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(54) Title: TREATMENT OF PATIENTS WITH HYPONATREMIA AND CARDIAC DISEASE

(57) Abstract: Compositions are directed to biased ligands which modulate V_{1A}R-G protein signaling and simultaneously activating GRK2/beta-arrestin-dependent signaling. Other compositions comprise agents which modulate expression or activity of an arginine vasopressin receptor (AVP-R) molecule. The biased ligands and agents may be contained in pharmaceutical compositions for administration to patients, for example, patients with heart failure and/or elevated levels of arginine vasopressin. We describe methods of treatment which may include administration of the compositions, alone or in combination with other treatments. Methods of identifying candidate therapeutic agents are also provided.

WO 2014/153044 A1

TREATMENT OF PATIENTS WITH HYPONATREMIA AND CARDIAC DISEASE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of the filing dates of U.S. Provisional Application No. 61/783,571, which was filed on March 14, 2013 and U.S. Provisional Application No. 61/836,351, which was filed on June 18, 2013. For the purpose of any U.S. application that may claim the benefit of U.S. Provisional Application No. 61/783,571 and U.S. Provisional Application No. 61/836,351, the contents of these earlier filed applications are hereby incorporated by reference in their entirety.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

[0002] This invention was made with U.S. government support under grant numbers HL105414 and HL091799 awarded by the National Institutes of Health. The U.S. government may have certain rights in the invention.

FIELD OF THE INVENTION

[0003] Embodiments of the invention are directed to compositions for the treatment of cardiac diseases or disorders, such as heart failure, with or without hyponatremia, and methods of treatment. Assays for the identification of novel therapeutic agents are also provided.

BACKGROUND

[0004] Heart failure encompasses conditions in which the heart is unable to pump suffcient blood and oxygen to meet the body's needs. The basis for heart failure can vary, but the consequences of heart failure are extremely serious. In the United States alone heart failure contributes to nearly 300,000 deaths per year, and according the Center for Disease Control and Prevention, about half the people who develop heart failure die within five years of diagnosis. The societal costs of heart failure are also substantial. The costs of health care services,

medications, and missed days of work are estimated at about $32 billion annually. There is a continuing need for new therapeutic strategies for heart failure treatment.

[0005] Circulating levels of arginine vasopressin (AVP), which are elevated during hypovolemia and during cardiac stress, mediate important physiological functions such as osmotic regulation, vasoconstriction, and release of adrenocorticotropic hormone (ACTH). These physiological effects are mediated through the interaction of AVP to specific membrane G protein-coupled receptors (GPCR) in different target tissues: $V_{1A}R$ (Gαq-coupled; heart and vasculature), V_2R (Gαs-coupled; renal collecting tubule) and $V_{1B}R$ (Gαq-coupled; anterior pituitary), also termed V_3R. While administration of AVP to neonatal mouse cardiomyocytes has been reported to increase cell hypertrophy. AVP has variable effects on the heart during injury associated with heart failure, heart muscle disease, ischemia and reperfusion. Recently, it was found that both constitutive and controlled cardiac-specific overexpression of $V_{1A}R$ induced the development of left ventricular hypertrophy, dilatation, diminished contractile performance and reprogramming of the heart failure (HF) gene program in transgenic mice, effects that were found to be mediated via Gαq protein-dependent signaling (Li X, *et al.* (2011) *Circulation* 124(5):572-581).

SUMMARY

[0006] Embodiments of the invention are directed to methods for identifying agents for treatment of cardiac diseases or disorders. In particular, these agents comprise biased ligands; GPCR antagonists that activate unique G protein-independent pathways. Conditions that are treated include, for example, heart failure, hyponatremia and the like.

[0007] Levels of arginine vasopressin are elevated in patients with heart failure and there is a direct relationship between elevated levels and mortality. Over-expression of the cardiac vasopressin receptor ($V_{1A}R$) resulted in the development of heart failure through G protein-dependent signaling. However, activation of the $V_{1A}R$ in cloned heart-like cells (H9c2 cells) also promotes cardioprotective effects through a G protein-coupled receptor kinase 2 (GRK2)- and beta-arrestin-dependent pathway, completely independent of $V_{1A}R$-G protein signaling.

[0008] In embodiments, a biased ligand inhibits $V_{1A}R$-G protein signaling while simultaneously activating GRK2/beta-arrestin-dependent signaling. The biased ligand further comprises a pharmaceutical composition for administration to patients, for example, patients with heart failure and/or elevated levels of arginine vasopressin or copeptin. In other

embodiments, the biased ligand for the $V_{1A}R$ could be combined with a V2 (kidney) arginine vasopressin receptor antagonist. The antagonist could be administered concurrently with the one or more biased ligands or consecutively in one or more doses.

[0009] In other embodiments, a biased ligand inhibits the expression and/or function of arginine vasopressin receptor (AVP-R) or fragments thereof. In embodiments, an arginine vasopressin receptor (AVP-R) or fragments thereof comprises: peptides, polypeptides, oligonucleotides, polynucleotides, or combinations thereof.

[0010] In other embodiments, a pharmaceutical agent inhibits V1A expression or function in patients with cardiac disease or disorders and/or patients having elevated having elevated levels of arginine vasopressin or copeptin as compared to normal baseline controls.

[0011] In other embodiments, the biased ligand inhibits $V_{1A}R$ expression or function.

[0012] In other embodiments, the biased ligand inhibits $V_{1A}R$ and V2 expression or function. In embodiments, the biased ligand inhibits $V_{1A}R$ expression or function to a greater degree than V2 expression or function.

[0013] Other aspects are described *infra*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] Figures 1A -1E show that AVP protects H9c2 cells against hypoxia/reoxygenation-induced cell death via $V_{1A}R$- and ERK1/2-dependent signaling. Figure 1A: Cell survival in response to serum starvation under conditions of H/R or normoxia, *P<0.05 vs starvation + normoxia, #P<0.05 vs FBS in same group, one-way ANOVA. Figure 1B: Caspase 3/7 activity in response to serum starvation under conditions of H/R or normoxia, **P<0.01 vs starvation + normoxia, ##P<0.01 vs FBS in same group, one-way ANOVA. Figure 1C: AVP concentration-dependently increased cell survival and decreased caspase 3/7 activity under conditions of H/R, *P<0.05 for cell survival, **P<0.01 for caspase 3/7 activity, repeated two-way ANOVA. Figures 1D & 1E: Pretreatment of cells with SR49059 (0.1 μM) or PD98059 (10 μM) for 30 min blunted the cardioprotection of AVP, evidenced by the enhanced caspase 3/7 activity (Figure 1D) and reduced cell survival (Figure 1E), *P<0.05 vs control, one-way ANOVA. All data are presented as the mean ± SEM from 3 independent experiments.

[0015] Figures 2A-2C show that $V_{1A}R$, not $V_{1B}R$ or V2R, mediates ERK1/2 phosphorylation in H9c2 cells. Figure 2A: Cells were stimulated with increasing concentrations of AVP for 5 minutes and the P-ERK1/2 response determined via immunoblotting. Figure 2B: Inhibitory concentration responses for $V_{1A}R$-, $V_{1B}R$- or V2R-selective antagonists (30 min pretreatment at 1nM-10μM) against AVP (10nM)-induced P-ERK1/2. Figure 2C: MEK1/2 inhibition with PD98059 (10 μM) blunted the AVP-dependent phosphorylation of ERK1/2, **P<0.01 vs control at the same timepoints, unpaired t-test. Left panels: Representative immunoblots; right panels: The averaged data (mean ± SEM) of 3-4 independent experiments.

[0016] Figures 3A-3D Inhibition of Gαq protein/PKC signaling blunts the AVP-induced acute P-ERK1/2 response but has no effect on the AVP-induced cell survival response. (Figure 3A) Overexpression of GqI-GFP (vs GFP alone) with adenovirus or inhibition of PKC with Ro-31 (1 μM) significantly blunted the acute (2-10 min), but not prolonged (30-60 min) phosphorylation of ERK1/2 evoked by AVP (10 nM) in H9c2 cells. (Figure 3B) The averaged data of (Figure 3A), *P<0.05, **P<0.01 GqI vs GFP at the same timepoints, and #P<0.05, ##P<0.01 Ro-31 vs vehicle (0.1% DMSO) at the same timepoints, unpaired t-test. (Figure 3C) Overexpression of GqI failed to block the AVP-mediated decrease in caspase 3/7 activity and increase in cell survival, **p<0.01 vs appropriate non-stimulated control, one-way ANOVA. (Figure 3D) Pretreatment of H9c2 cells with Ro-31 (1 μM) did not impact the ability of AVP to protect cells from H/R, as AVP concentration-dependently decreased caspase 3/7 activity and increased cell survival. *P<0.05 for cell survival, **P<0.01 for caspase 3/7 activity, repeated two-way ANOVA. All data are presented as the mean ± SEM from at least 3 independent experiments.

[0017] Figures 4A-4D show that the overexpression of βARK_{CT} blunts persistent AVP-induced P-ERK1/2 and abrogates AVP-mediated H9c2 cell survival. Figures 4A, 4B: Overexpression of βARK_{CT} significantly blunted the ERK1/2 phosphorylation response (from 10-60 min) following AVP stimulation, whereas treatment with the Gβγ protein inhibitor gallein (10 μM) had no effect on AVP-mediated ERK1/2 phosphorylation. *P<0.05, **P<0.01 vs the same timepoint, unpaired t-test. Figures 4C, 4D: Overexpression of βARK_{CT} significantly blocked AVP-dependent decrease in caspase 3/7 activity (Figure 4C) and increase in cell survival (Figure 4D), *P<0.05 vs no AVP addition in appropriate control (β-gal or βARK_{CT}), one-way ANOVA. All data are presented as the mean ± SEM from 3 independent experiments.

[0018] Figures 5A-5G show that siRNA-mediated deletion of GRK2 and β-arrestin1, but not GRK5, blunts persistent AVP-induced P-ERK1/2 and cell survival responses in H9c2 cells. Representative immunoblots showing GRK2 (Figure 5A), GRK5 (Figure 5B) and β-arrestin1 (Figure 5C) protein expression after siRNA transfection and the corresponding ERK1/2 phosphorylation responses to AVP stimulation (10nM, 0-60 min) in H9c2 cells. Figure 5D: The averaged data of GRK2, GRK5, or β-arrestin1 knockdown, compared to scrambled siRNA, **P<0.01 vs scrambled siRNA control, one-way ANOVA. Figure 5E: Summary of the effects of siRNA-mediated deletion of GRK2, GRK5 and βarrestin1 on the P-ERK1/2 response to AVP (10nM, 0-60 min), **P<0.01 (GRK2 siRNA), ##P<0.01 (βarrestin1 siRNA) vs scrambled siRNA at the same timepoint, unpaired t-test. The effects of siRNA-mediated knockdown of GRK2, GRK5 or β-arrestin1 on AVP-mediated caspase 3/7 activity (Figure 5F) and cell survival (Figure 5G) during H/R in H9c2 cells are shown. *P<0.05 between scrambled siRNA vs GRK2 siRNA (cell survival, caspase 3/7 activity), #P<0.05 between scrambled siRNA vs β-arrestin1 siRNA (cell survival) and ##P<0.01 between scrambled siRNA vs β-arrestin1 siRNA (caspase 3/7 activity), repeated two-way ANOVA. All data are presented as mean ± SEM of 3 independent experiments.

[0019] Figures 6A, 6B show that $V_{1B}R$ and V_2R antagonists do not prevent AVP-mediated effects on caspase 3/7 activity and cell survival. H9c2 cells underwent H/R in the presence or absence of $V_{1B}R$- (SSR14941, 0.1μM) or V_2R- (OPC41061, 0.1μM) selective antagonists and AVP (10nM) and caspase 3/7 activity (Figure 6A) or cell survival (Figure 6B) were assessed. *P<0.05 vs control, one-way ANOVA. The data are presented as the mean ± SEM from 3 independent experiments.

[0020] Figure 7 shows that pretreatment of H9c2 cells with Ro-31 prior to H/R leads to increased caspase 3/7 activity and decreased cell survival. H9c2 cells were pretreated with Ro-31 (1μM) and then underwent H/R, *P<0.05 vs control, one-way ANOVA. The data are presented as mean ± SEM of at least 3 independent experiments.

[0021] Figures 8A-8D shows that PKC inhibition does not impact the effects of βARK_{CT} overexpression on AVP-mediated P-ERK1/2 and protection. Figures 8A, 8B: The PKC inhibitor Ro-31 alone blunted the acute (5 min), but not the persistent (30 min) P-ERK1/2 responses to AVP (10 nM), while the combination of Ro-31 and βARK_{CT} overexpression did not further alter the acute or persistent P-ERK1/2 responses to each agent alone, *P<0.05, **P<0.01 vs βgal

alone at same timepoint, ##P<0.01 vs βARK_{CT} at same timepoint, one-way ANOVA. Figures 8C, 8D: Pretreatment of H9c2 cells with Ro-31 (1 μM) prior to H/R did not impact the effect of βARK_{CT} overexpression on AVP-protection in decreasing caspase 3/7 activity and in increasing in cell survival, *P<0.05 vs no AVP addition in corresponding β-gal + Ro-31 or βARK_{CT} + Ro-31 groups, one-way ANOVA. The data are presented as mean ± SEM of 3 independent experiments.

[0022] Figures 9A-9D shows that inhibition of Gβγ with gallein fails to suppress AVP-induced P-ERK1/2 and cell survival in H9c2 cells. Figures 9A, 9B: Gallein does not inhibit AVP-induced P-ERK1/2 at any concentration tested. Figures 9C, 9D: Pretreatment of cells with increasing concentrations of gallein (0-10 μM) did not suppress the AVP (10nM)-induced caspase 3/7 activity and cell survival responses, *P<0.05 vs no AVP, one-way ANOVA. All data are presented as mean ± SEM of 3 independent experiments.

[0023] Figures 10A, 10B show that H/R in the presence or absence of AVP did not impact siRNA-mediated silencing of GRK2. Figure 10A: A representative immunoblot showing the expression of GRK2 in H9c2 cells 96 h after transfection of GRK2 siRNA and the H/R protocol in the presence or absence of AVP at 0.1-1000 nM. Figure 10B: The averaged data are expressed as mean ± SEM of 3 independent experiments. **P<0.01 vs. scrambled siRNA control at the same concentration of AVP, one-way ANOVA.

[0024] Figure 11 shows that β-arrestin1 is the dominate β-arrestin expressed in H9c2 cells. Both H9c2 and HEK293 cell lysates (15 μg protein/lane) were employed to determine the relative protein expression of β-arrestins 1 and 2, with GAPDH as an internal control.

[0025] Figures 12A-12C show that $V_{1A}R$ expression is increased in human heart failure. Figure 12A: Realtime PCR reveals a significant increase in *AVPR1A* expression in failing human hearts versus non-failing human hearts. *AVPR1A* expression is normalized to 18S and data are presented as RQ with RQmin and RQmax as error bars, 2-tailed t-test. Saturation radioligand binding analysis with [^{125}I]-p-AVP indicates a significant increase in V1AR plasma membrane expression in failing hearts over non-failing hearts (B_{max}, Figure 12B), while $V_{1A}R$ affinity for ligand was not different between failing and non-failing hearts (K_d, Figure 12C), 2-tailed t-test.

[0026] Figures 13A, 13B show that AVP reduces βAR ligand affinity and Ca^{2+} mobilization in adult mouse cardiomyocytes. Figure 13A: Competition radioligand binding of ^{125}I-CYP in crude adult mouse cardiomyocyte membrane preparations with increasing concentrations of ISO alone or in the presence of AVP (0.5 μM). While AVP alone did not displace ^{125}I-CYP from βAR, AVP reduced the affinity of βAR for ISO. Figure 13B: ISO (50nM)-mediated Ca2+ mobilization was significantly reduced by pretreatment of AVP (10nM) as detected in Fura-2-loaded adult mouse cardiomyocytes; mean ±sem, ***P<0.001, One-way ANOVA.

[0027] Figures 14A-14D show that AVP impairs βAR-dependent cAMP generation. Figure 14A: cAMP generation in response to ligand stimulation was assessed in HEK 293 cells stably expressing β1AR and transiently transfected with V_{1A}R and the cAMP FRET biosensor ICUE3. ISO (1nM) induced a rapid and sustained FRET response, while AVP alone (1 μM) had no effect. Figure 14B: ISO concentration-FRET response curves were generated in the absence or presence of 1 μM AVP, revealing a log unit reduction in the potency of ISO for producing cAMP in the presence of AVP. Figure 14C: At the EC_{50} of ISO (100pM) for cAMP production, AVP (1 μM) is shown to significantly reduce the ISO-mediated response by ~50%, which can be fully reduced with the addition of the β-blocker propranolol; **P<0.001, One-way ANOVA. Figure 14D: Adult feline cardiomyocytes expressing endogenous levels of βAR and V_{1A}R and infected with adenovirus encoding ICUE3 were pretreated with rolipram 5 min prior to initiation of FRET recording. After 30 second baseline, the cells were treated with vehicle or 1 μM AVP (1) for 1 min, then with 1 μM ISO (2). ISO produced a biphasic increase in cAMP production in the endogenous adult cardiomyocyte system that was largely abolished by AVP pretreatment.

[0028] Figures 15A-15C show that AVP/V_{1A}R signaling decreases the impact of ISO perfusion on cardiac function in the whole heart. *Ex vivo* perfused hearts from V_{1A}R-TG mice and their wild type littermates (WT) were treated with increasing concentrations of ISO alone or in the presence of 1 nM AVP (WT + AVP (10^{-9})) and their contractile parameters measured. ISO-mediated LVDP (Figure 15A) and +dP/dt (Figure 15B) responses were each reduced in either the V_{1A}R -TG or W± AVP (10^{-9}) hearts. Figure 15C: Treatment of WT hearts with increasing concentrations of of AVP did not alter the impact of forskolin (Fsk) on contractility. Data is mean±sem, *P<0.05, **P<0.01, ***P<0.001 (V_{1A}R-TG vs. WT), †P<0.05, ††P<0.01 (WT + AVP (10^{-9}) vs. WT), two-way repeated ANOVA.

[0029] Figures 16A-16D show that $V_{1A}R$ antagonist SR 49059 protects against TAC-induced cardiac dysfunction. In response to TAC, fractional shortening (A) and ejection fraction (B) were significantly decreased in vehicle-treated mice (TAC+V) compared to vehicle-treated sham surgery mice (Sham+V). Treatment of the TAC mice with SR 49059 (1mg/kg/day, starting 1 week post-TAC) prevented these changes. †p<0.05, ††p<0.01, †††p<0.001 versus Sham+V, *p<0.05, **p<0.01 versus TAC+SR at corresponding weeks, two-way ANOVA with Bonferonni multiple comparisons test. N = 9 (Sham+V), 8 (Sham+SR), 13 (TAC+V), 14 (TAC+SR). Radioligand binding analysis of left ventricular tissue of the Sham or TAC ± SR 49059 mice indicate that TAC increased $V_{1A}R$ membrane density (C, N = 7 (Sham+V), 4 (Sham+SR), 7 (TAC+V), 5 (TAC+SR)) and decreased βAR membrane density (D, N = 4 (Sham+V), 4 (Sham+SR), 6 (TAC+V), 6 (TAC+SR)), effects that were reversed by treatment with SR 49059. *p<0.05, **p<0.01, one-way ANOVA with Bonferonni multiple comparisons test.

[0030] Figure 17 show heart rate to body weight ratios of TAC mice ± SR 49059. ***p<0.01, one-way ANOVA with Bonferonni multiple comparisons test.

[0031] Figures 18A-18F show that AVP negatively impacts mouse cardiac βAR ligand binding and Ca2+ transients in adult mouse cardiomyocytes. A) Competition radioligand binding on membrane preparations from left ventricular tissue of adult mice revealed that although increasing concentrations of AVP (green) do not alter βAR ligand binding, the presence of AVP (0.5 μM) decreased the affinity of isoproterenol (ISO) for βAR (red) compared to ISO alone (blue). N = 4 per ligand concentration. Representative tracings from field stimulation-induced Ca2+ transients in Fura2-loaded adult mouse left ventricular myocytes in response to non-stimulated control (B), AVP (10 nM, C), ISO (50 nM, D) or AVP+ISO (E). F) Summary of the changes in Ca2+ transients indicateds a significant decrease in ISO-mediated transients in the presence of AVP. **p<0.01, ***p<0.001, one-way ANOVA with Bonferonni multiple comparisons test. N = 16 (NS), 8 (AVP, ISO, AVP+ISO).

[0032] Figure 19 shows a summary of the changes in Ca2+ transients in adult mouse cardiomyocytes in response to increasing concentrations of AVP. N = 5 (0-100 nM AVP, 3 (1000 nM AVP).

[0033] Figures 20A-20F show that AVP reduces ISO-mediated cAMP generation in adult feline ventricular myocytes. cAMP generation responses were monitored in AFVM infected with the fluorescent biosensor ICUE3 in response to vehicle (Veh) + ISO (100 nM, A), rolipram (Rol, 1 μM) + Veh + ISO (B), Rol + AVP (1 μM) + ISO (C), Veh + forskolin (Frk 1 μM, D) or AVP + Frk (E). F) Summarized area under the curve (AUC) data relative to the Rol+ Veh+ ISO condition showed that AVP pretreatment significantly reduces ISO-mediated cAMP generation, while not affecting Frk-mediated cAMP generation. ***$p<0.001$, one-way ANOVA with Bonferonni multiple comparisons test. N = 20 (ISO), 31 (Rol+ISO), 58 (Rol+AVP+ISO), 13 (Frk), 11 (AVP+Frk).

[0034] Figures 21A-21K show that AVP effects on βAR-mediated cAMP generation are Gq protein-independent. cAMP and DAG generation responses were monitored in HEK 293 cells stably expressing β1AR and transiently transfected with $V_{1A}R$ and either ICUE3 or DAGR. ISO (100 pM, A), but not AVP (1 μM, B), increases cAMP generation. AVP pretreatment reduces ISO-mediated cAMP production (C) in a competitive manner (D). E) AVP (1 μM) induced a rapid DAG generation response that was blocked by SR 49059 (10 μM). Pretreatment of the cells with SR 49059 did not block ISO-induced cAMP generation (F) but did prevent the AVP-dependent reduction in the ISO response (G). H) The rapid DAG response to AVP stimulation was blocked by UBO-QIC (10 nM). Pretreatment of the cells with UBO-QIC neither blocked ISO-induced cAMP generation (I) nor the AVP-dependent reduction in the ISO response (J). K) Summarized area under the curve (AUC) results relative to ISO treatment alone, ***$p<0.001$, one-way ANOVA with Bonferonni multiple comparisons test. N = 34 (ISO), 40 (AVP+ISO), 18 (SR+AVP+ISO), 18 (QIC+AVP+ISO).

[0035] Figures 22A-22F show that GRK phosphorylation-deficient $V_{1A}R$ retains Gq protein-coupling and augments βAR responsiveness. A) Schematic of the GRK- $V_{1A}R$, where red circles indicate serine/threonine sites mutated to alanine, corresponding to those in Table 1. HEK 293 cells stably expressing β1AR were transiently transfected with DAGR or ICUE3 with either WT $V_{1A}R$ or GRK- $V_{1A}R$. Both WT $V_{1A}R$ (B) and GRK- $V_{1A}R$ (C) induced DAG formation responses to AVP (1 μM). D) DAG formation area under the curve (AUC) responses relative to WT $V_{1A}R$ indicated stimulation of GRK- $V_{1A}R$ leds to more DAG accumulation. *$p<0.05$, two-tailed t-test. N = 10 each. E) Stimulation of GRK- $V_{1A}R$ with AVP (1 μM) leds to enhanced

ISO-mediated cAMP formation, an effect blocked by the Gq protein inhibitor UBO-QIC. F) Summarized area under the curve (AUC) results relative to ISO treatment of GRK- $V_{1A}R$, ***p<0.001, one-way ANOVA with Bonferonni multiple comparisons test. N = 48 (ISO), 50 (AVP+ISO), 50 (UBO-QIC+AVP+ISOS).

[0036] Figure 23 shows radioligand binding of transiently transfected WT $V_{1A}R$ versus GRK- $V_{1A}R$ in HEK 293 cells shows similar levels of expression (Bmax, A) and ligand affinity (Kd, B). N = 3 each.

[0037] Figures 24A-24F show that $V_{1A}R$ overexpression blocks basal cardiac contractility ex vivo, but $V_{1A}R$ activation does not impact adenylyl cyclase activity. Cardiac contractile parameters were measured in ex vivo Langendorff preparations from wild-type (WT) mice or mice with cardiac-restricted $V_{1A}R$ expression ($V_{1A}R$ -TG). Infusion of increasing concentrations of the non-selective phosphodiesterase inhibitor IBMX led to an increase in contractility in WT hearts as measured by LVDP (A), +dP/dt (B) and –dP/dt (C), expressed as % of baseline, effects that were absent in $V_{1A}R$ -TG hearts. *P<0.05, **P<0.01, ***p<0.001 versus WT hearts at corresponding concentration, two-way ANOVA with Bonferonni multiple comparisons test. N = 5 hearts each. WT hearts received increasing concentrations of the adenylyl cyclase activator forskolin (Frk), which enhanced LVDP (D), +dP/dt (E) and –dP/dt (F) in either the presence or absence of AVP (1 nM) pretreatment. **P<0.01 versus Veh-treated hearts at corresponding concentration, two-way ANOVA with Bonferonni multiple comparisons test. N = 5 hearts each.

[0038] Figure 25 shows that radioligand binding of WT versus $V_{1A}R$ -TG mouse heart membrane preparations shows a five-fold increase in $V_{1A}R$ expression in the $V_{1A}R$ -TG hearts over WT levels (A) with similar ligand affinities in each heart genotype (B). ***p<0.001, two-tailed t-test. N = 5 each.

[0039] Figures 26A-26C show that either $V_{1A}R$ overexpression or stimulation can block ISO-mediated cardiac contractility ex vivo, even in the presence of genetic Gq protein inhibition. Cardiac contractile parameters were measured in ex vivo Langendorff preparations from WT or $V_{1A}R$ -TG mice. Infusion of increasing concentrations of ISO led to an increase in contractility in WT hearts as measured by LVDP (A), +dP/dt (B) and –dP/dt (C), expressed as % of baseline. These effects were blocked in $V_{1A}R$ -TG hearts, in WT hearts pretreated with AVP (1 nM) and in

GqI-expressing hearts (genetic inhibitor of Gq protein) pretreated with AVP. *P<0.05, **P<0.01, ***p<0.001 WT+AVP versus WT+Veh hearts, †p<0.05, ††p<0.01, †††p<0.001 $V_{1A}R$ -TG versus WT+Veh hearts, and ‡‡p<0.01, ‡‡‡p<0.001 GqI-TG+AVP versus WT+Veh hearts at corresponding concentration, two-way ANOVA with Bonferonni multiple comparisons test. N = 5 (WT+Veh), 5 (WT+AVP), 8 ($V_{1A}R$ -TG), 7 (GqI-TG+AVP) hearts each.

[0040] Figure 27 shows that cardiac contractile parameters were measured in ex vivo Langendorff preparations from WT mice. Infusion of increasing concentrations of AVP led to a small decrease in contractility in WT hearts as measured by LVDP (A), +dP/dt (B) and –dP/dt (C), expressed as % of baseline. N = 3 hearts.

DETAILED DESCRIPTION

[0041] In the heart, Gαq protein-coupled receptors induce the activation of protein kinase C (PKC) leading to cellular hypertrophy and activation of the heart failure (HF) gene program. Gαq protein-coupled receptors, including $V_{1A}R$, also undergo agonist-induced phosphorylation by PKC and G-protein coupled receptor kinase isoforms (GRK2 and GRK5) leading to internalization and desensitization of the $V_{1A}R$. The GRKs can also alter cellular physiology independent of PKC through phosphorylation-dependent recruitment of β-arrestins and subsequent activation of downstream pathways including extracellular-regulated kinase 1/2 (ERK1/2) signaling, which can act to inhibit the ubiquitous responses to cell stress including apoptosis. Indeed, in the heart, G protein-independent signaling promotes cardioprotection downstream of angiotensin II- and β-adrenergic receptor activation during conditions of myocardial stress. These findings led to the development of biased ligands; GPCR antagonists that activate unique G protein-independent pathways. However, the role of GRK/β-arrestin-mediated $V_{1A}R$ signaling in promoting cell survival was heretofore unknown.

[0042] The present invention is based in part on our discovery that $V_{1A}R$ signalling inhibits β-adrenergic receptor activity through a GRK-dependent, Gq protein independent signalling pathway. We have found that the neurohormone arginine vasopressin (AVP), which is elevated in patients with worsening symptoms of heart failure, decreased the ability of norepinphine to augment cardiac contractility. Patients admitted to the hospital with an exacerbation of heart failure have elevated circulating levels of both norepinephrine, which binds to both β1- and β2-

adrenergic receptors in the heart in order to increase cardiac contractility, and AVP. Our findings suggest that high levels of AVP can inhibit the ability of the adrenergic nervous system to support cardiac contractility and can explain why patients admitted with an exacerbation of heart failure do not respond to exogenous adrenergic agonists such as dobutamine or dopamine. Our data indicate that AVP plays a dual role in exacerbation of heart failure. AVP not only inhibits cardiac contractility through activation of Gq, but more importantly it inhibits the ability of norepinephrine to support the failing heart. Finally, our data show that the negative effect of AVP can be mitigated by blocking the effects of AVP with a highly selective AVP antagonist.

[0043] Accordingly, the methods of the invention feature methods of administering compositions comprising agents that modulate the $V_{1A}R$, e.g., $V_{1A}R$ antagonists. The $V_{1A}R$ modulating agent can inhibit $V_{1A}R$-G protein signaling and simultaneously activate G-protein coupled receptor kinase (GRK)/beta-arrestin signaling. The $V_{1A}R$ modulating agent can be administered alone or along with an agent that targets the β-adrenergic receptor (βAR), e.g., a βAR agonist, or an agent that targets the vasopressin 2 receptor (V_2R). In some embodiments, the $V_{1A}R$ modulating agent can be administered along with an agent that targets the β-adrenergic receptor (βAR), e.g., a βAR agonist and an agent that targets the vasopressin 2 receptor (V_2R). The therapeutic methods described herein can be carried out in connection with other cardiovascular disease therapies (*e.g.*, lifestyle changes, drug, antibody or hormone-based therapy, implantable devices). The methods also include methods of screening for agents that modulate the $V_{1A}R$. The screening methods can include both cell-based and invitro assays and can be configured in a high-throughput format. The compositions of the invention can include agents that modulate the $V_{1A}R$, e.g., $V_{1A}R$ antagonists, and optionally, an agent that targets the β-adrenergic receptor (βAR), e.g., a βAR agonist, or an agent that targets the vasopressin 2 receptor (V_2R), The compositions can be formulated as pharmaceuticals according to their use.

[0044] As used herein, the term "modulating" refers to an increase or decrease in the level or activity of $V_{1A}R$ relative to the levels of $V_{1A}R$ in a biological sample that has not been exposed to the $V_{1A}R$ -modulator. Preferred modulators are inhibitors of one or more activities of $V_{1A}R$. A $V_{1A}R$ modulating agent can be a small molecule compound, an antisense reagent, an siRNA reagent, an antibody, an enzyme, a polypeptide, an organic or inorganic molecule, a natural or synthetic compound, or any combination of a small molecule compound, an antisense reagent, an

siRNA reagent, an antibody, an enzyme, a polypeptide, an organic or inorganic molecule, a natural or synthetic compound. An exemplary $V_{1A}R$ modulating agent is the $V_{1A}R$ antagonist SR49059. Exemplary V_2R modulating agents include conivaptan, tolvaptan. lixivaptan. An exemplary selective $V_{1B}R$ modulating agent is nelivaptan.

[0045] The $V_{1A}R$ modulating agents can be selective. By "selective" is meant that the compound binds to or inhibits the $V_{1A}R$ with greater affinity or potency, respectively, compared to at least one other arginine vasopressin receptor. In some embodiments, the $V_{1A}R$ modulating agents of the invention are selective inhibitors of $V_{1A}R$ over $V_{1B}R$ and/or V_2R. Selectivity can be at least about 5-fold, at least about 10-fold, at least about 20-fold, at least about 50-fold, at least about 100-fold, at least about 200-fold, at least about 500-fold or at least about 1000-fold. Selectivity can be measured by methods routine in the art. In some embodiments, selectivity can be tested at the K_m AVP concentration of each receptor. In some embodiments, selectivity of $V_{1A}R$ modulating agents can be determined at the physiological AVP concentration. In some embodiments, the selectivity of compounds of the invention can be determined by cellular assays associated with particular $V_{1A}R$ activity.

[0046] The NCBI reference amino acid sequence for the human $V_{1A}R$ polypeptide (GenBank accession number NP_000697.1 GI:4502331) is shown in Example 10. Other representative forms of $V_{1A}R$ can have an amino acid sequence that has 1, 2, 3, 4, 5, 10 or more amino acid changes compared to the amino acid sequence of GenBank Accession No. NP_000697.1 GI:4502331. Other amino acid sequences that have been identified for $V_{1A}R$ include for example, without limitation, GenBank accession number AAH74803.1GI:50959688 and GenBank accession number AAP84363.1 GI:32482021.

[0047] Arginine vasopressin is a nine amino acid peptide that is synthesized as a precursor protein that includes arginine vasopressin and two associated proteins, neurophysin 2 and a glycopeptide, copeptin. The NCBI reference amino acid sequence for the human precursor protein (GenBank accession number NP_000481.2 GI:13259533) is shown in Example 11. The segment corresponding to the mature arginine vasopressin spans animo acids 20-28 of the precursor protein, i.e., CYFQNCPRG (SEQ ID NO.: 25). The segment corresponding to the

mature copeptin spans animo acids 126-164 of the precursor protein, i.e., SDRS NATQLDGPAGALLLRLVQLAGAPEPFEPAQPDAY (SEQ ID NO.: 26).

[0048] Several aspects of the invention are described below with reference to example applications for illustration. It should be understood that numerous specific details, relationships, and methods are set forth to provide a full understanding of the invention. One having ordinary skill in the relevant art, however, will readily recognize that the invention can be practiced without one or more of the specific details or with other methods. The present invention is not limited by the illustrated ordering of acts or events, as some acts may occur in different orders and/or concurrently with other acts or events. Furthermore, not all illustrated acts or events are required to implement a methodology in accordance with the present invention.

[0049] Embodiments of the invention may be practiced without the theoretical aspects presented. Moreover, the theoretical aspects are presented with the understanding that Applicants do not seek to be bound by the theory presented.

[0050] All genes, gene names, and gene products disclosed herein are intended to correspond to homologs from any species for which the compositions and methods disclosed herein are applicable. Thus, the terms include, but are not limited to genes and gene products from humans and mice. It is understood that when a gene or gene product from a particular species is disclosed, this disclosure is intended to be exemplary only, and is not to be interpreted as a limitation unless the context in which it appears clearly indicates. Thus, for example, for the genes disclosed herein, which in some embodiments relate to mammalian nucleic acid and amino acid sequences are intended to encompass homologous and/or orthologous genes and gene products from other animals including, but not limited to other mammals, fish, amphibians, reptiles, and birds. In preferred embodiments, the genes or nucleic acid sequences are human.

Definitions

[0051] The terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention. As used herein, the singular forms "a", "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise. Furthermore, to the extent that the terms "including", "includes", "having",

"has", "with", or variants thereof are used in either the detailed description and/or the claims, such terms are intended to be inclusive in a manner similar to the term "comprising."

[0052] As used herein, the terms "comprising," "comprise" or "comprised," and variations thereof, in reference to defined or described elements of an item, composition, apparatus, method, process, system, etc. are meant to be inclusive or open ended, permitting additional elements, thereby indicating that the defined or described item, composition, apparatus, method, process, system, etc. includes those specified elements--or, as appropriate, equivalents thereof--and that other elements can be included and still fall within the scope/definition of the defined item, composition, apparatus, method, process, system, etc.

[0053] The term "about" or "approximately" means within an acceptable error range for the particular value as determined by one of ordinary skill in the art, which will depend in part on how the value is measured or determined, *i.e.*, the limitations of the measurement system. For example, "about" can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, "about" can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value. Where particular values are described in the application and claims, unless otherwise stated the term "about" meaning within an acceptable error range for the particular value should be assumed.

[0054] "Optional" or "optionally" means that the subsequently described circumstance may or may not occur, such that the description includes instances where the circumstance occurs and instances where it does not.

[0055] As used herein, the term "agent" is meant to encompass any molecule, chemical entity, composition, drug, therapeutic agent, chemotherapeutic agent, or biological agent capable of preventing, ameliorating, or treating a disease or other medical condition. The term includes small molecule compounds, antisense reagents, siRNA reagents, antibodies, enzymes, peptides organic or inorganic molecules, natural or synthetic compounds and the like. An agent can be assayed in accordance with the methods of the invention at any stage during clinical trials, during pre-trial testing, or following FDA-approval.

[0056] As defined herein, a "therapeutically effective" amount of a compound or agent (i.e., an effective dosage) means an amount sufficient to produce a therapeutically (e.g., clinically) desirable result. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors can influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of the compounds of the invention can include a single treatment or a series of treatments.

[0057] Unless otherwise indicated, the terms "peptide", "polypeptide" or "protein" are used interchangeably herein, although typically they refer to peptide sequences of varying sizes.

[0058] The term "variant," when used in the context of a polynucleotide sequence, may encompass a polynucleotide sequence related to a wild type gene. This definition may also include, for example, "allelic," "splice," "species," or "polymorphic" variants. A splice variant may have significant identity to a reference molecule, but will generally have a greater or lesser number of polynucleotides due to alternate splicing of exons during mRNA processing. The corresponding polypeptide may possess additional functional domains or an absence of domains. Species variants are polynucleotide sequences that vary from one species to another. Of particular utility in the invention are variants of wild type gene products. Variants may result from at least one mutation in the nucleic acid sequence and may result in altered mRNAs or in polypeptides whose structure or function may or may not be altered. Any given natural or recombinant gene may have none, one, or many allelic forms. Common mutational changes that give rise to variants are generally ascribed to natural deletions, additions, or substitutions of nucleotides. Each of these types of changes may occur alone, or in combination with the others, one or more times in a given sequence.

[0059] The terms "determining", "measuring", "evaluating", "detecting", "assessing" and "assaying" are used interchangeably herein to refer to any form of measurement, and include determining if an element is present or not. These terms include both quantitative and/or qualitative determinations. Assessing may be relative or absolute. "Assessing the presence of"

includes determining the amount of something present, as well as determining whether it is present or absent.

[0060] The term "assay" used herein, whether in the singular or plural shall not be misconstrued or limited as being directed to only one assay with specific steps but shall also include, without limitation any further steps, materials, various iterations, alternatives etc., that can also be used. Thus, if the term "assay" is used in the singular, it is merely for illustrative purposes.

[0061] A "label" or a "detectable label" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include radio labeled molecules fluorophores, luminescent compounds, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins which can be made detectable, e.g., by incorporating a label into the peptide or used to detect antibodies specifically reactive with the peptide.

[0062] The term "fluorophore" includes any compound, composition or molecule capable of emitting light in response to irradiation. In many instances, fluorophores emit light in the visible region of light. In other instances, the fluorophores can emit light in the non-visible regions of light, such as ultraviolet, near-ultraviolet, near-infrared, and infrared. For example and without limitation, examples of fluorophores include: quantum dots; nanoparticles; fluorescent proteins, such as green fluorescent protein and yellow fluorescent protein; heme-based proteins or derivatives thereof; carbocyanine-based chromophores, such as IRDye 800CW, Cy 3, and Cy 5; coumarin-based chromophores, such as (7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin) (CPM); fluorine-based chromophores, such as fluorescein, fluorescein isothiocyanate (FITC); and numerous ALEXA FLUOR^{TM} chromophores and ALEXA FLUOR^{TM} bioconjugates, which absorb in the visible and near-infrared spectra. The emission from the fluorophores can be detected by any number of methods, including but not limited to, fluorescence spectroscopy, fluorescence microscopy, fluorimeters, fluorescent plate readers, infrared scanner analysis, laser scanning confocal microscopy, automated confocal nanoscanning, laser spectrophotometers, fluorescent-activated cell sorters (FACS), image-based analyzers and fluorescent scanners (e.g., gel/membrane scanners).

[0063] As used herein, the term "chromophore" refers to a substituent which, with another chromophore, can be used for energy transfer (e.g., FRET assay).

[0064] The term "chemiluminescent compound" includes any compound, composition or molecule capable of emitting light in response to a chemical reaction. A "bioluminescent compound" refers to a naturally occurring form of a chemiluminescent compound. Examples of chemiluminescent compounds include: lucigenin, luminol. Examples of bioluminescent compounds include: luciferins, coelenterazines. The emission from chemiluminescent compounds can be detected by luminometers or scanning spectrometers.

[0065] The term "luminescent component" or "luminescent compound" as used herein refers to a component capable of absorbing energy, such as electrical (e.g., electro-luminescence), chemical (e.g., chemi-luminescence) or acoustic energy and then emitting at least some fraction of that energy as light over time. The term "component" as used herein includes discrete compounds, molecules, bioluminescent proteins and macro-molecular complexes or mixtures of luminescent and non-luminescent compounds or molecules that act to cause the emission of light.

[0066] The term "high-throughput screening" or "HTS" refers to a method drawing on different technologies and disciplines, for example, optics, chemistry, biology or image analysis to permit rapid, highly parallel biological research and drug discovery. HTS methods are known in the art and they are generally performed in multiwell plates with automated liquid handling and detection equipment; however it is envisioned that the methods of the invention may be practiced on a microarray or in a microfluidic system.

[0067] The term "library" or "drug library" as used herein refers to a plurality of chemical molecules (test compound), a plurality of nucleic acids, a plurality of peptides, or a plurality of proteins, organic or inorganic compounds, synthetic molecules, natural molecules, or combinations thereof.

[0068] As used herein, the term "target" or "target molecule" refers to any type of molecule, or structure to be detected or characterized. The molecule can be an intracellular molecule, such as for example, nucleic acid sequences, peptides, structures (e.g. intracellular membranes, ribosomes, etc.), surface molecules (e.g. receptors), extracellular molecules (e.g. cytokines, enzymes, viral particles, organisms, biological samples and the like.

[0069] As used herein, "biological samples" include solid and body fluid samples. The biological samples used in the present invention can include cells, protein or membrane extracts of cells, blood or biological fluids such as ascites fluid or brain fluid (e.g., cerebrospinal fluid). Examples of solid biological samples include, but are not limited to, samples taken from tissues of the central nervous system, bone, breast, kidney, cervix, endometrium, head/neck, gallbladder, parotid gland, prostate, pituitary gland, muscle, esophagus, stomach, small intestine, colon, liver, spleen, pancreas, thyroid, heart, lung, bladder, adipose, lymph node, uterus, ovary, adrenal gland, testes, tonsils, thymus and skin, or samples taken from tumors. Examples of "body fluid samples" include, but are not limited to blood, serum, semen, prostate fluid, seminal fluid, urine, feces, saliva, sputum, mucus, bone marrow, lymph, and tears.

[0070] As used herein, "cardiac disease" refers to any type of heart disease including heart failure, heart muscle disease, cardiomyopathy, hypertrophic cardiomyopathy, dilated cardiomyopathy, atherosclerosis, coronary artery disease, ischemic heart disease, myocarditis, viral infection, wounds, hypertensive heart disease, valvular disease, congenital heart disease, myocardial infarction, congestive heart failure, arrhythmias, diseases resulting in remodeling of the heart, etc., or disorders resulting from traumatic injury. Diseases of the heart can be due to any reason, such as for example, damage to cardiac tissue such as a loss of contractility (e.g., as might be demonstrated by a decreased ejection fraction).

[0071] Cardiac damage or disorder characterized by insufficient cardiac function includes any impairment or absence of a normal cardiac function or presence of an abnormal cardiac function. Abnormal cardiac function can be the result of disease, injury, and/or aging. As used herein, abnormal cardiac function includes morphological and/or functional abnormality of a cardiomyocyte, a population of cardiomyocytes, or the heart itself. Non-limiting examples of morphological and functional abnormalities include physical deterioration and/or death of cardiomyocytes, abnormal growth patterns of cardiomyocytes, abnormalities in the physical connection between cardiomyocytes, under- or over-production of a substance or substances by cardiomyocytes, failure of cardiomyocytes to produce a substance or substances which they normally produce, and transmission of electrical impulses in abnormal patterns or at abnormal times. Abnormalities at a more gross level include dyskinesis, reduced ejection fraction, changes as observed by echocardiography (e.g., dilatation), changes in EKG, changes in exercise tolerance, reduced capillary perfusion, and changes as observed by angiography. Abnormal

cardiac function is seen with many disorders including, for example, ischemic heart disease, e.g., angina pectoris, myocardial infarction, chronic ischemic heart disease, hypertensive heart disease, pulmonary heart disease (cor pulmonale), valvular heart disease, e.g., rheumatic fever, mitral valve prolapse, calcification of mitral annulus, carcinoid heart disease, infective endocarditis, congenital heart disease, myocardial disease, e.g., myocarditis, dilated cardiomyopathy, hypertensive cardiomyopathy, cardiac disorders which result in congestive heart failure, and tumors of the heart, e.g., primary sarcomas and secondary tumors. Heart damage also includes wounds, such as for example, knife wound; biological (e.g. viral; autoimmune diseases) or chemical (e.g. chemotherapy, drugs); surgery; transplantation and the like.

[0072] As used herein the phrase "diagnostic" means identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

[0073] As used herein the phrase "diagnosing" refers to classifying a disease or a symptom, determining a severity of the disease, monitoring disease progression, forecasting an outcome of a disease and/or prospects of recovery. The term "detecting" may also optionally encompass any of the above. Diagnosis of a disease according to the present invention can be effected by determining a level of a polynucleotide or a polypeptide of the present invention in a biological sample obtained from the subject, wherein the level determined can be correlated with predisposition to, or presence or absence of the disease. It should be noted that a "biological sample obtained from the subject" may also optionally comprise a sample that has not been physically removed from the subject, as described in greater detail below.

[0074] "Treatment" is an intervention performed with the intention of preventing the development or altering the pathology or symptoms of a disorder. Accordingly, "treatment" refers to both therapeutic treatment and prophylactic or preventative measures. "Treatment" may

also be specified as palliative care. Those in need of treatment include those already with the disorder as well as those in which the disorder is to be prevented. Accordingly, "treating" or "treatment" of a state, disorder or condition includes: (1) preventing or delaying the appearance of clinical symptoms of the state, disorder or condition developing in a human or other mammal that may be afflicted with or predisposed to the state, disorder or condition but does not yet experience or display clinical or subclinical symptoms of the state, disorder or condition; (2) inhibiting the state, disorder or condition, *i.e.*, arresting, reducing or delaying the development of the disease or a relapse thereof (in case of maintenance treatment) or at least one clinical or subclinical symptom thereof; or (3) relieving the disease, *i.e.*, causing regression of the state, disorder or condition or at least one of its clinical or subclinical symptoms. The benefit to an individual to be treated is either statistically significant or at least perceptible to the patient or to the physician.

[0075] The terms "patient" or "individual" or "subject" are used interchangeably herein, and refers to a mammalian subject to be treated, with human patients being preferred. In some cases, the methods of the invention find use in experimental animals, in veterinary application, and in the development of animal models for disease, including, but not limited to, rodents including mice, rats, and hamsters; and primates.

[0076] As used herein, the term "kit" refers to any delivery system for delivering materials. In the context of reaction assays, such delivery systems include systems that allow for the storage, transport, or delivery of reaction reagents (e.g., oligonucleotides, enzymes, etc. in the appropriate containers) and/or supporting materials (e.g., buffers, written instructions for performing the assay etc.) from one location to another. For example, kits include one or more enclosures (e.g., boxes) containing the relevant reaction reagents and/or supporting materials. As used herein, the term "fragmented kit" refers to a delivery systems comprising two or more separate containers that each contain a subportion of the total kit components. The containers may be delivered to the intended recipient together or separately. For example, a first container may contain an enzyme for use in an assay, while a second container contains oligonucleotides. The term "fragmented kit" is intended to encompass kits containing Analyte specific reagents (ASR's) regulated under section 520(e) of the Federal Food, Drug, and Cosmetic Act, but are not limited thereto. Indeed, any delivery system comprising two or more separate containers that each contains a subportion of the total kit components are included in the term "fragmented kit."

In contrast, a "combined kit" refers to a delivery system containing all of the components of a reaction assay in a single container (e.g., in a single box housing each of the desired components). The term "kit" includes both fragmented and combined kits.

Assay Description

[0077] Provided herein are methods and compositions for identifying candidate therapeutic agents that target the arginine vasopressin receptor. The methods, which we may also refer to as "assays" or "screening assays" can be configured in a variety of formats that include cell-based assays, which permit the user to observe the effect of a candidate therapeutic agent in the context of the whole cell, and in vitro assays, in which the target of interest has been paritally or substatially purified.

[0078] In embodiments, a method of identifying a candidate therapeutic agent comprises contacting a cell expressing an arginine vasopressin receptor (AVP-R), a G-protein coupled receptor kinase (GRK) and β-arrestin; assaying for modulation, in the presence or absence of the candidate therapeutic agent, of G-protein and GRK2/beta-arrestin dependent signaling and or expression or function of an arginine vasopressin receptor (AVP-R).

[0079] In preferred embodiments, the arginine vasopressin receptor comprises V_{1A} receptor ($V_{1A}R$), V2 receptor (V_2R), V_{1B} (V3) receptors or combinations thereof. In other preferred embodiments, the arginine vasopressin receptor is $V_{1A}R$.

[0080] In other embodiments, the candidate therapeutic agent inhibits $V_{1A}R$-G protein signaling and simultaneously activates GRK2/beta-arrestin-development.

[0081] In other embodiments, the candidate therapeutic agent inhibits $V_{1A}R$ expression or function.

[0082] In other embodiments, the candidate therapeutic agent inhibits$V_{1A}R$ and V2 expression or function. In embodiments, the candidate therapeutic agent inhibits$V_{1A}R$ expression or function to a greater degree than V2 expression or function.

[0083] In some embodiments, a composition comprises a therapeutically effective amount of a combination of two or more candidate therapeutic agents which inhibit $V_{1A}R$-G protein signaling and simultaneously activate GRK2/beta-arrestin and/or expression or function of an arginine vasopressin receptor (AVP-R). In other embodiments, at least one agent inhibits $V_{1A}R$-G protein signaling and a second agent activates GRK2/beta-arrestin signaling.

[0084] In other preferred embodiments, the candidate therapeutic agent is a non-selective V_{1A}-V_2 receptor antagonist whereby the agent inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling.

[0085] In other preferred embodiments, the candidate therapeutic agent is a non-selective V_{1A}-V_2 receptor antagonist whereby the agent inhibits expression and or function of V_{1A}-V_2 receptors.

[0086] The Examples section which follows, details methods for determining whether an agent modulates V_{1A}R-G protein signaling and/or activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling. In embodiments, signaling is identified by modulation of function, expression or activity of ERK1/2, βARK_{CT}, caspase 3/7, βarrestins, GRKs, angiotensin and receptors thereof, angiotensin type 1A receptor (AT1R), adrenergic receptors, or combinations thereof.

[0087] In other preferred embodiments, a method of identifying a candidate therapeutic agent comprises contacting a biological sample with a candidate therapeutic agent wherein the sample comprises a G-protein dependent and a G-protein independent signaling receptor; and, assaying for modulation, in the presence or absence of the candidate therapeutic agent, of G-protein dependent signaling and G-protein independent signaling as compared to a baseline control.

[0088] In preferred embodiments, the candidate therapeutic agent inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin.

[0089] In other preferred embodiments, the candidate therapeutic agent is a non-selective V_{1A}-V2 receptor antagonist whereby the agent inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin.

[0090] In other embodiments, a high throughput screening method of identifying a candidate therapeutic agent comprises contacting a support surface comprising an arginine vasopressin receptor (AVP-R) or fragments thereof, and/or G-protein dependent and a G-protein independent signaling molecule with a candidate therapeutic agent; assaying for modulation, in the presence or absence of the candidate therapeutic agent, AVP-R expression or function and/or G-protein dependent signaling and G-protein independent signaling as compared to a baseline control, thereby, identifying a candidate therapeutic agent.

[0091] In embodiments, a G-protein dependent and G-protein *in*dependent signaling molecule comprises: peptides, polypeptides, oligonucleotides, polynucleotides, or combinations thereof.

[0092] In embodiments, an arginine vasopressin receptor (AVP-R) or fragments thereof comprises: peptides, polypeptides, oligonucleotides, polynucleotides, or combinations thereof.

[0093] In other embodiments, the candidate therapeutic agent inhibits $V_{1A}R$ expression or function.

[0094] In other embodiments, the candidate therapeutic agent inhibits$V_{1A}R$ and V2 expression or function. In embodiments, the candidate therapeutic agent inhibits$V_{1A}R$ expression or function to a greater degree than V2 expression or function.

[0095] In other embodiments, a candidate therapeutic agent comprises a $V_{1A}R$ antagonist.

[0096] In other embodiments, a candidate therapeutic agent comprises a beta adrenergic receptor agonist.

[0097] In other embodiments, a candidate therapeutic agent is a V1A receptor antagonist and a beta adrenergic receptor agonist.

[0098] In embodiments, the support surface is any support for conducting assays. For example, if the assay is an immunoassay, the support is a typical multi-well plate. In other cases, the support is a bead to which molecules can be attached. The variations and types of assays are not limited. Thus, the support comprises: plastic, glass, beads, fibers, gels, electrochemical detectors, nanotubes, porous strips, paper, matrices or combinations thereof.

[0099] In other embodiments, a pharmaceutical composition comprises an agent in a therapeutically effective amount, identified by any of the methods embodied herein. In some embodiments, the pharmaceutical composition optionally comprises a V2-selective antagonist.

[00100] In other embodiments, a therapeutic agent inhibits signaling of $V_{1A}R$-associated Gq protein and simultaneously activates $V_{1A}R$-dependent G-protein coupled receptor kinase (GRK) and β-arrestin.

[00101] *Candidate/Test Agents*: Candidate agents include numerous chemical classes, though typically they are organic compounds including small organic compounds, nucleic acids including oligonucleotides, and peptides. Small organic compounds suitably may have e.g. a molecular weight of more than about 40 or 50 yet less than about 2,500. Candidate agents may comprise functional chemical groups that interact with proteins and/or DNA.

[00102] Candidate agents may be obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides. Alternatively, libraries of natural compounds in the form of e.g. bacterial, fungal and animal extracts are available or readily produced.

[00103] *Chemical Libraries*: Developments in combinatorial chemistry allow the rapid and economical synthesis of hundreds to thousands of discrete compounds. These compounds are typically arrayed in moderate-sized libraries of small molecules designed for efficient screening. Combinatorial methods, can be used to generate unbiased libraries suitable for the identification of novel compounds. In addition, smaller, less diverse libraries can be generated that are descended from a single parent compound with a previously determined biological activity. In either case, the lack of efficient screening systems to specifically target therapeutically relevant biological molecules produced by combinational chemistry such as inhibitors of important enzymes hampers the optimal use of these resources.

[00104] A combinatorial chemical library is a collection of diverse chemical compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks," such as reagents. For example, a linear combinatorial chemical library, such as a polypeptide library, is formed by combining a set of chemical building blocks (amino acids) in a large number of combinations, and potentially in every possible way, for a given compound length (i.e., the number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

[00105] A "library" may comprise from 2 to 50,000,000 diverse member compounds. Preferably, a library comprises at least 48 diverse compounds, preferably 96 or more diverse compounds, more preferably 384 or more diverse compounds, more preferably, 10,000 or more diverse compounds, preferably more than 100,000 diverse members and most preferably more than 1,000,000 diverse member compounds. By "diverse" it is meant that greater than 50% of the compounds in a library have chemical structures that are not identical to any other member of the library. Preferably, greater than 75% of the compounds in a library have chemical structures that are not identical to any other member of the collection, more preferably greater than 90% and most preferably greater than about 99%.

[00106] The preparation of combinatorial chemical libraries is well known to those of skill in the art. For reviews, see Thompson *et al.*, Synthesis and application of small molecule libraries, *Chem Rev* 96:555-600, 1996; Kenan *et al.*, Exploring molecular diversity with combinatorial shape libraries, *Trends Biochem Sci* 19:57-64, 1994; Janda, Tagged versus untagged libraries: methods for the generation and screening of combinatorial chemical libraries, *Proc Natl Acad Sci* USA. 91:10779-85, 1994; Lebl *et al.*, One-bead-one-structure combinatorial libraries, *Biopolymers* 37:177-98, 1995; Eichler *et al.*, Peptide, peptidomimetic, and organic synthetic combinatorial libraries, *Med Res Rev.* 15:481-96, 1995; Chabala, Solid-phase combinatorial chemistry and novel tagging methods for identifying leads, *Curr Opin Biotechnol.* 6:632-9, 1995; Dolle, Discovery of enzyme inhibitors through combinatorial chemistry, *Mol Divers.* 2:223-36, 1997; Fauchere *et al.*, Peptide and nonpeptide lead discovery using robotically synthesized soluble libraries, Can J. Physiol Pharmacol. 75:683-9, 1997; Eichler *et al.*, Generation and utilization of synthetic combinatorial libraries, *Mol Med Today* 1: 174-80, 1995; and Kay *et al.*, Identification of enzyme inhibitors from phage-displayed combinatorial peptide libraries, *Comb Chem High Throughput Screen* 4:535-43, 2001.

[00107] Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to, peptoids (PCT Publication No. WO 91/19735); encoded peptides (PCT Publication WO 93/20242); random bio-oligomers (PCT Publication No. WO 92/00091); benzodiazepines (U.S. Pat. No. 5,288,514); diversomers, such as hydantoins, benzodiazepines and dipeptides (Hobbs, *et al., Proc. Nat. Acad. Sci.* USA, 90:6909-6913 (1993)); vinylogous polypeptides (Hagihara, *et al., J. Amer. Chem. Soc.* 114:6568 (1992)); nonpeptidal peptidomimetics with β-D-glucose scaffolding (Hirschmann, *et al., J. Amer. Chem. Soc.*, 114:9217-9218 (1992)); analogous organic syntheses of small compound libraries (Chen, *et al., J. Amer. Chem. Soc.*, 116:2661 (1994)); oligocarbamates (Cho, *et al., Science,* 261:1303 (1993)); and/or peptidyl phosphonates (Campbell, *et al., J. Org. Chem.* 59:658 (1994)); nucleic acid libraries (see, Ausubel, Berger and Sambrook, all *supra*); peptide nucleic acid libraries (see, e.g., U.S. Pat. No. 5,539,083); antibody libraries (see, e.g., Vaughn, *et al., Nature Biotechnology,* 14(3):309-314 (1996) and PCT/US96/10287); carbohydrate libraries (see, e.g., Liang, *et al., Science*, 274:1520-1522 (1996) and U.S. Pat. No. 5,593,853); small organic molecule libraries (see, e.g., benzodiazepines, Baum C&E News, January 18, page 33 (1993); isoprenoids (U.S. Pat. No. 5,569,588); thiazolidinones and metathiazanones (U.S. Pat. No. 5,549,974); pyrrolidines

(U.S. Pat. Nos. 5,525,735 and 5,519,134); morpholino compounds (U.S. Pat. No. 5,506,337); benzodiazepines (U.S. Pat. No. 5,288,514); and the like.

[00108] Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem. Tech, Louisville Ky., Symphony, Rainin, Woburn, Mass., 433A Applied Biosystems, Foster City, Calif., 9050 Plus, Millipore, Bedford, Mass.). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, Ru, Tripos, Inc., St. Louis, Mo., ChemStar, Ltd., Moscow, RU, 3D Pharmaceuticals, Exton, Pa., Martek Bio sciences, Columbia, Md., etc.).

[00109] The screening assays of the invention suitably include and embody, animal models, cell-based systems and non-cell based systems.

[00110] In another preferred embodiment, a method of identifying candidate therapeutic agents for treatment of disease, comprises culturing an isolated cell expressing a target molecule, administering a candidate therapeutic agent to the cultured cell; correlating the target molecules expression, activity and/or function in the presence or absence of a candidate therapeutic agent as compared to control cells, wherein a drug is identified based on desirable therapeutic outcomes. For example, a drug which modulates expression of the target molecule whereby expression levels are responsible for the disease state or the target molecule modulates the activity of another molecule whether upstream or downstream in a pathway. In other examples the assays measure kinase activity. In other examples, the assay measures binding partners.

[00111] Another suitable method for diagnosis and candidate drug discovery includes contacting a test sample with a cell expressing a target molecule e.g. $V_{1A}R$, and detecting interaction of the test agent with the target molecule.

[00112] In another preferred embodiment, a cell from a patient is isolated and contacted with a candidate therapeutic molecule. The genes, expression products thereof, are monitored to identify which genes or expression products are regulated by the drug. For example, modulation of function, expression or activity of ERK1/2, βARK_{CT}, caspase 3/7, βarrestins, G protein coupled receptor kinase (GRK), angiotensin and receptors thereof, angiotensin type 1A receptor (AT1R), adrenergic receptors, or combinations thereof.

[00113] *High-Throughput Screening:* The assays embodied herein are suitable for drug screening in a high throughput screening of compounds having suitable binding affinity to the target of interest. In this method, large numbers of different small test compounds are synthesized on a solid substrate. The test compounds are reacted with target molecules, or fragments thereof, and washed. Bound molecules are then detected by the methods embodied herein.

[00114] The methods of screening of the invention comprise using screening assays to identify, from a library of diverse molecules, one or more compounds having a desired activity. A "screening assay" is a selective assay designed to identify, isolate, and/or determine the structure of, compounds within a collection that have a preselected activity. By "identifying" it is meant that a compound having a desirable activity is isolated, its chemical structure is determined (including without limitation determining the nucleotide and amino acid sequences of nucleic acids and polypeptides, respectively) the structure of and, additionally or alternatively, purifying compounds having the screened activity). Biochemical and biological assays are designed to test for activity in a broad range of systems ranging from protein-protein interactions, enzyme catalysis, small molecule-protein binding, to cellular functions. Such assays include automated, semi-automated assays and HTS (high throughput screening) assays.

[00115] In HTS methods, many discrete compounds are preferably tested in parallel by robotic, automatic or semi-automatic methods so that large numbers of test compounds are screened for a desired activity simultaneously or nearly simultaneously. It is possible to assay and screen up to about 6,000 to 20,000, and even up to about 100,000 to 1,000,000 different compounds a day using the integrated systems of the invention.

[00116] Typically in HTS, target molecules are administered or cultured with isolated cells with modulated receptors, including the appropriate controls.

[00117] In one embodiment, screening comprises contacting each cell culture with a diverse library of member compounds, some of which are ligands of the target, under conditions where complexes between the target and ligands can form, and identifying which members of the libraries are present in such complexes. In another non limiting modality, screening comprises contacting a target with a diverse library of member compounds, some of which are inhibitors (or activators) of the target, under conditions where a product or a reactant of the reaction produce a

detectable signal. In the latter modality, inhibitors of target molecules decrease the signal from a detectable product or increase a signal from a detectable reactant (or *vice-versa* for activators).

[00118] The methods disclosed herein can be used for screening a plurality of test compounds. In certain embodiments, the plurality of test compounds comprises between 1 and 200,000 test compounds, between 1 and 100,000 test compounds, between 1 and 1,000 test compounds, between 1 and 100 test compounds, or between 1 and 10 test compounds. In certain embodiments, the test compounds are provided by compound libraries, whether commercially available or not, using combinatorial chemistry techniques. In certain embodiments, the compound libraries are immobilized on a solid support.

[00119] As discussed above, the target can be present in any substrate as the assay parameters can be manipulated or optimized for each type of substrate. For example, if the target is at the surface of, or in a cell, or secreted by a cell, the following parameters would be determined: the optimal cell line, cell density, culture medium, serum concentration, final reagents volumes, compound incubation times (for example 12, 24 or 36 hours). If the target is in a cell-free solution, the optimal composition of the solution can be determined as well as the range of concentrations of the positive control standard. Other parameters that can be determined are ligand concentrations, temperature of incubation and incubation times of the ligands (for example 1 to 4 hours). The set-up of the reading instrument, for example a time-resolved fluorimeter, is optimized for the measurement window and time delay, excitation parameters (e.g. number of flashes delivered), gain adjustment, and reader head positioning with respect to the receptacle. The proper pharmacological control, if available, needs to be determined.

[00120] High throughput screening can be used to measure the effects of drugs on complex molecular events such as signal transduction pathways, as well as cell functions including, but not limited to, cell function, apoptosis, cell division, cell adhesion, locomotion, exocytosis, and cell-cell communication. Multicolor fluorescence permits multiple targets and cell processes to be assayed in a single screen. Cross-correlation of cellular responses will yield a wealth of information required for target validation and lead optimization.

[00121] In another aspect, the present invention provides a method for analyzing cells comprising providing an array of locations which contain multiple cells wherein the cells contain one or more fluorescent reporter molecules; scanning multiple cells in each of the locations

containing cells to obtain fluorescent signals from the fluorescent reporter molecule in the cells; converting the fluorescent signals into digital data; and utilizing the digital data to determine the distribution, environment or activity of the fluorescent reporter molecule within the cells.

[00122] *Microarrays*: Identification of a nucleic acid sequence capable of binding to a target molecule can be achieved by immobilizing a library of nucleic acids onto the substrate surface so that each unique nucleic acid is located at a defined position to form an array. In general, the immobilized library of nucleic acids are exposed to a biomolecule or candidate agent under conditions which favored binding of the biomolecule to the nucleic acids. The nucleic acid array would then be analyzed by the methods embodied herein to determine which nucleic acid sequences bound to the biomolecule. Preferably the biomolecules would carry a pre-determined label for use in detection of the location of the bound nucleic acids.

[00123] An assay using an immobilized array of nucleic acid sequences may be used for determining the sequence of an unknown nucleic acid; single nucleotide polymorphism (SNP) analysis; analysis of gene expression patterns from a particular species, tissue, cell type, etc.; gene identification; etc.

[00124] In further embodiments, oligonucleotides or longer fragments derived from any of the polynucleotide sequences, may be used as targets in a microarray. The microarray can be used to monitor the identity and/or expression level of large numbers of genes and gene transcripts simultaneously to identify genes with which target genes or its product interacts and/or to assess the efficacy of candidate therapeutic agents in regulating expression products of genes that mediate, for example, neurological disorders. This information may be used to determine gene function, and to develop and monitor the activities of therapeutic agents.

[00125] Microarrays may be prepared, used, and analyzed using methods known in the art (see, e.g., Brennan *et al.*, 1995, U.S. Pat. No. 5,474,796; Schena *et al.*, 1996, *Proc. Natl. Acad. Sci.* U.S.A. 93: 10614-10619; Baldeschweiler *et al.*, 1995, PCT application WO95/251116; Shalon, *et al.*, 1995, PCT application WO95/35505; Heller *et al.*, 1997, *Proc. Natl. Acad. Sci.* U.S.A. 94: 2150-2155; and Heller *et al.*, 1997, U.S. Pat. No. 5,605,662). In other embodiments, a microarray comprises peptides, or other desired molecules which can be assayed to identify a candidate agent.

[00126] In another preferred embodiment a method for screening candidate compounds for the treatment or prevention of a cardiac disease or disorder comprises contacting a sample with a candidate therapeutic agent and measuring the effects the compound has on a target. For example if it is a cellular product such as a receptor, the compound may regulate the receptor expression and the compound can then be further studied for any possible therapeutic effects (increase or decrease parameter being monitored e.g. expression, oxidation level, apoptic markers). An abnormal expression state may be caused by pathology such as disease, cancer, genetic defects and/or a toxin.

[00127] *Methods of Treatment*

[00128] In other preferred embodiments, a method of treating a patient having a cardiac disease or disorder, wherein the patient has elevated arginine vasopressin (AVP) and/or copeptin levels as compared to a baseline level, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V1A receptor antagonist.

[00129] In other embodiments, a method of preventing or treating a subject at risk of or suffering from a cardiac disease or disorder comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits an arginine vasopressin receptor (AVP-R) expression or function, and/or inhibitsV_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling. In some embodiments, a V2 arginine receptor antagonist is optionally administered. In preferred embodiments the cardiac disease and/or disorder is heart failure and/or hyponatremia.

[00130] In another preferred embodiment, a method of preventing or treating a subject at risk of or suffering from a cardiac disease or disorder comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits signaling of V_{1A}R-associated Gq protein and simultaneously activates V_{1A}R-dependent G-protein coupled receptor kinase (GRK) and β-arrestin signaling, and/or inhibits expression or function of an arginine vasopressin receptor (AVP-R) or fragments thereof; thereby, preventing or treating a subject at risk of or suffering a cardiac disease or disorder.

[00131] In another preferred embodiment, a method of preventing or treating a subject at risk of or suffering from heart failure and/or hyponatremia comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent

which inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling and/or inhibits expression and/or function of an arginine vasopressin receptor (AVP-R). In some embodiments, the method further comprises administering a V2 arginine receptor antagonist.

[00132] In other embodiments, a method of treating heart failure in a patient, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of V1A receptor antagonist and a beta adrenergic receptor agonist.

[00133] In other embodiments, a method of preventing or treating a cardiac disease or disorder in a subject, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V1A receptor antagonist and/or at least one beta adrenergic receptor agonist.

[00134] In yet other embodiments, a method of preventing or treating a cardiac disease or disorder in a subject, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V1A receptor antagonist. In some embodiments, the pharmaceutical composition further comprising at least one beta adrenergic receptor agonist and is administered to the patient. In embodiments, the at least one V1A receptor antagonist and at least one beta adrenergic receptor agonist are administered consecutively or at the same time. In some embodiments, a pharmaceutical composition comprising a therapeutically effective amount of V1A receptor antagonist and a beta adrenergic receptor agonist is also administered as part of the treatment.

Administration of Compositions

[00135] The agents identified by the methods embodied herein can be formulated and compositions of the present invention may be administered in conjunction with one or more additional active ingredients, pharmaceutical compositions, or other compounds. The therapeutic agents of the present invention may be administered to an animal, preferably a mammal, most preferably a human.

[00136] In some embodiments, a pharmaceutical composition comprises a therapeutically effective amount of V1A receptor antagonist and a beta adrenergic receptor agonist is also administered as part of the treatment.

[00137] In some embodiments, a pharmaceutical composition comprises a therapeutically effective amount of V1A receptor antagonist and/or a beta adrenergic receptor agonist.

[00138] In other embodiments, a pharmaceutical composition comprises at least one or more candidate therapeutic agents embodied herein.

[00139] The pharmaceutical formulations may be for administration by oral (solid or liquid), parenteral (intramuscular, intraperitoneal, intravenous (IV) or subcutaneous injection), transdermal (either passively or using ionophoresis or electroporation), transmucosal and systemic (nasal, vaginal, rectal, or sublingual), or inhalation routes of administration, or using bioerodible inserts and can be formulated in dosage forms appropriate for each route of administration.

[00140] The agents may be formulated in pharmaceutically acceptable carriers or diluents such as physiological saline or a buffered salt solution. Suitable carriers and diluents can be selected on the basis of mode and route of administration and standard pharmaceutical practice. A description of exemplary pharmaceutically acceptable carriers and diluents, as well as pharmaceutical formulations, can be found in Remington's Pharmaceutical Sciences, a standard text in this field, and in USP/NF. Other substances may be added to the compositions to stabilize and/or preserve the compositions.

[00141] The compositions of the invention may be administered to animals by any conventional technique. The compositions may be administered directly to a target site by, for example, surgical delivery to an internal or external target site, or by catheter to a site accessible by a blood vessel. Other methods of delivery, e.g., liposomal delivery or diffusion from a device impregnated with the composition, are known in the art. The compositions may be administered in a single bolus, multiple injections, or by continuous infusion (e.g., intravenously). For parenteral administration, the compositions are preferably formulated in a sterilized pyrogen-free form.

[00142] The compounds identified by this invention may also be administered orally to the patient, in a manner such that the concentration of drug is sufficient to inhibit bone resorption or to achieve any other therapeutic indication as disclosed herein. Typically, a pharmaceutical composition containing the compound is administered at an oral dose of between about 0.1 to about 50 mg/kg in a manner consistent with the condition of the patient. Preferably the oral dose would be about 0.5 to about 20 mg/kg.

[00143] An intravenous infusion of the compound in 5% dextrose in water or normal saline, or a similar formulation with suitable excipients, is most effective, although an intramuscular bolus injection is also useful. Typically, the parenteral dose will be about 0.01 to about 100 mg/kg; preferably between 0.1 and 20 mg/kg, in a manner to maintain the concentration of drug in the plasma at a concentration effective to inhibit a cysteine protease. The compounds may be administered one to four times daily at a level to achieve a total daily dose of about 0.4 to about 400 mg/kg/day. The precise amount of an inventive compound which is therapeutically effective, and the route by which such compound is best administered, is readily determined by one of ordinary skill in the art by comparing the blood level of the agent to the concentration required to have a therapeutic effect. Prodrugs of compounds of the present invention may be prepared by any suitable method. For those compounds in which the prodrug moiety is a ketone functionality, specifically ketals and/or hemiacetals, the conversion may be effected in accordance with conventional methods.

[00144] No unacceptable toxicological effects are expected when compounds, derivatives, salts, compositions etc., of the present invention are administered in accordance with the present invention. The compounds of this invention, which may have good bioavailability, may be tested in one of several biological assays to determine the concentration of a compound which is required to have a given pharmacological effect.

[00145] In another preferred embodiment, there is provided a pharmaceutical or veterinary composition comprising one or more identified compounds and a pharmaceutically or veterinarily acceptable carrier. Other active materials may also be present, as may be considered appropriate or advisable for the disease or condition being treated or prevented.

[00146] The carrier, or, if more than one be present, each of the carriers, must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient.

[00147] The compounds identified by the methods herein would be suitable for use in a variety of drug delivery systems described above. Additionally, in order to enhance the *in vivo* serum half-life of the administered compound, the compounds may be encapsulated, introduced into the lumen of liposomes, prepared as a colloid, or other conventional techniques may be employed which provide an extended serum half-life of the compounds. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka, *et al.*, U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028 each of which is incorporated herein by reference. Furthermore, one may administer the drug in a targeted drug delivery system, for example, in a liposome coated with a tissue-specific antibody. The liposomes will be targeted to and taken up selectively by the organ.

[00148] The formulations include those suitable for rectal, nasal, topical (including buccal and sublingual), vaginal or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration, but preferably the formulation is an orally administered formulation. The formulations may conveniently be presented in unit dosage form, e.g. tablets and sustained release capsules, and may be prepared by any methods well known in the art of pharmacy.

[00149] Such methods include the step of bringing into association the above defined active agent with the carrier. In general, the formulations are prepared by uniformly and intimately bringing into association the active agent with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

[00150] The compound identified using these methods can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby the compound is combined in admixture with a pharmaceutically acceptable carrier vehicle. Therapeutic formulations are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate,

citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™. (ICI Americas Inc., Bridgewater, N.J.), PLURONICS™. (BASF Corporation, Mount Olive, N.J.) or PEG.

[00151] The formulations to be used for *in vivo* administration must be sterile and pyrogen free. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

[00152] Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary physician. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. "The use of interspecies scaling in toxicokinetics" In Toxicokinetics and New Drug Development, Yacobi *et al.*, Eds., Pergamon Press, New York 1989, pp. 42-96.

[00153] Formulations for oral administration in the present invention may be presented as: discrete units such as capsules, cachets or tablets each containing a predetermined amount of the active agent; as a powder or granules; as a solution or a suspension of the active agent in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water in oil liquid emulsion; or as a bolus etc.

[00154] For compositions for oral administration (e.g. tablets and capsules), the term "acceptable carrier" includes vehicles such as common excipients e.g. binding agents, for example syrup, acacia, gelatin, sorbitol, tragacanth, polyvinylpyrrolidone (Povidone), methylcellulose, ethylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, sucrose and starch; fillers and carriers, for example corn starch, gelatin, lactose, sucrose, microcrystalline cellulose, kaolin, mannitol, dicalcium phosphate, sodium chloride and alginic

acid; and lubricants such as magnesium stearate, sodium stearate and other metallic stearates, glycerol stearate stearic acid, silicone fluid, talc waxes, oils and colloidal silica. Flavoring agents such as peppermint, oil of wintergreen, cherry flavoring and the like can also be used. It may be desirable to add a coloring agent to make the dosage form readily identifiable. Tablets may also be coated by methods well known in the art.

[00155] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active agent in a free flowing form such as a powder or granules, optionally mixed with a binder, lubricant, inert diluent, preservative, surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may be optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active agent.

[00156] Other formulations suitable for oral administration include lozenges comprising the active agent in a flavored base, usually sucrose and acacia or tragacanth; pastilles comprising the active agent in an inert base such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active agent in a suitable liquid carrier.

[00157] Parenteral formulations will generally be sterile.

[00158] *Dose:* An effective dose of a composition of the presently disclosed subject matter is administered to a subject in need thereof. A "therapeutically effective amount" or a "therapeutic amount" is an amount of a therapeutic composition sufficient to produce a measurable response (e.g., a biologically or clinically relevant response in a subject being treated). The response can be measured in many ways, as discussed above, e.g. cytokine profiles, cell types, cell surface molecules, etc. Actual dosage levels of active ingredients in the compositions of the presently disclosed subject matter can be varied so as to administer an amount of the active compound(s) that is effective to achieve the desired therapeutic response for a particular subject. The selected dosage level will depend upon the activity of the therapeutic composition, the route of administration, combination with other drugs or treatments, the severity of the condition being treated, and the condition and prior medical history of the subject being treated. However, it is within the skill of the art to start doses of the compound at levels lower than required to achieve the desired therapeutic effect and to gradually increase the dosage until the desired effect is

achieved. The potency of a composition can vary, and therefore a "treatment effective amount" can vary. However, using the assay methods described herein, one skilled in the art can readily assess the potency and efficacy of a candidate compound of the presently disclosed subject matter and adjust the therapeutic regimen accordingly.

[00159] The compositions disclosed herein are generally and variously useful for treatment of heart disease, e.g., heart failure. A patient is effectively treated whenever a clinically beneficial result ensues. This may mean, for example, a complete resolution of the symptoms of a disease, a decrease in the severity of the symptoms of the disease, or a slowing of the disease's progression. These methods can further include the steps of a) identifying a subject (e.g., a patient and, more specifically, a human patient) who has heart failure; and b) providing to the subject a composition comprising a compound described herein, such as any pharmaceutically acceptable salt of such a compound. An amount of such a compound provided to the subject that results in a complete resolution of the symptoms of a disease, a decrease in the severity of the symptoms of the disease, or a slowing of the disease's progression is considered a therapeutically effective amount. The present methods may also include a monitoring step to help optimize dosing and scheduling as well as predict outcome. In some methods of the present invention, one can first determine whether a patient has elevated levels of AVP and then make a determination as to whether or not to treat the patient with one or more of the compositions described herein. AVP levels can be assayed using any standard method and then compared to a reference level to determine whether the patient has elevated levels of AVP. Monitoring can also be used to rapidly distinguish responsive patients from nonresponsive patients.

[00160] Cardiovascular disorders amenable to the therapeutic, and/or prognostic methods of the invention can be disorders that are responsive to the modulation $V_{1A}R$. While we believe we understand certain events that occur in the course of treatment, the compositions of the present invention are not limited to those that work by affecting any particular cellular mechanism. Any form of cardiovascular disorder which is associated with misregulation of AVP, $V_{1A}R$ (e.g., overexpression or altered binding or activity) is within the scope of the invention.

[00161] The methods of the invention can be expressed in terms of the preparation of a medicament. Accordingly, the invention encompasses the use of the agents and compositions

described herein in the preparation of a medicament. The compounds described herein are useful in therapeutic compositions and regimens or for the manufacture of a medicament for use in treatment of diseases or conditions as described herein (e.g., a cardiovascular disorder disclosed herein).

[00162] Any composition described herein can be administered to any part of the host's body for subsequent delivery to a target cell. A composition can be delivered to, without limitation, the brain, the cerebrospinal fluid, joints, nasal mucosa, blood, lungs, intestines, muscle tissues, skin, or the peritoneal cavity of a mammal. In terms of routes of delivery, a composition can be administered by intravenous, intracranial, intraperitoneal, intramuscular, subcutaneous, intramuscular, intrarectal, intravaginal, intrathecal, intratracheal, intradermal, or transdermal injection, by oral or nasal administration, or by gradual perfusion over time. In a further example, an aerosol preparation of a composition can be given to a host by inhalation.

[00163] The dosage required will depend on the route of administration, the nature of the formulation, the nature of the patient's illness, the patient's size, weight, surface area, age, and sex, other drugs being administered, and the judgment of the attending clinicians. Suitable dosages are in the range of 0.01-1,000 mg/kg. Wide variations in the needed dosage are to be expected in view of the variety of cellular targets and the differing efficiencies of various routes of administration. Variations in these dosage levels can be adjusted using standard empirical routines for optimization, as is well understood in the art. Administrations can be single or multiple (e.g., 2- or 3-, 4-, 6-, 8-, 10-, 20-, 50-, 100-, 150-, or more fold). Encapsulation of the compounds in a suitable delivery vehicle (e.g., polymeric microparticles or implantable devices) may increase the efficiency of delivery.

[00164] The duration of treatment with any composition provided herein can be any length of time from as short as one day to as long as the life span of the host (e.g., many years). For example, a compound can be administered once a week (for, for example, 4 weeks to many months or years); once a month (for, for example, three to twelve months or for many years); or once a year for a period of 5 years, ten years, or longer. It is also noted that the frequency of treatment can be variable. For example, the present compounds can be administered once (or twice, three times, etc.) daily, weekly, monthly, or yearly.

[00165] An effective amount of any composition provided herein can be administered to an individual in need of treatment. The term "effective" as used herein refers to any amount that induces a desired response while not inducing significant toxicity in the patient. Such an amount can be determined by assessing a patient's response after administration of a known amount of a particular composition. In addition, the level of toxicity, if any, can be determined by assessing a patient's clinical symptoms before and after administering a known amount of a particular composition. It is noted that the effective amount of a particular composition administered to a patient can be adjusted according to a desired outcome as well as the patient's response and level of toxicity. Significant toxicity can vary for each particular patient and depends on multiple factors including, without limitation, the patient's disease state, age, and tolerance to side effects.

[00166] Any method known to those in the art can be used to determine if a particular response is induced. Clinical methods that can assess the degree of a particular disease state can be used to determine if a response is induced. The particular methods used to evaluate a response will depend upon the nature of the patient's disorder, the patient's age, and sex, other drugs being administered, and the judgment of the attending clinician.

[00167] Concurrent administration of two or more therapeutic agents does not require that the agents be administered at the same time or by the same route, as long as there is an overlap in the time period during which the agents are exerting their therapeutic effect. Simultaneous or sequential administration is contemplated, as is administration on different days or weeks. The compositions may also be administered with another standard therapeutic agent for treatment of cardivascular disease.

[00168] *Kits and Methods*

[00169] The present invention further provides systems and kits (e.g., commercial therapeutic, diagnostic, or research products, reaction mixtures, etc.) that contain one or more or all components sufficient, necessary, or useful to practice any of the methods described herein. These systems and kits may include buffers, detection/imaging components, positive/negative control reagents, instructions, software, hardware, packaging, or other desired components.

[00170] The kits provide useful tools for screening test compounds capable of modulating the effects of a compound on a target molecule. The kits can be packaged in any suitable manner to aid research, clinical, and testing labs, typically with the various parts, in a suitable container along with instructions for use.

[00171] Provided herein are kits for identifying a compound that modulates the interaction between a target molecule and a test agent. In certain embodiments, the kits comprise (a) a target molecule labeled with a first detectable label; and (b) a test agent labeled with a second detectable label. In certain embodiments, the kits may further comprise lipids and/or solvents. In certain embodiments, the kits may further comprise buffers and reagents needed for the procedure, and instructions for carrying out the assay. In certain embodiments, the kits may further comprise, where necessary, agents for reducing the background interference in a test, positive and negative control reagents, apparatus for conducting a test, and the like.

[00172] In certain embodiments of the methods and kits provided herein, solid phase supports are used for purifying proteins, labeling samples or carrying out the solid phase assays. Examples of solid phases suitable for carrying out the methods disclosed herein include beads, particles, colloids, single surfaces, tubes, multiwell plates, microtiter plates, slides, membranes, gels and electrodes. When the solid phase is a particulate material (e.g., beads), it is, in one embodiment, distributed in the wells of multi-well plates to allow for parallel processing of the solid phase supports.

[00173] Methods and kits disclosed herein may be carried out in numerous formats known in the art. In certain embodiments, the methods provided herein are carried out using solid-phase assay formats. In certain embodiments, the methods provided herein are carried out in a well of a plate with a plurality of wells, such as a multi-well plate or a multi-domain multi-well plate. The use of multi-well assay plates allows for the parallel processing and analysis of multiple samples distributed in multiple wells of a plate. Multi-well assay plates (also known as microplates or microtiter plates) can take a variety of forms, sizes and shapes (e.g., round- or flat-bottom multi-well plates). Exemplary multi-well plate formats that can be used in the methods provided herein include those found on 96-well plates (12 × 8 array of wells), 384-well plates (24 × 16 array of wells), 1536-well plate (48 × 32 array of well), 3456-well plates and 9600-well plates. Other formats that may be used in the methods provided herein include, but

are not limited to, single or multi-well plates comprising a plurality of domains, cuvettes, microarrays etc..

[00174] The methods provided herein, when carried out in standardized plate formats can take advantage of readily available equipment for storing and moving these plates as well as readily available equipment for rapidly dispensing liquids in and out of the plates (e.g., robotic dispenser, multi-well and multi-channel pipettes, plate washers and the like).

[00175] The invention has been described in detail with reference to preferred embodiments thereof. However, it will be appreciated that those skilled in the art, upon consideration of this disclosure, may make modifications and improvements within the spirit and scope of the invention.

[00176] All documents mentioned herein are incorporated herein by reference. All publications and patent documents cited in this application are incorporated by reference for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

EXAMPLES

[00177] While various embodiments of the present invention have been described above, it should be understood that they have been presented by way of example only, and not limitation. The following non-limiting examples are illustrative of the invention.

Example 1: Arginine Vasopressin Enhances Cell Survival Via a GRK2-βarrestin1-ERK1/2-Dependent Pathway in H9c2 Cells

[00178] *Materials and Methods*

[00179] *Materials:* Penicillin and streptomycin were purchased from Invitrogen (Gaithersburg, MD). AVP (V9879) was from Sigma-Aldrich. Ro 31-82425 (Ro-31) and PD 98059 were from Calbiochem (San Diego, CA). V1A receptor selective antagonist, SR45059, and Gβγ subunit inhibitor, gallein, were from Tocris Bioscience (Minneapolis, MN). V_{1B} receptor selective antagonist SSR149415 and V_2 receptor selective antagonist OPC41061 were purchased from Axon Medchem (Groningen, Netherlands). Cell viability assay kit and Caspase

3/7 assay kit were purchased from Promega (Madison, WI, USA). The hypoxia chamber was purchased from Billups-Rothenberg, Inc. (CA, USA). M199 and Opti-MEM I reduced serum medium were purchased from Invitrogen. Silencer FAM-labeled negative control siRNA (Ambion, AM4620), GRK 2 siRNA (Ambion, AM16708), GRK5 siRNA (Thermo Scientific, L-080156), and βarrestin1 siRNA (Thermo Scientific, L-080156) were from Ambion). Anti-P-ERK1/2, anti-total-ERK1/2, anti-β-arrestin1/2 and anti-GAPDH antibodies were purchased from Cell Signaling. Anti-GFP, Anti-GRK2 and anti-GRK5 were from Santa Cruz.

[00180] *Cell culture:* H9c2 cells were purchased from ATCC (Rockville, MD), maintained in M199 supplemented with 10% Fetal calf serum (FCS), 100 U/ml penicillin, and 10 μl/ml streptomycin, and grown in an atmosphere of 5% CO /95% humidified air at 37°C on 6-well plates, 100,000 cells seeded per well. An H9c2 cell is a cloned heart cell that behaves very much like heart cells, for example, both H9c2 cells and isolated heart cells hypertrophy in response to angiotensin. Adenoviral infection with β-gal, GFP, Gq-I-ires-GFP or βARK_{CT} was performed at 100 MOI when cell confluence reached 60-70%. 24-36 h after infection, cells were treated with the reagents under normal conditions or following hypoxia/re-oxygenation (H/R).

[00181] *Transfection of siRNA and adenovirus:* Cells (1×10^5 and 5000 cells per well in 6 well-plate and 96-well plate, respectively) were seeded in plates incubated in M199 with 10% FBS. When cell confluent reached to 50-60%, the medium was replaced by 1800 μl (6-well plate) or 60 μl (96-well plate) of Opti-MEM I reduced serum medium. The negative control I FAM (2.4 μg in 100 μl of Opti-MEM I per well in 6-well plate and 0.083 μg in 3.2 μl of Opti-MEM I per well in 96-well plate) and same amount of scrambled, GRK2, GRK5 and βarrestin1 I were mixed with the same volume of transfection reagent diluted with Opti-MEM I. The mixture (200 μl and 20 ul per well in 6-well and 96-well plates, respectively) were added into the cells according to the instructions (at a final concentration of 100 nM). The protein level of GRK2, GRK5 and βarrestin1 were determined by western blot at 48-96 hours after transfection, as indicated.

[00182] *Hypoxia/re-oxygenation:* 5000 cells per well were seeded in 96-well plates. When the cell confluence reached 80-90%, cells were starved with FCS-free medium overnight. Prior to H/R, the cells were pretreated with specific reagents and the culture cell plates were placed in the hypoxia-chamber. The chamber was sealed and aerated with 5% CO_2 and 95% Nitrogen at 3

liters per minute for 30 minutes. The cells were maintained in the hypoxic environment for 24 hrs at 37°C and then the cells were re-oxygenated for 24 hrs with 5% CO_2/95% humidified air.

[00183] *Cell viability and caspase 3/7 assay:* Sub-confluent cultures grown in 96-well plates were starved by withdrawing serum at least 6 h. After serum starvation, cells were pretreated with 10 pM to 0.1 μM of vasopressin for 30 minutes in the presence or absence of SR49059 (0.1μM), PD98059 (10 μM), Ro-31 (1μM), β-gal, Gq-I-ires-GFP or βARK_{CT}. Cells were then incubated in the hypoxia chamber for 24 h followed by re-oxygenation for 24 hours. The relative number of live cells and caspase 3/7 activity were measured following the manufacturer's instructions. Within each assay, triplicates were performed for each condition to attain a single n value.

[00184] *Immunoblot analysis of phosphorylated ERK1/2*: After cells were treated for 2-60 min with 1 nM to 10 μM AVP, they were rapidly washed 2 times with ice-cold PBS and lysed with 250 μl of ice-cold lysis buffer (50 mM Tris-HCl, 1 mM EGTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethlysulfonylflouride, 5 mg/ml leupeptin, 20 mg/ml aprotinin, 1 mM NaF, and 1 mM Na3VO4). After centrifugation at 13,000 x g for 10 min, equal amounts of total cell lysate (20 μg protein) were subjected to 4-12% SDS-PAGE, followed by immunoblotting for phosphorylated ERK1/2 (1:1000), total ERK1/2 (1:1000) or GFP (1:200). T-ERK1/2 was used to normalize P-ERK1/2 responses.

[00185] *Statistical Analysis:* A commercial software package was used for statistical analysis (Graph Pad Software Inc). Comparison of means ± SE was analyzed by unpaired t-test, one- or two-way ANOVA followed by Tukey's post hoc analysis, as indicated in figure legends. A value of $P<0.05$ was considered as statistically significant. Number of independent experiments performed in each assay is indicated in the figure legends.

[00186] *Results*

[00187] *AVP protects H9c2 cells against hypoxia/reoxygenation-induced cell death via V_{1A}R- and ERK1/2-dependent signaling*: To test the ability of AVP to mediate protective signaling, an *in vitro* model of cellular stress was established wherein H9c2 myoblasts, derived from embryonic rat ventricle, underwent hypoxia for 24 hr followed by re-oxygenation for 24 hr (H/R). Under normoxic and serum-free conditions H9c2 cell survival decreased and caspase 3/7 activity increased, responses that were significantly enhanced when the cells underwent H/R (Figures 1A and 1B). The addition of 10% fetal calf serum was sufficient to prevent these

responses under either normoxic or H/R conditions. Since H/R performed in the absence of serum induced the most significant alteration in H9c2 cell survival and caspase 3/7 activity, these conditions were used for all subsequent experiments. As seen in Figure 1C, pretreatment of the H9c2 cells with AVP significantly attenuated the H/R-induced changes in both cell survival and caspase 3/7 activity in a concentration-dependent manner.

[00188] To confirm the role for the cardiac-expressed AVP-receptor ($V_{1A}R$) in mediating AVP-dependent protection against cell death, H9c2 cells underwent H/R in the presence or absence of AVP (10 nM) and the $V_{1A}R$ selective antagonist, SR49059 (0.1 μM). Antagonism of $V_{1A}R$ with SR49059 abolished the ability of AVP to reduce both cell death and caspase 3/7 activity (Figures 1D and 1E). Neither $V_{1B}R$- nor V_2R-selective antagonists (SSR14941 and OPC41061, respectively) had an effect on the cellular response to AVP (Figure 6). Additionally, to assess the potential role for MEK1/2-ERK1/2 signaling in mediating the protective effect of AVP, H9c2 cells were treated with the MEK1/2 inhibitor PD98059 (10 μM) prior to H/R induction. Inhibition of MEK1/2 completely blocked the AVP-mediated effects on cell survival and caspase 3/7 activity (Figures 1D and 1E).

[00189] *AVP-mediated ERK1/2 phosphorylation dynamics:* Since MEK1/2 inhibition abolished the ability of AVP to enhance cell survival during H/R, it was sought to define the ERK1/2 signaling response to AVP. AVP stimulation of H9c2 cells for 5 min elicited ERK1/2 phosphorylation (P-ERK1/2) in a concentration-dependent manner (Figure 2A) with an EC_{50} of 12.5 ±1.3 nM. This response could only be blocked by $V_{1A}R$-selective antagonism, but not by $V_{1B}R$- or V_2R-selective antagonism. The IC_{50} of SR49059 ($V_{1A}R$), SSR14941 ($V_{1B}R$), and OPC41061 (V_2R) for inhibition of AVP (10 nM)-induced P-ERK1/2 were 0.38 ± 0.08, 104.4 ±10.2, and 374.9 ± 23.4 nM, respectively (Figure 3B). ERK1/2 signaling often persists long after receptor stimulation, therefore the P-ERK1/2 response to AVP was assessed at both acute (2-10 min) and prolonged (30-60 min) timepoints. AVP-induced P-ERK1/2 peaked at 5 min and was sustained at ~50% peak activation even to 60 min post-stimulation (Figure 2C). Pretreatment of H9c2 cells with the MEK1/2 inhibitor PD98059 (10 μM) significantly attenuated both the acute and prolonged P-ERK1/2 responses to AVP stimulation (Figure 2C). Thus far, these results demonstrate that $V_{1A}R$ stimulation enhances H9c2 cell survival via MEK1/2-ERK1/2 signaling.

[00190] *Inhibition of Gαq protein/PKC signaling blunts the AVP-induced acute P-ERK1/2 response but has no effect on the AVP-induced cell survival response:* GPCR stimulation induces

the simultaneous activation of both G protein-dependent and -independent signaling pathways that can each act to modulate ERK1/2 activity (Tilley D.G. (2011) *Circ. Res* 109(2):217-230). Since $V_{1A}R$ -Gαq protein coupling induces the activation of protein kinase C (PKC), regulating the hypertrophic gene program (Li X., *et al.* (2011) *Circulation* 124(5):572-581), it was tested whether Gαq protein/PKC-mediated signaling is involved in AVP-induced P-ERK1/2 and cell survival responses. Overexpression of the peptide inhibitor of Gαq (GqI) inhibited the acute P-ERK1/2 response to AVP (2 and 5 min, by 90±3% and 52±6%, respectively), but had no effect on the AVP-induced prolonged responses at 30 and 60 min (Figures 3A & 3B). Further, suppression of Gαq protein by GqI overexpression did not impact AVP-induced effects on caspase3/7 activity or cell viability (Figure 3C). Similarly, pretreatment of H9c2 cells with the PKC inhibitor Ro-31 (1 μM) inhibited acute phosphorylation of ERK1/2 (90±6% and 62±3% inhibition at 2 and 5 min, respectively), but had no effect on persistent ERK1/2 phosphorylation (30-60 min) (Figures 3A &3B). Pretreatment of H9c2 cells with Ro-31 prior to H/R led to a significant enhancement of H/R-induced cell death and caspase 3/7 activity (Figure 7). Interestingly, despite the enhanced negative effect on survival mediated by chronic inhibition of PKC by Ro-31, AVP was still capable of inducing a concentration-dependent reduction in cell death and caspase 3/7 activity (Figure 3D). These results provide evidence of a Gαq protein/PKC-*in*dependent component to $V_{1A}R$-mediated cell survival signaling.

[00191] *Overexpression of βARK_{CT} blunts persistent AVP-induced P-ERK1/2 and abrogates AVP-mediated H9c2 cell survival*: To begin to assess the contribution of G protein-independent signaling to $V_{1A}R$ -mediated P-ERK1/2 and survival responses in H9c2 cells, βARK_{CT}, the c-terminal peptide of GRK2 that prevents its recruitment and activation upon GPCR stimulation (Koch W.J. *et al.* (1994) *J. Biol. Chem.* 269(8):6193-6197) was overexpressed, or β-gal as a control. Overexpression of βARK_{CT} predominantly blunted the persistent P-ERK1/2 responses at 30 and 60min by 45±2% and 39±4%, respectively (Figures 4A and 4B). Additionally, AVP-elicited cell survival was completely abolished by overexpression of βARK_{CT} (Figures 4C and 4D). Comparison of the effects of the combined inhibition of AVP signaling by simultaneous treatment of H9c2 cells with Ro-31 and βARK_{CT} were consistent with these results. The combination of both Ro-31 and βARK_{CT} did not further impact the acute (PKC-sensitive) or persistent (primarily GRK2-sensitive) P-ERK1/2 responses (Figures 8A and 8B) or cell survival responses (Figures 8C and 8D) to AVP.

[00192] In relation to its inhibition of GRK2, βARK_{CT} acts to sequester Gβγ subunits, thus to determine if the AVP-mediated P-ERK1/2 and survival responses are Gβγ-sensitive, H9c2 cells underwent P-ERK1/2 and H/R assays following pretreatment with the Gβγ inhibitor gallein (Lehmann D.M. *et al.* (2008) *Mol. Pharm.* 73(2):410-418). Gallein neither suppressed AVP-elicited P-ERK1/2 even at high concentrations, nor altered the timecourse of AVP-induced P-ERK1/2 (Figures 4A and 4B, and Supplemental Figures 9A and 9B). Further, pretreatment of the cells with gallein did not block AVP-evoked cell survival responses under conditions of H/R (Figure 9C and 9D). These results indicate a role for GRK2-dependent $V_{1A}R$ signaling in the promotion of ERK1/2 phosphorylation and cell survival under conditions of cellular stress.

[00193] *siRNA-mediated deletion of GRK2 and β-arrestin1, but not GRK5, blunts persistent AVP-induced P-ERK1/2 and cell survival responses in H9c2 cells:* To confirm the role of GRK2 in mediating the effects of AVP on persistent ERK1/2 phosphorylation and survival in H9c2 cells, siRNA-mediated knockdown of GRK2 was performed versus a scrambled siRNA control. After a 48hr knockdown, GRK2 protein expression was significantly reduced by 68.5 ± 6.5% (Figures 5A and 5D), at which timepoint the P-ERK1/2 response to AVP was assessed. Consistent with the effects of βARKct on $V_{1A}R$ -mediated signaling, deletion of GRK2 significantly inhibited the persistent P-ERK1/2 response to AVP (Figures 5A and 5E). After 96 hr, including the H/R assay, GRK2 deletion was shown to impair the ability of AVP to promote H9c2 survival during H/R (Figures 5F and 5G). Of note, the H/R procedure did not alter siRNA-mediated silencing of GRK2 in the presence or absence of AVP (Figure 10). In addition to GRK2, GRK5 is the other predominant GRK cardiac isoform that has been shown to modulate ERK1/2 activity and survival signaling. (Huang Z.M. *et al.* (2102) *Front. Biosci.* 17:3047-3060). To determine if GRK5 contributes to G protein-independent effects of AVP on ERK1/2 phosphorylation and cell survival, GRK5 siRNA-mediated knockdown experiment were performed. Although knockdown of GRK5 protein expression was attained to a similar extent as that of GRK2 (Figures 5B and 5D), neither the P-ERK1/2 response (Figures 5B and 5E) nor the survival response (Figures 5F and 5G) to AVP were altered in comparison to the scrambled siRNA controls.

[00194] β-arrestins are recruited to GRK-phosphorylated GPCR to induce a number of cellular processes including persistent ERK1/2 activation, and promotes cardiac protection under conditions of stress (Kim KS, *et al.* (2012) *American J. Physiology.* 303(8):H1001-1010; Noma

T, *et al.* (2007) *J Clin Invest* 117(9):2445-2458; Luttrell LM, *et al.* (2001) *Proc Natl Acad Sci* U S A 98(5):2449-2454). Thus, it was sought to determine whether β-arrestins are required for the $V_{1A}R$ -GRK2-dependent effects on ERK1/2 and survival signaling. β-arrestin1 is the predominant isoform in H9c2 cells (Figure 11), thus the cells were transfected with β-arrestin1-selective versus scrambled siRNA. 48 hours later, β-arrestin1 protein expression was significantly reduced by 79.5 ± 3.5% (Figures 5C and 5D). As observed with GRK2 knockdown, deletion of β-arrestin1 inhibited the AVP-induced P-ERK1/2 response, again primarily at the persistent (30-60 min) timepoints (Figures 5C and 5E). Further, β-arrestin1 knockdown significantly impaired the ability of AVP to promote H9c2 cell survival and decrease caspase 3/7 activity during H/R (Figures 5F and 5G). Altogether, these results reveal that under conditions of hypoxic stress, engagement of a $V_{1A}R$ -GRK2-β-arrestin1-ERK1/2-mediated signaling pathway promotes survival.

[00195] *Discussion*

[00196] G protein-independent signaling through GRKs and β-arrestins mediate a number of beneficial effects on cardiac function and survival during heart failure (Kim K.S *et al.* (2012) *J. Clin Invest* 117(9):2455-2458). GRKs were initially identified as desensitizing regulators of GPCR, terminating their acute signaling responses to agonist stimulation via phosphorylation of the C-terminal tail of the receptor. It has since been demonstrated that beyond desensitization, GRK also initiate G protein-*in*dependent signaling events that act to regulate ERK1/2 activity, for instance via the recruitment of the scaffolding proteins β-arrestins through differential phosphorylation of GPCRs (Heitzler D. *et al.*(2102) *Mol. Sys. Biol.* 8:590). These events commonly promote more persistent ERK1/2 activity than that mediated by G proteins, and have been demonstrated to impact numerous cellular processes, including regulation of apoptosis and cell survival in numerous cells and, importantly, in the heart.

[00197] Although relatively little was known about the pathophysiologic effects of cardiac-expressed $V_{1A}R$, the inventors' study utilizing transgenic mice with inducible cardiac-restricted overexpression of $V_{1A}R$ was the first to conclusively demonstrate that enhanced $V_{1A}R$ signaling in the heart itself directly leads to the development of left ventricular hypertrophy, dilatation, diminished contractile performance and reprograming of the HF gene profile in a $G\alpha_q$ protein-dependent manner (Li X. *et al.* (2011) *Circulation* 124(5):572-581), though a role for Gαq protein-independent signaling in the control of cardiac function and/or survival was not

investigated. In the present study, using H9c2 myoblasts as an *in vitro* model of hypoxia/reoxygenation, H/R, it was demonstrated that AVP enhances cell survival during stress in a V_{1A}R-GRK2-β-arrestin1-ERK1/2-dependent manner. H9c2 myoblasts, derived from embryonic rat ventricle, were an excellent model system in which to directly test the effects of V_{1A}R signaling during *in vitro* stress as they express cardiac and skeletal isoforms of L-type Ca^{2+} channels, sarcolemmal ATPase splice variants characteristic of a normal heart and endogenous V_{1A}R, which respond to AVP with typical $G\alpha_q$ protein-coupled receptor responses.

[00198] This finding that V_{1A}R-mediated cell survival is mediated via G protein-independent regulation of ERK1/2 activity is consistent with other reports describing the molecular mechanisms of GPCR-induced survival signaling, including the cardiac-expressed β-adrenergic receptors (βAR) and the angiotensin type 1A receptor (AT1R). As with the V_{1A}R, the AT1R is a $G\alpha_q$-coupled receptor, and the survival signaling response to AT1R stimulation depends not on rapid $G\alpha_q$ protein-mediated ERK1/2 signaling, but on prolonged GRK/β-arrestin-mediated ERK1/2 signaling (Kim KS, *et al.* (2012). *American J. Physiology. Heart and Circulatory Physiology* 303(8):H1001-1010; Ahn S. *et al.* (2009). *J Biol Chem* 284(13):8855-8865). The data herein, demonstrates that this pattern of ERK1/2 activation is similar for V_{1A}R in H9c2 myoblasts, wherein Gαq protein/PKC-dependent signaling mediates the acute P-ERK1/2 response and GRK2-β-arrestin1-dependent signaling primarily regulates the persistent phase of the P-ERK1/2 response. This could account for the previous observation in the V_{1A}R transgenic mice that although inhibition of Gαq protein blocked the development of the heart failure phenotype, it only partially inhibited activation of ERK1/2 (Li X, *et al.* (2011). *Circulation* 124(5):572-581). Thus, although ERK1/2 signaling is required for the AVP-mediated survival response, the G protein-dependent cohort did not contribute to this effect.

[00199] A distinctive finding in this study is that GRK2, not GRK5, is responsible for driving the β-arrestin1-ERK1/2 signaling response to AVP, whereas previous studies have found that GRK2 mainly induces GPCR desensitization while other GRK isoforms, including GRK5, are responsible for mediating β-arrestin-dependent ERK1/2 signaling responses (Noma T, *et al.* (2007). *J Clin Invest* 117(9):2445-2458; Heitzler D, *et al.* (2012). *Mol. Sys. Biol.* 8:590). This is an important finding as inhibition of GRK2 is cardioprotective in models of heart failure (Lymperopoulos A, *et al.* (2012) *Curr. Pharm. Des.* 18(2):186-191), but could limit the ability of V_{1A}R to exert survival signaling. In fact, in light of the findings herein, inhibition of GRK2 could

exacerbate negative $V_{1A}R$-mediated effects by preventing receptor desensitization and augmenting deleterious $G\alpha_q$ protein-dependent signaling during heart failure in which there are elevated levels of circulating AVP.

[00200] The importance of understanding the relative contributions of both G protein-dependent and -independent pathways in the regulation of cell survival mechanisms has been recently highlighted in a number of studies investigating biased GPCR ligands, that is, ligands that have agonist-like effects but that are biased toward the activation of one particular pathway over another. A number of AT1R biased agonists that lack the ability to activate $G\alpha_q$ protein-dependent signaling but do promote GRK/β-arrestin-dependent ERK1/2 signaling have been shown capable of regulating apoptotic pathways *in vitro* and enhancing cardiac contractility and improved cardiac performance *in vivo*, one of which is undergoing Phase II evaluation for the treatment of heart failure (Violin JD, *et al.* (2010). *J Pharmacol Exp Ther* 335(3):572-579; Boerrigter G, *et al.* (2011) *Circ Heart Fail* 4(6):770-778). Late-stage heart failure is often associated with high levels of AVP that has been associated with symptomatic hyponatremia and increased mortality. However, the V2-selective antagonist tolvaptan had no effect on survival and actually increased circulating levels of AVP (Lanfear DE, *et al.* (2013) *Circulation. Heart failure* 6(1):47-52) while the V2-selective antagonist lixivaptan was associated with increased mortality in patients hospitalized with acute heart failure and hyponatremia. The present data provide evidence that the use of a biased ligand that blocks both V_2R and $V_{1A}R$-Gαq protein-dependent signaling, while at the same time activating $V_{1A}R$-mediated GRK2/β-arrestin signaling would provide a novel new therapeutic for the treatment of patients with chronic heart failure and elevated levels of AVP.

Example 2: Increased Vasopressin 1A Receptor Expression in Failing Human Heart

[00201] Plasma levels of the neurohormone arginine vasopressin (AVP) are increased in patients with heart failure (HF) and there is a direct relationship between a rise in AVP levels and increased morbidity and mortality. AVP activates a family of distinct G protein-coupled receptors: V1A receptors (V1A-R) in the heart and vasculature are coupled to Gq and V2 receptors (V2-R) in the renal parenchymal cells are coupled to Gs. Activation of V2-Rs causes reabsorption of free water leading to hyponatremia. Because of the clinical relevance of hyponatremia, basic science investigations have focused on the biology of the V2-R and V2-

selective antagonists have been developed for the treatment of both euvolemic and hypervolemic hyponatremia. Studies, conducted by the inventors, in transgenic mice with cardiac-restricted and controlled over-expression of the V1A-R demonstrate that V1A-mediated signaling can recapitulate the heart failure phenotype. However, little was known about the regulation of the V1A-R in normal or failing heart. The present study was therefore undertaken to evaluate the expression of V1A-R in failing and non-failing human heart.

[00202] Human left ventricular myocardium was obtained from 25 subjects with end-stage heart failure undergoing heart transplant (19 male, 6 female, age 51.2 ± 2.7) or from 9 organ donors (1 male, 8 female, age 60 ±2.9 years) whose hearts were unsuitable for donation owing to blood type, age or size incompatibility. Nine transplant recipients had HF secondary to ischemic cardiomyopathy, 10 had non-ischemic cardiomyopathy and one had HF secondary to valvular heart disease. All had severe left ventricular dysfunction: the mean left ventricular ejection fraction was 11.9 ± 0.8%. Eleven of the transplant recipients were receiving dobutamine at the time of surgery, 10 were receiving milrinone and 8 were receiving both milrinone and dobutamine. The non-failing hearts from the organ donors demonstrated normal left ventricular function by echocardiography (EF 60.4 ± 2.2%) . Tissue aliquots were removed from the left ventricular free wall and rapidly placed in liquid nitrogen and stored at -70°C as described previously (Bristow, MR, Feldman AM, *JCI*, 92, 2737,1993). The Temple University Institutional Review Board approved the study and consent was obtained for all subjects.

[00203] Reverse-transcribed cDNA from human myocardial mRNA was used to determine the expression of human V1A (*AVPR1A*) using ribosomal protein S18 (*RPS18*) as a reference for normalization of gene expression data. cDNA was reverse transcribed from 1 μg of total RNA extracted from the human left ventricular myocardium with RNeasy Fibrous Tissue Midi Kit (Qiagen, Valencia, CA). The primers for *AVPRIA* were F- 5'-CTTGAAGGAGATGGCCACTAAA-3' (SEQ ID NO: 1) and R- 5'-GTGATCGTGACGGCTTACAT-3' (SEQ ID NO: 2). The primers for *RS18* were F- 5'-CTTTGCCATCACTGCCATTAAG-3' (SEQ ID NO: 3) and R - 5'-ATCACACGTTCCACCTCATC-3' (SEQ ID NO: 4). Analysis of gene expression was performed using the delta-delta CT method (Applied Biosystems, Carlsbad, CA) to calculate relative quantitation (RQ) values.

[00204] Membrane for radioligand binding assays were prepared as previously described. (Böhm M, *et al.*, *Effects of xamoterol on inotropic and lusitropic properties of the human myocardium and on adenylate cyclase activity. Am Heart* J. 1990, 120(6 Pt 1):1381-92). The protein concentration was determined by the method of Lowry. Cleaned and minced myocardium was homogenized in ice-cold buffer (mmol/L: Tris-HCL 10, pH 7.4, EDTA 10) with a Polytron homogenizer (Brinkmann) at a setting of 9, two times for 10 sec and once for 5 sec. The homogenate was filtered through three layers of cheesecloth and centrifuged at 1000g for 10 min at 4°C. The supernatant was filtered through two layers of cheesecloth and centrifuged at 45,000g for 30 min at 4°C to yield membranes. The plasma membrane pellet was re-suspended to give a final concentration of 1 mg/mL protein with binding buffer (mmol/L: Tris 50, EDTA 1, pH 7.4). The protein concentration was determined by the method of Lowry.

[00205] V1A receptors levels were measured by saturation of radioligand ^{125}I-p-AVP (PerkinElmer, NEX310010UC, Waltham, MA) binding. Membrane preparations (approximately 40 μg protein) were incubated with ^{125}Ip-AVP (Perkin Elmer, NEX3011UC, Waltham, MA: 4 to 300 pmol/L) in buffer (mmol/L: Tris 50, EDTA 5, 0.1% BSA) either alone or with 5 μmol/L of the V1A selective blocker SR49059 which was used for determination of nonspecific binding. The incubation was carried out at 25°C for 2 hours in a volume of 100 μL. The reaction was terminated by the addition of ice-cold incubation buffer and rapid vacuum filtration through glass fiber filters (Whatman GF/C, Brandel, Inc). Each filter was washed three times with 7 ml of ice-cold 10 mM Tris-HCl plus 0.1% BSA. The radioactivity of the wet filters was determined in a Gamma counter. All assays were performed in duplicate. Receptor density was normalized to membrane protein. The dissociation constant (K_d) and the maximal number of binding sites (B_{max}) for ^{125}I-p-AVP were determined by Scatchard analysis of saturation binding isotherms with PrismGraph.

[00206] As seen in Figures 12A-12C, the density of V1A-Rs was significantly increased in failing human heart when compared with non-failing controls without a change in the affinity of the ligand for the receptor. That the change in V1A-R density was due to an increase in receptor expression was supported by the fact that there was a comparable increase in the levels of the mRNA encoding the V1A-R. (Figure 12A, 12B and 12C). This represents the first report demonstrating changes in V1A-R expression in either a model of HF or in the failing human heart. AVP-mediated signaling has maladaptive effects on the heart independent of effects on

the vasculature. Patients on LVAD support were excluded from the study. Interestingly, our findings are in marked contrast with the decrease in the expression of B1-adrenergic and angiotensin type 1 receptors that characterize animal models of HF and the failing human heart, including patients receiving inotropic support. (Asano K, *Circulation* 1997;95:1193-1200). Further studies are ongoing to better understand the molecular mechanisms responsible for the increase in AVP-R expression in failing human heart and in models of heart failure. None the less, these results evidence that increased expression of the V1A-R adversely affect cardiac function in patients with end stage disease and therefore may provide a new therapeutic target in patients with acute HF.

Example 3: β-Adrenergic receptor and Vasopressin interactions

[00207] Patients admitted to the hospital with an exacerbation of heart failure are characterized by an increase in sympathetic drive as evidenced by an increase in circulating levels of norepinephrine. Norepinephrine binds to both β1- and β2-adrenergic receptors in the heart in order to increase cardiac contractility. Patients admitted to the hospital with worsening symptoms of heart failure also have increased levels of the neurohormone arginine vasopressin (AVP); however, the role of AVP in patients admitted with an acute exacerbation of heart failure was heretofore, less well known. For example, it has been controversial as to whether the increased levels of AVP have a major effect on the vasculature and cause vasoconstriction or whether they have a direct effect on the heart. Indeed, most information comes from transgenic mice in which the V1A receptor is over-expressed just in the muscle cells of the heart.

[00208] Without wishing to be bound by theory, it was hypothesized that AVP can have a deleterious effect on the heart. In summary, it was found for the first time, that AVP markedly decreases the ability of adrenergic drive (norepinephrine) to augment cardiac contractility. The first information to support this finding is seen in Figures 13A, 13B. AVP reduced the ability of isoproterenol to bind to the β-Adrenergic receptor and decreased the ability of isoproterenol to mobilize calcium in isolated adult myocytes. These findings were important as they evidence that high levels of AVP can inhibit the ability of the adrenergic nervous system to support cardiac contractility and can explain why patients admitted with an exacerbation of heart failure do not respond to exogenous adrenergic agonists such as dobutamine or dopamine.

[00209] As seen in Figures 14A-14D, AVP decreases the ability of isoproterenol to activate adenylyl cyclase and to therefore increase cellular levels of cyclic AMP. This can be seen in both HEK cells that are transfected with the V1A receptor as well as in adult feline myocytes. Since cyclic AMP is a major mediator of adrenergic (isoproterenol)-mediated cardiac contractility. These results further evidence that AVP negatively impacts patients with acute heart failure.

[00210] Finally, as seen in Figures 15A-15C, it was shown in isolated murine hearts that infusions of norepinephrine will significantly increase cardiac contractility. However, if the hearts are pre-treated with AVP, or if hearts are studied in which the V1A receptor is over-expressed, the increase in contractility elicited by norepinephrine is substantially attenuated.

[00211] Taken together, this data provides evidence that cardiac contractility is markedly diminished by elevated levels of AVP and by the marked increase in V1A receptors in the failing human heart. AVP not only inhibits cardiac contractility through activation of Gq, but more importantly it inhibits the ability of norepinephrine to support the failing heart. For the first time, it was shown that the negative effect of AVP and the high levels of V1A receptors can only be treated by blocking the effects of AVP with a highly selective AVP antagonist in patients who are hospitalized with an acute exacerbation of heart failure. The traditional/common treatment for this group of patients – a beta-adrenergic receptor agonist such as dobutamine or dopamine– will not work because its effects are blocked by the high levels of AVP. Thus, the ideal pharmacologic management of patients with an acute exacerbation of heart failure and elevated levels of AVP would be, for example, administering a V1A antagonist, or to combine a V1A receptor antagonist with a beta adrenergic receptor agonist, or administer a V1A antagonist followed up at some point in the treatment with a beta adrenergic receptor agonist. There are various ways in which to combine the treatments.

Example 4: The $V_{1A}R$ -selective antagonist SR 49059 preserves cardiac contractile function and restores both $V_{1A}R$ and βAR expression levels during the development of pressure overload-induced hypertrophy in vivo

[00212] We have previously reported that $V_{1A}R$ expression is increased in end-stage human heart failure and that mice with cardiac-restricted inducible overexpression of $V_{1A}R$ ($V_{1A}R$ -TG) undergo progressive development of cardiomyopathy and decreased βAR responsiveness with

age. To determine whether changes in endogenous $V_{1A}R$ expression are associated with the development of cardiomyopathy and βAR dysfunction, WT mice underwent TAC in conjunction with osmotic minipump-mediated delivery of the $V_{1A}R$ -selective antagonist SR 49059. As expected, TAC decreased cardiac function as monitored by echocardiography and increased cardiac hypertrophy, however co-administration of SR 49059 preserved both fractional shortening (Fig. 16A) and ejection fraction (Fig. 16B) without impacting hypertrophy (Figure 17). SR 49059 administration in sham-operated mice did not impact cardiac function or hypertrophy. Cardiac $V_{1A}R$ and βAR expression were also assessed in each of the surgical and treatment groups via radioligand binding. TAC induced a significant 2-fold increase in $V_{1A}R$ expression, similar to that observed in human hearts, which was inhibited by SR 49059 (Fig. 16C). βAR downregulation is associated with the development and progression of heart failure21, and in response to 7 weeks of TAC in this study was reduced by ~30% (Fig. 16D). Interestingly, concomitant treatment of TAC mice with SR 49059 completely restored cardiac βAR expression to normal levels. These results confirmed the involvement of endogenous $V_{1A}R$ signaling in the progression of heart failure and indicated that a loss of βAR density in the presence of chronically enhanced $V_{1A}R$ expression may account for the diminished βAR responsiveness we previously observed in $V_{1A}R$ -TG mice.

Example 5: AVP negatively regulates endogenous cardiac βAR activation

[00213] To begin to understand a possible influence of cardiac-expressed $V_{1A}R$ on βAR signaling more acutely, we began to study the molecular relationship between these receptors. Initially, we performed ^{125}I-CYP competition binding analysis with increasing concentrations of isoproterenol (ISO) ± AVP in membrane preparations from the hearts of adult mice. Although AVP alone did not alter ^{125}I-CYP binding to βAR, the addition of AVP induced a significant rightward shift in the ability of ISO to displace ^{125}I-CYP from βAR (IC_{50} of ISO from 0.14μM to 1.4μM, $p<0.0001$ [2-tailed t-test], Fig. 18A), indicative of a loss in affinity of βAR for ISO. Loss of affinity for agonist binding can be associated with a loss in receptor coupling to downstream G protein-dependent signaling, therefore an alteration in βAR/Gs protein-dependent signaling would be expected with AVP. Since Gs protein-dependent Ca2+ mobilization is enhanced downstream of βAR in cardiomyocytes, we measured field stimulation-induced Ca2+ transient responses in Fura2-loaded adult mouse left ventricular myocytes in the presence or absence of

AVP and ISO (Fig. 18B-E). AVP alone did not alter Ca2+ handling even at μmolar concentrations (Figure 19), while ISO alone induced a substantial increase in the magnitude of the Ca2+ transients. Although, AVP did not evoke a change in Ca2+ mobilization alone, ISO-induced Ca2+ transients were significantly reduced in the presence of AVP, as summarized in Figure 18F.

[00214] βAR-induced Ca2+ transients occur in response to Gs protein-dependent generation of cAMP, thus we tested whether AVP stimulation impacts ISO-mediated cAMP production in adult feline left ventricular myocytes (AFVM) infected with adenovirus encoding the fluorescent cAMP biosensor ICUE323. Stimulation of AFVM with ISO produced only a small increase in cAMP production (Fig. 20A). Since βAR-dependent cAMP signaling is tightly controlled by phosphodiesterase 4 (PDE4) variants, we pretreated AFVM with the PDE4-selective antagonist rolipram to unmask the signal. Indeed, pretreatment of the cells with rolipram greatly enhanced the ISO-induced cAMP generation (Fig. 20B). Treatment of AFVM with AVP one minute prior to ISO addition blocked the cAMP response regardless of PDE4 inhibition (Fig. 20C), suggesting that $V_{1A}R$ stimulation impacted βAR signaling at least at the level of cAMP generation and not cAMP degradation. To test whether cAMP generation was altered by AVP, AFVM were stimulated with forskolin, a direct activator of adenylyl cyclase, in the absence (Fig. 3D) or presence (Fig. 20E) of AVP. As summarized in Figure 20F, although AVP significantly reduced ISO-dependent cAMP generation, AVP did not alter the ability of AFVM to produce cAMP in response to direct activation of adenylyl cyclase, further suggesting that $V_{1A}R$ stimulation impacted βAR at the receptor-G protein level.

Example 6: $V_{1A}R$ -mediated regulation of βAR signaling is Gq protein-independent

[00215] To assess the mechanism by which $V_{1A}R$ stimulation decreases βAR signaling, we used HEK 293 cells stably expressing β1AR and transiently expressing $V_{1A}R$ and either ICUE3 or the fluorescent diacylglycerol (DAG) biosensor DAGR. In these cells, ISO stimulation rapidly enhanced cAMP production (Fig. 21A) while AVP had no impact on cAMP production on its own (Fig. 21B). As observed in AFVM, AVP pretreatment greatly reduced the ISO-mediated cAMP production (Fig. 21C), and similar to the binding data above, AVP induced a competitive rightward shift in ISO-mediated cAMP generation (Fig. 21D), altering the EC_{50} of ISO-dependent cAMP generation from 43 pM to 256 pM. To confirm that AVP effects on βAR signaling occur through $V_{1A}R$, cells were pretreated with the $V_{1A}R$ -selective antagonist SR

49059. SR 49059 completely blocked AVP-dependent DAG production (Fig. 21E) and did not prevent ISO-mediated cAMP production in the absence of AVP (Fig. 21F). However, SR 49059 pretreatment was able to restore ISO-dependent cAMP production in the presence of AVP (Fig. 21G). Gq protein-dependent signaling has been shown to mediate βAR desensitization, therefore we investigated whether $V_{1A}R$ -Gq protein signaling accounted for the effects of AVP on βAR-mediated cAMP generation. We used the small molecule inhibitor of Gq protein UBO-QIC27 to assess the impact of Gq protein-dependent $V_{1A}R$ signaling on βAR responsiveness. As expected, UBO-QIC blocked AVP-Gq protein-dependent DAG production (Fig. 21H) and did not impact ISO-mediated cAMP production alone (Fig. 21I). UBO-QIC pretreatment was unable to restore ISO-mediated cAMP responsiveness in the presence of AVP (Fig. 21J). Thus, although AVP signaling through $V_{1A}R$ negatively regulated β1AR-dependent cAMP formation, it did so in a Gq protein-independent manner (Fig. 21K).

Example 7: $V_{1A}R$ -mediated regulation of βAR signaling is GRK-dependent

[00216] Since inhibition of Gq protein-dependent $V_{1A}R$ signaling did not ablate the effect of AVP on βAR signaling, we next tested whether Gq protein-independent $V_{1A}R$ signaling through G protein-coupled receptor kinases (GRKs) was responsible. Multiple GRK isoforms have been shown to play distinct roles in mediating receptor signaling responses in different tissues, but generalized ablation of GRKs would impact βAR signaling regardless of the role of $V_{1A}R$ in regulating βAR desensitization. Thus, to specifically explore the impact of GRK-dependent $V_{1A}R$ signaling, we developed a mutant $V_{1A}R$ lacking all possible C-terminal GRK phosphorylation sites (GRK- $V_{1A}R$, Fig. 22A, Table 1).

Table 1: GRK phosphorylation-deficient $V_{1A}R$ primers

Phospho-site	**Nucleotide mutations**	**Sequence** (sense top/antisense bottom)
S352	a1054g, g1055c	5'-gctgtaatccctggatatacatgttttttgctggccatctccttca-3' (SEQ ID NO: 5) 5'-tgaaggagatggccagcaaaaaacatgtatatccagggattacagc-3' (SEQ ID NO: 6)
S362	a1084g, g1085c	5'-ccttcaagactgtgttcaagccttcccatgctgccaaaac-3' (SEQ ID NO: 7) 5'-gttttggcagcatgggaaggcttgaacacagtcttgaagg-3' (SEQ ID NO: 8)
T378, S380	a1132g, a1138g, g1139c	5'-acatgaaggaaaaattcaacaaagaagatgctgacgctatgagcagaagacagacttttttattc-3' (SEQ ID NO: 9) 5'-gaataaaaagtctgtcttctgctcatagcgtcagcatcttctttgttgaatttttccttcatgt-3' (SEQ ID NO: 10)
S382	a1144g, g1145c	5'-aagaagatgctgacgctatggccagaagacaggctttttatg-3' (SEQ ID NO: 11) 5'-cataaaaagcctgtcttctggccatagcgtcagcatcttctt-3' (SEQ ID NO: 12)

T386, S389	a1156g, t1165g	5'-agaagatactgacagtatggccagaagacaggctttttatgctaacaatcgaagcccaac-3' (SEQ ID NO: 13) 5'-gttgggcttcgattgttagcataaaaagcctgtcttctggccatactgtcagtatcttct-3' (SEQ ID NO: 14)
S393, T395	a1177g, g1178c, a1183g	5'-cacatacccgcagcgtttgctggggctcgattgttagcataaaaa-3' (SEQ ID NO: 15) 5'-tttttatgctaacaatcgagccccagcaaacgctgcgggtatgtg-3' (SEQ ID NO: 16)
S397, T398	a1189g, g1190c, a1192g	5'-aacaatcgaagcccaacaaacgctgcgggtatgtggaaggactc-3' (SEQ ID NO: 17) 5'-gagtccttccacatacccgcagcgtttgttgggcttcgattgtt-3' (SEQ ID NO: 18)
S404, S407, S408, S410	t1210g, t1219g, t1222g, t1228g	5'-cgggtatgtggaaggacgcgcctaaagctgccaaggccatcaaattcattcct-3' (SEQ ID NO: 19) 5'-aggaatgaatttgatggccttggcagctttaggcgcgtccttccacatacccg-3' (SEQ ID NO: 20)
S417, T418	t1249g, a1252g	5'-ccatcaaattcattcctgttgcagcttgactcgagtctagaggg-3' (SEQ ID NO: 21) 5'-ccctctagactcgagtcaagctgcaacaggaatgaatttgatgg-3'(SEQ ID NO: 22)

[00217] Transfection of HEK 293 cells stably expressing β1AR with either the wild-type (WT) $V_{1A}R$ or the GRK- $V_{1A}R$ mutant resulted in similar levels of membrane expression and affinity for AVP (Figure 23). While stimulation of the WT $V_{1A}R$ (Fig. 22B) and GRK- $V_{1A}R$ (Fig. 22C) with AVP each induced rapid DAG formation, the GRK- $V_{1A}R$ caused a significantly greater accumulation of DAG (Fig. 22D), consistent with prolonged Gq protein-dependent activity of the receptor in the absence of GRK phosphorylation. Interestingly, in contrast to the WT $V_{1A}R$, where AVP stimulation reduced ISO-mediated cAMP generation (Fig. 21C), AVP stimulation of the GRK- $V_{1A}R$ did not decrease βAR-dependent cAMP production. Rather GRK- $V_{1A}R$ stimulation significantly enhanced ISO-mediated cAMP formation, an effect that was blocked by Gq protein inhibition with UBO-QIC (Fig. 22E, and summarized in Fig. 22F), suggesting that in the absence of GRK-dependent regulation of $V_{1A}R$ signaling, Gq protein-dependent $V_{1A}R$ signaling promotes βAR sensitivity to adrenergic stimulation. These results also suggested that when both Gq protein- and GRK-dependent $V_{1A}R$ signaling mechanisms are present, the GRK-dependent branch is dominant with regard to the regulation of βAR responsiveness.

Example 8: Both endogenous and overexpressed $V_{1A}R$ decrease βAR-dependent ex vivo cardiac contractility

[00218] Since ISO-induced Ca2+ transients were diminished in the presence of AVP, and we previously reported diminished ISO-dependent cardiac contractility in hearts with $V_{1A}R$ overexpression, we sought to determine the acute impact of $V_{1A}R$ stimulation on ISO responsiveness. To assess the impact of $V_{1A}R$ signaling on βAR function in the whole heart, ex

vivo Langendorff analysis was performed using WT versus $V_{1A}R$ -TG mouse hearts. $V_{1A}R$ -TG hearts contain approximately 5-fold higher expression of $V_{1A}R$ than WT hearts but with a similar affinity for AVP (Figure 25). Initially, contractility, including LVDP, +dP/dt and -dP/dt, was compared between WT and $V_{1A}R$ -TG hearts in response to increasing concentration of the non-selective PDE inhibitor IBMX. While IBMX infusion at higher concentrations increased contractility in WT hearts, $V_{1A}R$ -TG hearts were resistant to IBMX even at the highest concentrations tested (Figs. 24A-C), suggesting that basal cAMP generation in the heart is suppressed by $V_{1A}R$ overexpression. To determine if adenylyl cyclase activity is negatively regulated by $V_{1A}R$ signaling, WT hearts underwent infusion with forskolin in the presence or absence of 1nM AVP pretreatment. In accordance with the FRET experiments, pretreatment of WT hearts with AVP had little to no significant impact on forskolin-mediated effects on contractility (Figs. 24D-F), confirming that in the whole heart, $V_{1A}R$ -mediated effects on contractile signaling occur proximal to adenylyl cyclase activation.

[00219] Next, to directly assess βAR responsiveness, hearts were perfused with sequentially increasing concentrations of ISO alone (WT and $V_{1A}R$ -TG) or with 1 nM AVP pretreatment (WT + AVP). In comparison to the WT hearts alone, both $V_{1A}R$ -TG hearts and WT+AVP hearts produced significantly diminished responses to ISO perfusion, including LVDP, +dP/dt and -dP/dt (Figs. 26A-C), suggesting that cardiac overexpression of $V_{1A}R$ is sufficient to mimic the effects of exogenous addition of AVP on βAR responsiveness. The decrease in ISO responsiveness could not be explained by independent AVP effects, as AVP alone produced only a small concentration-dependent decrease in LVDP, +dP/dt and -dP/dt (Figure 27) that does not account for all of the diminished ISO responses observed. Additionally, we tested the effect of AVP on ISO responsiveness in isolated hearts of mice overexpressing the Gq protein inhibitory peptide GqI. Similar to the FRET data above where Gq protein inhibition was unable to block the AVP-mediated effect on ISO-induced cAMP formation, GqI overexpression did not prevent AVP from dampening ISO-mediated contractility in the isolated hearts (Figs. 26A-C). In fact, GqI overexpression resulted in a more statistically significant AVP-dependent ablation of ISO-mediated contractility compared to WT+Veh-treated hearts than in the WT+AVP or $V_{1A}R$ -TG hearts. Altogether our data suggest a role for cardiac $V_{1A}R$ -mediated desensitization of βAR signaling via a novel Gq protein-independent, GRK-dependent mechanism.

[00220] In this study we demonstrated for the first time that AVP is a potent inhibitor of βAR signaling in cultured adult cardiomyocytes and in a murine ex vivo Langendorff preparation through a GRK-dependent but Gq protein-independent manner. Furthermore, we showed that mice with HF secondary to TAC recapitulate the $V_{1A}R$ molecular phenotype found in humans with HF: a two-fold increase in myocardial $V_{1A}R$ density and decreased βAR expression. Interestingly, while the hypertrophic response to TAC was unchanged in the presence of the $V_{1A}R$ antagonist SR 49059, likely due to enhanced activity of other neurohormone pathways, SR 49059 normalized expression levels of both $V_{1A}R$ and βAR and significantly improved the ejection fraction in TAC mice compared with vehicle controls.

[00221] Using multiple approaches, we confirmed that $V_{1A}R$ signaling inhibits βAR activity through a GRK-dependent, but Gq protein-independent, signaling pathway. For example, pharmacologic inhibition of Gq protein by UBO-QIC had no effect on the ability of AVP to inhibit β1AR-mediated cAMP accumulation; however, over-expression of a mutant $V_{1A}R$ lacking all possible C-terminal GRK phosphorylation sites (GRK- $V_{1A}R$) not only blocked the AVP effect, but led to an enhancement of βAR-dependent cAMP accumulation. Additionally, genetic ablation of Gq protein activity in GqI-TG mice did not impact the ability of AVP to inhibit βAR responsiveness in isolated perfused hearts, but led to a more significant reduction in βAR-induced cardiac contractility than in mice with intact Gq protein-dependent signaling. The acute effects of AVP- $V_{1A}R$ signaling on βAR-mediated cardiac contractility and the chronic effects of AVP- $V_{1A}R$ signaling on cardiac morphology appear to occur by different mechanisms: the former through GRK-dependent signaling and the latter through Gq protein-dependent signaling.

[00222] Our finding that activation of the $V_{1A}R$ inhibited cardiac βAR-mediated cAMP signaling through a GRK-dependent but Gq protein-independent pathway contrasts with studies of other cardiac GPCRs. For instance, although both the angiotensin II type 1A receptor (AT1R) and $V_{1A}R$ have been shown to enhance V2R-Gs protein-mediated cAMP formation in Cho cells, this effect was Gq protein-dependent. Similarly, β2AR-mediated cAMP formation was increased by stimulation of several Gq protein-coupled receptors in cardiac fibroblasts, effects that have been attributed to PKC-dependent effects such as enhanced Gs protein-adenylyl cyclase coupling. Although desensitization of one GPCR upon activation of another distinct GPCR has been shown, for instance opioid receptor-like 1 (ORL1)-mediated desensitization of

the μ opioid receptor and M3 muscarinic receptor-mediated desensitization of β2AR, these effects have also been shown to be PKC-dependent, even when involving GRK signaling33, 34. Similarly, while mechanical stretch activates the AT1R with subsequent up-regulation of GRK2 and reduction in βAR signaling in neonatal rat ventricular myocytes, inhibition of either AT1R-Gq protein coupling or PKC-dependent phosphorylation of GRK2 restored normal βAR signaling35. Here, GRK-mediated regulation of βAR signaling occurred even in the presence of Gq protein inhibition and we only observed a $V_{1A}R$ -mediated increase in βAR signaling when all possible C-terminal GRK phosphorylation sites were mutated to alanine. Our results suggest that GRK-dependent $V_{1A}R$ signaling predominately reduces βAR activity and that βAR-enhancing Gq protein-dependent $V_{1A}R$ effects are either not normally present or unable to significantly impact βAR activity in the presence of intact V1AR coupling to GRK.

Example 9: Materials and Methods

[00223] *Materials*. Arginine vasopressin (V9879), dimethyl sulfoxide (D4540), forskolin (F6886), 3-isobutyl-1-methylxanthine (I5879), isoproterenol (I6504), rolipram (R6520) and SR 49059 (S5701) were purchased from Sigma-Aldrich (St. Louis, MO). Xtremegene 9 DNA transfection reagent was purchased from Roche Applied Science (Indianapolis, IN). UBO-QIC was purchased from Prof. Evi Kostenis, University of Bonn, Germany and dissolved to 1 mM in DMSO.

[00224] *Animals*. Transgenic (TG) mice used for the experiments described herein were developed by the investigators and maintained in the vivarium of the Temple University School of Medicine. All experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and were approved by the Institutional Animal Care and Use Committee at Temple University (ACUP#4031). TG mice included: GqI-TG mice with cardiac-restricted over-expression of a peptide derived from a carboxyl-terminal peptide of the α-subunit of Gq protein; and V1AR-TG mice with controlled and cardiac-restricted over-expression of the V1AR15. Wild-type (WT, 75%C57Bl6/J/25%FVB) littermate controls we used where appropriate.

[00225] *Transverse Aortic Constriction (TAC)*. Pressure overload was produced by transverse aortic constriction (TAC) as follows. In brief, after anesthesia with isoflurane (2.5%), the chest was opened and an aortic band was created in 8 wk old wild-type mice by placing a ligature (7-0

nylon suture) securely between the origin of the right innominate and left common carotid arteries with a 27-gauge needle as a guide. The sham procedure was identical except that the aorta was not ligated. Post-procedure the chest was closed and the animal was allowed to recover after anesthesia. Doppler velocity was measured in the right (RCA) and the left (LCA) carotid arteries and RCA/LCA velocity ratio was calculated to ensure that TAC produced equal aortic pressure gradient in all experimental groups.

[00226] *Subcutaneous implantation of ALZET minipumps.* 1 week after sham or TAC surgery, the mice were anesthetized with isoflurane (1.5-2.5%) and underwent subcutaneous implantation with minipumps (ALZET, Cupertino CA, model #2006) containing SR49059 (1 mg/kg/day) or vehicle control (0.1% DMSO in sterile saline) and were subsequently monitored for 6 weeks.

[00227] *Echocardiography.* Global left ventricular (LV) function was evaluated in mice before TAC and at weekly intervals after TAC using a VisualSonic Vevo 770 imaging system and a 707 scan head (Miami, FL) as previously described45. In brief, after mice were lightly sedated with isoflurane (2.0%), a parasternal short-axis view was obtained for LV M-mode imaging at the papillary muscle level. Three independent M-mode images were used for measurements of LV end-diastolic internal diameter (LVEDD) and LV end-systolic internal diameter (LVESD) in two consecutive beats according to the American Society of Echocardiography leading edge method. Fractional shortening (FS) was calculated as FS% = [(LVEDD – LVESD)/LVEDD]×100. Anterior (AWT) and Posterior Wall thickness (PWT) were also measured.

[00228] *Cardiac membrane preparation and radioligand binding for V1AR and βAR.* Membrane preparation and radioligand binding assays were performed as follows. In brief, cleaned and minced myocardium was homogenized in ice-cold buffer (mmol/L: Tris-HCl 10, pH 7.4, EDTA 10) with a Polytron homogenizer (Brinkmann Instruments). The homogenate was filtered through three layers of cheesecloth and centrifuged at 1000 X g for 10 minutes at 4°C. The supernatant was then filtered through two layers of cheesecloth and centrifuged at 45,000 X g for 30 minutes at 4°C to yield membranes. The plasma membrane pellet was re-suspended to give a final concentration of 1 mg/mL protein with binding buffer (mmol/L: Tris 50, EDTA 1, pH 7.4). The protein concentration was determined by the method of Lowry using bovine albumin serum as the standard.

[00229] Membrane preparations (25ug protein for βAR-binding or 40 μg protein for V1AR-binding) were incubated with 125I-cyanopindolol (Cyp, PerkinElmer, Waltham, MA; 4 to 300 pmol/L) or ^{125}I-p-AVP (PerkinElmer, Waltham, MA; 0.67 to 150 pmol/L) in incubation buffer (mmol/L: Tris 50, EDTA 5, 0.1% BSA). Incubations were performed either alone or with propranolol (βAR non-specific antagonist, 10μmol/L) or SR49059 (V1AR-selective antagonist, 5 μmol/L), which were used for determination of nonspecific binding that were then subtracted from total binding for calculation of specific binding. The incubation was carried out at room temperature (25°C) for 2 hours in a total volume of 250μL (βAR) or 100 μL (V1AR) in which steady state kinetics were achieved in specific binding. The reaction was terminated by the addition of ice-cold incubation buffer and rapid vacuum filtration through glass fiber filters (Whatman GF/C, Brandel, Inc). Each filter was washed three times with an additional 7 ml of ice-cold 10 mmol/L Tris-HCl plus 0.1% BSA. The radioactivity of the wet filters was determined in a Gamma counter. All assays were performed in duplicate. Receptor density was normalized to milligrams of membrane protein. Kd and the maximal number of binding sites (Bmax) were determined by Scatchard analysis of saturation binding isotherms with PrismGraph.

[00230] *Isolation of adult murine cardiac myocytes and intracellular Ca2+ transient measurements.* Cardiac myocytes were isolated from the septum and LV free wall of 8-12 week old male WT mice as follows. Briefly, mice were heparinized (1,500 U/kg ip) and anesthetized (pentobarbital sodium, 50 mg/kg ip). Excised hearts were mounted on a steel cannula and retrograde perfused (100 cmH2O, 37°C) with Ca2+-free bicarbonate buffer followed by enzymatic digestion (collagenases B and D, protease XIV). Isolated myocytes were plated on laminin-coated glass coverslips, and the Ca2+ concentration of the buffer was incrementally increased from 0.05 to 0.125 to 0.25 to 0.5 mmol/L with 10 min of exposure at each Ca2+ concentration. The 0.5 mol/L Ca2+ buffer was then aspirated and replaced with MEM (Sigma-Aldrich) containing 1.2 mmol/L Ca2+, 2.5% FBS, and antibiotics (1% penicillin/streptomycin). The myocytes were exposed to 0.67 μmol/L Fura 2-AM for 15 min at 37°C, then field-stimulated to evoke the intracellular Ca2+ transient (1 Hz, 37°C) in medium 199 containing 1.8 mmol/L extracellular Ca2+ concentration ([Ca2+]o). Intracellular Ca2+ transient measurements were performed as previously described.

[00231] Adult feline left ventricular myocyte (AFVM) isolation and infection. Adult feline left ventricular myocytes were isolated as follows. Briefly, felines were anesthetized with

sodium pentobarbital and hearts were rapidly excised, cannulated, and mounted on a constant flow Langendorff apparatus. Hearts were rinsed with a physiological Krebs-Henseleit buffer (KHB) and then retrograde perfused with collagenase containing KHB. When the tissue softened, the left ventricle was isolated and gently minced, filtered, and equilibrated in KHB with 0.2 mmol/L CaCl2, and 1% bovine serum albumin (BSA) at room temperature. Isolated myocytes were washed with serum-free culture medium (Medium 199, Sigma) supplemented with penicillin-streptomycin-glutamine (Gibco) and seeded on 10mm glass coverslip-containing 35mm culture dishes (MatTek Corporation, MA) coated with laminin (BD Bioscience). $5x10^4$ cells/10mm insert were infected with adenovirus containing the cAMP FRET reporter ICUE323 (Ad-ICUE3) at a multiplicity of infection (MOI) of 40 for 36 hr.

[00232] *Site-directed mutagenesis of human V1AR to attain GRK phosphorylation-deficient V1AR (GRK-V1AR).* The nucleotide sequences encoding the 17 carboxy-terminal serine (S352, S362, S380, S382, S389, S393, S393, S404, S407, S408, S410, S417) and threonine (T378, T386, T395, T398, T418) residues in HA-tagged human V1AR underwent mutagenesis to alanine-encoding sequences using the Stratagene Quikchange II XL Site-Directed Mutagenesis Kit as per manufacturer's instructions. Primers are listed in Table 1.

[00233] *HEK 293 cell culture and transfection.* HEK 293 cells stably expressing FLAG-tagged β1AR25 were grown in 10% FBS and 1% PSF-containing MEM. $5x10^4$ cells/coverslip were seeded on 10mm glass coverslip-containing 35mm dishes as described above and transfected for 24 hr with 1μg WT-V1AR or GRK-V1AR with 1 μg of either ICUE3 or the diacylglycerol FRET reporter (DAGR) using a 3:1 ratio of X-tremeGENE 9 to DNA.

[00234] *Fluorescent resonance energy transfer (FRET) measurements.* AFVM or HEK 293 cells expressing FRET reporters were rinsed and media was replaced with imaging buffer, as previously described, prior to imaging using a Leica DMI4000B inverted microscope with a Leica DFC365 FX 1.4-megapixel monochrome digital camera with CFP excitation and CFP and YFP emissions measured every 2 sec. Cells were pretreated for 5 min with buffer or antagonists. After 30 sec of baseline reads the cells were stimulated with buffer or AVP, followed by ISO at 90 sec. Single cell measurements at 20X magnification were used to assess changes in FRET and each treatment condition was performed in a minimum of 3 independent cell preparations. Quantification of the changes in FRET ratio were calculated as change in CFP emission/YFP emission over time, normalized to baseline.

[00235] *Langendorff perfusion system*. The isolated heart perfusion technique wasperformed as follows. In brief, hearts perfused under constant pressure (80 mmHg) with a solution containing (in mmol/L:) NaCl 13.8, NaHCO3 22, KCl 4.7, KH2PO4 1.2, MgSO4 1.1, glucose 1.1, CaCl2 2, Na pyruvate 2. A balloon was placed in the LV, connected to a Millar pressure transducer and an ADInstruments Physiograph (Colorado Springs, CO) and filled with H2O to set the LV end-diastolic pressure (LVEDP) at 10 mmHg. Hearts were maintained at a temperature of 37 °C and were paced at a rate of 480 beats per min. After a 15 min stabilization period, pharmacologic agents were added to the perfusion media at 5 min intervals. LV pressure (LVP), LVEDP, and the maximum rate of positive and negative change in LV pressure (±LV dP/dt) were recorded. LVDP was calculated by subtracting the LVEDP from the LV systolic pressure. Data were analyzed with LabChart Pro-6.0 (ADInstruments).

[00236] *Statistical Analysis*. A commercial software package was used for statistical analysis (Graph Pad Software Inc). Comparison of means ± SE was analyzed by unpaired t-test or one- or two-way ANOVA followed by Bonferroni test, where appropriate. A value of $P<0.05$ was considered statistically significant.

Example 10: Representative $V_{1A}R$ amino acid sequence (GenBank accession number NP_000697.1 GI:4502331) (SEQ ID NO: 23)

```
1 mrlsagpdag psgnsspwwp latgagntsr eaealgegng pprdvrneel akleiavlav
61 tfavavlgns svllalhrtp rktsrmhlfi rhlsladlav affqvlpqmc wdityrfrgp
121 dwlcrvvkhl qvfgmfasay mlvvmtadry iavchplktl qqparrsrlm iaaawvlsfv
181 lstpqyfvfs mievnnvtka rdcwatfiqp wgsrayvtwm tggifvapvv ilgtcygfic
241 yniwcnvrgk tasrqskgae qagvafqkgf llapcvssvk sisrakirtv kmtfvivtay
301 ivcwapffii qmwsvwdpms vwtesenpti titallgsln sccnpwiymf fsghllqdcv
361 qsfpccqnmk ekfnkedtds msrrqtfysn nrsptnstgm wkdspkssks ikfipvst  SE
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Example 11: vasopressin-neurophysin 2-copeptin preproprotein amino acid sequence (GenBank accession number NP_000481.2 GI:13259533) (SEQ ID NO: 24)

1 mpdtmlpacf lgllafssac yfqncprggk ramsdlelrq clpcgpggkg rcfgpsicca

61 delgcfvgta ealrcqeeny lpspcqsgqk acgsggrcaa fgvccndesc vtepecregf

121 hrrarasdrs natqldgpag alllrlvqla gapepfepaq pday

What is claimed is:

1. A method of identifying a candidate therapeutic agent comprising:

contacting a cell expressing an arginine vasopressin receptor (AVP-R), a G-protein coupled receptor kinase (GRK) and β-arrestin with a candidate therapeutic agent;

assaying for modulation, in the presence or absence of the candidate therapeutic agent, of G-protein and GRK2/beta-arrestin dependent signaling, thereby

identifying a candidate therapeutic agent.

2. The method of claim 1, wherein the arginine vasopressin receptor comprises V1A receptor (V1AR), V2 receptor (V2R), V_{1B} (V3) receptors or combinations thereof.

3. The method of claim 2, wherein the arginine vasopressin receptor is $V_{1A}R$.

4. The method of claim 2, wherein the candidate therapeutic agent inhibits $V_{1A}R$-G protein signaling and simultaneously activates GRK2/beta-arrestin-development.

5. The method of claim 2, wherein a composition comprising a therapeutically effective amount of a combination of two or more candidate therapeutic agents inhibits $V_{1A}R$-G protein signaling and simultaneously activates GRK2/beta-arrestin.

6. The method of claim 2, wherein the candidate therapeutic agent is a non-selective V_{1A}-V2 receptor antagonist whereby the agent inhibits $V_{1A}R$-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin.

7. The method of claim 1, wherein an assay to measure G-protein and GRK2/beta-arrestin dependent signaling comprises: immunoassays, phosphorylation assays, enzyme assays, bioassays, biochip assays, blots, hybridization assays, cell-based assays, high-throughput screening assays, chromatography, chemical assays, phage display assays, lab-on-a-chip, microfluidics based assays, microarrays, microchips, nanotube based assays, colorimetric assays, spectrophotometric assays or combinations thereof.

8. The method of claim 1, wherein signaling is identified by modulation of function, expression or activity of ERK1/2, βARKCT, caspase 3/7, βarrestins, GRKs, angiotensin and receptors thereof, angiotensin type 1A receptor (AT1R), adrenergic receptors, or combinations thereof.

9. A method of identifying a candidate therapeutic agent comprising:

contacting a biological sample with a candidate therapeutic agent wherein the sample comprises a G-protein dependent and a G-protein independent signaling receptor;

assaying for modulation, in the presence or absence of the candidate therapeutic agent, of G-protein dependent signaling and G-protein independent signaling as compared to a baseline control, thereby,

identifying a candidate therapeutic agent.

10. The method of claim 9, wherein the G-protein dependent signaling and G-protein independent signaling is mediated by an arginine vasopressin receptor.

11. The method of claim 10, wherein the arginine vasopressin receptor comprises V_{1A} receptor ($V_{1A}R$), V2 receptor (V2R) , V1B (V3) receptors or combinations thereof.

12. The method of claim 11, wherein the arginine vasopressin receptor is $V_{1A}R$.

13. The method of claim 9, wherein the candidate therapeutic agent inhibits $V_{1A}R$-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin.

14. The method of claim 9, wherein the candidate therapeutic agent is a non-selective V_{1A}-V2 receptor antagonist whereby the agent inhibits $V_{1A}R$-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin.

15. The method of claim 9, wherein a biological sample comprises: fluids, peptides, polypeptides, oligonucleotides, polynucleotides, cells, tissues or combinations thereof.

16. The method of claim 9, wherein an assay to measure G-protein dependent signaling and G-protein independent signaling comprises: immunoassays, phosphorylation assays, enzyme

assays, bioassays, biochip assays, blots, hybridization assays, cell-based assays, high-throughput screening assays, chromatography, chemical assays, phage display assays, lab-on-a-chip, microfluidics based assays, microarrays, microchips, nanotube based assays, colorimetric assays, spectrophotometric assays or combinations thereof.

17. The method of claim 9, wherein signaling is identified by modulation of function, expression or activity of ERK1/2, βARKCT, caspase 3/7, βarrestins, G protein coupled receptor kinase (GRK), angiotensin and receptors thereof, angiotensin type 1A receptor (AT1R), adrenergic receptors, or combinations thereof.

18. A high throughput screening method of identifying a candidate therapeutic agent comprising:

contacting a support surface comprising a G-protein dependent and a G-protein independent signaling molecule, with a candidate therapeutic agent;

assaying for modulation, in the presence or absence of the candidate therapeutic agent, of G-protein dependent signaling and G-protein independent signaling as compared to a baseline control, thereby,

identifying a candidate therapeutic agent.

19. The method of claim 18, wherein the G-protein dependent and G-protein independent signaling molecule comprises: peptides, polypeptides, oligonucleotides, polynucleotides, or combinations thereof.

20. The method of claim 18, wherein the support surface comprises: plastic, glass, beads, fibers, gels, electrochemical detectors, nanotubes, porous strips, paper, matrices or combinations thereof.

21. The method of claim 18, wherein the G-protein dependent signaling and G-protein independent signaling molecule is an arginine vasopressin receptor or fragments thereof.

22. The method of claim 21, wherein the arginine vasopressin receptor comprises V_{1A} receptor ($V_{1A}R$), V2 receptor (V2R) , V1B (V3) receptors or combinations thereof.

23. The method of claim 18, wherein the candidate therapeutic agent inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling.

24. The method of claim 18, wherein an assay to measure G-protein dependent signaling and G-protein independent signaling comprises: immunoassays, phosphorylation, enzyme assays, bioassays, biochip assays, blots, hybridization assays, cell-based assays, high-throughput screening assays, chromatography, chemical assays, phage display assays or combinations thereof.

25. A pharmaceutical composition comprising an agent in a therapeutically effective amount, identified by the methods of claims 1, 9 or 18.

26. The pharmaceutical composition of claim 25, optionally comprising a V2-selective antagonist.

27. A therapeutic agent wherein the agent inhibits signaling of V_{1A}R-associated Gq protein and simultaneously activates V_{1A}R-dependent G-protein coupled receptor kinase (GRK) and β-arrestin.

28. A method of preventing or treating a subject at risk of or suffering from a cardiac disease or disorder comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling.

29. The method of claim 28, further comprising administering a V2 arginine receptor antagonist.

30. A method of preventing or treating a subject at risk of or suffering from a cardiac disease or disorder comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits signaling of V_{1A}R-associated Gq protein while simultaneously activating V_{1A}R-dependent G-protein coupled receptor kinase (GRK) and β-arrestin signaling; thereby, preventing or treating a subject at risk of or suffering a cardiac disease or disorder.

31. A method of preventing or treating a subject at risk of or suffering from heart failure and/or hyponatremia comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits V_{1A}R-G protein signaling and simultaneously activates G-protein coupled receptor kinase (GRK)/beta-arrestin signaling.

32. The method of claim 31, further comprising administering a V2 arginine receptor antagonist.

33. A method of preventing or treating a subject at risk of or suffering from heart failure comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits expression or function of an arginine vasopressin receptor (AVP-R) or fragments thereof.

34. The method of claim 33, wherein the agent wherein the agent is a selective antagonist of V_{1A} receptors.

35. The method of claim 33, wherein the agent inhibits or decreases expression of the V_{1A} receptors.

36. A method of identifying a therapeutic agent for heart failure, comprising: contacting a cell expressing an arginine vasopressin receptor (AVP-R) or fragments thereof, with a candidate therapeutic agent;

assaying for modulation of expression and/or function of AVP-R, in the presence or absence of the candidate therapeutic agent, thereby,

identifying a candidate therapeutic agent.

37. The method of claim 36, wherein the arginine vasopressin receptor comprises V_{1A} receptor (V_{1A}R), V2 receptor (V2R), V1B (V3) receptors or combinations thereof.

38. The method of claim 37, wherein the arginine vasopressin receptor is V_{1A}R.

39. The method of claim 37, wherein the candidate therapeutic agent selectively inhibits $V_{1A}R$ expression and/or function.

40. The method of claim 36, wherein an assay to measure expression and/or function of an arginine vasopressin receptor (AVP-R) or fragments thereof, comprises: immunoassays, phosphorylation assays, enzyme assays, bioassays, biochip assays, blots, hybridization assays, cell-based assays, high-throughput screening assays, chromatography, chemical assays, phage display assays, lab-on-a-chip, microfluidics based assays, microarrays, microchips, nanotube based assays, colorimetric assays, spectrophotometric assays or combinations thereof.

41. A method of identifying a candidate therapeutic agent comprising:

contacting a biological sample with a candidate therapeutic agent wherein the sample comprises arginine vasopressin receptor (AVP-R) molecules;

assaying for modulation of AVP-R expression or function in the presence or absence of the candidate therapeutic agent, as compared to a baseline control, thereby,

identifying a candidate therapeutic agent.

42. The method of claim 41, wherein the arginine vasopressin receptor comprises V_{1A} receptor ($V_{1A}R$), V2 receptor (V2R), V1B (V3) receptor molecules or combinations thereof.

43. The method of claim 42, wherein the arginine vasopressin receptor molecule is $V_{1A}R$.

44. The method of claim 42, wherein the candidate therapeutic agent modulates expression or function of $V_{1A}R$ molecules.

45. The method of claim 42, wherein the $V_{1A}R$ molecules comprise peptides, polypeptides, oligonucleotides, polynucleotides, or combinations thereof.

46. The method of claim 41, wherein a biological sample comprises: fluids, peptides, polypeptides, oligonucleotides, polynucleotides, cells, tissues or combinations thereof.

47. The method of claim 41, wherein an assay to measure $V_{1A}R$ molecule expression and/or function comprises: immunoassays, phosphorylation assays, enzyme assays, bioassays, biochip

assays, blots, hybridization assays, cell-based assays, high-throughput screening assays, chromatography, chemical assays, phage display assays, lab-on-a-chip, microfluidics based assays, microarrays, microchips, nanotube based assays, colorimetric assays, spectrophotometric assays or combinations thereof.

48. A high throughput screening method of identifying a candidate therapeutic agent comprising:

contacting a support surface comprising an arginine vasopressin receptor (AVP-R) molecule with a candidate therapeutic agent;

assaying for modulation, in the presence or absence of the candidate therapeutic agent, for expression or function of the AVP-R molecule as compared to a baseline control, thereby,

identifying a candidate therapeutic agent.

49. The method of claim 48, wherein the arginine vasopressin receptor (AVP-R) molecule comprises: peptides, polypeptides, oligonucleotides, polynucleotides, or combinations thereof.

50. The method of claim 48, wherein the support surface comprises: plastic, glass, beads, fibers, gels, electrochemical detectors, nanotubes, porous strips, paper, matrices or combinations thereof.

51. The method of claim 48, wherein the arginine vasopressin receptor comprises V_{1A} receptor ($V_{1A}R$), V2 receptor (V2R) , V1B (V3) receptors or combinations thereof.

52. The method of claim 51, wherein the candidate therapeutic agent inhibits or decreases $V_{1A}R$ expression or function.

53. A pharmaceutical composition comprising an agent in a therapeutically effective amount, identified by the methods of claims 36, 41 or 48.

54. A therapeutic agent wherein the agent modulates expression or function of an arginine vasopressin receptor (AVP-R) molecule, in vitro or in vivo.

55. The therapeutic agent of claim 54, wherein the agent is an antagonist of V_{1A} receptor molecules.

56. The therapeutic agent of claim 54, wherein the agent inhibits or decreases the expression of the V_{1A} receptor molecules.

57. A method of preventing or treating a subject at risk of or suffering from a cardiac disease or disorder comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits expression or function of an arginine vasopressin receptor (AVP-R) molecule.

58. A method of preventing or treating a subject at risk of or suffering from heart failure and/or hyponatremia comprising: administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of at least one agent which inhibits expression or function of an arginine vasopressin receptor (AVP-R) molecule and/or modulates arginine vasopressin (AVP) and/or copeptin levels.

59. A method of treating heart failure in a patient, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of V_{1A} receptor antagonist and a beta adrenergic receptor agonist.

60. A method of preventing or treating a cardiac disease or disorder in a subject, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V_{1A} receptor antagonist and, optionally as additive therapy for patients in need thereof, at least one beta adrenergic receptor agonist.

61. A method of preventing or treating a cardiac disease or disorder in a subject, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V_{1A} receptor antagonist.

62. The method of 61, wherein a pharmaceutical composition comprising at least one beta adrenergic receptor agonist is optionally administered to the patient.

63. The method of claim 62, wherein the at least one V_{1A} receptor antagonist and at least one beta adrenergic receptor agonist are administered consecutively or at the same time.

64. The method of claim 61, wherein the pharmaceutical composition further comprises at least one beta adrenergic receptor agonist.

65. A method of treating a patient suffering from heart failure and/or hyponatremia, a surrogate marker for elevated arginine vasopressin (AVP), comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V_{1A} receptor antagonist.

66. The method of claim 65, wherein the pharmaceutical composition optionally comprises at least one beta adrenergic receptor agonist.

67. A method of treating a patient having a cardiac disease or disorder, wherein the patient has elevated arginine vasopressin (AVP) and/or copeptin levels as compared to a baseline level, comprising administering a pharmaceutical composition comprising a therapeutically effective amount of at least one V_{1A} receptor antagonist.

68. The method of 67, wherein a pharmaceutical composition comprising at least one beta adrenergic receptor agonist is optionally administered to the patient.

69. A composition comprising at least one V_{1A} receptor antagonist and/or at least one beta adrenergic receptor agonist.

70. A composition comprising at least one V_{1A} receptor antagonist.

71. A method of treating a subject at risk of or suffering from heart failure, the method comprising:

a) identifying a subject in need of treatment;

b) administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a V_{1A} receptor modulating agent.

72. The method of claim 71, wherein the identifying step comprises determining whether the patient has an elevated level of arginine vasopressin or copeptin compared to a reference level.

72. The method of claim 71, wherein the V_{1A} receptor modulating agent comprises a selective V_{1A} receptor antagonist.

73. The method of claim 71, further comprising the step of administering a β-adrenergic receptor agonist.

74. The method of claim 71, further comprising the step of adminiustering a selective V2 receptor antagonist.

75. The method of claim 71 wherein the V_{1A} receptor antagonist, β-adrenergic receptor agonist, and the V2 receptor antagonist are administered simultaneously.

76. The method of claim 71, wherein the subject is human.

76. The method of claim 71, wherein the heart failure is accompanied by hyponatremia.

77. The method of claim 76, wherein the hyponatremia is hypervolemic hyponatremia.

78. The method of claim 76, wherein the hyponatremia is euvolemic hyponatremia.

79. The method of claim 71, wherein the V_{1A} receptor modulating agent is administered until a symptom of heart failure improves.

80. The method of claim 79, wherein the symptom of heart failure comprises fatigue, dyspnea, rapid or irregular heartbeat, or elevated blood pressure.

81. A method of identifying an agent that inhibits the binding of arginine vasopressin or an analogue thereof to the V_{1A} receptor, the method comprising:

a) providing a cell expressing a V_{1A} receptor,

b) contacting the cell with arginine vasopressin or an analogue thereof and a candidate therapeutic agent;

c) determining whether the binding of arginine vasopressin to the V_{1A} receptor is decreased in the presence of the candidate therapeutic agent, wherein a decrease is an indication that the candidate therapeutic agent inhibits the binding of arginine vasopressin or an analogue thereof to the V_{1A} receptor.

82. A method of identifying an agent that modulates the activity of the V_{1A} receptor, the method comprising:

a) providing a cell expressing a V_{1A} receptor,

b) contacting the cell with arginine vasopressin or an analogue thereof and a candidate therapeutic agent;

c) determining whether the activity of the V_{1A} receptor is modulated in the presence of the candidate therapeutic agent, wherein a modulation is an indication that the candidate therapeutic agent modulates the activity of the V_{1A} receptor.

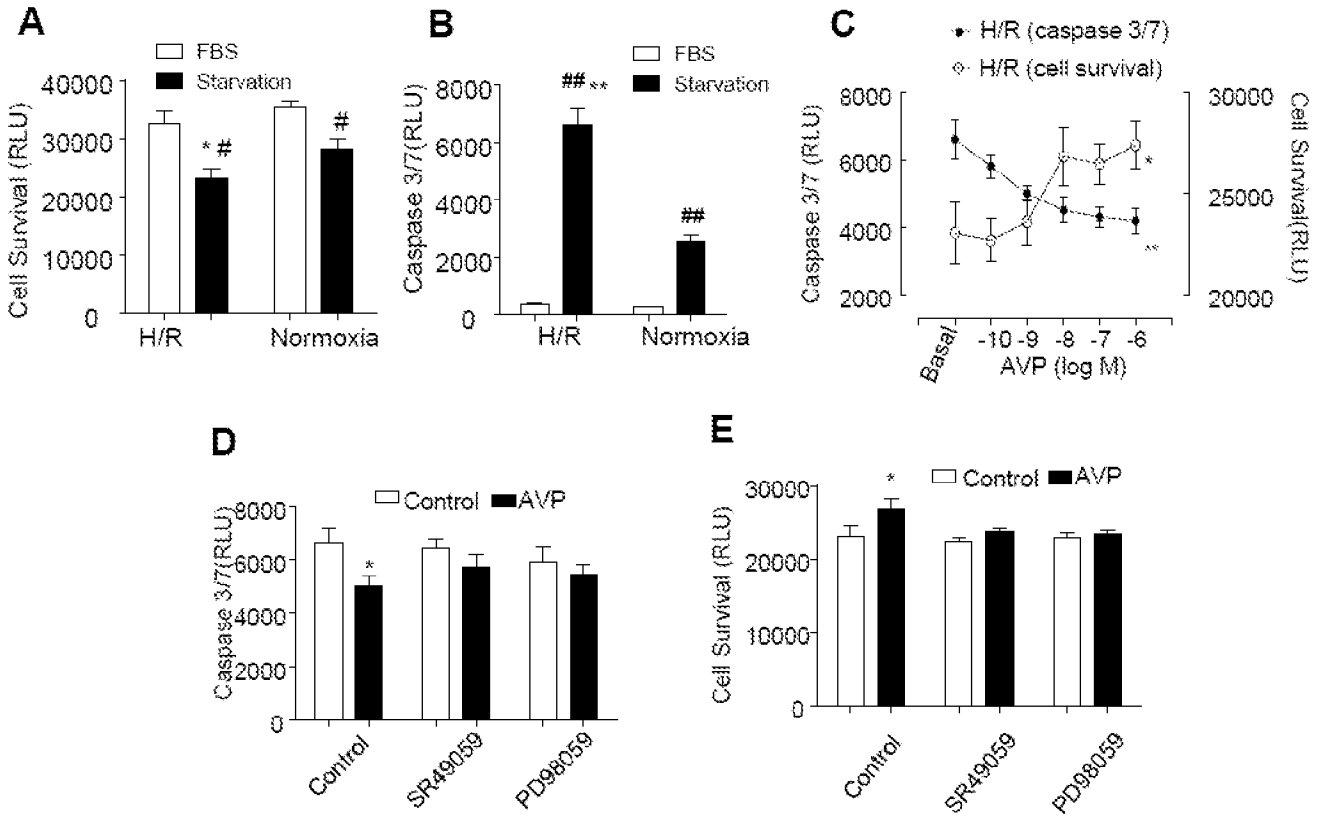
83. The method of claim 82, wherein the activity of the V_{1A} receptor is G-protein coupled receptor kinase dependent signaling.

84. The method of claim 82, wherein the activity of the V_{1A} receptor is V_{1A}R-G protein independent signaling.

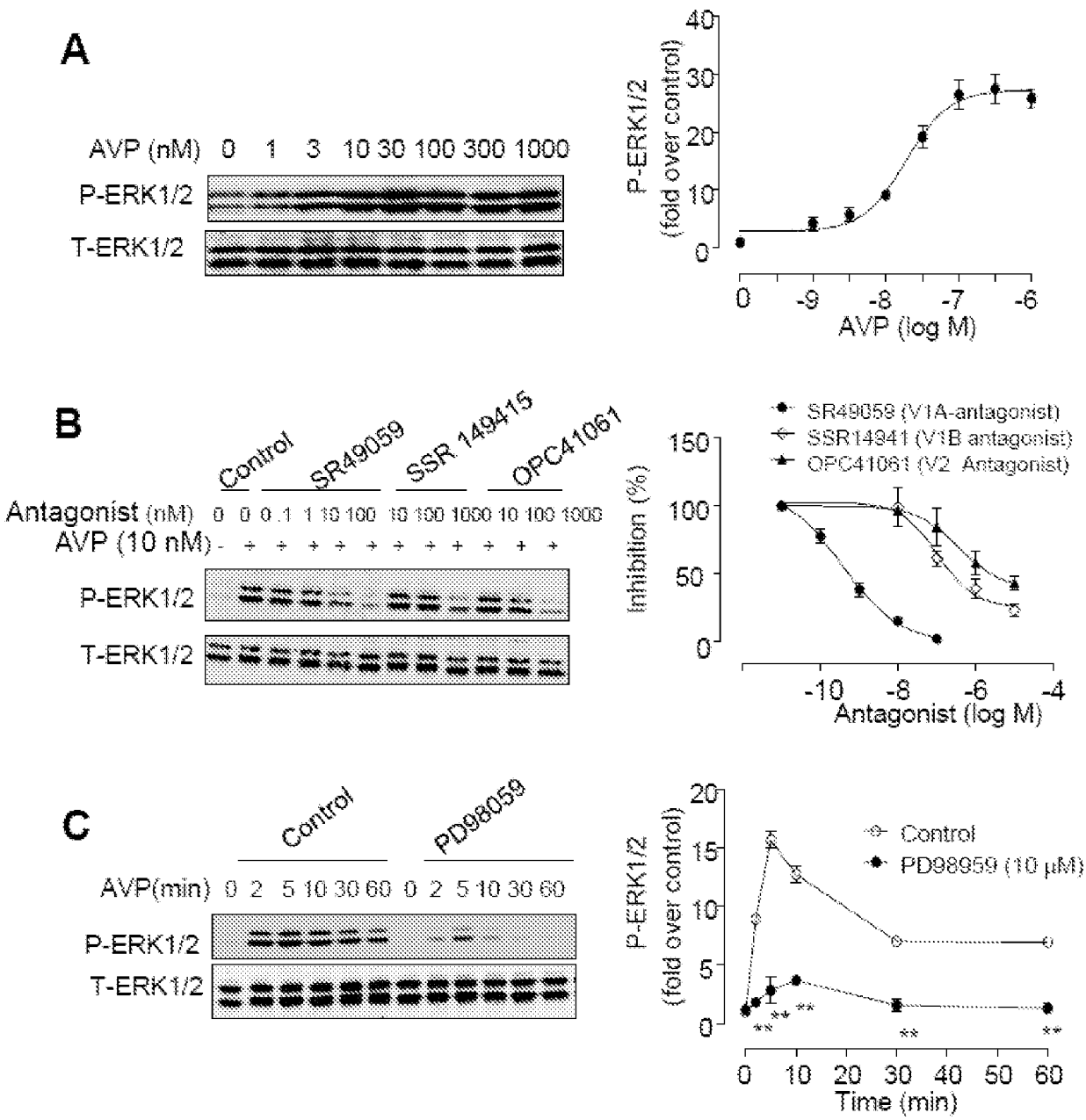
85. The method of claim 83 or 84, wherein the determining step comprises measuring the expression or activity of ERK1/2, βARKCT, caspase 3/7, βarrestins, GRK, angiotensin and receptors thereof, angiotensin type 1A receptor (AT1R), adrenergic receptor, or combinations thereof.

86. The method of claim 85, wherein the adrenergic receptor is the β-adrenergic receptor.

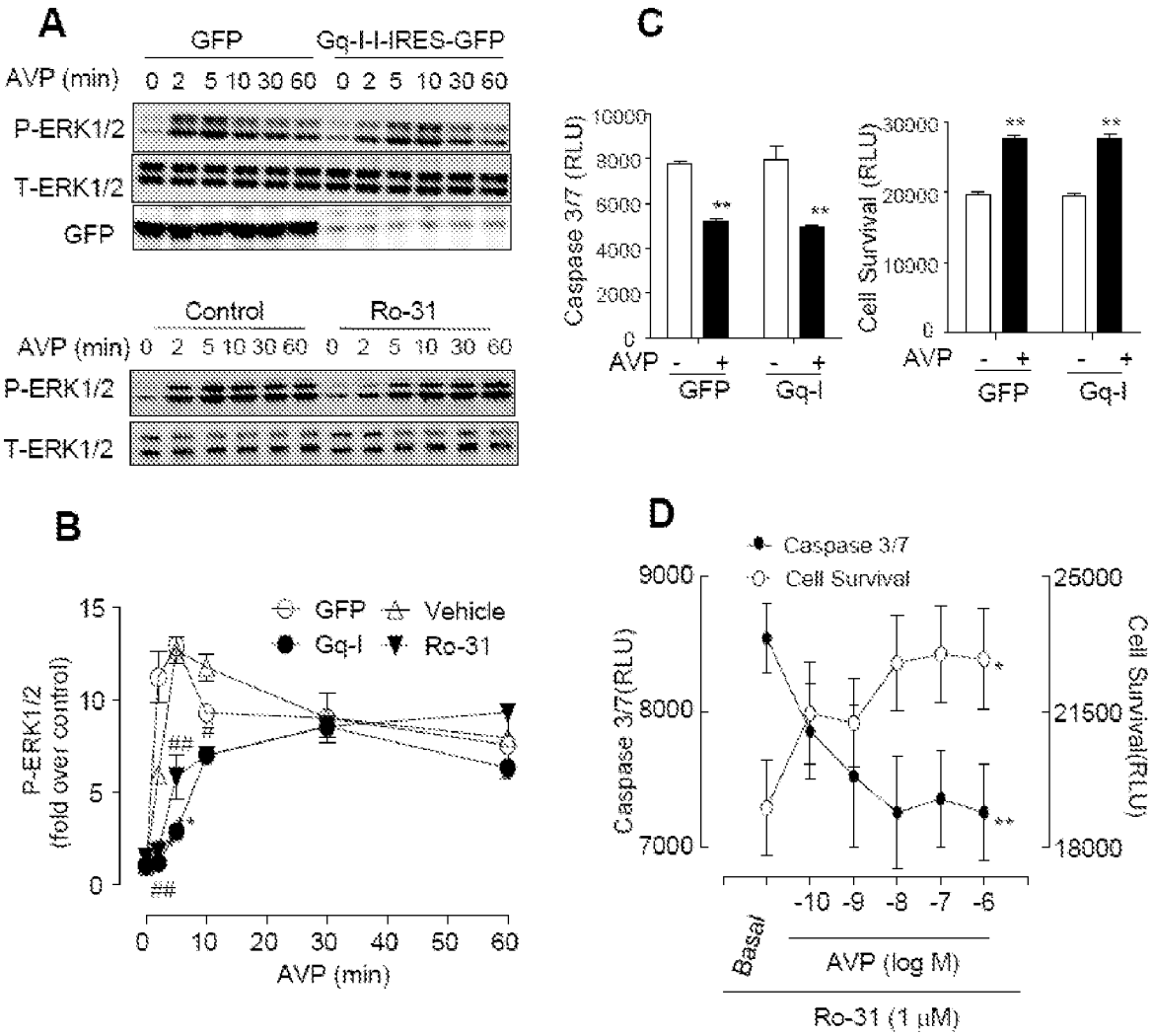
87. The method of claim 82, wherein the method comprises identifying an antagonist of the V_{1A} receptor.



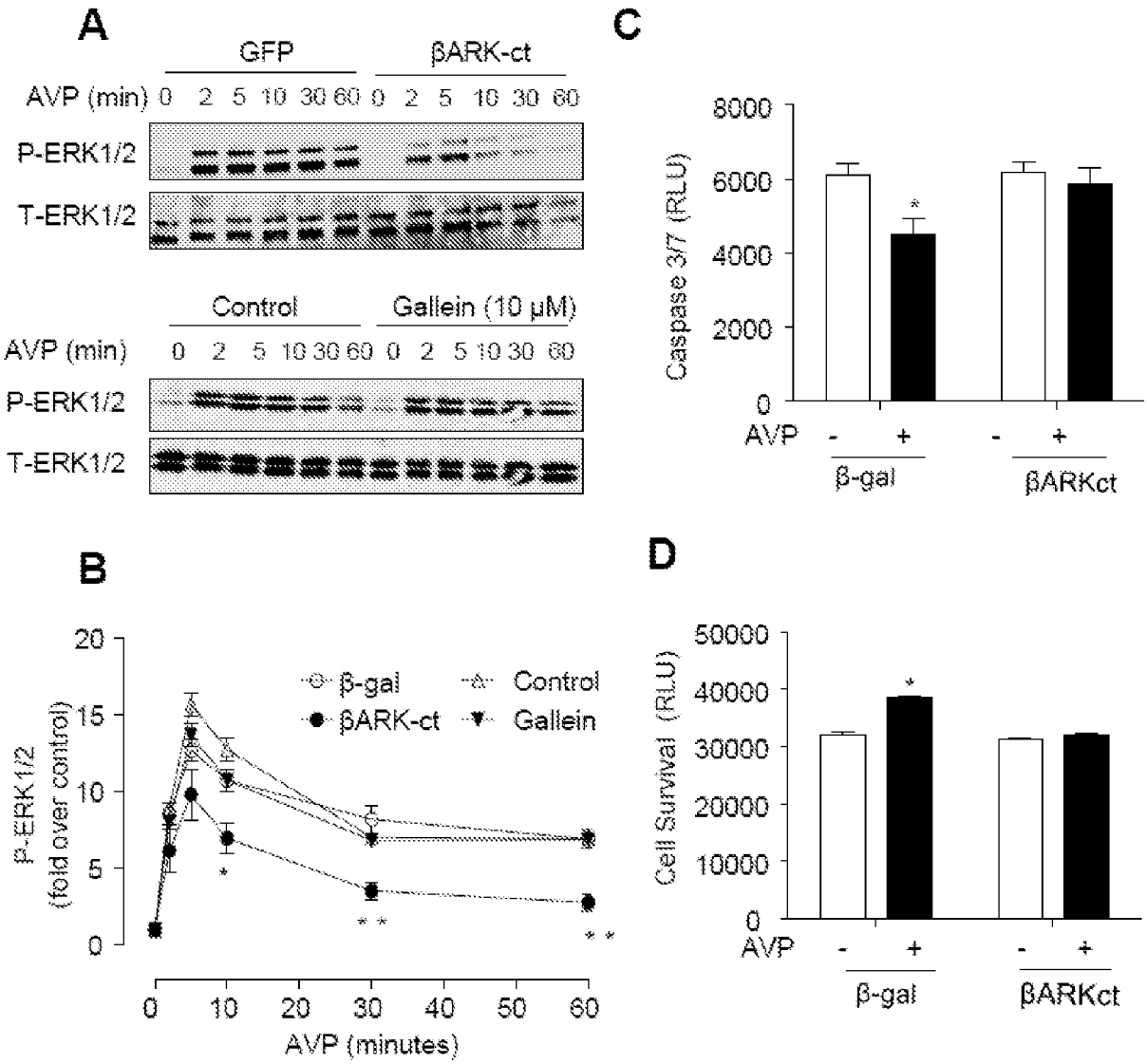
FIGURES 1A-1E



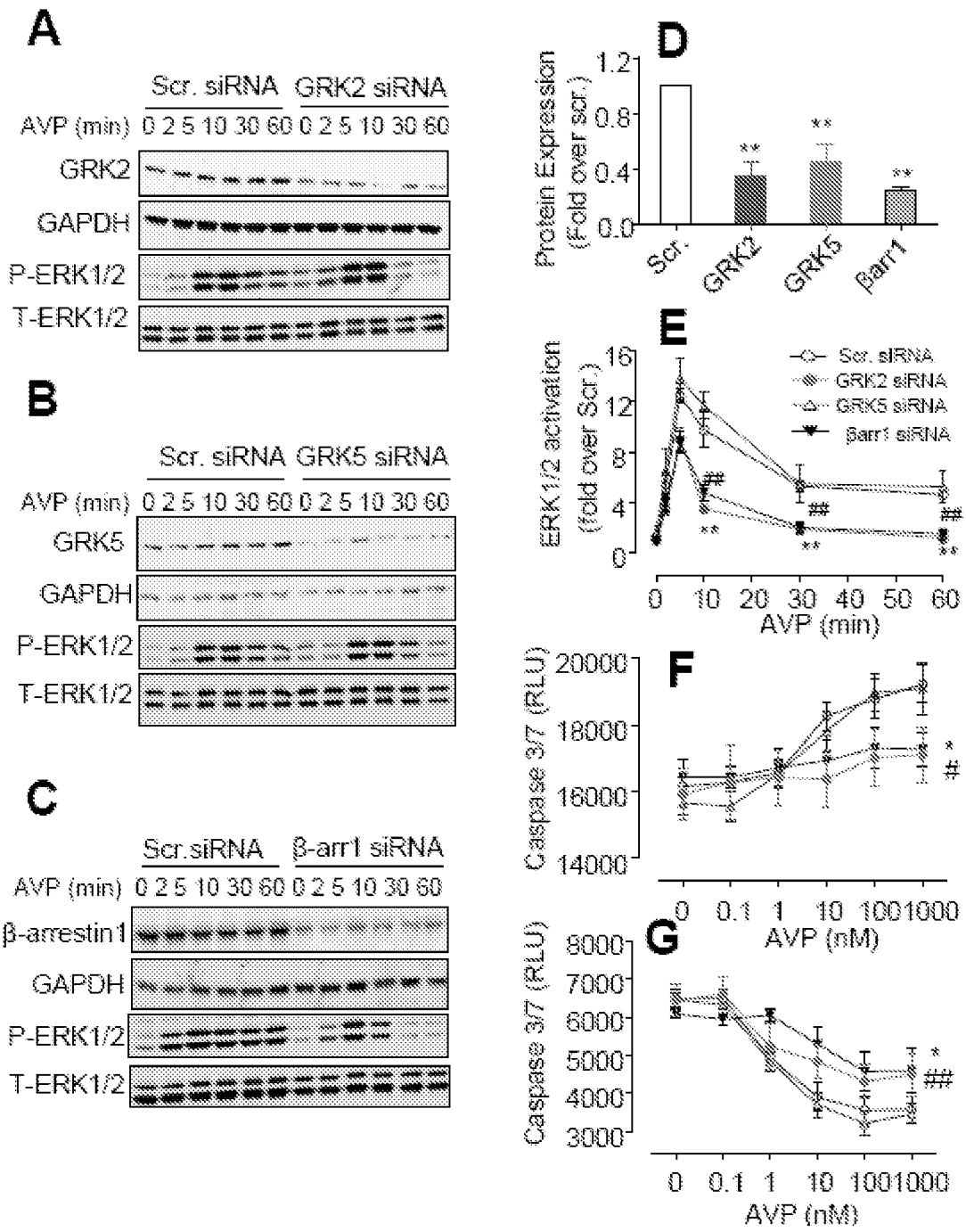
FIGURES 2A-2C



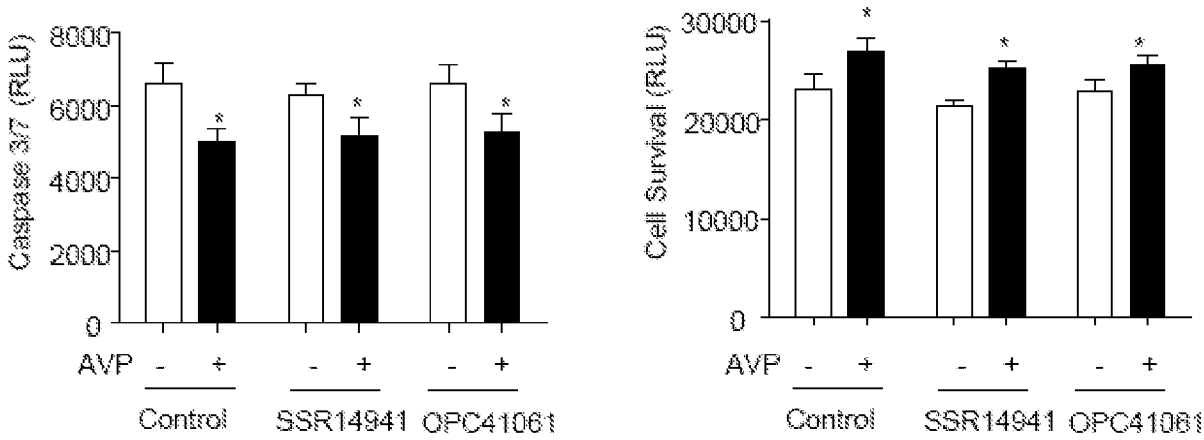
FIGURES 3A-3D



FIGURES 4A-4D



FIGURES 5A-5G



A. B.

FIGURES 6A, 6B

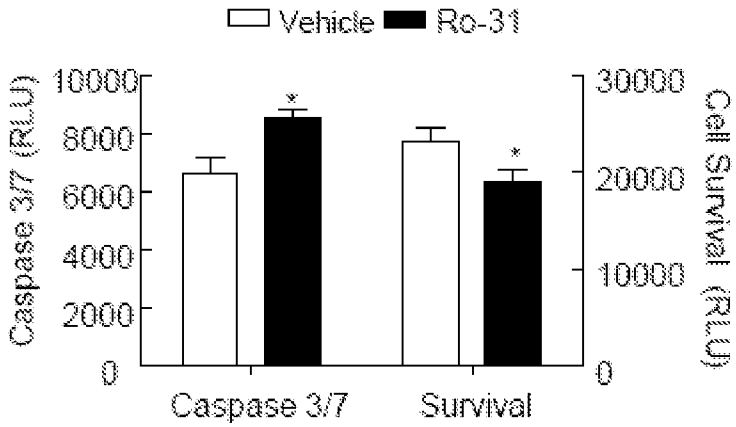
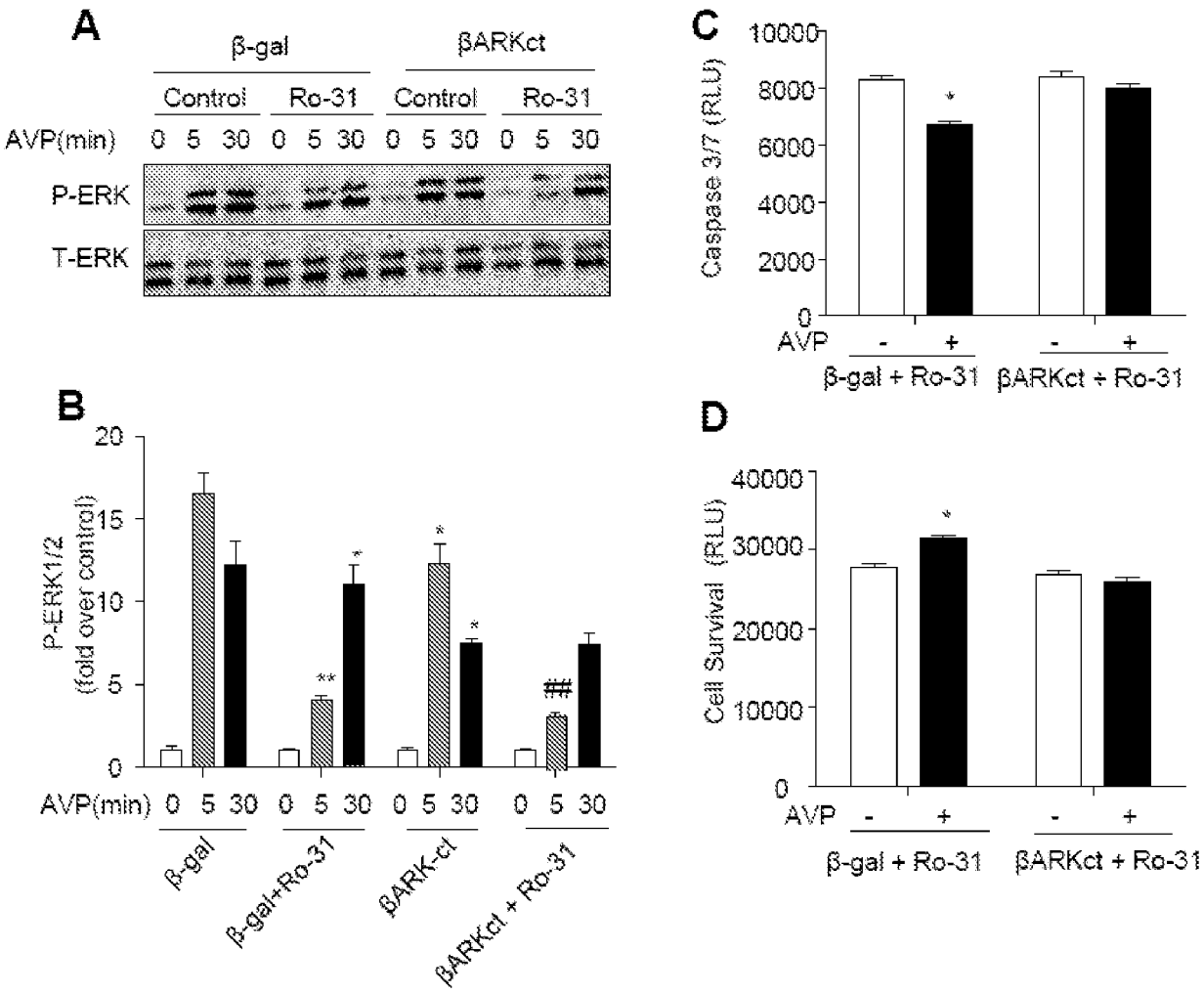
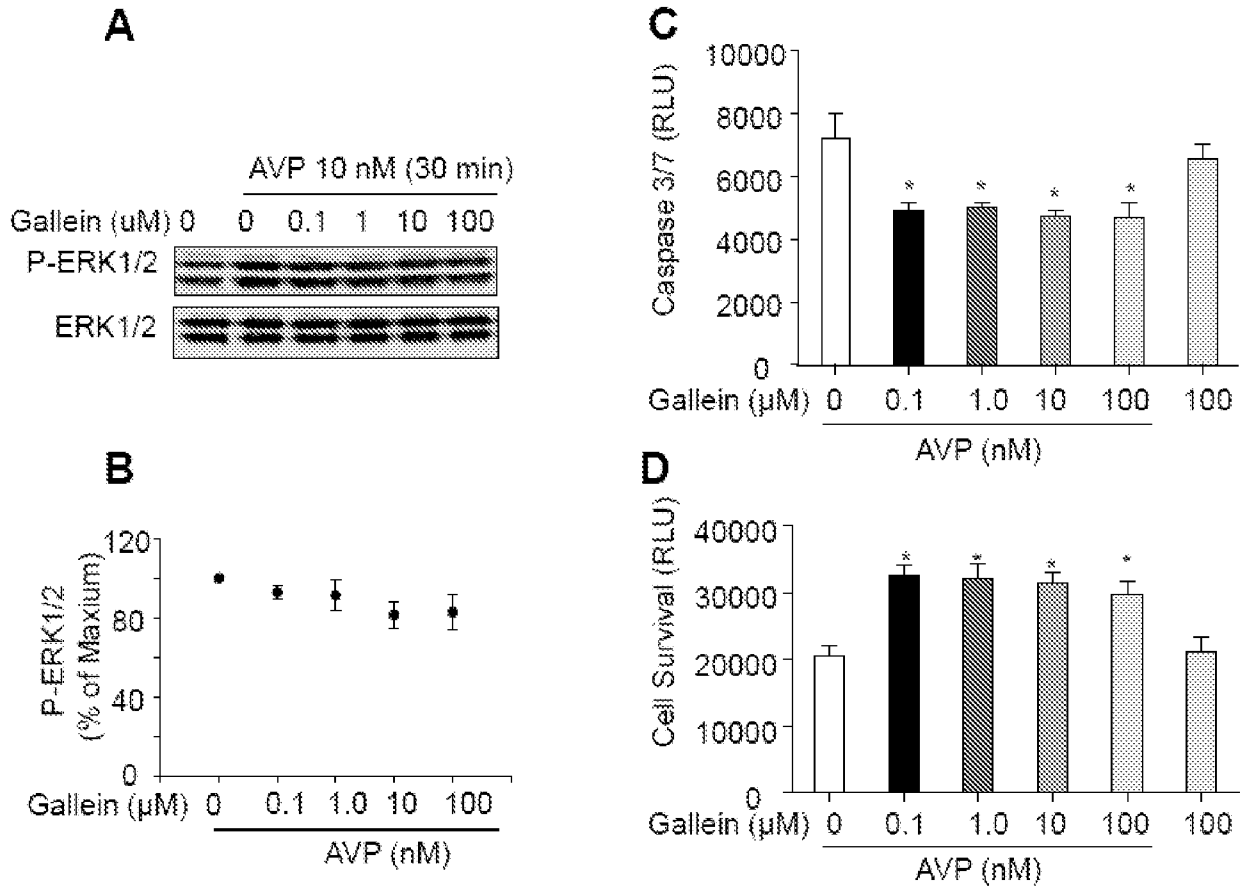


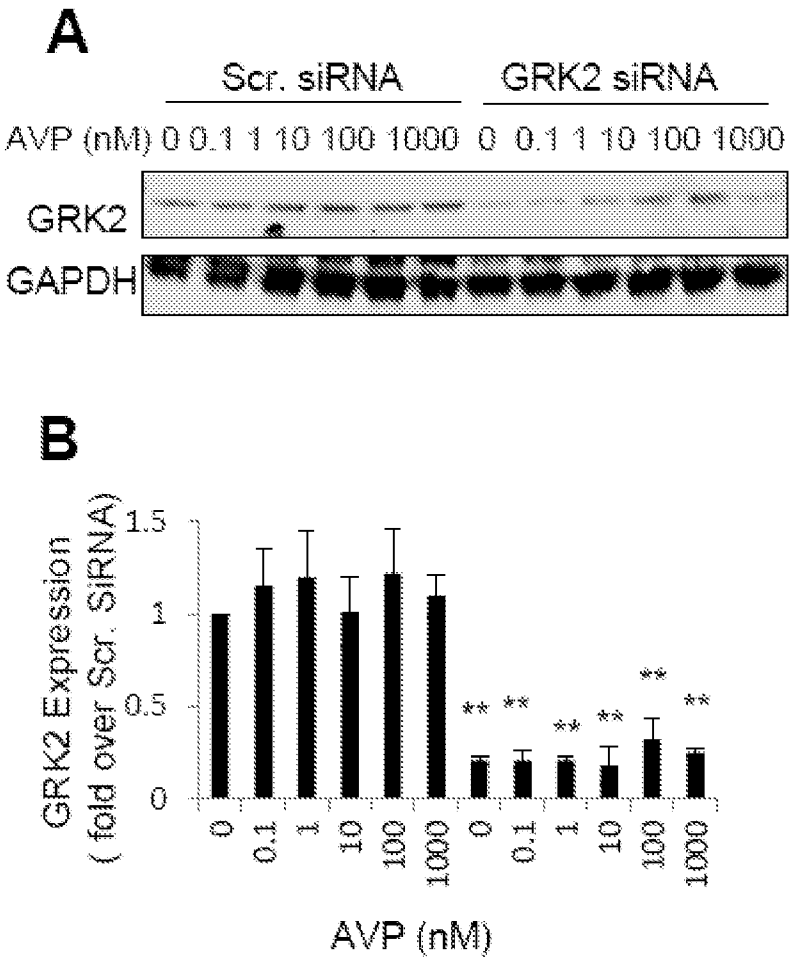
FIGURE 7



FIGURES 8A-8D



FIGURES 9A-9D



FIGURES 10A-10B

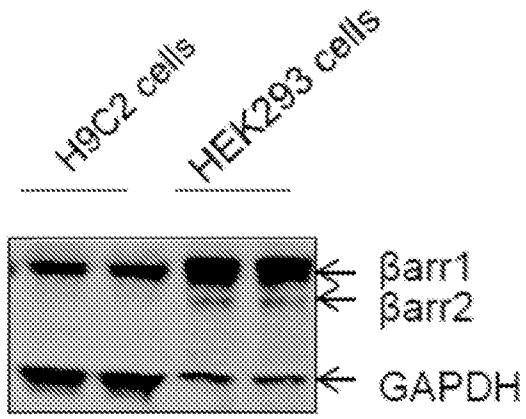
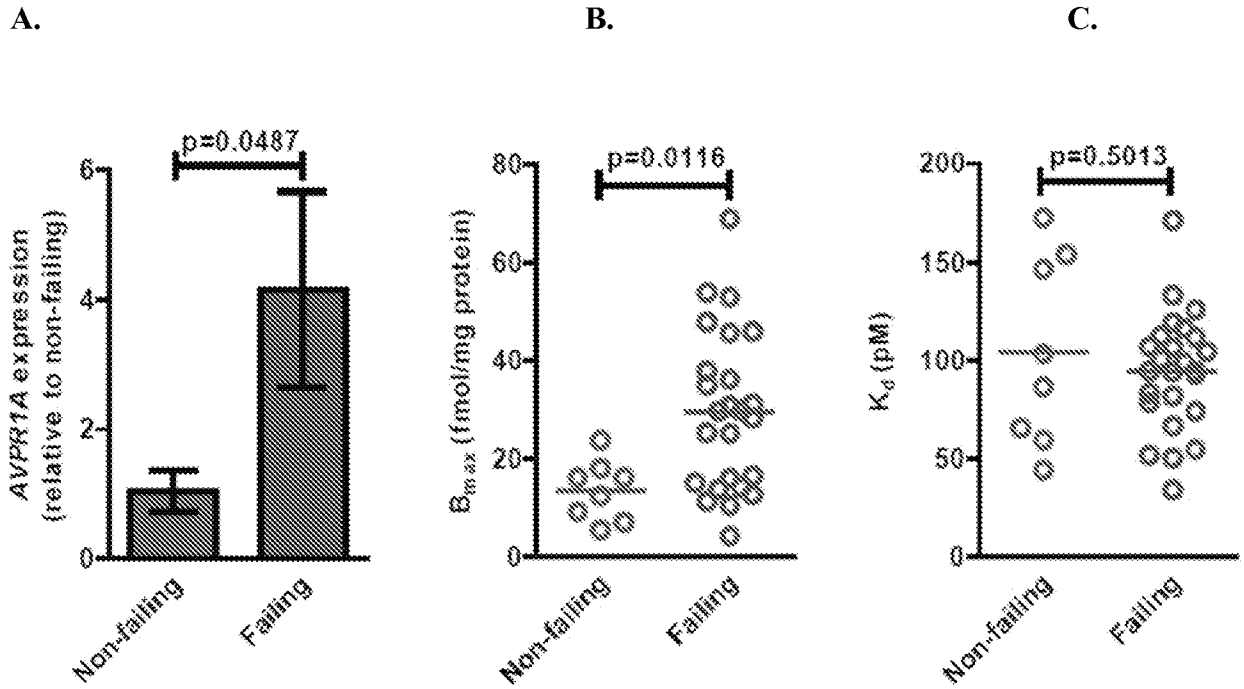
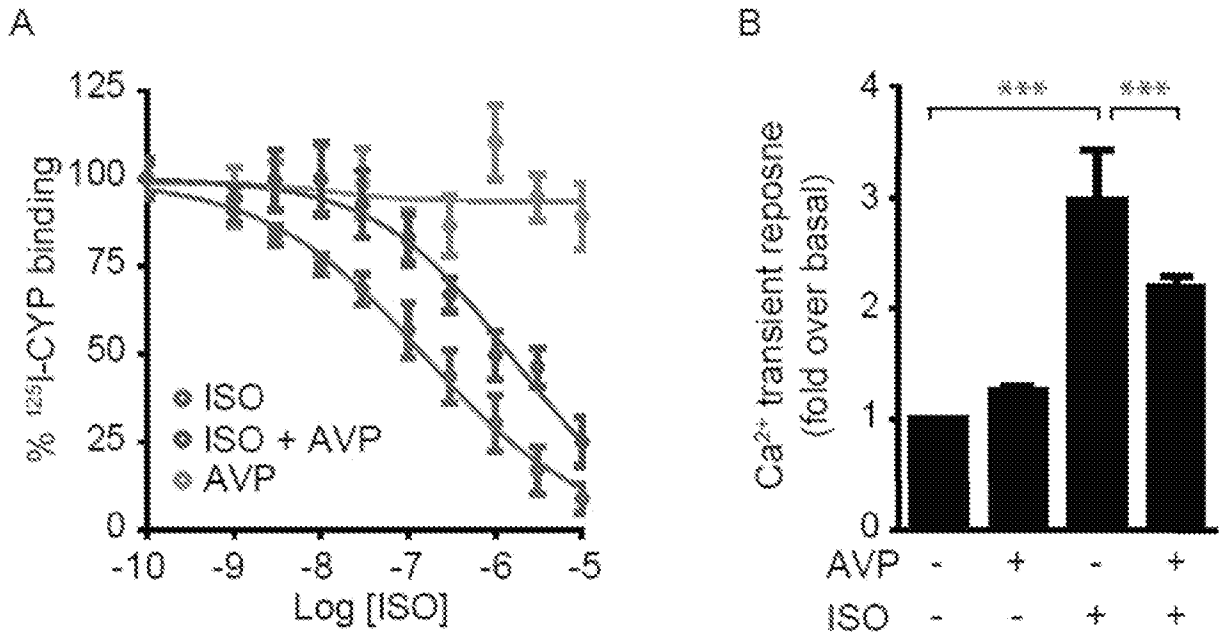


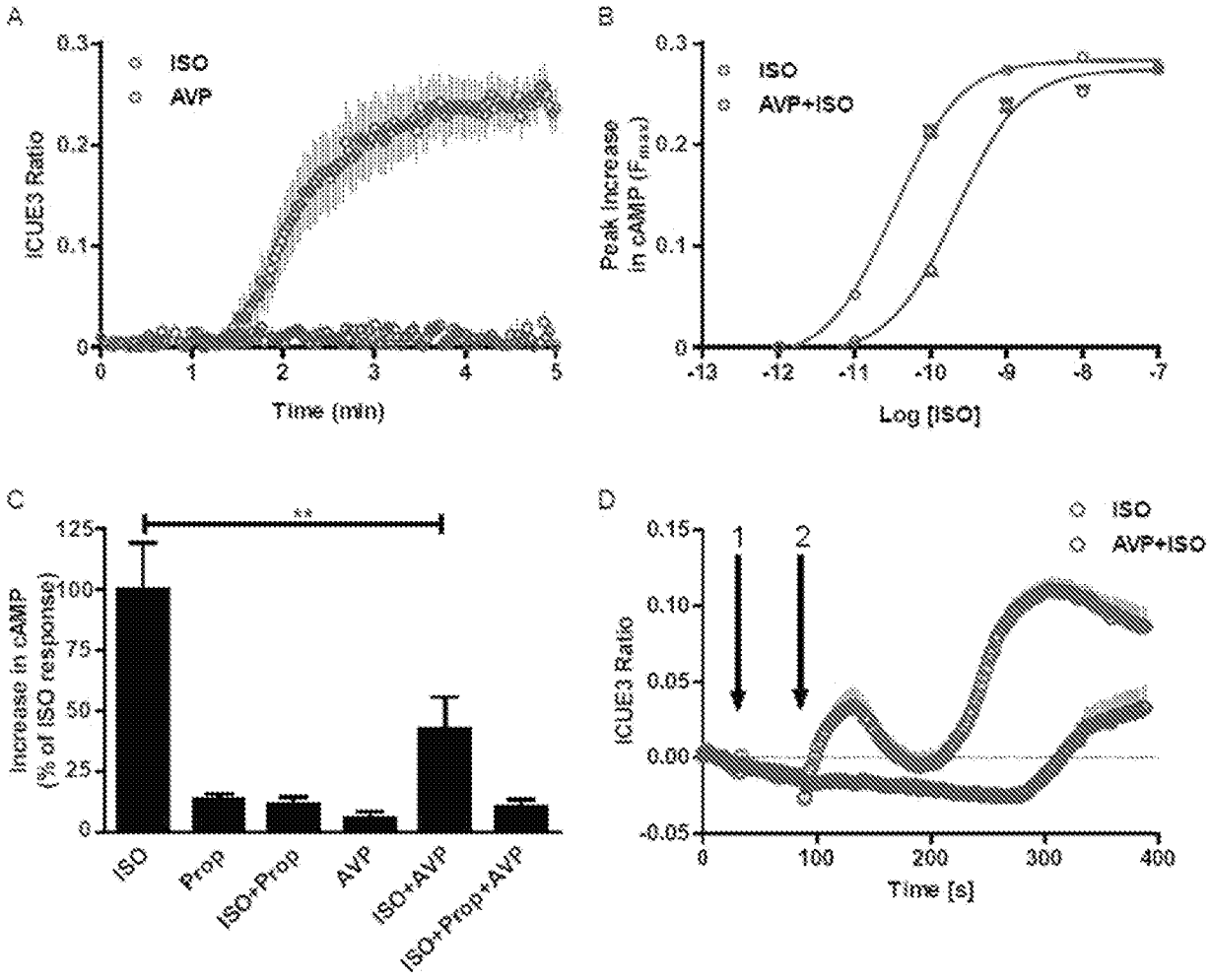
FIGURE 11



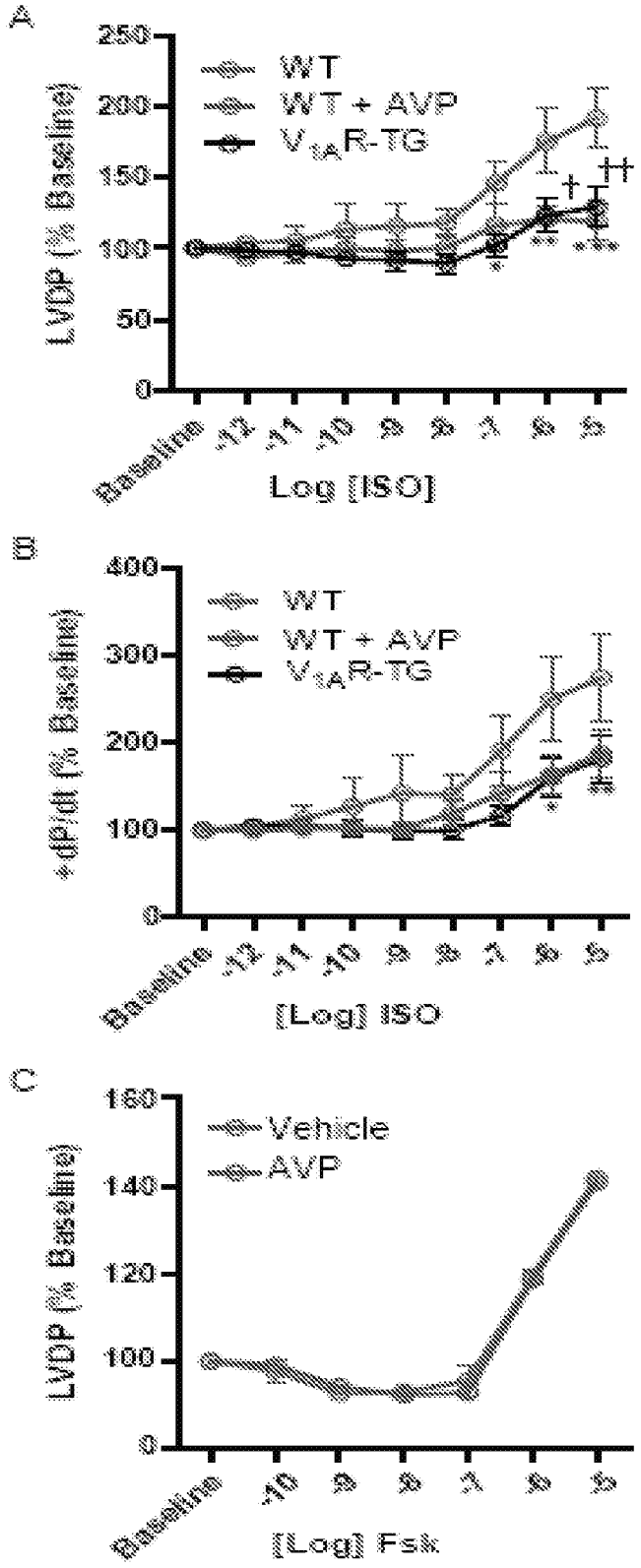
FIGURES 12A, 12B, 12C



FIGURES 13A, 13B



FIGURES 14A-14D



FIGURES 15A-15C

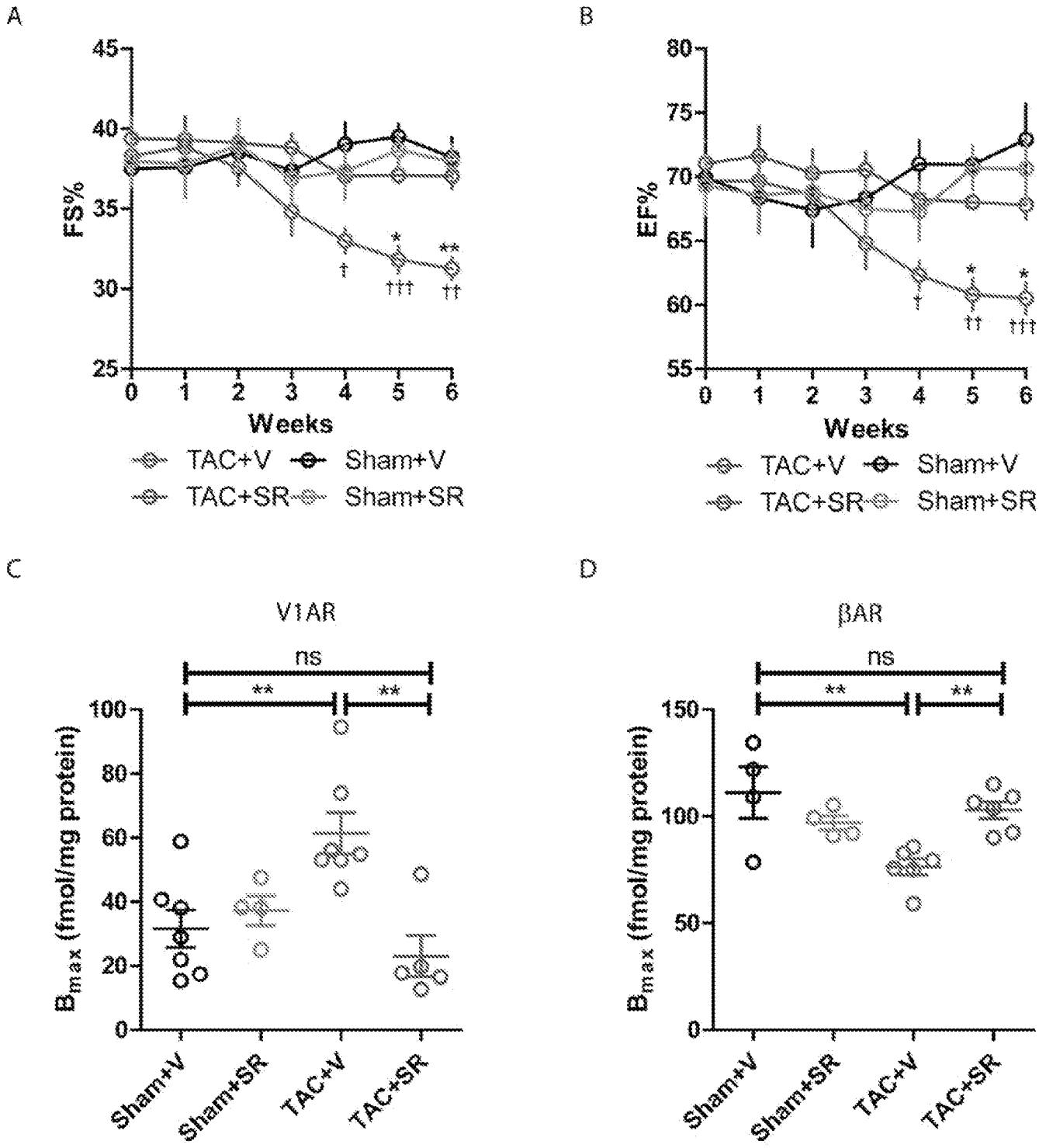

A
FS%
Weeks
TAC+V
Sham+V
TAC+SR
Sham+SR
B
EF%
Weeks
TAC+V
Sham+V
TAC+SR
Sham+SR
C
V1AR
ns
**
**
Bmax (fmol/mg protein)
Sham+V
Sham+SR
TAC+V
TAC+SR
D
βAR
ns
**
**
Bmax (fmol/mg protein)
Sham+V
Sham+SR
TAC+V
TAC+SR

Figure 16

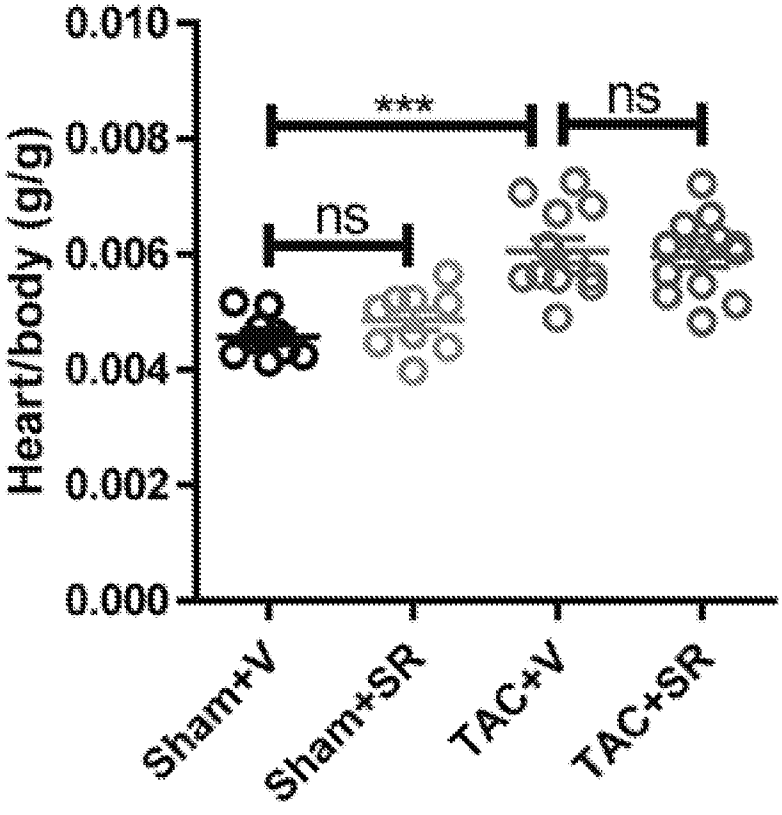

0.010
0.008
0.006
0.004
0.002
0.000
Heart/body (g/g)
***
ns
ns
a
b
c
d
Sham+V
Sham+SR
TAC+V
TAC+SR

Figure 17

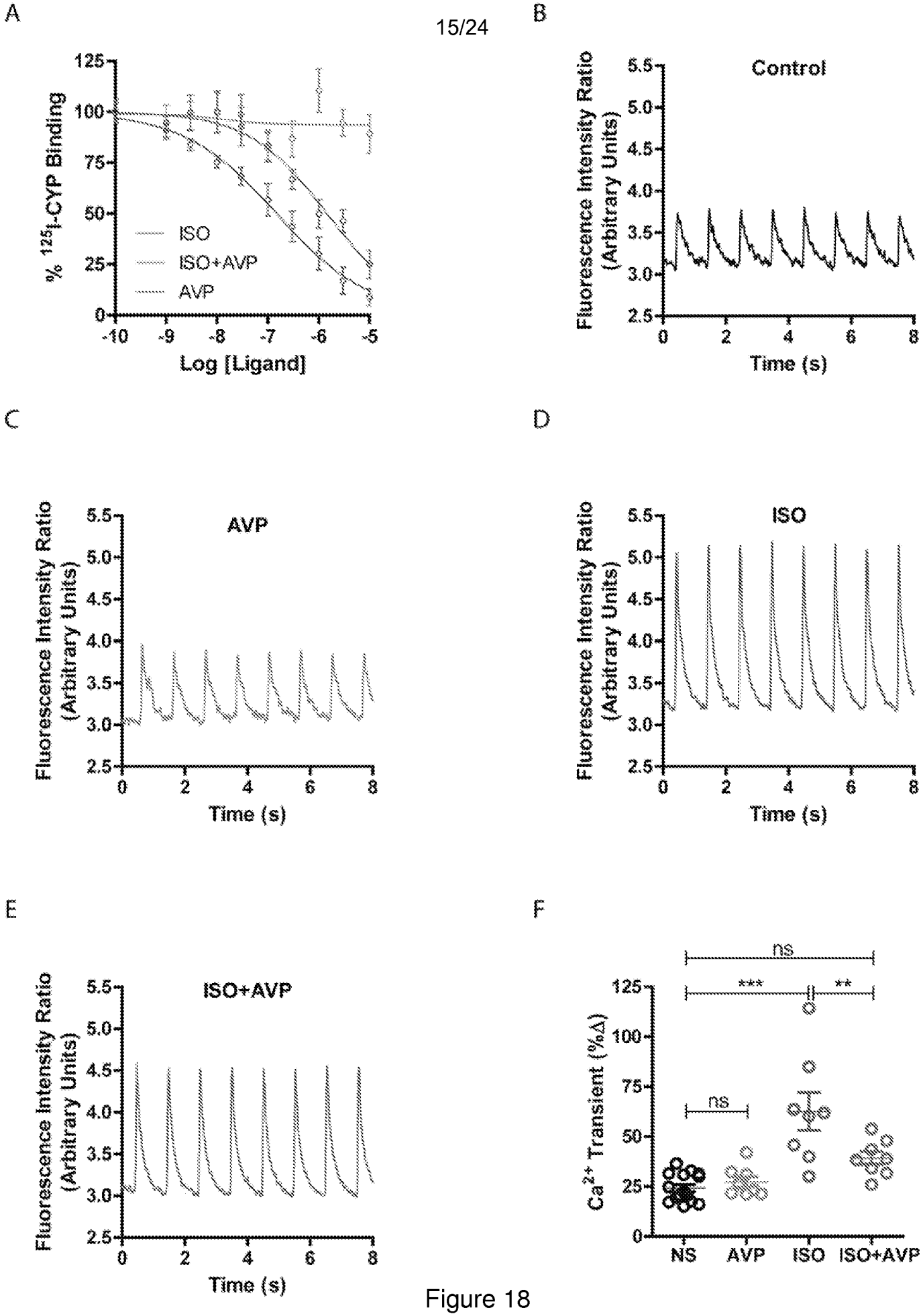

A
% 125I-CYP Binding
Log [Ligand]
ISO
ISO+AVP
AVP
B
Control
Fluorescence Intensity Ratio (Arbitrary Units)
Time (s)
C
AVP
D
ISO
E
ISO+AVP
F
Ca2+ Transient (%Δ)
ns
***
**
NS
AVP
ISO
ISO+AVP

Figure 18

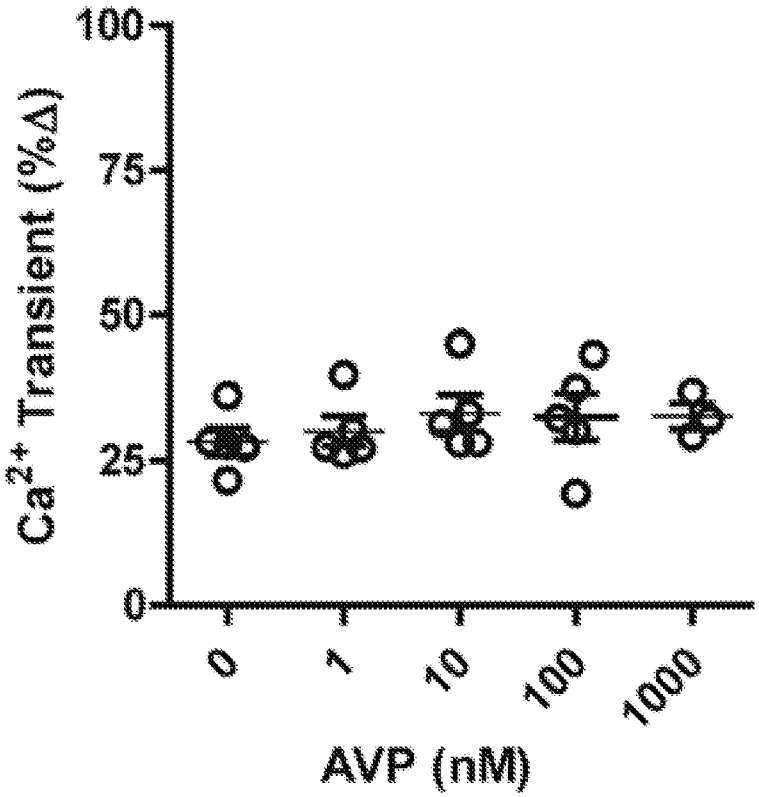


Figure 19

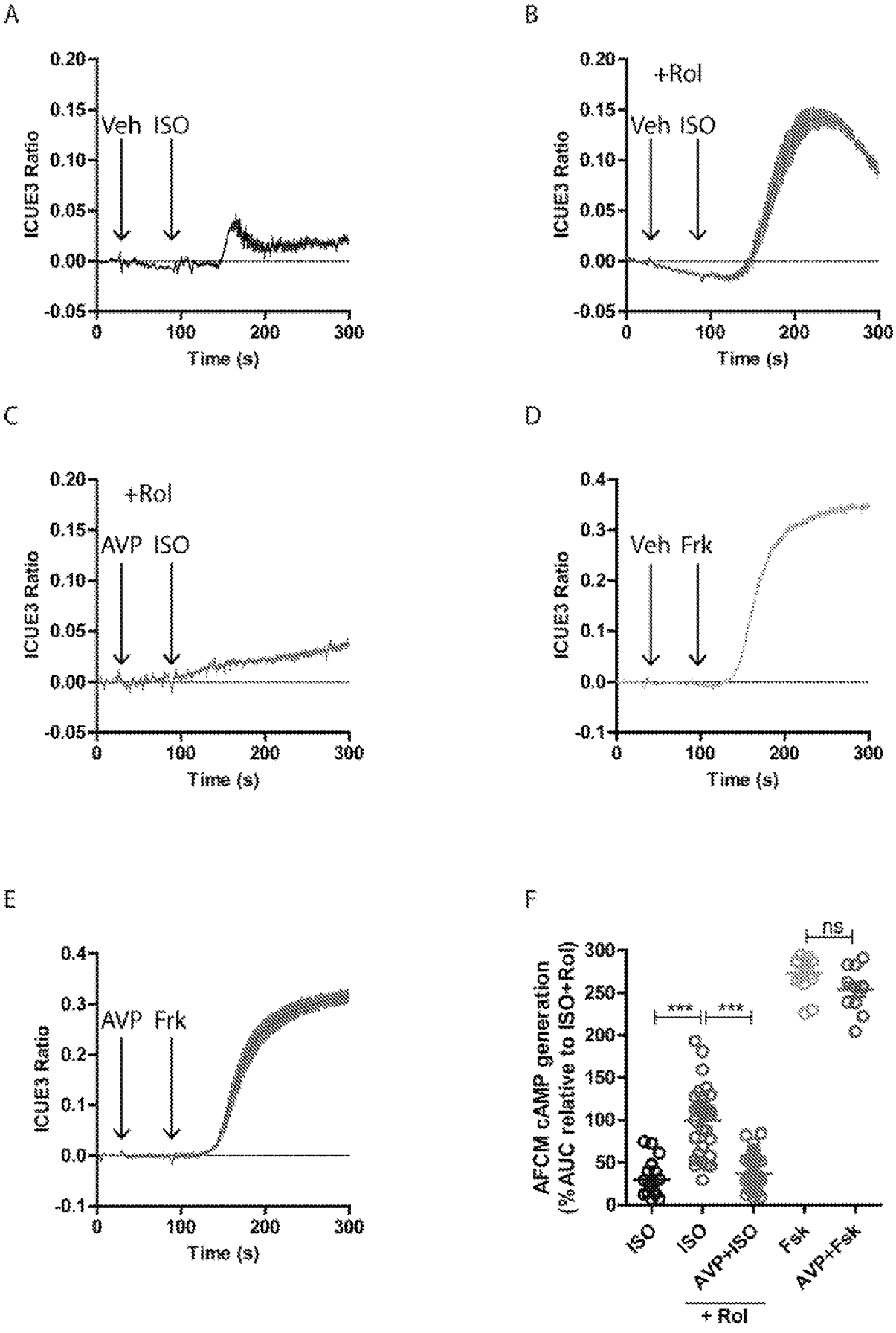

A
ICUE3 Ratio
Veh ISO
Time (s)
B
+Rol
Veh ISO
ICUE3 Ratio
Time (s)
C
+Rol
AVP ISO
ICUE3 Ratio
Time (s)
D
Veh Frk
ICUE3 Ratio
Time (s)
E
AVP Frk
ICUE3 Ratio
Time (s)
F
AFCM cAMP generation
(%AUC relative to ISO+Rol)
ns
***
***
ISO
ISO
AVP+ISO
Fsk
AVP+Fsk
+ Rol

Figure 20

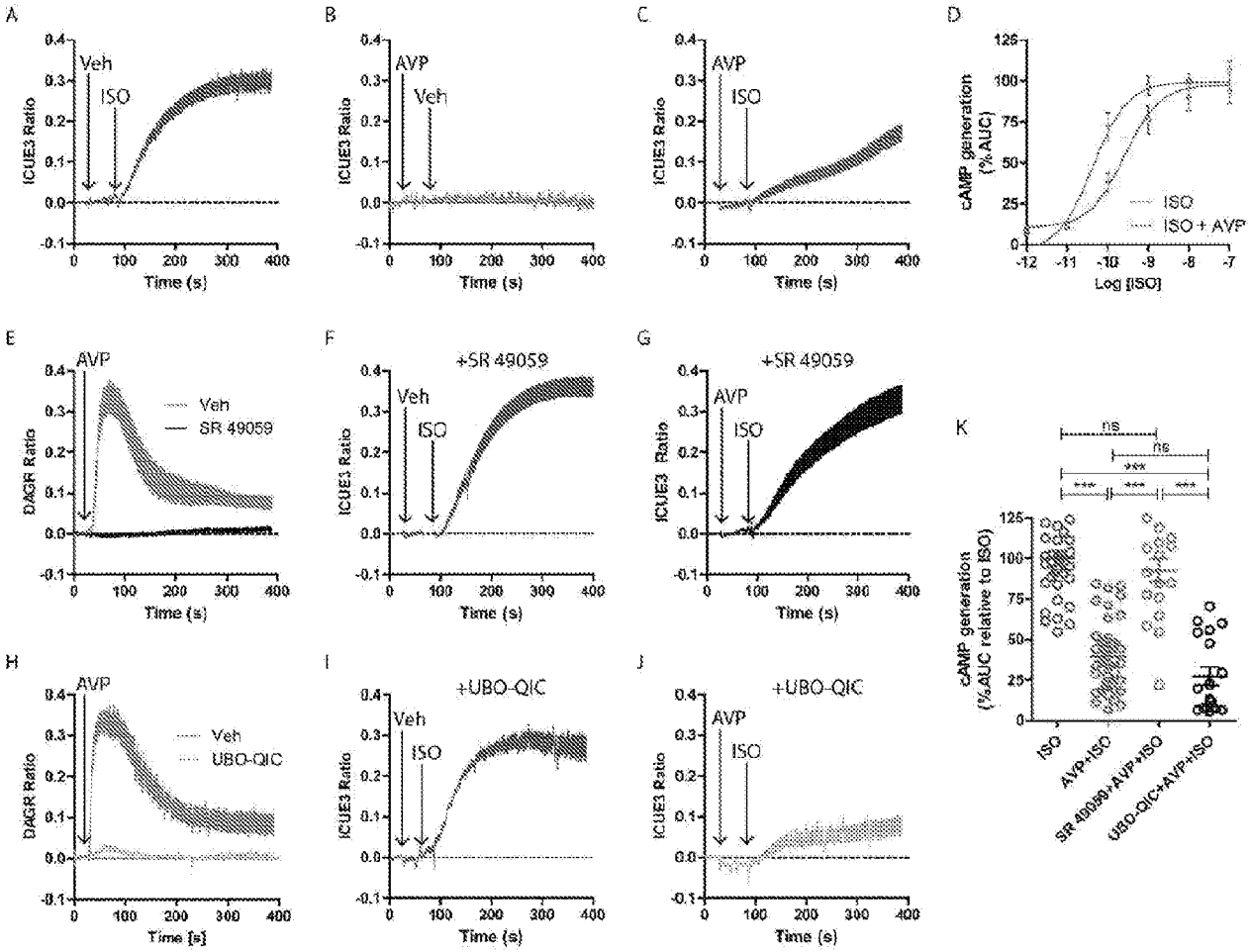

A
Veh
ISO
ICUE3 Ratio
Time (s)
B
AVP
Veh
ICUE3 Ratio
Time (s)
C
AVP
ISO
ICUE3 Ratio
Time (s)
D
cAMP generation (%AUC)
ISO
ISO + AVP
Log [ISO]
E
AVP
Veh
SR 49059
DAGR Ratio
Time (s)
F
+SR 49059
Veh
ISO
ICUE3 Ratio
Time (s)
G
+SR 49059
AVP
ISO
ICUE3 Ratio
Time (s)
H
AVP
Veh
UBO-QIC
DAGR Ratio
Time (s)
I
+UBO-QIC
Veh
ISO
ICUE3 Ratio
Time (s)
J
+UBO-QIC
AVP
ISO
ICUE3 Ratio
Time (s)
K
ns
ns
***
***
***
***
cAMP generation (%AUC relative to ISO)
ISO
AVP+ISO
SR 49059+AVP+ISO
UBO-QIC+AVP+ISO

Figure 21

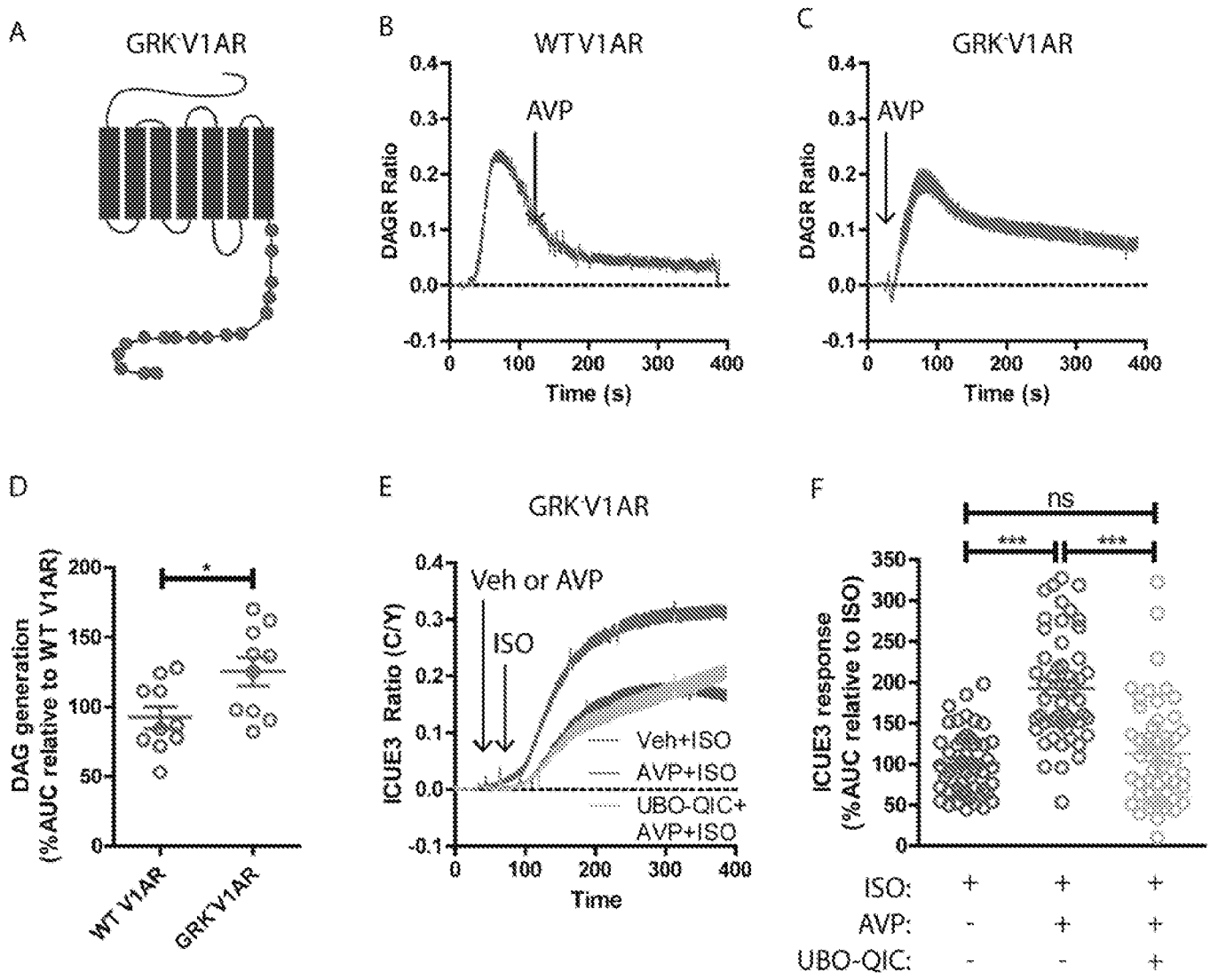

A
GRK-V1AR
B
WT V1AR
AVP
DAGR Ratio
Time (s)
C
GRK-V1AR
AVP
DAGR Ratio
Time (s)
D
DAG generation
(%AUC relative to WT V1AR)
*
WT V1AR
GRK-V1AR
E
GRK-V1AR
Veh or AVP
ISO
ICUE3 Ratio (C/Y)
Veh+ISO
AVP+ISO
UBO-QIC+
AVP+ISO
Time
F
ns
***
***
ICUE3 response
(%AUC relative to ISO)
ISO: + + +
AVP: - + +
UBO-QIC: - - +

Figure 22

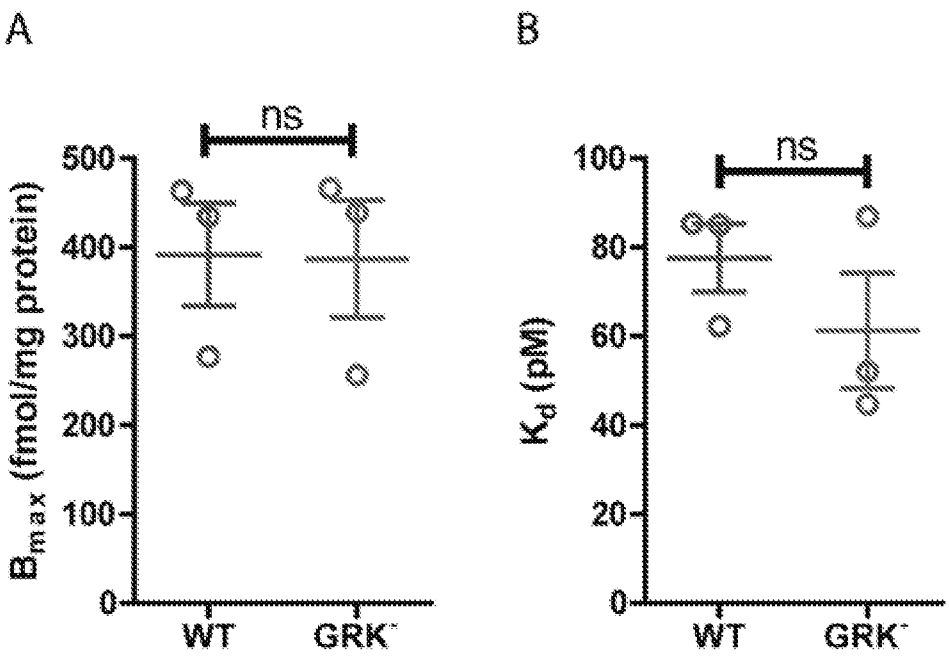

A
B
ns
ns
500
400
300
200
100
0
100
80
60
40
20
0
B_{max} (fmol/mg protein)
K_d (pM)
WT
GRK-
WT
GRK-

Figure 23

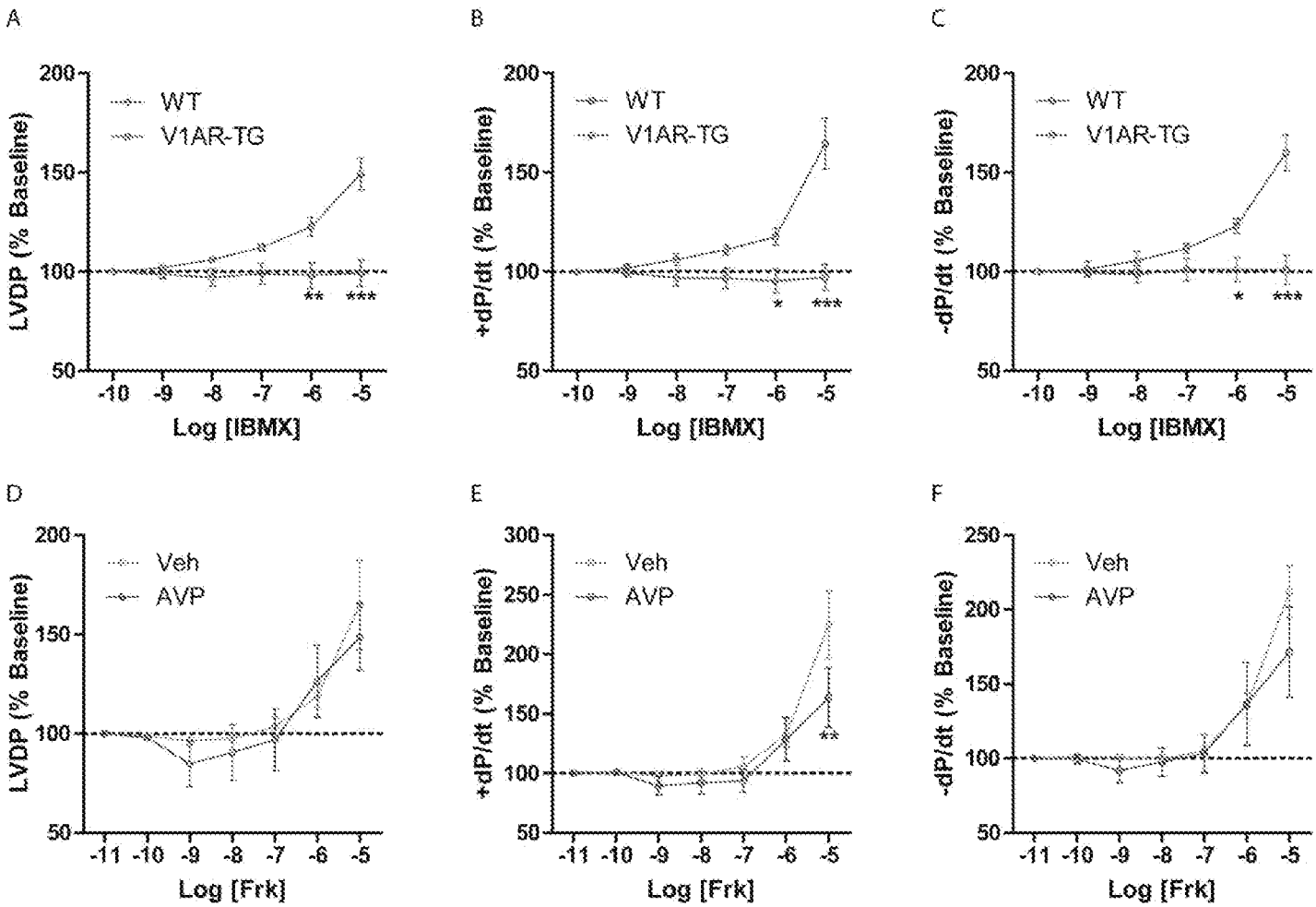

A
B
C
WT
V1AR-TG
LVDP (% Baseline)
+dP/dt (% Baseline)
-dP/dt (% Baseline)
Log [IBMX]
D
E
F
Veh
AVP
Log [Frk]

Figure 24

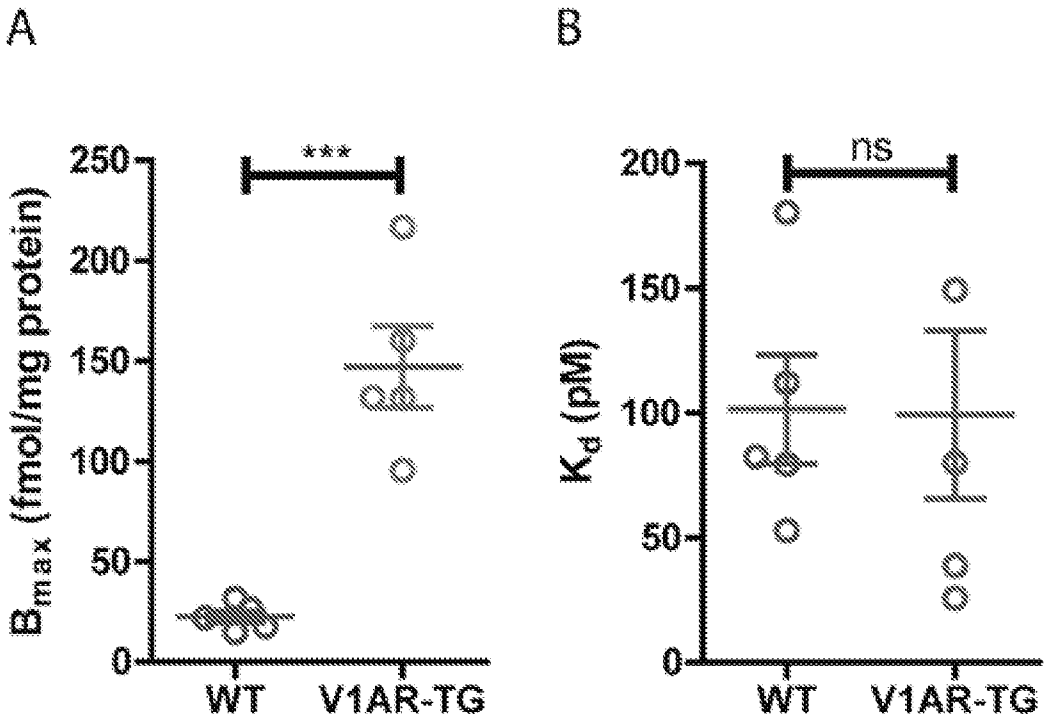

A
B
***
ns
B_{max} (fmol/mg protein)
K_d (pM)
250
200
150
100
50
0
200
150
100
50
0
WT
V1AR-TG
WT
V1AR-TG

Figure 25

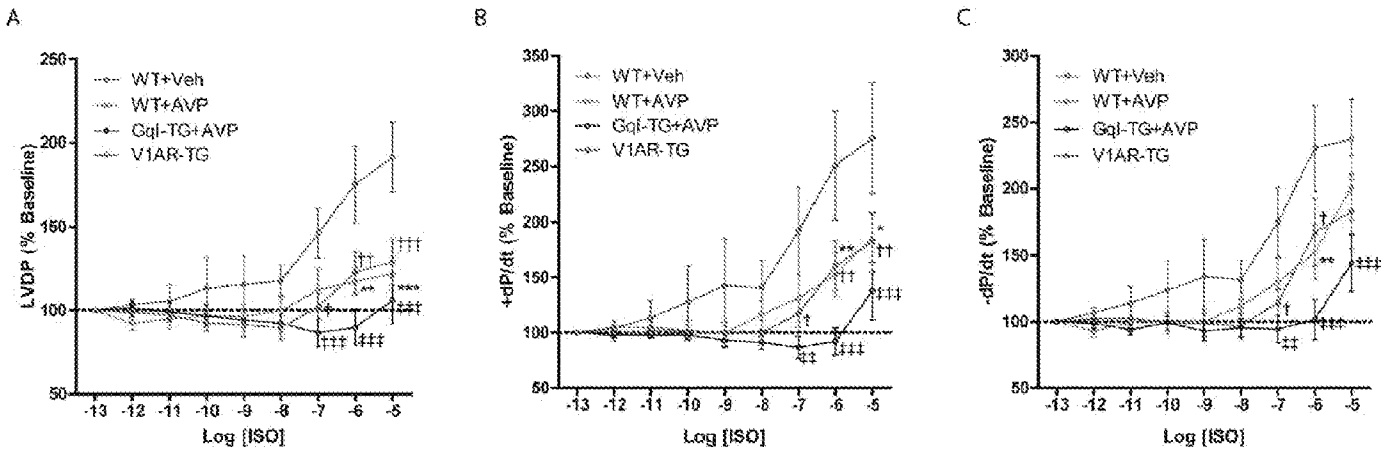

A
B
C
WT+Veh
WT+AVP
Gqi-TG+AVP
V1AR-TG
LVDP (% Baseline)
+dP/dt (% Baseline)
-dP/dt (% Baseline)
Log [ISO]

Figure 26

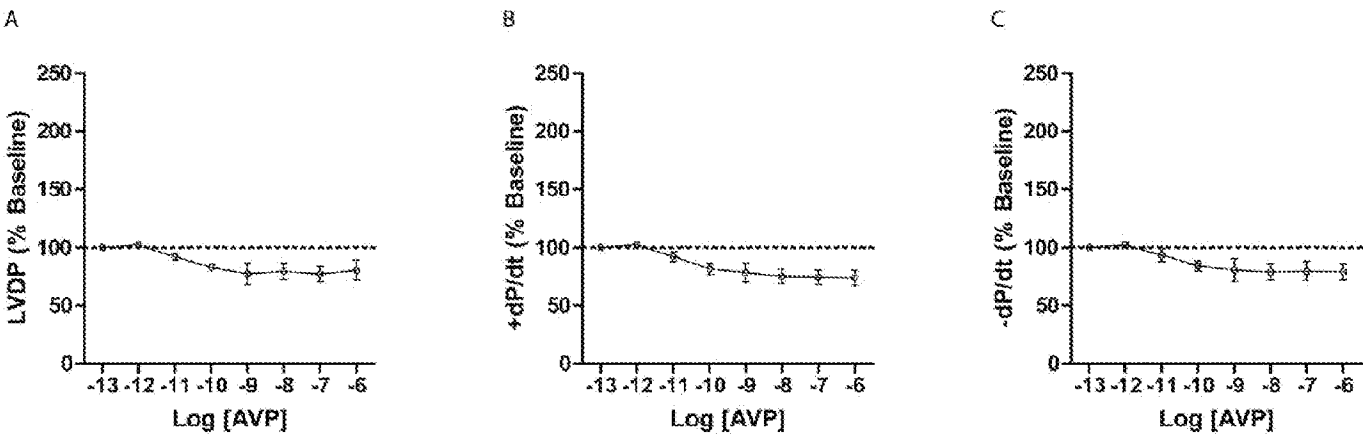

A
LVDP (% Baseline)
250
200
150
100
50
0
-13 -12 -11 -10 -9 -8 -7 -6
Log [AVP]
B
+dP/dt (% Baseline)
250
200
150
100
50
0
-13 -12 -11 -10 -9 -8 -7 -6
Log [AVP]
C
-dP/dt (% Baseline)
250
200
150
100
50
0
-13 -12 -11 -10 -9 -8 -7 -6
Log [AVP]

Figure 27

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 2014/028817

A. CLASSIFICATION OF SUBJECT MATTER

(see extra sheet)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

G01N 33/68, A61K 38/00, A61K 31/7088, A61P 9/00, A61P 7/08

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

PatSearch (RUPTO internal), EMBL, NCBI, PAJ, Espacenet, DWPI, PCT Online, USPTO DP, CIPO (Canada PO), SIPO DB

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y A	WO 2012/007594 A1 (VIB VZW et al.) 19.01.2012, p.1-4, 7, 25-26, 47-63, example 12, claims	9, 15-20, 24-25 1-3, 7-8, 10-12, 21-22, 26, 48-52, 83-86 4-6, 13-14, 23, 27-32
X Y	US 2012/053218 A1 (BRUEGGEMEIER ULF. et al.) 01.03.2012, paragraphs [0004]-[0010],[0120]-[0190], example 25	33-47, 53-82, 87 1-3, 7-8, 10-12, 21-22, 26, 48-52, 83-86
Y	NAKAYA M. et al. Induction of cardiac fibrosis by β-blocker in G protein-independent and G protein-coupled receptor kinase 5/β -arrestin2-dependent Signaling pathways, J. Biol. Chem., 2012, Vol.287, no.42, p.35669-35677, especially p.35669-35676	1-3, 7-8

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 July 2014 (14.07.2014)	07 August 2014 (07.08.2014)
Name and mailing address of the ISA/RU: FIPS, Russia, 123995, Moscow, G-59, GSP-5, Berezhkovskaya nab., 30-1 Facsimile No. +7 (499) 243-33-37	Authorized officer J.Goretova Telephone No. 495 531 65 15

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 2014/028817

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2005/0100934 A1 (LEE KEVIN J. et al.) 12.05.2005, abstract, paragraphs [0010]-[0016], [0081]-[0116], examples 1-6, 15-29	1-87

INTERNATIONAL SEARCH REPORT
Classification of subject matter

International application No.
PCT/US 2014/028817

G01N 33/68 *(2006.01)*
A61K 38/00 *(2010.01)*
A61K 31/7088 *(2006.01)*
A61P 9/00 *(2006.01)*
A61P 7/08 *(2006.01)*