Accepted Manuscript

Simultaneous adrenal and cardiac GPCR-Gβ γ inhibition halts heart failure progression

Fadia A. Kamal, PharmD, PhD Deanne M. Mickelsen, BS Katherine M. Wegman, Joshua G. Travers, BS Jacob Moalem, MD Stephen R. Hammes, MD, PhD Alan V. Smrcka, PhD Burns C. Blaxall, PhD

PII: S0735-1097(14)01656-8

DOI: 10.1016/j.jacc.2014.02.587

Reference: JAC 20012

To appear in: *Journal of the American College of Cardiology*

Received Date: 19 December 2013

Revised Date: 10 February 2014

Accepted Date: 25 February 2014

Please cite this article as: Kamal FA, Mickelsen DM, Wegman KM, Travers JG, Moalem J, Hammes SR, Smrcka AV, Blaxall BC, Simultaneous adrenal and cardiac GPCR-Gβ γ inhibition halts heart failure progression, *Journal of the American College of Cardiology* (2014), doi: 10.1016/j.jacc.2014.02.587.

Simultaneous adrenal and cardiac GPCR-Gβ γ inhibition halts heart failure progression

Fadia A. Kamal, PharmD, PhD[*]; Deanne M. Mickelsen, BS[†]; Katherine M. Wegman[*]; Joshua G. Travers, BS[*]; Jacob Moalem, MD[‡,§]; Stephen R. Hammes, MD, PhD[§]; Alan V. Smrcka, PhD[‖]; Burns C. Blaxall, PhD[*,¶]

[*] The Heart Institute, Cincinnati Children's Hospital Medical Center, Cincinnati, OH

[†] Aab Cardiovascular Research Institute, Department of Medicine, University of Rochester Medical Center, Rochester, NY

[‡] Department of Surgery, [§] Department of Medicine, [‖] Department of Pharmacology and Physiology, University of Rochester Medical Center, Rochester, NY

Disclosures: none

[¶] Corresponding author
Burns C. Blaxall, PhD, FAHA
Professor, Director of Translational Science
The Heart Institute, Molecular Cardiovascular Biology
Cincinnati Children's Hospital Medical Center
240 Albert Sabin Way, MLC 7020
Cincinnati, OH 45229-3039

burns.blaxall@cchmc.org
Ph. 513.803.4005 Fax: 513.636.5958

Brief title: Inhibiting the cardio-adrenal axis in heart failure

Sources of funding
This work was funded by NIH R01 HL089885 and NIH R01 HL091475 (BCB) and by an AHA postdoctoral fellowship (FAK).

Abstract
Background: elevated sympathetic nervous system activity is a salient characteristic of heart failure (HF) progression. It causes pathologic desensitization of β -adrenergic receptors (β -AR), facilitated predominantly through Gβ γ-mediated signaling. The adrenal glands are key contributors to the chronically elevated plasma catecholamine levels observed in HF, where adrenal α2-AR feedback inhibitory function is impaired also through Gβ γ-mediated signaling. Objective: we propose simultaneous inhibition of Gβ γ signaling in the heart and the adrenal gland as a novel therapeutic approach for HF. Methods and results: we investigated the efficacy of a small molecule Gβ γ inhibitor, gallein, in a clinically relevant, pressure-overload model of HF. Daily gallein treatment (10 mg/kg/day), initiated four weeks following transverse aortic constriction, improved survival and cardiac function, and attenuated cardiac remodeling. Mechanistically, gallein restored β-AR membrane density in cardiomyocytes, attenuated Gβ γ-mediated GRK2-PI3Kγ membrane recruitment, and reduced Akt and GSK-3β phosphorylation. Gallein also reduced circulating plasma catecholamine levels as well as catecholamine production in isolated mouse adrenal glands by restoring adrenal α2-AR feedback inhibition. In human adrenal endocrine tumors (pheochromocytoma), gallein attenuated catecholamine secretion, as well as GRK2 expression and membrane translocation. Conclusions: these data suggest small molecule Gβ γ inhibition as a systemic pharmacologic therapy for HF by simultaneously normalizing pathologic adrenergic/Gβ γ signaling in both the heart and the adrenal gland. Our data also suggest important endocrine/cardiovascular interactions and a possible role for small molecule Gβ γ inhibition in treating endocrine tumors such as pheochromocytoma, in addition to HF.

Key Words: catecholamines; fibrosis; heart failure; hypertrophy; sympathetic nervous system.

List of abbreviations
Heart failure (HF)
Sympathetic nervous system (SNS)
Catecholamines (CA)
Adrenergic receptors (AR)
G-protein coupled receptor (GPCR)
G-protein coupled receptor kinase 2 (GRK2)
Phosphoinositide 3-kinaseγ (PI3Kγ□
A truncated Gβ γ-binding GRK2 peptide that lacks the kinase domain (β ARKct)
Transverse aortic constriction (TAC)
Isoproterenol (ISO)

1. Introduction

Heart failure (HF) is a progressive disease with poor prognosis. A common feature of HF is elevated sympathetic nervous system (SNS) activity to compensate for poor cardiac output, including excess adrenal production of the catecholamines (CA) epinephrine and norepinephrine (1-3). Chronic CA-mediated stimulation of β -adrenergic receptors (β -AR, a G-protein coupled receptor (GPCR)), elicits pathologic upregulation of G-protein coupled receptor kinase 2 (GRK2) that is recruited to membrane Gβ γ subunits to phosphorylate β -AR, leading to their desensitization (4,5). Prior reports suggest a therapeutic role for inhibiting Gβ γ-GRK2 interaction in HF to restore β -AR expression and function, utilizing a truncated Gβ γ-binding GRK2 peptide that lacks the kinase domain (β ARKct) (6-13), as well as a truncated Gβ γ-binding phosducin (14), and a truncated phosphoinositide 3-kinaseγ (PI3Kγ (15-17). Interestingly, Gβ γ-GRK2 was recently found to desensitize the adrenal α2-AR, another GPCR responsible for feedback inhibition of CA release from the adrenal gland. Importantly, adrenal α2-AR desensitization contributes to elevated plasma CA and consequent β -AR desensitization in HF (18,19).

We recently identified and validated novel compounds that selectively inhibit Gβ γ binding interactions(20), namely M119 and its highly homologous structural analog, gallein. These compounds competitively antagonize the binding of GRK2 to a specific protein-protein interaction domain of the Gβ γ subunit, thus inhibiting the pathologic Gβ γ-GRK2 interaction. Both compounds bind to Gβ γ with equal affinity in vitro, and have equivalent efficacies in enhancing contractility in isolated cardiomyocytes as well as in acute pharmacologic and genetic mouse models of HF (21). The advantages of these compounds include their convenient route of administration (i.p. injection or oral gavage), and their bioavailability and cell permeability (22).

Additionally, they inhibit the Gβ γ-GRK2 interaction without disturbing various other aspects of basal or agonist stimulated G-protein signaling (20). We were the first to report early proof-of-concept studies regarding possible beneficial effects of these newly identified compounds in acute pharmacologic and transgenic animal models of HF (21).

In the current study, we report the therapeutic efficacy of the selective small molecule Gβ γ inhibitor, gallein, when delivered following the establishment of HF in a clinically relevant surgical model. Further, we report concomitant salutary effects of gallein on the adrenal gland, including direct inhibition of adrenal CA synthesis and release both in HF animal models and in primary human adrenal pheochromocytoma tissue. We propose that simultaneous small molecule inhibition of Gβ γ signaling in the heart and the adrenal gland is a novel therapeutic approach for HF, and possibly for other diseases of excess catecholamine release, such as pheochromocytoma.

2. Material and Methods

Detailed materials and methods are included in the online supplement.

3. Results

3. 1. The small molecule Gβ γ inhibitor "gallein" dose-dependently ameliorates cardiac dysfunction and hypertrophy in HF mice

To test the possible therapeutic effects of small molecule Gβ γ inhibition in treating established HF, daily gallein administration was initiated four weeks following transverse aortic constriction (TAC) and continued for eight weeks (12 weeks post-TAC). Gallein dose-dependently reduced ventricular hypertrophy (VW/TL) and improved cardiac function (Figure 1 and supplementary figure 1). Administration of gallein at a dose of 10 mg/kg/day was found to be the optimal therapeutic dose, and was thus utilized for subsequent studies (identified as TAC+G).

3. 2. Gallein improves survival and preserves cardiac contractility in pressure overload

hypertrophy

Following eight weeks of daily treatments initiated four weeks post-TAC (i.e. at 12 weeks post-TAC), gallein significantly enhanced survival to 80%, compared to 54% survival in TAC+V (Figure 2A). Cardiac function in TAC+V mice declined at 8 weeks post-TAC, with further deterioration at week 12. Gallein treatment initiated after establishment of HF prevented such deterioration and preserved cardiac function (Tables 1 and 2, and Figure 2D).

3. 3. Gallein restores β -AR density, down-regulates GRK2 expression, and inhibits GRK2 and PI3Kγ membrane recruitment

To evaluate mechanisms underlying the beneficial effects of gallein on cardiac function, we measured β -AR density, GRK2 and PI3Kγ membrane recruitment, and GRK2 gene expression. TAC mice exhibited a significant reduction in β -AR density that was normalized by gallein treatment (Figure 2B). This was accompanied by a reduction in cardiac GRK2 gene expression and GRK2-PI3K110γ membrane translocation in TAC+G mice compared to TAC+V (Figure 2C, E, and F respectively).

3. 4. Gallein attenuates cardiac remodeling and inflammation in pressure overload HF

Gallein treatment attenuated the progression of cardiac hypertrophy in TAC mice, as reflected by reduced ventricular weight to tibia length ratio (Figure 3A) and cardiomyocyte cross-sectional area (Figure 3B and C). This protective effect of gallein on cardiac hypertrophy was accompanied by reduced phosphorylation of cardiac Akt (Figure 3D) and its downstream signal, GSK-3β (Figure 3E), and a parallel reduction in myocardial fibrosis (Figure 4A and B). This may be attributed to the significantly reduced expression of the fetal genes ANP and BNP (Figure 4C & D), the inflammatory cytokines IL-1β , IL-6, and TNF-α (Figure 4E, F, & G), and the pro-fibrotic marker α-SMA (Figure 4H). Moreover, we observed less myocardial apoptosis in

TAC+G mice evidenced by less apoptotic nuclei and reduced caspase-3 cleavage in cardiac lysates (supplementary figure 2).

3. 5. Gallein attenuates CA production and adrenal remodeling and restores adrenal α2-AR feedback inhibition in TAC mice

Heart failure is associated with chronically elevated plasma CA concentrations. At 12 weeks post-TAC, gallein significantly reduced plasma epinephrine and norepinephrine concentrations to 1.5-fold and 2.5-fold of baseline, respectively whereas vehicle treated mice showed significant elevations of 2.8-fold and 7.5-fold, respectively (Figure 5A and B). Adrenal medulla hypertrophy is a common feature in HF that occurs concurrent with elevated plasma CA levels (23); this was also reduced by gallein treatment (Figure 5C). Further, adrenal glands from TAC+G mice cultured in vitro showed significantly lower levels of basal CA secretion (Figure 5D and E). Adrenal α2-AR normally provide feedback inhibition of excess CA release, a function that is desensitized in HF. Gallein also restored α2-AR feedback inhibitory function as compared to TAC+V mice (Figure 5F and G). Further, gallein attenuated the overexpression of tyrosine hydroxylase (rate limiting enzyme of CA production) and chromogranin A (neurokine that is synthesized, co-stored, and co-secreted with vesicular CA) (Figure 5C and supplementary figure 5).

3.6. Gallein directly restores α2-AR feedback inhibitory function in adrenal glands from untreated ISO-pump mice

Ex-vivo cultured adrenal glands from untreated mice chronically exposed to the β -AR agonist, Isoproterenol (ISO) exhibit impaired adrenal α2-AR function, similar to other HF models. Importantly, in vitro gallein treatment directly restored the α2-AR feedback inhibitory function in these adrenal glands (Supplementary figure 3).

3. 7. Gallein directly attenuates CA secretion and GRK2 protein expression and membrane translocation in cultured human pheochromocytoma

Pheochromocytoma is a tumor of the adrenal gland characterized by excessive CA production (24). Gallein treatment significantly reduced CA production in cultured pheochromocytoma slices (Figure 6A and B) with attenuated GRK2 protein expression and membrane translocation (Figure 6D and E respectively). Interestingly, expression of tyrosine hydroxylase and chromogranin A was significantly downregulated by gallein treatment in cultured pheochromocytoma slices (Figure 6C). Further, α2-AR feedback inhibitory function trended towards improvement in gallein treated cultured pheochromocytomas (supplementary figure 4).

4. Discussion

4.1. Small molecule Gβ γ inhibition halts HF progression post-TAC

In the present study, we demonstrate the efficacy of the small molecule Gβ γ inhibitor, gallein, in ameliorating established HF through simultaneous inhibition of Gβ γ in the heart and the adrenal gland. Importantly, gallein partially restores normal cardiac function and halts or reverses pathologic cardiac remodeling when administered after the establishment of chronic HF in a clinically relevant animal model. It is noteworthy that gallein treatment in sham animals mildly increases cardiac contractility as we have previously shown (21) and in supplementary figure 6. We observed a dose-dependent therapeutic effect of gallein on cardiac function and hypertrophy in TAC mice in vivo, with 10mg/kg/day identified as the optimum dose. Concomitantly, gallein treatment attenuated cardiac fibrosis and apoptosis indicating attenuated cardiac remodeling. Further, gallein treatment attenuated cardiac inflammatory cytokine expression in TAC HF mice (Figure 4E, F, & G). Our preliminary studies have demonstrated possible systemic anti-inflammatory effects of Gβ γ inhibitors in a mouse carrageenan-induced paw inflammation

model (25). Prior studies have correlated inflammation and loss of cardiac function in pressure overload hypertrophy (26),(27). Thus, attenuated cardiac inflammation in TAC mice by gallein may provide an additional mechanism for its therapeutic effect in pressure overload HF.

In summary, gallein increased survival, improved cardiac function, and reduced cardiac remodeling and inflammation when administered daily to mice with established HF, i.e. four weeks through twelve weeks post-TAC.

4.2. Small molecule Gβ γ inhibition preserves cardiac membrane β -AR density by attenuating GRK2-PI3Kγ signaling

Excess SNS activity and outflow are salient characteristics of HF, where reduced cardiac output triggers compensatory SNS over-activity (elevated local and circulating CA) that acutely rescues cardiac function. However, chronic stimulation of β -AR by CA leads to their desensitization and attenuated contractile signaling (1-3). Receptor desensitization is mainly mediated by the Gβ γ-subunits, which recruit receptor-desensitizing kinases such as GRK2 and PI3Kγ to agonist-stimulated β -AR, resulting in receptor phosphorylation and the recruitment of arrestins that initiate receptor internalization, desensitization and down-regulation (5,28-30). Previous data from our lab and others suggests that inhibiting Gβ γ signaling and its interaction with GRK2 and PI3Kγ could be of therapeutic value in HF (5,16,29,30). This has been demonstrated by large peptide inhibitors of Gβ γ binding in both cell culture and in animal models of HF (2,31), (6), (14), (15-17). In our study, small molecule Gβ γ inhibition by gallein reduced GRK2 expression and GRK2-PI3K110γ membrane recruitment, thus preserving β AR membrane expression and cardiac function.

4.3. Gallein interrupts activation of the cardiac Akt-GSK-3β hypertrophic pathway

In addition to forming a complex with GRK2 that desensitizes β -AR, PI3Kγ is of specific

interest in HF because it mediates a myriad of pathological effects. PI3Kγ activates Akt/PKB following GPCR activation, leading to pathological hypertrophy (28,32,33). Conversely, loss or reduction of activity is associated with increased contractility, relaxation and reduced hypertrophy (34). Akt is a well-known player in cardiac hypertrophy with a large number of downstream effectors. In particular, phosphorylated Akt mediates GSK-3β Ser-9 phosphorylation thus inhibiting its anti-hypertrophic effects. Our results suggest that attenuated Akt activation in gallein treated TAC mice results in elevated levels of active (non-phosphorylated) GSK-3β that attenuates cardiac hypertrophy by negatively regulating hypertrophic gene transcription and protein translation (35).

4.4. Systemic gallein treatment normalizes sympathetic tone and adrenal function in TAC mice

The adrenal glands, specifically adrenal medullary chromaffin cells, are the site of CA synthesis and secretion into the circulation. Adrenal chromaffin cell α2-AR play a crucial role in feedback regulation of circulating levels of plasma CA (see Figure 7). Recent reports demonstrate that HF prognosis is worse in patients with an α2-AR deletion polymorphism that impairs feedback inhibition of CA release (36,37). Further, recent data demonstrate that in HF adrenal α2-AR are also desensitized by pathologic Gβ γ-GRK2 signaling (19) (18), and that this desensitization can possibly be mitigated by adrenal β ARKct (38).

In our study, the salutary extra-cardiac effects of systemic Gβ γ inhibition by gallein in chronic murine HF included reduced plasma CA levels, limited adrenal medullary hypertrophy, normalized adrenal α2-AR function, and attenuated expression of both tyrosine hydroxylase, the rate-limiting enzyme in CA synthesis, and chromogranin A, a neurokine that is synthesized, co-stored, and co-secreted with CA in chromaffin cell vesicles. To investigate whether these adrenal

effects resulted directly from gallein treatment or were merely the result of HF amelioration, we examined the direct effects of gallein treatment in ex-vivo cultured adrenal glands isolated from vehicle treated ISO-pump HF mice, where gallein treatment directly restored adrenal α2-AR function (Supplementary figure 3).

4.5. Small molecule Gβ γ inhibition directly reduces CA secretion in ex-vivo cultured human pheochromocytoma

To further investigate the salutary effect of gallein on CA secretion in adrenal chromaffin cells, we utilized ex-vivo cultured human pheochromocytoma explants. Pheochromocytoma is a CA secreting tumor of the adrenal medulla that is accompanied by cardiovascular complications (24). Importantly, gallein treatment reduced CA secretion and attenuated GRK2 expression and membrane translocation in ex vivo pheochromocytoma culture with attenuated protein expression of tyrosine hydroxylase and chromogranin A, suggesting a direct inhibitory effect on CA synthesis and secretion by chromaffin cells. To date, pharmacological management of pheochromocytoma is poor at best, and surgical resection remains the final option. Our results suggest a possible therapeutic role for small molecule Gβ γ inhibition in pheochromocytoma through its direct effect on CA secretory mechanisms. Importantly, these experiments were conducted immediately on explanted pheochromocytomas obtained from human subjects.

5. Conclusion

Recent reports have suggested that, if it were possible, systemic pharmacologic therapy to simultaneously normalize pathologic Gβ γ-GRK2 signaling in both the heart and the adrenal gland could be of substantial therapeutic benefit in HF. Our current study suggests gallein to be such a systemic pharmacologic therapy. Administration of gallein to animals after the establishment of HF in a clinically relevant surgical model appears to normalize dampened AR

signaling in both the heart and the adrenal gland. Our data also suggest a possible therapeutic role for small molecule Gβ γ inhibition in other diseases of elevated catecholamine release, such as pheochromocytoma. Future studies will seek to further establish the therapeutic efficacy of gallein in larger animal models of HF.

References

1. Bristow MR, Ginsburg R, Minobe W et al. Decreased catecholamine sensitivity and beta-adrenergic-receptor density in failing human hearts. The New England journal of medicine 1982;307:205-11.

2. Rockman HA, Koch WJ, Lefkowitz RJ. Seven-transmembrane-spanning receptors and heart function. Nature 2002;415:206-12.

3. Kamal FA, Smrcka AV, Blaxall BC. Taking the heart failure battle inside the cell: small molecule targeting of Gbetagamma subunits. Journal of molecular and cellular cardiology 2011;51:462-7.

4. Koch WJ, Lefkowitz RJ, Rockman HA. Functional consequences of altering myocardial adrenergic receptor signaling. Annual review of physiology 2000;62:237-60.

5. Naga Prasad SV, Barak LS, Rapacciuolo A, Caron MG, Rockman HA. Agonist-dependent recruitment of phosphoinositide 3-kinase to the membrane by beta-adrenergic receptor kinase 1. A role in receptor sequestration. The Journal of biological chemistry 2001;276:18953-9.

6. Blaxall BC, Spang R, Rockman HA, Koch WJ. Differential myocardial gene expression in the development and rescue of murine heart failure. Physiological genomics 2003;15:105-14.

7. Harding VB, Jones LR, Lefkowitz RJ, Koch WJ, Rockman HA. Cardiac beta ARK1 inhibition prolongs survival and augments beta blocker therapy in a mouse model of severe heart failure. Proc Natl Acad Sci U S A 2001;98:5809-14.

8. Koch W, Rockman H, Samama P et al. Cardiac function in mice overexpressing the b-adrenergic receptor kinase or a bARK inhibitor. Science 1995;268:1350-1353.

9. Rockman HA, Chien KR, Choi DJ et al. Expression of a beta-adrenergic receptor kinase 1 inhibitor prevents the development of myocardial failure in gene-targeted mice. Proceedings of the National Academy of Sciences of the United States of America 1998;95:7000-5.

10. Rengo G, Lymperopoulos A, Zincarelli C et al. Myocardial adeno-associated virus serotype 6-betaARKct gene therapy improves cardiac function and normalizes the neurohormonal axis in chronic heart failure. Circulation 2009;119:89-98.

11. Shah AS, White DC, Emani S et al. In vivo ventricular gene delivery of a beta-adrenergic receptor kinase inhibitor to the failing heart reverses cardiac dysfunction. Circulation 2001;103:1311-6.

12. White DC, Hata JA, Shah AS, Glower DD, Lefkowitz RJ, Koch WJ. Preservation of myocardial beta-adrenergic receptor signaling delays the development of heart failure after myocardial infarction. Proc Natl Acad Sci U S A 2000;97:5428-33.

13. Williams ML, Hata JA, Schroder J et al. Targeted beta-adrenergic receptor kinase (betaARK1) inhibition by gene transfer in failing human hearts. Circulation 2004;109:1590-3.

14. Hippe HJ, Lutz S, Cuello F et al. Activation of heterotrimeric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and Gbeta subunits. Specific activation of Gsalpha by an NDPK B.Gbetagamma complex in H10 cells. J Biol Chem 2003;278:7227-33.

15. Nienaber JJ, Tachibana H, Naga Prasad SV et al. Inhibition of receptor-localized PI3K preserves cardiac beta-adrenergic receptor function and ameliorates pressure overload heart failure. J Clin Invest 2003;112:1067-79.

16. Perrino C, Naga Prasad SV, Patel M, Wolf MJ, Rockman HA. Targeted inhibition of beta-adrenergic receptor kinase-1-associated phosphoinositide-3 kinase activity preserves beta-

adrenergic receptor signaling and prolongs survival in heart failure induced by calsequestrin overexpression. J Am Coll Cardiol 2005;45:1862-70.

17. Perrino C, Naga Prasad SV, Schroder JN, Hata JA, Milano C, Rockman HA. Restoration of beta-adrenergic receptor signaling and contractile function in heart failure by disruption of the betaARK1/phosphoinositide 3-kinase complex. Circulation 2005;111:2579-87.

18. Lymperopoulos A, Rengo G, Funakoshi H, Eckhart AD, Koch WJ. Adrenal GRK2 upregulation mediates sympathetic overdrive in heart failure. Nature medicine 2007;13:315-23.

19. Brede M, Nagy G, Philipp M, Sorensen JB, Lohse MJ, Hein L. Differential control of adrenal and sympathetic catecholamine release by alpha 2-adrenoceptor subtypes. Mol Endocrinol 2003;17:1640-6.

20. Bonacci TM, Mathews JL, Yuan C et al. Differential targeting of Gbetagamma-subunit signaling with small molecules. Science 2006;312:443-6.

21. Casey LM, Pistner AR, Belmonte SL et al. Small molecule disruption of G beta gamma signaling inhibits the progression of heart failure. Circulation research 2010;107:532-9.

22. Mathews JL, Smrcka AV, Bidlack JM. A novel Gbetagamma-subunit inhibitor selectively modulates mu-opioid-dependent antinociception and attenuates acute morphine-induced antinociceptive tolerance and dependence. The Journal of neuroscience : the official journal of the Society for Neuroscience 2008;28:12183-9.

23. Schneider J LA, Hein L, Gilsbach R. Chronic cardiac pressure overload induces adrenal medulla hypertrophy and increased catecholamine synthesis. Basic Res Cardiol 2011;4:591-602.

24. Eisenhofer G, Timmers HJ, Lenders JW et al. Age at diagnosis of pheochromocytoma differs according to catecholamine phenotype and tumor location. The Journal of clinical endocrinology and metabolism 2011;96:375-84.

25. Lehmann DM, Seneviratne AM, Smrcka AV. Small molecule disruption of G protein beta gamma subunit signaling inhibits neutrophil chemotaxis and inflammation. Molecular pharmacology 2008;73:410-8.

26. Velten M, Duerr GD, Pessies T et al. Priming with synthetic oligonucleotides attenuates pressure overload-induced inflammation and cardiac hypertrophy in mice. Cardiovascular research 2012;96:422-32.

27. Nagai T, Anzai T, Kaneko H et al. C-reactive protein overexpression exacerbates pressure overload-induced cardiac remodeling through enhanced inflammatory response. Hypertension 2011;57:208-15.

28. Naga Prasad SV, Esposito G, Mao L, Koch WJ, Rockman HA. Gbetagamma-dependent phosphoinositide 3-kinase activation in hearts with in vivo pressure overload hypertrophy. The Journal of biological chemistry 2000;275:4693-8.

29. Naga Prasad SV, Jayatilleke A, Madamanchi A, Rockman HA. Protein kinase activity of phosphoinositide 3-kinase regulates beta-adrenergic receptor endocytosis. Nature cell biology 2005;7:785-96.

30. Naga Prasad SV, Laporte SA, Chamberlain D, Caron MG, Barak L, Rockman HA. Phosphoinositide 3-kinase regulates beta2-adrenergic receptor endocytosis by AP-2 recruitment to the receptor/beta-arrestin complex. The Journal of cell biology 2002;158:563-75.

31. Hata JA, Koch WJ. Phosphorylation of G protein-coupled receptors: GPCR kinases in heart disease. Mol Interv 2003;3:264-72.

32. Oudit GY, Sun H, Kerfant BG, Crackower MA, Penninger JM, Backx PH. The role of phosphoinositide-3 kinase and PTEN in cardiovascular physiology and disease. Journal of molecular and cellular cardiology 2004;37:449-71.

33. Patrucco E, Notte A, Barberis L et al. PI3Kgamma modulates the cardiac response to chronic pressure overload by distinct kinase-dependent and -independent effects. Cell 2004;118:375-87.

34. Oudit GY, Crackower MA, Eriksson U et al. Phosphoinositide 3-kinase gamma-deficient mice are protected from isoproterenol-induced heart failure. Circulation 2003;108:2147-52.

35. Sussman MA, Volkers M, Fischer K et al. Myocardial AKT: the omnipresent nexus. Physiological reviews 2011;91:1023-70.

36. Neumeister A, Charney DS, Belfer I et al. Sympathoneural and adrenomedullary functional effects of alpha2C-adrenoreceptor gene polymorphism in healthy humans. Pharmacogenetics and genomics 2005;15:143-9.

37. Small KM, McGraw DW, Liggett SB. Pharmacology and physiology of human adrenergic receptor polymorphisms. Annual review of pharmacology and toxicology 2003;43:381-411.

38. Lymperopoulos A, Rengo G, Gao E, Ebert SN, Dorn GW, 2nd, Koch WJ. Reduction of sympathetic activity via adrenal-targeted GRK2 gene deletion attenuates heart failure progression and improves cardiac function after myocardial infarction. The Journal of biological chemistry 2010;285:16378-86.

Figure Legends

Figure 1. Dose-response efficacy of gallein following TAC. A). A schematic representation of the experimental time-line showing initiation of gallein treatment after the establishment of HF, i.e. four weeks post-TAC. A dose-dependent cardioprotective effect of daily i.p. gallein was observed by both: B) cardiac morphometry (ventricular weight to tibia length, VW/TL) and C) cardiac function (echocardiography, % fractional shortening). 10 mg/kg/day appears to be the optimum therapeutic dose. ### $P < 0.001$ vs sham; ** $P < 0.01$ and * $P < 0.05$ vs TAC+V (using one-way ANOVA and Bonferroni's post-hoc analysis); § $P < 0.05$ vs baseline TAC+V; €€ $P < 0.01$ vs all groups at baseline; and ¥¥ $P < 0.05$ vs 12 weeks TAC+V (using repeated measures ANOVA with Bonferroni's post-hoc analysis).

Figure 2. Salutary effect of gallein post-TAC. A). Gallein (10 mg/kg/day) treated mice showed enhanced survival (80%; 8/10), while mice receiving vehicle injection showed lower survival rate (54.55%; 6/11) relative to a 100% survival in the sham group. B). Cardiac β -AR density was significantly reduced in TAC mice and was recovered to almost normal levels by gallein treatment. C). GRK2 gene expression was elevated in TAC+V mice and was reduced by gallein treatment. D). M-mode echocardiographic images showing impaired contractile function in TAC+V group and recovered function in gallein treated animals. This likely resulted from Gallein-mediated recovery of β -AR function due to attenuation of GRK2 and PI3Kγ membrane recruitment (E and F). # $P < 0.05$ vs sham; *** $P< 0.001$, ** $P< 0.01$, and * $P< 0.05$ vs TAC+V (using one-way ANOVA with Bonferroni's post-hoc analysis). Nonparametric analysis of β -AR binding utilizing Kruskal Wallis test yielded $P < 0.05$ for sham vs TAC+V.

Figure 3. Gallein reduces ventricular hypertrophy and Akt phosphorylation. A). Hypertrophy (ventricular weight to tibia length, VW/TL) was attenuated in gallein treated animals. B). Reduced cardiomyocyte cross-sectional area (CM CA) in gallein treated mice as a quantification of C) wheat germ agglutinin staining (WGA: green and nuclear DAPI: blue; scale bar= 50 µm). D). Reduced cytosolic Ser473-Akt phosphorylation as compared to total Akt protein expression and E). Ser9-GSK-3β phosphorylation relative to total GSK-3β protein expression in gallein treated mice (densitometric analysis and fold change), parallel with VW/TL and CM CA data. ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$ vs sham; * $P< 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs TAC+V (using one-way ANOVA with Bonferroni's post-hoc analysis). Nonparametric analysis of pGSK/GSK utilizing Kruskal-Wallis test yielded $P < 0.05$ for sham and $P < 0.01$ for TAC+G vs TAC+V.

Figure 4. Reduced cardiac fibrosis and inflammatory markers in gallein treated mice post-TAC. A). Picro-sirius red and B). Masson's trichrome staining shows less cardiac fibrosis in gallein treated mice. C-H). Real time PCR analysis of inflammatory and profibrotic gene expression (normalized to GAPDH as housekeeping gene) in cardiac RNA extracts show attenuated gene expression of these markers by gallein treatment. ### $P < 0.001$, ## $P < 0.01$, and # $P < 0.05$ vs sham; *** $P < 0.001$ & * $P< 0.05$ vs TAC+V (using one-way ANOVA with Bonferroni's post-hoc analysis). Nonparametric analysis of Nppb and Il6 utilizing Kruskal-Wallis test yielded $P < 0.05$ for sham vs TAC+V and $P < 0.01$ for TAC+G vs TAC+V, respectively.

Figure 5. Gallein reduces plasma catecholamines, adrenal hypertrophy, and restores adrenal α2-AR feedback inhibition of catecholamine release. A-B). Gallein treatment reduces circulating plasma catecholamines. C). Gallein attenuates adrenal medullae hypertrophy post-TAC (Hematoxylin-eosin staining of paraffin-fixed adrenal sections; scale bar= 25 □m) and attenuates tyrosine hydroxylase (TH) as well as chromogranin A (CgA) protein expression in adrenal chromaffin cells (immunofluorescent staining; scale bar= 50 µm). D-E). Gallein reduces chronically elevated catecholamine secretion in ex vivo cultures of post-TAC adrenal medullae. F-G). Adrenal α2-AR feedback inhibitory function is recovered by gallein treatment in TAC mice. $^{\#\#\#\#}P < 0.0001$, $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$, and $^{\#}P < 0.05$ vs sham; $^{**}P < 0.01$ and $^{*}P < 0.05$ vs TAC+V (using one-way ANOVA with Bonferroni's post-hoc analysis). Nonparametric analysis of plasma epinephrine utilizing Kruskal-Wallis test yielded $P < 0.001$ for sham vs TAC+V.

Figure 6. Gallein reduces catecholamine secretion and normalizes α2-AR feedback inhibition in diseased human adrenal medullae. Ex vivo cultured human adrenal pheochromocytoma slices were treated with gallein (G; 10 □M) or vehicle (V) for 48 hrs. A-B). Gallein treatment attenuates catecholamines secretion. C). Gallein attenuates tyrosine hydroxylase (TH) and chromogranin A (CgA) protein expression levels and D-E) attenuates GRK2 protein expression and membrane translocation (densitometric analysis and fold change). $^{****}P < 0.0001$, $^{***}P < 0.001$, $^{**}P < 0.01$ vs group V (using student's t-test and non-parametric utilizing Mann-Whitney test).

Figure 7. Model for dual efficacy of small molecule Gβ γ inhibition in the heart and the adrenal gland during HF. A). In cardiomyocytes, Gβγ-GRK2 interaction, triggered by elevated sympathetic nervous system (SNS) activity in HF, signals β-AR desensitization. B). In adrenal chromaffin cells, the site of catecholamine (CA) production: (1) central nicotinic stimulation triggers (2) synthesis and (3) secretion of CA into plasma. High levels of plasma CA (4) stimulate α2-AR mediated (5) feedback inhibition of CA synthesis and secretion, C). In HF, continuous CA stimulation of α2-AR trigger its Gβγ-GRK2 mediated desensitization and the loss of feedback inhibition of CA release, contributing to SNS over-activity in HF. D). Small molecule Gβγ inhibitors may provide a novel therapeutic approach for HF by inhibiting Gβγ-GRK2 signaling simultaneously in the heart and the adrenal gland, thus breaking this vicious cycle.

Table 1. Echocardiographic measurements of mice at baseline and weeks 4, 8, and 12 post-TAC.

	Baseline		**4 Weeks**		**8 Weeks**		**12 Weeks**	
	TAC+V	TAC+G	TAC+V	TAC+G	TAC+V	TAC+G	TAC+V	TAC+G
% EF	80.42±2.01	81.97±0.51	70.4±5.04	74.08±2.48	58.40±9.29	73.82±2.29	51.26±9.84$^{\#\#}$	75.77±3.47*
%FS	50.91±0.68	49.37±0.51	39.90±4.04	42.26±2.00	32.16±6.07$^{\#}$	41.94±1.88	26.14±5.28$^{\#\#}$	44.08±2.81**
LVID; s (mm)	1.50±0.07	1.54±0.06	1.92±.25	1.92±0.14	2.55±0.52	1.91±0.13	2.94±0.71	1.80±0.13**
LVID; d (mm)	3.16±0.08	3.06±0.09	3.44±0.23	3.37±0.13	4.04±0.37	3.25±0.12	4.41±0.58$^{\#}$	3.25±0.12*
Volume; s (□l)	7.20±0.44	6.83±0.55	16.22±4.56	12.31±2.02	38.65±15.69	11.57±1.98	62.34±28.75$^{\#}$	11.19±2.38**
Volume; d (□l)	39.76±1.99	37.62±2.37	50.17±7.21	46.09±3.97	74.64±16.87	42.77±3.60	98.08±30.08$^{\#}$	43.88±3.61**

$^{\#\#}P < 0.01$, and $^{\#}P < 0.05$ vs baseline TAC+V, $^{**}P < 0.01$ and $^{*}P < 0.05$ vs TAC+V at 12 weeks post-TAC (using repeated measures ANOVA and Bonferroni's post-hoc analysis).

Table 2. Hemodynamic measurements of Sham, TAC+V, and TAC+G mice at the end of the study.

	Sham	**TAC+V**	**TAC+G**
LVEDP (mmHg)	6.73±1.83	23.94±4.91$^{\#\#}$	8.57±2.15*
+dP/dt (mmHg/sec)	8777±860.3	5249±913.3$^{\#}$	8677±743.0*
-dP/dt (mmHg/sec)	-8027±926.5	-4022±570.6$^{\#}$	-7792±824.9*
LV min (mm Hg)	4.99±1.31	18.32±5.03$^{\#}$	5.92±2.33*
LV max (mm Hg)	129.3±3.98	109.9±14.57	166.4±13.35*

$^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ vs sham, and $*P < 0.05$ vs TAC+V (using one-way ANOVA and Bonferroni's post-hoc analysis).