Small Molecule Disruption of Gβγ Signaling Inhibits the Progression of Heart Failure

Liam M. Casey,* Andrew R. Pistor, Stephen L. Belmonte, Dmitriy Migdalovich, Olga Stolpnik, Frances E. Nwakanma, Gabriel Vorobiof, Olga Dunaevsky, Alessandra Matavel, Coeli M.B. Lopes, Alan V. Smrcka, Burns C. Blaxall

Rationale: Excess signaling through cardiac Gβγ subunits is an important component of heart failure (HF) pathophysiology. They recruit elevated levels of cytosolic G protein–coupled receptor kinase (GRK)2 to agonist-stimulated β-adrenergic receptors (β-ARs) in HF, leading to chronic β-AR desensitization and downregulation; these events are all hallmarks of HF. Previous data suggested that inhibiting Gβγ signaling and its interaction with GRK2 could be of therapeutic value in HF.

Objective: We sought to investigate small molecule Gβγ inhibition in HF.

Methods and Results: We recently described novel small molecule Gβγ inhibitors that selectively block Gβγ-binding interactions, including M119 and its highly related analog, gallein. These compounds blocked interaction of Gβγ and GRK2 in vitro and in HL60 cells. Here, we show they reduced β-AR–mediated membrane recruitment of GRK2 in isolated adult mouse cardiomyocytes. Furthermore, M119 enhanced both adenyl cyclase activity and cardiomyocyte contractility in response to β-AR agonist. To evaluate their cardiac-specific effects in vivo, we initially used an acute pharmacological HF model (30 mg/kg per day isoproterenol, 7 days). Concurrent daily injections prevented HF and partially normalized cardiac morphology and GRK2 expression in this acute HF model. To investigate possible efficacy in halting progression of preexisting HF, calsequestrin cardiac transgenic mice (CSQ) with extant HF received daily injections for 28 days. The compound alone halted HF progression and partially normalized heart size, morphology, and cardiac expression of HF marker genes (GRK2, atrial natriuretic factor, and β-myosin heavy chain).

Conclusions: These data suggest a promising therapeutic role for small molecule inhibition of pathological Gβγ signaling in the treatment of HF. (Circ Res. 2010;107:532-539.)

Key Words: G proteins ■ adrenergic receptor ■ G protein–coupled receptor kinases ■ cardiomyopathy ■ heart failure ■ cardiomyocyte

Heart failure (HF) is a devastating disease with poor prognosis, and remains a leading cause of death worldwide.1,2 Excess signaling through cardiac G protein Gβγ subunits is an important component of HF pathophysiology. In particular, they recruit elevated levels of cytosolic G protein–coupled receptor kinase 2 (GRK2) (β-adrenergic receptor kinase [β-ARK1]) to agonist-stimulated β-ARs in HF, leading to the chronic β-AR desensitization, downregulation and pathological signaling that are hallmarks of HF.4,5

Increasing evidence suggests a critical role for Gβγ-mediated signaling in HF. In particular, GRK2 is significantly upregulated in cardiomyocytes of animal models of HF and human HF patients; this elevates Gβγ-GRK2 interactions and contributes to chronic desensitization of β-AR signaling6,7; interestingly, levels of GRK2 appear to correlate with the severity of HF.6,8 Enhancing Gβγ-GRK2 interaction by cardiac targeted overexpression of GRK2(s) can directly cause HF in experimental animal models;9 its genetic ablation has generally proven to be cardioprotective.10–12

Several studies suggest targeting Gβγ-mediated signaling as an effective treatment for HF. First, studies using a Gβγ-sequestering peptide derived from the Gβγ-binding domain of GRK2, known as βARKct or GRK2ct, have demonstrated that interfering with Gβγ interactions in a variety of animal models of HF improves cardiac function, normalizes β-AR receptor levels and restores signaling responsiveness.13,14 Similarly, overexpression of a truncated dominant-negative form of phosducin, another Gβγ-binding
protein, can also restore cardiac function when virally delivered to failing rabbit hearts. Also, disruption of phosphatidylinositol 3-kinase (PI3Kγ) recruitment to the membrane-associated Gβγ-GRK2 complex by βARKct or expression of the GRK2-binding PIK domain of PI3Kγ is cardioprotective in a variety of HF models. These large peptide inhibitor studies highlight the therapeutic potential of Gβγ inhibitors in HF.

We recently developed a novel small molecule targeting strategy to selectively inhibit Gβγ-binding interactions. Studies from our laboratories have identified a number of small molecules that inhibit peptide binding to the protein interaction domain of the Gβγ subunit, suggesting possible salutary modulation of pathological Gβγ-dependent signaling in HF. Here we demonstrate that 2 of these compounds, M119 and gallein, influence β-AR–dependent, Gβγ-mediated signaling in isolated mouse cardiomyocytes. Furthermore, we show that these compounds block the progression of cardiac dysfunction and hypertrophy in 2 murine models of HF. We conclude that M119 and gallein, and other related Gβγ-targeting compounds, will provide valuable tools for dissecting the functional importance of different Gβγ-protein interactions and may lead to development of Gβγ-targeted small molecule therapeutics for the treatment of HF.

Methods

Animals

Isoproterenol Infusion

32 12-week-old C57Bl6-J wild-type male mice (The Jackson Laboratory) were divided into 4 groups of 8 mice each: V-V (isoproterenol [Iso] vehicle/M119 vehicle), V-M (Iso vehicle/M119), I-V (Iso/M119 vehicle), and I-M (Iso/M119). M119 or M119 vehicle (1× PBS, pH 7.7) was delivered at 100 mg/kg per day via intraperitoneal injection (200 µL/injection). Filtered solutions of Iso or Iso vehicle (0.002% ascorbic acid in saline) were delivered via implantable miniosmotic pump at a concentration of 30 mg/kg per day. All animal procedures were performed in accordance with the guidelines of the Department of Laboratory Animal Medicine and the University Committee on Animal Resources at the University of Rochester Medical Center.

Calsequestrin

Ten 8- to 12-week-old CSQ overexpressing mice were divided into 2 groups: gallein and vehicle. Gallein or vehicle (1× PBS, pH 7.7) was delivered at 30 mg/kg per day via daily peritoneal injection (200 µL/injection). All animal procedures were performed in accordance with the guidelines of the Department of Laboratory Animal Medicine and the University Committee on Animal Resources at the University of Rochester Medical Center.

Echocardiography

Transthoracic 2D and M-mode echocardiography analysis was used to assess heart function in trained, conscious mice with a VisualSonics Vevo 770 echocardiography machine equipped with a 30MHz probe (VisualSonics). At least 5 animals from each group were measured and the pooled data were analyzed for statistical significance.

Myocyte Isolation

Wild-type male C57Bl6-J mice aged 12 to 16 weeks were anesthetized with 0.5 mL of heparin (100 U/mL) and 0.5 mL of ketamine/midazolam in saline combination via intraperitoneal injection. Once anesthetized, the heart was removed, immediately suspended on a Langendorff apparatus by cannulation of the aortic root and perfused at constant rate of 4 mL/min at 37°C starting with 4 minutes of perfusion buffer (5 mmol/L NaHCO3, 30 mmol/L taurine, 10 mmol/L BDM, 5 mmol/L glucose, pH 7.4). Subsequently, enzymatic digestion was achieved by the infusion of calcium-free digestion buffer (120 mg collagenase type II in 50 mL perfusion buffer) for 3 minutes followed by 10 minutes of perfusion with calcium containing digestion buffer (digestion buffer+40 mmol/L CaCl2). The heart was then removed and placed in a dish filled with 2 mL of stopping buffer (10% FBS, 12.5 µmol/L CaCl2 in perfusion buffer). Following removal of the atria, the ventricles were teased apart and pipetted into small pieces. To remove undigested tissue, the cell suspension was filtered through a 200-µm mesh and allowed to settle by gravity for 10 minutes at 37°C. The supernatant was discarded and the cardiomyocyte pellet was resuspended in 10 mL of stopping buffer. CaCl2 (100 mM) was added incrementally to a final concentration of 1.2 mmol/L and the cardiomyocytes were again allowed to settle. The final pellet was resuspended in 5 mL of plating medium (MEM, 2.5% FBS, 1× penicillin/streptomycin, 2 mM/L L-glutamine) and plated onto laminin-coated BT-CS glass chamber slides (Cell Micro Controls) for 1 to 2 hours in a 37°C incubator at 2% CO2 before contractility studies.

Myocyte Contractility

The glass chamber slides were placed on a microscope stage (Olympus IX71) connected to a field stimulator specifically designed for driving isolated cardiomyocytes (MyoPacer, IonOptix). Cardiomyocytes were stimulated at 0.5 Hz and imaged with a variable field-rate camera (MYO100 MyoCam, Ionoptix) using both edge detection and sarcomere length technology. Peak contraction was measured as the percentage of peak cell shortening. Cardiomyocytes were treated with one of 8 solutions: vehicle (control), Iso (1 µmol/L), M119 (10 µmol/L), gallein (10 µmol/L), propranolol (1 µmol/L), Iso/M119 (1 µmol/L and 10 µmol/L, respectively), propranolol/Iso (1 µmol/L each), or propranolol/M119 (1 µmol/L and 10 µmol/L, respectively) for 10 minutes each. Treatment order was varied between experiments to control for time-dependent changes in cardiomyocyte contractility. All studies were recorded and saved in grayscale and numeric formats. Each data point represents cardiomyocytes isolated from a different animal with at least 7 individual cardiomyocytes averaged per treatment and an equal number of contractions (>6) included for each cardiomyocyte.

Additional information and methods for morphometry/histology, protein preparation, immunoblotting, RNA extraction, real-time PCR, cAMP assay, statistical analysis, and small molecule information are all included in the expanded Methods section, available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Small Molecule Gβγ Inhibition Influences β-AR Signaling in Isolated Cardiomyocytes

We recently identified compounds M119 and gallein as highly related small molecules with similar efficacy that
modulate GPCR signaling by interfering with Gβγ-binding interactions. M119 and gallein block peptide binding to Gβγ with equal efficacy, they block Gβγ-GRK2 association in vitro and in HL-60 leukocytes in cell culture and produce equivalent dose–response curves in an inflammatory model in vivo (and Online Figure I). Previous studies using peptide inhibitors of Gβγ indicate that targeting of β-AR–dependent, Gβγ-mediated signaling can lead to dramatic improvement in cardiac function in diverse models of HF. Here, we tested whether cell-permeant small molecules could influence β-AR signaling in cardiomyocytes and improve cardiac function in murine models of HF. To begin, we examined whether M119 affected basal and β-AR–elicited cAMP generation in adult mouse cardiomyocytes. Elevation of cAMP production elicited by the β-AR agonist Iso was significantly enhanced in the presence of M119 (Figure 1A), which also induced a slight increase in cAMP at baseline.

Next, we examined whether M119 or gallein influence β-AR–mediated GRK2 membrane recruitment. Adult mouse cardiomyocytes from wild type mice were pretreated with 10 μmol/L gallein or vehicle followed by 1 μmol/L Iso or vehicle, after which cardiomyocytes were subjected to subcellular fractionation. Immunoblot analysis of the membrane fraction demonstrated increased GRK2 with Iso alone compared to vehicle-treated cells (Figure 1B and 1C; Online Figure II, A and B). Gallein or M119 alone did not significantly affect the subcellular distribution of GRK2 compared to control treated cells. However, both M119 and gallein reduced β-AR–mediated GRK2 membrane translocation, because cells treated with either compound, as well as Iso, show only ~1.3-fold increase in membrane bound GRK2 compared to control cells (Figure 1B and 1C; Online Figure II, A and B). Thus, consistent with our prior in vitro studies using purified proteins and in HL-60 leukocytes stimulated with fMLP, M119 and gallein can reduce β-AR–mediated GRK2 recruitment to the membrane in adult cardiomyocytes.

The specificity of M119 on Gβγ signaling was also determined by assessing electrophysiology in Xenopus oocytes expressing the cardiac G protein–coupled inward rectifying Kα channel (GIRK) subunits Kir3.1 and Kir3.4, together with the muscarinic type 2 receptor. M119 showed no affect on both the basal and ACh activated Kir3.1/Kir3.4 current (Online Figure III), supporting previous data indicating these compounds selectively interfere with a subset of Gβγ interactions.

Based on these results, we sought to determine whether M119 could influence cardiomyocyte contractility both at baseline and in response to β-AR stimulation by measuring sarcomere shortening of isolated adult mouse cardiomyocytes. Compared to untreated cardiomyocytes, Iso significantly enhanced contractility demonstrating these cells were responsive to β-AR agonist (Figure 2A and 2B). Combined treatment with Iso and M119 resulted in a further significant increase in contraction over Iso alone. In addition to affecting the length of sarcomere shortening, M119 alone and in combination with Iso also increased the rate of cardiomyocyte contraction (Figure 2C). The effect of M119 alone on cardiomyocyte contractility was not unexpected because M119 could enhance signaling downstream from constitutive signaling activity of β-ARs reported to account for a portion of baseline cardiomyocyte contractility. Consistent with previous reports of this phenomenon, we found that propranolol alone slightly reduced the rate of contraction (Figure 2C). Furthermore, we found that propranolol completely inhibited the effect of M119 (and Iso) on baseline contractility (Figure 2B and 2C), suggesting compound specificity for β-AR-Gβγ signaling in cardiomyocytes. Collectively these data demonstrate that small molecule Gβγ inhibition enhances β-AR–mediated signaling and contractility in isolated adult cardiomyocytes.
M119 Improves Cardiac Function in an Acute Pharmacological Model of HF

Based on our in vitro experiments, we next examined the effect of M119 on cardiac function in vivo. Chronic stimulation of β-ARs by Iso delivered via implantable miniosmotic pumps is an established acute pharmacological murine model of HF12,23 and this model was used to examine the effects of M119 on HF. Echocardiographic measurements were recorded from all mice before initiating the treatment regimen. Miniosmotic pumps containing Iso or saline were implanted and the mice were concurrently injected daily with M119 or vehicle for 7 days. Following 7 days of treatment, cardiac function was again analyzed by echocardiography. As shown in Figure 3, Iso-treated mice showed significant signs of cardiac dysfunction after 7 days with a dramatic reduction in percent fractional shortening compared to vehicle controls. Mice treated with M119 alone showed no significant decrease in percent fractional shortening and no alteration in heart rate compared to vehicle controls, indicating that M119 by itself does not alter cardiac function (Figure 3 and Online Table I).

Figure 3. M119 improves cardiac function, and reduces cardiac hypertrophy and interstitial fibrosis, in an acute pharmacological HF model. A, Representative M-mode tracings of vehicle-only, Iso-treated, and M119+Iso-treated animals. B, Average fractional shortening data of animals in each treatment group (n=6 to 8 per group) shows significant normalization of cardiac function in Iso-treated animals that also received M119. C, HW:BW ratios of animals following 7-day treatment. Iso-treated animals show significant cardiac hypertrophy compared to vehicle controls. M119 treatment significantly reduces cardiac hypertrophy when administered to Iso-pumped animals. D, Masson’s trichrome staining of tissue sections showed that Iso+M119 animals exhibit reduced interstitial fibrosis compared to Iso-only animals. **P<0.01, ***P<0.001.
compared to significantly decreased cardiac function observed in Iso-pumped animals that received vehicle control injections (Figure 3 and Online Table I). Iso-pumped animals treated with M119 also showed significant normalization of LV volumes and mean velocity of circumferential fiber shortening (Online Table I).

Concurrent M119 Reduces Iso-Induced Cardiac Dysfunction and Hypertrophy

Cardiac morphometry was also examined to determine whether M119 influences hypertrophic growth of the heart. Following echocardiographic analysis of treated mice, body weight (BW) and heart weight (HW) were determined for all mice. As shown in Figure 3C, Iso-treated animals exhibited a \( \approx 33\% \) increase in mean HW:BW ratio compared to vehicle controls, indicating that chronic Iso infusion induced significant cardiac hypertrophy. By contrast, M119 treatment of Iso-pumped animals significantly reduced cardiac hypertrophy. These morphometric data were substantiated by significant normalization of ventricular wall thickness with M119 treatment of Iso-pumped animals as detected by echocardiography (Online Table I). Animals treated with M119 alone showed no significant change in HW:BW ratio, indicating that this drug does not by itself induce cardiac hypertrophy.

Isolated hearts were examined using histological staining techniques. Hematoxylin and eosin staining revealed that hearts from Iso-pumped animals were visibly larger than vehicle-pumped animals, consistent with the observed effect on HW:BW ratios (data not shown). In addition, Masson’s trichrome staining revealed that Iso-pumped, M119-treated animals demonstrate reduced interstitial and perivascular fibrosis compared to Iso-pumped animals (Figure 3D). No evidence of tissue pathology was found in any group on staining of lung, liver, or brain tissue (not shown).

Gallein Halts the Progression of HF in Mice With Established HF

Cardiac restricted calsequestrin overexpressing mice (CSQ) replicate many hallmarks of HF, including dysfunctional \( \beta\)-AR signaling. To investigate the efficacy of G\( \beta\gamma\)-targeting compounds in halting HF progression, CSQ mice (a generous gift from Dr. Larry Jones) with established HF at 8 weeks of age were treated with the compound gallein, which is highly related and similarly efficacious to M119, administered daily for 1 month by intraperitoneal injection. Echocardiographic analysis showed that gallein treatment completely prevented the progression of HF, maintaining essentially all echocardiographic measures, including functional data for both fractional shortening and ejection fraction (Figure 4 and Online Table II). Conversely, vehicle-treated animals demonstrated progressive worsening of cardiac function and of numerous measures associated with the progression of eccentric hypertrophy and dilated cardiomyopathy, including chamber dilation and ventricular wall thinning (Figure 4 and Online Table II). Additionally, morphometric analysis showed that gallein inhibited the progression of pathological cardiac hypertrophy associated with this genetic HF model (Figure 5A and 5C). Gallein also numerically (but not significantly) enhanced cardiac \( \beta\)-AR cell surface expression toward normal, determined by radioligand binding (Online Figure IV). Finally, improved cardiac function in CSQ mice following gallein treatment was accompanied by a concomitant reduction in expression of both atrial natriuretic factor and \( \beta\)-myosin heavy chain (Figure 5B).
Small Molecule Gβγ Inhibitors Reduce GRK2 Expression in HF

In parallel experiments, age-, sex-, and background strain (DBA)-matched nontransgenic control animals received equivalent daily gallein or vehicle injections for one month. Compared to vehicle treatment, no histological abnormalities were observed in the gallein treated group (heart, lung, liver, brain; data not shown), nor were there any differences in cardiac morphology or morphometry (Online Figure V, B). Interestingly, a slight but significant elevation of cardiac contractility (percentage fractional shortening) was observed by echocardiography in the gallein group following 1 month of treatment (Online Figure V, A; Online Table III), mirroring prior reports that cardiac GRK2 expression in 2 distinct animal models of HF.6,7,27 Collectively these results suggest that directed targeting of Gβγ protein expression. As demonstrated previously,23 Iso-pumped animals show robust increase in GRK2 protein level after one week of treatment (Figure 6A). Interestingly, upregulation of GRK2, indicative of HF, was significantly reduced in Iso-pumped animals treated with M119 (Figure 6A). GRK2 expression was also significantly decreased in CSQ animals treated with Gallein (Figure 6B). Thus, in addition to the immediate effects of small molecule Gβγ inhibitor treatment on Gβγ-mediated GRK2 membrane recruitment demonstrated in cardiac myocytes in vitro, long-term small molecule Gβγ inhibitor treatment normalized cardiac GRK2 expression in 2 distinct animal models of HF.

Discussion

Recent studies from our laboratories identified a number of small molecules that bind Gβγ and modulate Gβγ-protein interactions.19,20 These compounds were able to inhibit a number of Gβγ-dependent signaling events, including Gβγ-GRK2 association. GRK2 protein levels are significantly elevated in HF, and substantial evidence indicates that blocking both Gβγ and the GRK2-Gβγ interaction in diverse HF models is cardioprotective.13,14 In this study, we tested whether M119 and its highly related and similarly efficacious Gβγ compound inhibitor gallein could be used to target Gβγ in cardiomyocytes and thereby influence aspects of β-AR signaling and improve cardiac function in animal models of HF. Our data indicate that M119 and gallein interfere with Gβγ-GRK2 interactions in cardiomyocytes, and may enhance β-AR signaling in vitro. M119 and gallein both halt HF progression and improve cardiac function, morphometry, histology, and gene expression in animal models of either new onset or established HF.

Recruitment of GRK2 to the membrane by Gβγ is among the more well-established functions of Gβγ downstream of β-AR signaling. General inhibition of Gβγ, and of the Gβγ-GRK2 interaction, using various Gβγ inhibitory peptides (eg, βARKct, nt-del-Phosducin, PI3Kγ Inact) has proven to be a highly effective treatment for a variety of animal HF models and in isolated failing human cardiomyocytes.28 The results of these studies have alternately been viewed as a specific effect of inhibiting GRK2 function and as a broader effect on Gβγ signaling. Consistent with the latter hypothesis, another Gβγ sequestering peptide, a truncated form of the Gβγ-binding protein phosducin (nt-del-Phosducin), has also been used successfully to restore β-AR signaling in cardiomyocytes.29 More recently, a peptide inhibitor of PI3Kγ, which is recruited to Gβγ in part through association with GRK2, has also proven to be effective in treating animal models of HF.16,17,27 Collectively these results suggest that generally inhibiting pathological Gβγ-dependent signaling in failing cardiomyocytes is salutary. Consistent with these observations, our novel small molecule based approach to targeting Gβγ signaling appears to function, at least in part, like peptide inhibitors of Gβγ signaling. Whether the observed effects are attributable specifically to Gβγ-GRK2 inhibition, or to other Gβγ signaling components, will be a target of our future studies. Importantly, the present study suggests selective interference with a subset of Gβγ interactions, as Gβγ-GIRK signaling was not affected (Online Figure III).

Our studies of isolated adult cardiomyocytes showed that small molecule Gβγ inhibition enhanced cAMP production and cardiomyocyte contractility following acute β-AR stimulation. These data are consistent with published results showing similar effects of βARKct expression in cardiac...
myocytes in culture and in vivo. Because M119 and gallein were also found to reduce Iso-induced GRK2 membrane recruitment, we conclude that these small molecules influence β-AR signaling in cardiomyocytes similar to βARKct.

Gβγ inhibitory compounds M119 and gallein partially normalized cardiac morphology and gene expression and halted HF progression in the acute ISO pump model of HF and in CSQ mice with established HF. These data suggest promising utility of small molecule Gβγ inhibition in treating both new onset and extant HF. Importantly, β-blockers are a standard therapy in the treatment of human HF. Combination of cardiac-restricted βARKct expression with the β-blocker metoprolol has previously demonstrated synergistic benefit for cardiac function in CSQ mice, suggesting a possible synergy of small molecule Gβγ inhibition with β-blocker therapy, which will be a target of our future investigation.

In addition to its role in the cardiomyocytes, recent evidence demonstrates that dysregulation of AR-dependent Gβγ signaling in other tissues may also contribute to cardiac dysfunction. Lymperopoulos et al recently showed a significant increase of GRK2 in the chromaffin cells of the adrenal medulla, suggesting that increased Gβγ-GRK2 association contributes to dysfunctional feedback inhibition of catecholamine release via α2-AR signaling. Interestingly, viral delivery of βARKct to the adrenal gland restored α2-AR feedback inhibition of catecholamine release and dramatically improved cardiac function, attributable, at least in part, to decreased chronic stimulation of cardiac β-ARs. Increased GRK2 expression is also associated with hypertension. β-AR signaling normally promotes vasodilation but 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. Because heart disease can result from dysfunctional signaling in multiple organs, we and others believe that systemic delivery of small molecule Gβγ 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.

In summary, we have demonstrated that small molecule Gβγ inhibitors function in vivo to improve cardiac function and halt HF progression in both new-onset and extant HF in mice. Small molecule Gβγ inhibitors appear to maintain β-AR responsiveness, in part by interfering with GRK2 membrane recruitment. Future studies will determine whether Gβγ inhibitor compounds modulate other known Gβγ-dependent signaling events, including Gβγ-mediated signaling via novel extracellular signal-regulated kinase 1/2 phosphorylation and whether these effects contribute to their effects in cardiac pathophysiology. Such studies will aim to determine whether small molecule targeting of Gβγ may be an effective therapeutic paradigm for the treatment of HF.

Acknowledgments
We thank Sundeen Malik for help with immunoblotting.

Sources of Funding
This work was supported in part by NIH R01 grants HL091475, HL084087, and HL089885 (to B.C.B.); NIH grant GM081772 (to A.V.S.); NIH T32 grant (GM07356) (to S.L.B.); and an American Heart Association postdoctoral fellowship (to S.L.B.).

Disclosures
None.

References
15. Hippe HJ, Lutz S, Cuello F, Knorr K, Vogt A, Jakobs KH, Wieland T, Nirooian M. Activation of heteroteric G proteins by a high energy phosphate transfer via nucleoside diphosphate kinase (NDPK) B and...
Novelty and Significance

What Is Known?

- Excess signaling through cardiac G protein βγ (Gβγ) subunits via β-adrenergic receptors (β-ARs) is a major component of the pathological changes associated with the progression of heart failure (HF).
- Numerous reports indicate that peptide inhibitors of β-AR/Gβγ signaling can block many of the molecular changes associated with HF and can improve cardiac function.
- We recently identified 2 membrane permeable, structurally related small molecules that selectively target Gβγ-protein interactions that can be used effectively in vivo.

What New Information Does This Article Contribute?

- Two structurally related small molecules that target specific Gβγ signaling pathways are cardioprotective in mouse models of both new onset and extant HF.

- Cardiac protection conferred by these Gβγ targeting compounds is associated with partial reversal of pathological molecular changes known to occur in HF.

Prior studies have demonstrated that interfering with Gβγ signaling downstream of β-AR activation is cardioprotective in HF models. Previous approaches generally used large peptides that required viral vector delivery to target cells/organisms. Here, we demonstrate that systemic delivery of small molecule inhibitors of Gβγ signaling can disrupt pathological molecular changes underlying HF. Furthermore, we demonstrate that these small molecules reduce the progression of HF in 2 distinct mouse models. Our study provides rationale for further development of Gβγ-targeting compounds as a therapeutic approach for HF. This could lead to new cardiac therapies that, in combination with existing drugs, may improve patient health.
Small Molecule Disruption of Gβγ Signaling Inhibits the Progression of Heart Failure
Liam M. Casey, Andrew R. Pistner, Stephen L. Belmonte, Dmitriy Migdalovich, Olga Stolpnik, Frances E. Nwakanma, Gabriel Vorobiof, Olga Dunaevsky, Alessandra Matavel, Coeli M.B. Lopes, Alan V. Smrcka and Burns C. Blaxall

Circ Res. 2010;107:532-539; originally published online June 24, 2010;
doi: 10.1161/CIRCRESAHA.110.217075

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/107/4/532

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2010/06/24/CIRCRESAHA.110.217075.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Supplement Material

Methods

Morphometry/Histology
After the specified drug treatment schedule, mini-pumps were removed where necessary and tissues were isolated from anesthetized mice immediately following conscious echocardiography. Briefly, anesthetized mice were weighed, sacrificed and then tissues including heart, lung and liver were isolated and individually weighed. The heart was bisected horizontally and the upper half including the atria and portions of the left and right ventricles were fixed in fresh 4% paraformaldehyde while the lower portion was snap frozen in liquid nitrogen for protein isolation. For histology, hearts were embedded in paraffin and then serial 5 mm sections were collected. Sections were used for routine hematoxylin and eosin staining or Masson’s trichrome staining.

Protein Preparation and Immunoblotting

Isolated cardiomyocytes
Proteins were isolated from cardiomyocytes using a modification of the method of DeFea et al\(^1\). Briefly, cardiomyocytes are plated onto laminin coated 6 cm plates at 2-3x10\(^5\) cells per plate and maintained for 2 hours in plating medium (MEM + 1% serum, 2 mM L-glutamine, 1X penicillin/streptomycin) prior to treatment. Cells were treated with 10 \(\mu\)M M119 or vehicle for 5 minutes followed by treatment with 1\(\mu\)M Iso or PBS (vehicle) for 5 minutes. Immediately following, cells were rinsed once with 2 mL of Tyrode’s buffer, lysed in 300 \(\mu\)l of HES buffer (10 mM HEPES, 1 mM EDTA, 250 mM Sucrose) containing protease inhibitors (Complete Mini; Sigma-Aldrich) and phosphatase inhibitors (20 \(\mu\)M \(\beta\)-glycerophosphate, 1 \(\mu\)M sodium orthovanadate, 50 mM okadaic acid), scraped from the plate and homogenized with 20 strokes in an ice cold Dounce homogenizer. The homogenate was centrifuged at 700Xg for 10 min and the supernatant was then cleared by centrifugation at 100,000Xg for 60 min to separate cytosolic and high-speed membrane fractions. Membrane pellets were resuspended in HES buffer + 1% Triton containing protease and phosphatase inhibitors.

Whole heart tissue
For whole-heart lysates, the nitrogen-frozen heart tissue was pulverized and mechanically homogenized in an equal volume of RIPA (1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium doedecyl sulfate, 150 mM NaCl, 50 mM Tris pH8.0). Following a clearing spin at 3000 rpm for 10 min at 4\(^\circ\)C, the supernatants were sonicated for 10 seconds and centrifuged at 15,000 rpm for 20 min at 4\(^\circ\)C. The supernatant was carefully removed and used for immunoblotting.

\(\beta\)-AR binding studies
Myocardial membrane and cytosol fractionation were performed, membranes were assayed with \([^{125}\text{I}]\)-CYP, non-specific binding determined with 1 \(\mu\)M alprenolol. Specific binding (Bmax) was normalized to membrane protein concentration\(^2\) \(^3\).

Immunoblotting
Protein concentrations were determined via the Bradford method with the BioRad DC protein assay (BioRad Inc.). Equal amounts of either cardiomyocyte membranes (10 mg) or whole heart lysate (50 \(\mu\)g) were loaded onto pre-cast NuPAGE 4-12% Bis-Tris gels (Invitrogen). Proteins were transferred to nitrocellulose membranes and reacted with primary antibodies to GRK2 (rabbit anti-GRK2, mouse anti-\(\beta\)-Arrestin, 1:500, Santa Cruz Biotechnology). Primary antibodies
were then detected using either HRP conjugated goat anti-rabbit secondary antibodies and ECL detection (Roche) or fluorescent-conjugated secondary antibodies (Rockland Immunochemicals, Molecular Probes) and a Li-COR fluorescent detection system (Li-COR Biosciences).

**RNA extraction and Real-Time PCR**

RNA was isolated and analyzed as we and others have previously described. Briefly, Total RNA extraction from murine left ventricle tissue was performed with the RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA integrity was analyzed using the Agilent RNA 6000 Nano assay for the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Reverse Transcription was carried out using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, Foster City, CA). Real Time PCR was performed using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems Inc, Foster City, CA). All assays were performed in a 384 well plate with each reaction volume mixture having a final volume of 10mL. All samples were run in triplicate. The reaction mixture for the ANF and β-MHC assays was comprised of 2.5ul of RT products diluted 1:100 in nuclease free water, 900nM forward primers, 900nM reverse primer, 1x Power SYBR Green PCR Master Mix (Applied Biosystems Inc, Foster City, CA) and nuclease free water. The reaction conditions for the ANF and β-MHC assays consisted of an initial denaturing step of 95°C for 10 minutes, followed by 40 cycles of amplification and quantitation steps of 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute. Following cycling a dissociation curve analysis was run consisting of conditions of 95°C for 15 seconds, 60°C for 15 seconds and 95°C for 15 seconds. Primers for the ANF and β-MHC assays were synthesized by Integrated DNA Technologies (Integrated DNA Technologies, Inc., Coralville, IA). The reaction mixture for GAPDH was comprised of 1X Mouse GAPDH Endogenous Control Assay P/N 4352932E (Applied Biosystems Inc, Foster City, CA), TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems Inc, Foster City, CA) and nuclease free water. The reaction conditions for the GAPDH assay consisted of an initial denaturing step of 95°C for 10 minutes, followed by 40 cycles of amplification and quantitation steps of 95°C for 15 seconds, 60°C for 1 minute. Threshold values were set automatically by the SDS2.3 software (Applied Biosystems Inc, Foster City, CA) and the threshold cycle number (Ct) was determined for each gene of interest and GAPDH. The ΔCt value was calculated (Ctene of interest – Ct GAPDH) and the ratio of gene of interest to GAPDH was calculated using the formula 2^-ΔCt . Fold changes were calculated by dividing the ratio of gene of interest to GAPDH for the experimental group by the ratio of gene of interest to GAPDH for the control group.

**cAMP Assay**

Freshly isolated cardiomyocytes were resuspended at a final concentration of ~20,000 rod-shaped cardiomyocytes per 100 mL of Krebs buffer containing 750mM IBMX to inhibit endogenous phosphodiesterase activity. Cells were pretreated with 10 mM M119 or vehicle for 5 minutes then stimulated for 15 minutes with 100 mM Iso (Sigma) or 100 mM Forskolin (Sigma) while rotating at room temperature. cAMP was extracted from the cells by adding ice cold 100% ethanol to a final concentration of 70% and incubating on ice for 15 minutes. Cells were then vortexed briefly, centrifuged for 10 minutes at 12,000 rpm and the resulting supernatant containing cAMP was transferred to a fresh eppendorff tube for concentration in a Speed-Vac. Resulting pellets were then assayed for cAMP using the Bridge-It cAMP Designer Fluorescence Assay (Mediomics Inc.) according to manufacturer’s recommendations. To control for interexperimental variability in cell number and viability, cAMP measurements were represented as relative fluorescence (RF) calculated as follows: RF= (fluorescence (baseline) – fluorescence
(sample)) / fluorescence (baseline). Results were derived from at least five experiments for each treatment.

**Statistical Analysis:** For single biochemical/physiological observations, unpaired students t-tests were used. Multiple responses of various physiological and biochemical and assays were analyzed using one-way or repeated measures ANOVA. Post-hoc analysis (ie. Newman-Keuls) was performed if significance was achieved, with $P<0.05$ required for significance in all tests.

**Small molecules**
M119 (NSC119910) kindly provided by the National Cancer Institute repository. Gallein from Acros Organics, Geel, Belgium.
Mini-osmotic pumps containing Iso in saline with .002% ascorbic acid (Iso vehicle) or Iso vehicle alone were implanted into 12-week old C57B6-J mice and Iso was delivered at a rate of 30mg/kg/day. Mice were simultaneously given daily 200µl injections of either M119 (100 mg/kg/day) or 1 x PBS pH8.6 (M119 vehicle). Treatment groups are abbreviated as follows: V-V (Iso vehicle/ M119 vehicle), V-M (Iso vehicle/ M119), I-V (Iso/ M119 vehicle), and I-M (Iso/ M119). Abbreviations used are as follows; LVID;d, left ventricular internal dimension at diastole; LVID;s, left ventricular internal dimension at systole; IVS;d, intraventricular septal thickness at diastole; LVPW;d, left ventricular posterior wall thickness at diastole; IVS;s, intraventricular septal thickness at systole; LVPW;s, left ventricular posterior wall thickness at systole; LV Vol;d, left ventricular volume at diastole; LV Vol;s, left ventricular volume at systole; %EF, Ejection fraction (calculated); %FS, percent fractional shortening; AoV ET, aortic valve ejection time; HR, heart rate; mVcf, mean velocity of fractional shortening. *P<.05, **P<.01, ***P<.001 vs. V-V. #P<.05, ##P<.01, ###P<.001 vs. I-V. All other pair wise comparisons, including I-M vs. V-V and I-M vs. V-M, were not significantly different.

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>V-V</th>
<th>V-M</th>
<th>I-V</th>
<th>I-M</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVII;d (mm)</td>
<td>3.04±0.11</td>
<td>2.87±0.10</td>
<td>3.42±0.16</td>
<td>3.03±0.18</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>1.40±0.02</td>
<td>1.29±0.08</td>
<td>2.39±0.22*</td>
<td>1.47±0.15**</td>
</tr>
<tr>
<td>IVS;d (mm)</td>
<td>0.87±0.03</td>
<td>0.90±0.04</td>
<td>0.88±0.04</td>
<td>0.90±0.03</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>0.78±0.01</td>
<td>0.83±0.02</td>
<td>0.80±0.03</td>
<td>0.84±0.03</td>
</tr>
<tr>
<td>IVS;s (mm)</td>
<td>1.17±0.08</td>
<td>1.19±0.04</td>
<td>1.10±0.05</td>
<td>1.25±0.03*</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.30±0.07</td>
<td>1.30±0.04</td>
<td>1.06±0.03*</td>
<td>1.18±0.04*</td>
</tr>
<tr>
<td>LV Vol;d (µl)</td>
<td>42.46±2.45</td>
<td>36.89±3.98</td>
<td>53.68±5.14</td>
<td>47.49±6.06</td>
</tr>
<tr>
<td>LV Vol;s(µl)</td>
<td>5.50±0.26</td>
<td>5.44±0.45</td>
<td>25.48±4.92*</td>
<td>10.76±2.64*</td>
</tr>
<tr>
<td>%EF</td>
<td>86.83±0.54</td>
<td>84.51±1.67</td>
<td>55.24±4.34**</td>
<td>79.73±2.89**</td>
</tr>
<tr>
<td>% FS</td>
<td>54.35±1.05</td>
<td>51.63±1.85</td>
<td>28.56±2.71***</td>
<td>48.03±2.60***</td>
</tr>
<tr>
<td>AoV ET (msec)</td>
<td>49.25±3.38</td>
<td>47.31±0.92</td>
<td>53.91±2.67</td>
<td>46.09±2.40</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>570±21</td>
<td>586±39</td>
<td>549±25</td>
<td>509±26</td>
</tr>
<tr>
<td>mVcf (circ/sec)</td>
<td>1123±55</td>
<td>1101±57</td>
<td>555±41***</td>
<td>1084±85***</td>
</tr>
</tbody>
</table>
## Online Table II. Echocardiographic data in CSQ mice at baseline and following four weeks daily vehicle or Gallein.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Vehicle</th>
<th></th>
<th></th>
<th>Gallein</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 Weeks</td>
<td>% Change at 4wks</td>
<td>Baseline</td>
<td>4 Weeks</td>
<td>% Change at 4wks</td>
</tr>
<tr>
<td>LVID;d (mm)</td>
<td>4.6±0.16</td>
<td>5.04±0.2</td>
<td>9.65</td>
<td>4.16±0.29</td>
<td>4.37±0.11*</td>
<td>4.88</td>
</tr>
<tr>
<td>LVID;s (mm)</td>
<td>3.15±0.13</td>
<td>3.99±0.22</td>
<td>26.65</td>
<td>2.86±0.23</td>
<td>2.94±0.24*</td>
<td>2.64</td>
</tr>
<tr>
<td>IVS;d (mm)</td>
<td>0.86±0.05</td>
<td>0.68±0.04</td>
<td>-20.88</td>
<td>0.8±0.05</td>
<td>0.66±0.02</td>
<td>-17.65</td>
</tr>
<tr>
<td>LVPW;d (mm)</td>
<td>0.86±0.05</td>
<td>0.67±0.03</td>
<td>-21.81</td>
<td>0.78±0.05</td>
<td>0.68±0.03</td>
<td>-13.15</td>
</tr>
<tr>
<td>IVS;s (mm)</td>
<td>1.13±0.03</td>
<td>0.89±0.03</td>
<td>-21.2</td>
<td>1.02±0.04</td>
<td>0.91±0.04</td>
<td>-11.67</td>
</tr>
<tr>
<td>LVPW;s (mm)</td>
<td>1.14±0.03</td>
<td>0.88±0.02</td>
<td>-22.71</td>
<td>1.02±0.07</td>
<td>0.93±0.04</td>
<td>-8.43</td>
</tr>
<tr>
<td>LV Vol:d (µl)</td>
<td>98.5±7.85</td>
<td>122.2±10.78</td>
<td>24.06</td>
<td>88.4±14.39</td>
<td>86.5±4.96*</td>
<td>-2.15</td>
</tr>
<tr>
<td>LV Vol;s (µl)</td>
<td>40.24±3.9</td>
<td>71.42±8.81</td>
<td>77.49</td>
<td>36.42±7.01</td>
<td>34.72±5.92*</td>
<td>-4.69</td>
</tr>
<tr>
<td>%EF</td>
<td>58.92±2.92</td>
<td>42.37±3.24</td>
<td>-28.09</td>
<td>54.26±3</td>
<td>60.87±5.51*</td>
<td>12.18</td>
</tr>
<tr>
<td>%FS</td>
<td>31.39±2.08</td>
<td>21.08±1.87</td>
<td>-32.84</td>
<td>28.89±2.08</td>
<td>33.07±4.2*</td>
<td>14.46</td>
</tr>
<tr>
<td>AoV ET (msec)</td>
<td>46.2±2.35</td>
<td>43.25±0.67</td>
<td>-6.49</td>
<td>41.97±1.71</td>
<td>39.38±1.68</td>
<td>-6.17</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>363±34.33</td>
<td>252.75±9.5</td>
<td>-30.37</td>
<td>364.4±32.01</td>
<td>260.5±4.32</td>
<td>-28.52</td>
</tr>
</tbody>
</table>

Two groups of five male CSQ mice at 8 weeks of age were initiated on once daily IP injections of vehicle or 30 mg/kg/day gallein for one month, and were followed by serial conscious echocardiography. Abbreviations: LVID;d, left ventricular internal dimension at diastole; LVID;s, left ventricular internal dimension at systole; IVS;d, intraventricular septal thickness at diastole; LVPW;d, left ventricular posterior wall thickness at diastole; IVS;s, intraventricular septal thickness at systole; LVPW;s, left ventricular posterior wall thickness at systole; LV Vol;d, left ventricular volume at diastole; LV Vol;s, left ventricular volume at systole; %EF, Ejection fraction (calculated); %FS, % fractional shortening; AoV ET, aortic valve ejection time; HR, heart rate; mVcf, mean velocity of circumferential shortening. *P<.05 4wk Vehicle vs. Gallein. No significant differences between groups at baseline.
## Online Table III. Echocardiographic data in wild-type DBA mice at baseline and following four weeks daily vehicle or Gallein.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Vehicle</th>
<th>Galilein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>4 Weeks</td>
</tr>
<tr>
<td>LVID(d) (mm)</td>
<td>2.85±0.11</td>
<td>2.89±0.11</td>
</tr>
<tr>
<td>IVS(d) (mm)</td>
<td>1.37±0.06</td>
<td>1.49±0.12</td>
</tr>
<tr>
<td>LVPW(d) (mm)</td>
<td>0.96±0.03</td>
<td>0.89±0.02</td>
</tr>
<tr>
<td>IVS(s) (mm)</td>
<td>1.13±0.05</td>
<td>1.16±0.07</td>
</tr>
<tr>
<td>LVPW(s) (mm)</td>
<td>1.34±0.06</td>
<td>1.29±0.04</td>
</tr>
<tr>
<td>LV Vol(d) (µl)</td>
<td>31.19±3.12</td>
<td>32.34±2.89</td>
</tr>
<tr>
<td>LV Vol(s) (µl)</td>
<td>4.87±0.57</td>
<td>6.36±1.33</td>
</tr>
<tr>
<td>%EF</td>
<td>84.44±1.02</td>
<td>80.96±2.66</td>
</tr>
<tr>
<td>%FS</td>
<td>51.93±1.16</td>
<td>48.57±2.73</td>
</tr>
<tr>
<td>AoV ET (msec)</td>
<td>42.33±1.01</td>
<td>39.33±1.28</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>537.8±9.03</td>
<td>564.91±22.5</td>
</tr>
</tbody>
</table>

Two groups of five male wild-type DBA mice at 8 weeks of age were initiated on once daily IP injections of vehicle or 30 mg/kg/d gallein for 1 month, and were followed by serial conscious echocardiography. Abbreviations: LVID\(d\), left ventricular internal dimension at diastole; LVID\(s\), left ventricular internal dimension at systole; IVS\(d\), intraventricular septal thickness at diastole; LVPW\(d\), left ventricular posterior wall thickness at diastole; IVS\(s\), intraventricular septal thickness at systole; LVPW\(s\), left ventricular posterior wall thickness at systole; LV Vol\(d\), left ventricular volume at diastole; LV Vol\(s\), left ventricular volume at systole; %EF, Ejection fraction (calculated); %FS, percent fractional shortening; AoV ET, aortic valve ejection time; HR, heart rate; mVcf, mean velocity of fractional shortening. *P<.05, No significant difference in any measure between groups at
Online Figure I. **Structures of M119 and Gallein.** Gallein and M119 are highly related compounds. Importantly, compounds that duplicate either M119 or gallein but lack the hydroxyl groups at positions 4 and 5 of the tricyclic ring structure are completely inactive (Lehmann et al, Mol Pharm, 2008).
Online Figure II. **Acute gallein treatment can modulate β-AR signaling.** A,B) Gallein interferes with β-AR induced GRK2 membrane recruitment. Representative western blot analysis of GRK2 protein level in membrane fractions of cardiomyocytes treated as indicated. Positive control lane of proteins from HEK293 cells transfected with GRK2 (B). Densitometric analysis of GRK2 membrane recruitment from four independent experiments demonstrates reduction in Iso-induced GRK2 membrane recruitment.
Online Figure III. M119 does not affect Gβγ-mediated activation of Kir3.1/Kir3.4 channels. Xenopus oocytes expressing the subunits underlying the cardiac K(+) channel, Kir3.1 and Kir3.4, were expressed in oocytes together with the muscarinic type 2 receptor. (A) Typical Kir3.1/Kir3.4 current measured before and after 10 μM ACh application. The K(+) channel blocker Ba2+ was applied to determine the zero current level. (B) Effect of M119 on Kir3.1/Kir3.4 currents. Typical Kir3.1/Kir3.4 current before and after ACh application for oocytes treated for 1 hour with M119. (C) Summary data. M119 showed no effect on both the basal and ACh activated Kir3.1/Kir3.4 current.
Online Figure IV. **Effect of Gallein treatment on cardiac β-AR expression.** Radioligand β-AR binding assays determined β-AR density on cardiac membranes isolated from non-transgenic littermate control mice, or from CSQ mice treated with vehicle or gallein once daily (30 mg/kg/day) for one month. *P<.05 vs. wt
Online Figure V. Gallein treatment of wild-type mice mildly elevates cardiac contractility with no effect on cardiac morphometry. Two groups of five male wild type DBA mice at 8 weeks of age were initiated on once daily injections of vehicle or 30 mg/kg/day gallein for one month, and were followed by serial conscious echocardiography. A) Quantitation of fractional shortening data. B) Heart weight to body weight ratio at 4 weeks. *P<0.05 vs. vehicle.
References


