β -AR specific activity of both the contractility and the $\text{G}\beta\gamma$ -mediated effects of M119 and gallein. Overall, the $\text{G}\beta\gamma$ -inhibitory molecules M119 and gallein enhance β -AR-mediated signaling and contractility in isolated adult cardiomyocytes [5].

We also recently reported that systemic M119 and gallein treatment initiated at the onset of HF normalized cardiac function and morphology, reduced interstitial cardiac fibrosis, and normalized elevated GRK2 expression in the chronic isoproterenol mini-osmotic pump mouse model of HF. While this data is encouraging, we also wished to determine whether $\text{G}\beta\gamma$ inhibitory treatment would be beneficial when initiated well after the onset of HF, to more accurately reflect the common human therapeutic paradigm. A transgenic mouse model of HF generated by cardiac-targeted overexpression of calsequestrin (CSQ) has been utilized in many HF studies, as the animals suffer from HF by 6–8 weeks, with HF-related mortality onset by ~12 – 14 weeks [47]. Four weeks of daily intraperitoneal gallein injections, initiated at 8 weeks (when CSQ mice suffer from advanced HF), completely halted the progression of HF, and partially normalized cardiac fibrosis, β -AR expression and HF marker gene expression (ANF, BNP, GRK2) [5]. Taken together, these data not only validate the therapeutic approach of general $\text{G}\beta\gamma$ inhibition in HF, but also suggest a role for therapeutic development of systemically available small molecule $\text{G}\beta\gamma$ inhibitor compounds for the treatment of HF (Figure 1).

Systemic effects of small molecule $\text{G}\beta\gamma$ inhibition in HF

Beyond a role in cardiomyocytes, desensitization of adrenergic receptor- $\text{G}\beta\gamma$ signaling in adrenal glands may also contribute to HF. Lymperopoulos et al recently showed a significant increase of GRK2 in adrenal chromaffin cells, suggesting that elevated $\text{G}\beta\gamma$ -GRK2 signaling elevates catecholamine release via desensitized α_2 -AR signaling [48]. Adrenal β ARKct delivery via adenovirus restored α_2 -AR feedback inhibition of catecholamine release and enhanced cardiac function, resulting in part from reduced cardiac inotropy via β -ARs. Increased GRK2 expression is also associated with hypertension [49,50]. β -AR signaling normally promotes vasodilation; overexpression of GRK2 within vascular smooth muscle cells leads to diminished β -AR signaling and elevated resting blood pressure, a major risk factor for HF [31]. Because heart disease can result from dysfunctional signaling in multiple organs, we and others [48] believe that systemic delivery of small molecule $\text{G}\beta\gamma$ inhibitors could simultaneously target multiple causes of this disease. This approach to modulating intracellular signaling is also consistent with the growing trend of therapeutics targeting intracellular components of pathological signaling in cardiovascular disease [51].

Other downstream effectors of Gβγ inhibition

ERK ½

ERK1/2 has been proposed to play an important role in HF pathogenesis. Activation of ERK1/2, via T202/Y204 phosphorylation in the TEY motif, occurs in response to nearly all forms of cardiac stress, and is generally thought to be adaptive, whereas absence of ERK1/2 appears to be maladaptive [52]. However, the specific role of ERK1/2, and that of Gβγ in ERK1/2 activation, remains somewhat unclear [53]. Lorenz et al recently demonstrated that Gβγ-mediated activation of ERK1/2 by direct protein-protein interaction leads to autophosphorylation on a novel site (murine T188) to facilitate cardiomyocyte hypertrophy [53]. Sustained ERK1/2 T188 phosphorylation was also shown to occur in cardiac tissue from both mouse and human HF [54]. Importantly, cardiac targeted expression of the ERK2 T188D phosphomimetic mutant exacerbated hypertrophy, fibrosis and cardiac dysfunction following transverse aortic constriction (TAC) or chronic angiotensin II (AngII) infusion [54]. In summary, these data suggest an important role for Gβγ-ERK1/2 in HF pathogenesis. Future studies will investigate whether targeted inhibition of Gβγ/ERK1/2-mediated cardiac hypertrophy may be a target for small molecule Gβγ inhibitors.

PI3Kγ

PI3Ks are a family of evolutionarily conserved lipid kinases that mediate many cellular responses to physiological and pathophysiological stimuli. Class IA PI3K are activated by receptor tyrosine kinase/cytokine receptor, while class IB PI3K are activated by GPCRs downstream of Gβγ[55,56], leading to the generation of phosphatidyl inositol (3,4,5)P3 and recruitment and activation of Akt/protein kinase B, 30-phosphoinositide-dependent kinase-1 (PDK1), or monomeric G-proteins, and phosphorylation of a wide range of downstream targets and several anti-apoptotic effectors. Class IA (PI3Kα, β, and δ) and class IB (PI3Kγ) PI3Ks mediate distinct phenotypes in the heart [57–59]. In the myocardium, GPCR-induced PI3K/Akt activation occur in response to several peptide agonists including urocortin, ghrelin, and adrenomedullin as well as β2-AR stimulation which enhances cell survival and antagonizes apoptosis [60–63]. PI3Kγ/Akt activation following GPCR stimulation typically leads to pathological (maladaptive) hypertrophy [13,63–66], whereas loss of PI3Kγ prevents activation of Akt/PKB and ERK1/2 pathways in response to β-AR stimulation, resulting in a marked reduction in hypertrophy [64]. Loss of PI3Kγ is associated with sustained increases in contractility and relaxation [67]. As described above, cytosolic PI3K in complex with GRK2 are also recruited by Gβγ subunits to mutually participate in β-AR desensitization, internalization and degradation. Thus it is possible that Gβγ inhibitory compounds may target Gβγ-PI3Kγ/Akt signaling in heart failure models to produce at least a portion of their beneficial effect.

Inflammatory effects mediated by Gβγ

Chemoattractant-mediated recruitment of leukocytes is responsible for many of the deleterious effects of chronic inflammatory diseases [68,69]. Many chemoattractants activate GPCRs in leukocytes and initiate critical Gβγ– PI3Kγ dependent activation of chemoattractant-dependent neutrophil functions including chemotaxis and superoxide production [69,70].

Recently, we utilized small-molecule inhibition of Gβγ-dependent signaling, including Gβγ-dependent activation of PI3Kγ and Rac1, to inhibit chemoattractant-dependent neutrophil migration in vitro and inflammation in vivo. Small-molecule Gβγ inhibitors suppressed fMLP-stimulated Rac activation, superoxide production, and PI3K activation in differentiated HL60 cells. Systemic administration inhibited paw edema and neutrophil infiltration in a mouse carrageenan-induced paw edema model [69]. These data demonstrate

that targeting G $\beta\gamma$ regulation may also be an effective strategy in inflammatory diseases. Considering the established importance of inflammation, inflammatory signaling, and positive results of anti-inflammatory therapies for HF in pre-clinical trials, these data also suggest that further investigation of small molecule G $\beta\gamma$ inhibitors in the treatment of HF is warranted.

Therapeutic issues: β -blockers, β ARKct, and small molecule G $\beta\gamma$ inhibitors

β -blockers are a standard component of the HF therapeutic paradigm, where they generally reduce both morbidity and mortality. Since β -blockers initially reduce HF patients' diminished sympathetic reserve, several steps of dose escalation/titration are critical. Paradoxically, although patients generally feel worse upon initiation of β -blocker therapy, dose escalation/titration ultimately results in stabilized or improved sympathetic reserve and cardiac function. This may be explained in part by a reduction of pathologic receptor desensitization, internalization and down-regulation, ultimately enhancing "normal" signaling by blocking chronic/pathologic receptor agonism. Indeed, recent evidence suggests that while receptors at the cell surface are responsible for beneficial/contractile signaling, those undergoing chronic signaling and/or internalization may scaffold to pathologic (e.g. hypertrophic, autophagic, apoptotic) signaling complexes [71,72]. Supporting this notion is the recent discovery of "biased ligands" for various GPCRs, including β -ARs. Such ligands have been found to bias GPCR signals, for example to either G-protein-dependent or G-protein independent signaling, as well as interaction of GPCRs with other signaling pathways or modulation of G-protein switching. Various conformational states of the multifunctional protein β -arrestin often appear to mediate the transmitted signal. [71,72]. This approach to understanding biased ligands is currently being utilized to screen for the next generation of GPCR-targeted therapeutics.

It may seem paradoxical that "improving" β -AR signaling via G $\beta\gamma$ inhibition (either with β ARKct or small molecules) could be therapeutic in HF tantamount to the benefits achieved by β -blocker therapy. Interestingly, and as mentioned above, β ARKct proved to be synergistic with β -blocker therapy in mouse HF [28]. Considering this data, and the emerging concepts of biased ligands and ligand-dependent receptor signaling, we hypothesize that β -blockers stabilize beneficial/contractile signaling by reducing extracellular β -AR overstimulation and subsequent activation of pathologic signals, whereas G $\beta\gamma$ inhibition reduces initiation of these same pathologic signals at the intracellular signaling interface (Figure 1). In effect, we view β -blocker therapy and G $\beta\gamma$ inhibition as complementary extra- and intra-cellular approaches to attenuate pathologic β -AR signaling while maintaining/improving signaling and β -AR-elicited contractile response.

Several hurdles remain before the pre-clinical data with G $\beta\gamma$ inhibition in HF can be translated to the clinical arena. In the case of β ARKct, obstacles include viral vectors and cardiac gene delivery, although great strides are being made in both arenas. In the case of small molecules, understanding the systemic effects of G $\beta\gamma$ inhibition is paramount. However, as mentioned above, we believe that systemic delivery of small molecule G $\beta\gamma$ inhibitors could simultaneously target multiple facets of cardiovascular disease. Once therapeutic targeting of G $\beta\gamma$ signaling is ready for clinical testing, comparative efficacy will be critical to the success of any cardiovascular clinical trial [73]. Fortunately, preclinical data support additive or synergistic benefit of combining G $\beta\gamma$ inhibition with β -blockers [28], a standard comparative efficacy therapy for HF.

SUMMARY

In summary, targeting $G\beta\gamma$ signaling has proven a promising therapeutic paradigm in the treatment of HF. Unfortunately, therapeutic targeting of cardiac $G\beta\gamma$ in HF to date has only been achieved by large peptides administered via viral gene therapy, which has historically faced developmental hurdles as a therapeutic modality. Identification of selective and differential small molecule compounds targeting a specific subset of $G\beta\gamma$ signaling, such as those outlined herein, will provide valuable new research tools to dissect the pathological pathways of HF, and may provide novel and readily bioavailable therapeutics for cardiovascular disease.

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Figure 1.
Therapeutic approaches to inhibit Gβγ signaling pathways in HF.

Table 1

A partial list of gain and loss of function of G $\beta\gamma$ -mediated signaling effectors in the heart.

β ARKct (cardiac restricted transgenesis, viral gene delivery)	Normalization of cardiac function and β -AR signaling	<ul style="list-style-type: none"> • mouse myocardial infarction model [20] • Genetic HF models [27,28] • failing human heart myocytes [29] • rat carotid artery restenosis model [30] • mouse GRK2 overexpressing hypertensive model [31] • Mouse myocardial ischemia reperfusion model [74]
Truncated phosducin (viral gene delivery to the heart)	<ul style="list-style-type: none"> • Similar to bARKct • No affect on cAMP level 	<ul style="list-style-type: none"> • rabbit rapid ventricular pacing HF model [34]
GRK2 knockout (inducible cardiac ablation)	Enhanced cardiac function and halted remodeling	<ul style="list-style-type: none"> • myocardial infarction mouse model [20]
GRK2 overexpression (transgenic mouse model)	<ul style="list-style-type: none"> • Deterioration of cardiac function • Hypertensive mouse model 	<ul style="list-style-type: none"> • mouse GRK2 overexpressing hypertensive model [31] • mouse myocardial ischemia reperfusion model [74]
PI3K domain negative peptide (cardiac specific disruption of PI3K membrane recruitment)	Restoration of cardiac function and β -AR signaling	<ul style="list-style-type: none"> • mouse PI3K overexpressing HF model [13,17] • mouse CSQ transgenic HF model [14]
Small molecule G $\beta\gamma$ inhibition (M119, Gallein)	Attenuate progression of cardiac dysfunction	<ul style="list-style-type: none"> • mouse isoproterenol HF model [5] • mouse CSQ transgenic HF model [5]