

# $\beta$ -Arrestin–Biased Allosteric Modulator Potentiates Carvedilol-Stimulated $\beta$ Adrenergic Receptor Cardioprotection<sup>§</sup>

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## ABSTRACT

$\beta_1$  adrenergic receptors ( $\beta_1$ ARs) are central regulators of cardiac function and a drug target for cardiac disease. As a member of the G protein–coupled receptor family,  $\beta_1$ ARs activate cellular signaling by primarily coupling to Gs proteins to activate adenylyl cyclase, cAMP-dependent pathways, and the multi-functional adaptor-transducer protein  $\beta$ -arrestin. Carvedilol, a traditional  $\beta$ -blocker widely used in treating high blood pressure and heart failure by blocking  $\beta$  adrenergic receptor–mediated G protein activation, can selectively stimulate Gs-independent  $\beta$ -arrestin signaling of  $\beta$  adrenergic receptors, a process known as  $\beta$ -arrestin–biased agonism. Recently, a DNA-encoded small-molecule library screen against agonist-occupied  $\beta_2$  adrenergic receptors ( $\beta_2$ ARs) identified Compound-6 (Cmpd-6) to be a positive allosteric modulator for agonists on  $\beta_2$ ARs. Intriguingly, it was further discovered that Cmpd-6 is positively cooperative with the  $\beta$ -arrestin–biased ligand carvedilol at  $\beta_2$ ARs. Here we describe the surprising finding that at  $\beta_1$ ARs unlike  $\beta_2$ ARs, Cmpd-6 is cooperative *only* with carvedilol and not agonists. Cmpd-6 increases the binding affinity of carvedilol for  $\beta_1$ ARs and potentiates carvedilol-stimulated,  $\beta$ -arrestin–dependent  $\beta_1$ AR signaling, such as epidermal growth factor receptor transactivation and extracellular signal-

regulated kinase activation, whereas it does not have an effect on Gs-mediated cAMP generation. In vivo, Cmpd-6 enhances the antiapoptotic, cardioprotective effect of carvedilol in response to myocardial ischemia/reperfusion injury. This antiapoptotic role of carvedilol is dependent on  $\beta$ -arrestins since it is lost in mice with myocyte-specific deletion of  $\beta$ -arrestins. Our findings demonstrate that Cmpd-6 is a selective  $\beta$ -arrestin–biased allosteric modulator of  $\beta_1$ ARs and highlight its potential clinical utility in enhancing carvedilol-mediated cardioprotection against ischemic injury.

## SIGNIFICANCE STATEMENT

This study demonstrates the positive cooperativity of Cmpd-6 on  $\beta_1$ ARs as a  $\beta$ -arrestin–biased positive allosteric modulator. Cmpd-6 selectively enhances the affinity and cellular signaling of carvedilol, a known  $\beta$ -arrestin–biased  $\beta$ -blocker for  $\beta_1$ ARs, whereas it has minimal effect on other ligands tested. Importantly, Cmpd-6 enhances the  $\beta$ -arrestin–dependent in vivo cardioprotective effect of carvedilol during ischemia/reperfusion injury–induced apoptosis. The data support the potential therapeutic application of Cmpd-6 to enhance the clinical benefits of carvedilol in the treatment of cardiac disease.

## Introduction

$\beta_1$  adrenergic receptors ( $\beta_1$ ARs) are key regulators of heart rate and myocardial contractility and a common therapeutic target for the treatment of cardiac diseases, such as hypertension and heart failure (Wang et al., 2018). As a member of the G protein–coupled receptor (GPCR) family,  $\beta_1$ ARs

primarily transduce signals through stimulatory guanine nucleotide-binding proteins (Gs proteins). Upon receptor activation by the endogenous catecholamine epinephrine or norepinephrine,  $\beta_1$ ARs couple to Gs proteins to activate the effector enzyme adenylyl cyclase to catalyze the generation of second messenger cAMP, leading to downstream signaling and a diverse array of cellular and physiologic responses (Wang et al., 2018). Sustained Gs signaling activated by chronic neurohumoral stimulation of the  $\beta_1$ AR can lead to lethal cardiac arrhythmias and deleterious maladaptive cardiac remodeling (Rockman et al., 2002). Therefore,  $\beta$ -blockers that prevent excessive  $\beta_1$ AR activation by blunting catecholamine-stimulated downstream Gs signaling, such as carvedilol, metoprolol, and bisoprolol, are widely used in the treatment of heart failure (Packer et al., 1996, 2001; MERIT-

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**ABBREVIATIONS:**  $\beta$ AR,  $\beta$  adrenergic receptor;  $\beta_1$ AR,  $\beta_1$  adrenergic receptor;  $\beta_2$ AR,  $\beta_2$  adrenergic receptor; BRET, bioluminescence resonance energy transfer; CHS, cholesterol hemisuccinate; Cmpd-6, Compound-6; CYP, cyanopindolol; DDM, *N*-dodecyl- $\beta$ -D-maltoside; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; GPCR, G protein–coupled receptor; HDL, high-density lipoprotein; HEK, human embryonic kidney; I/R, ischemia/reperfusion; miR, microRNA;  $\alpha$ MyHC,  $\alpha$ -myosin heavy chain; PAM, positive allosteric modulator; pERK, phosphorylated ERK; rGFP, recombinant Green Fluorescent Protein; RlucII, Renilla luciferase variant 2; TUNEL, terminal deoxynucleotidyl transferase–mediated digoxigenin–deoxyuridine nick-end labeling.

HF-Study-Group, 1999; Bhatt et al., 2017). However, despite the fact that  $\beta$ -blockers effectively decrease morbidity and mortality in heart failure, there is a large variation among patients in the level of responsiveness to these drugs (Bhatt et al., 2017). Moreover, the development of intolerable side effects often leads to difficulty in achieving the targeted drug doses in many patients even though evidence suggests a positive correlation between higher  $\beta$ -blocker doses and improved clinical outcomes (Bhatt et al., 2017). The major side effects of  $\beta$ -blockers involve fatigue because of their potent blockade of exercise-induced heart rate and the nonselective inhibition of both  $\beta_1$  adrenergic receptor ( $\beta_1$ AR) and  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) subtypes (carvedilol) leading to unwanted effects in other tissues, such as bronchial and blood vessel constriction (Cleland et al., 2000). Therefore, developing new  $\beta_1$ AR ligands with improved efficacy, higher receptor subtype specificity, and fewer side effects is a huge unmet need.

It is now appreciated that, in addition to Gs protein coupling,  $\beta_1$ ARs also transduce signaling through  $\beta$ -arrestins (Rajagopal et al., 2010), adaptor proteins initially discovered for their roles in interdicting Gs activation and desensitizing GPCRs (Benovic et al., 1989; Lohse et al., 1990). The awareness that a ligand-activated GPCR can selectively couple to different transducers (i.e., G protein or  $\beta$ -arrestin) has led to the current concept of biased agonism (Rajagopal et al., 2010; Wang et al., 2018). The  $\beta$ -blocker carvedilol has been shown to specifically stimulate  $\beta$  adrenergic receptor ( $\beta$ AR) to mediate  $\beta$ -arrestin-dependent extracellular signal-regulated kinase (ERK) signaling (Wisler et al., 2007) and epidermal growth factor receptor (EGFR) transactivation (Kim et al., 2008; Wang et al., 2017) and is therefore termed as a  $\beta$ -arrestin-biased agonist. Moreover, activation of  $\beta$ -arrestin-mediated pathways have been shown to promote cardioprotective effects (Noma et al., 2007; Kim et al., 2008; Wang et al., 2017).

An exciting area of GPCR pharmacology is the emerging field of allosteric modulation. Allosteric modulators bind to receptors in areas that are topographically distinct from the orthosteric site and cooperate with orthosteric ligands to modulate receptor activity (Hauser et al., 2017). Allosteric modulators may revolutionize drug discovery because of their potential advantages over the traditional orthosteric agents, such as greater specificity, precise spatial-temporal regulation of receptor activity, and the possibility to engage or enhance signaling bias by finely modulating potency and efficacy of orthosteric ligands (Hauser et al., 2017; Thal et al., 2018).

Given the cardioprotective effect of  $\beta_1$ AR-mediated  $\beta$ -arrestin-biased signaling and the potential therapeutic advantage of allosteric modulators, we set out to identify, characterize, and then translate in vivo novel ligands that function as  $\beta$ -arrestin-biased allosteric modulators on the  $\beta_1$ AR. In this study, we investigated the allosteric modulation activity on the  $\beta_1$ AR of Compound-6 (Cmpd-6) (Supplemental Fig. 1), a small-molecule agent identified as an unbiased positive allosteric modulator (PAM) for the  $\beta_2$ AR through an affinity screen of a DNA-encoded chemical library (Ahn et al., 2018; Liu et al., 2019). Recent work has shown that a unique property of Cmpd-6 is its positive cooperativity with only carvedilol on the  $\beta_2$ AR among a diverse panel of  $\beta$ -blockers (Pani et al., 2021). Here, we identify unique allosteric properties of Cmpd-6 on  $\beta_1$ AR ligand binding and cellular signaling and demonstrate that Cmpd-6 enhances the in vivo cardioprotective effects of carvedilol in response to cardiac injury. These

data suggest that drugs like Cmpd-6 may have therapeutic advantages by enhancing the effectiveness of orthosteric ligands while minimizing unwanted side effects.

## Materials and Methods

**Reagents.** Cmpd-6 was synthesized as previously described (Ahn et al., 2018; Liu et al., 2019). Carvedilol; isoproterenol; epinephrine; nor-epinephrine; metoprolol; carazolol; atenolol; bisoprolol; ICI-118,551; and epidermal growth factor (EGF) were purchased from Sigma-Aldrich (St. Louis, MO). Alprenolol was from Tocris Bioscience (Minneapolis, MN). Bucindolol was from Santa Cruz Biotechnology (Dallas, TX). Radiolabeled  $^{125}\text{I}$ -cyanopindolol was from PerkinElmer Life Sciences (Waltham, MA).

**$\beta_1$ AR Expression and Purification.** The  $\beta_1$ AR was expressed in and purified from Expi293F cells as previously described for the  $\beta_2$ AR (Staus et al., 2018) with small modifications. FLAG-tagged  $\beta_1$ AR was transfected into Expi293F cells (Invitrogen) with Expifectamine (Invitrogen) as described in the manufacturer's protocol. Cells were harvested 3 days after transfection and lysed by stirring in lysis buffer (10 mM Tris pH 7.4; 2 mM EDTA; 10 mM  $\text{MgCl}_2$ ; 5 units/ml benzonase, benzamidine, and leupeptin; and 1  $\mu\text{M}$  alprenolol) for 30 minutes at room temperature. Cell membrane was pelleted by centrifugation at  $32,000 \times g$  for 20 minutes at  $4^\circ\text{C}$ , homogenized in solubilization buffer [20 mM HEPES pH 7.4; 100 mM NaCl; 10 mM  $\text{MgCl}_2$ ; 1% *N*-dodecyl- $\beta$ -D-maltoside (DDM); 0.1% cholesterol hemisuccinate (CHS); 5 units/ml benzonase, benzamidine, and leupeptin; and 1  $\mu\text{M}$  alprenolol], stirred at room temperature and  $4^\circ\text{C}$  for 1 hour each, and centrifuged at  $32,000 \times g$  for 40 minutes at  $4^\circ\text{C}$ . The supernatant was supplemented with 2 mM  $\text{CaCl}_2$  and loaded on M1-FLAG column at 2–3 ml/min at  $4^\circ\text{C}$ . The M1-FLAG column was washed with 5 column volumes each of low-salt (100 mM NaCl) and high-salt (500 mM NaCl) wash buffer (20 mM HEPES pH 7.4; 2 mM  $\text{CaCl}_2$ ; 0.1% DDM; 0.01% CHS, benzamidine, and leupeptin; and 1  $\mu\text{M}$  alprenolol). The wash cycle was repeated an additional three times and then completed with 10 column volumes of low-salt wash buffer. Receptor was eluted in elution buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.1% DDM, 0.01% CHS, 0.2 mg/ml FLAG-peptide, 5 mM EDTA, and 1  $\mu\text{M}$  alprenolol) and cleaned up by size-exclusion chromatography with Superdex 200 Increase Column (GE Healthcare Life Sciences).

**High-Density Lipoprotein Reconstitution.** Reconstitution of purified  $\beta_1$ ARs into high-density lipoprotein (HDL) particles was performed as previously described for the  $\beta_2$ AR (Ahn et al., 2018). In brief, 2  $\mu\text{M}$  purified  $\beta_1$ AR was incubated with 100  $\mu\text{M}$  apolipoprotein A1 (Membrane Scaffold Protein 1) and 8 mM phosphatidylcholine/phosphatidylglycerol (3:2 molar ratio mixture) in buffer (20 mM HEPES pH 7.4, 100 mM NaCl, and 100 mM cholate) for 1 hour at  $4^\circ\text{C}$ . Biobeads (Bio-Rad) were then added and rotated overnight at  $4^\circ\text{C}$  to remove detergent.  $\beta_1$ AR-HDL particle was isolated from empty HDL particles using M1-FLAG chromatography.

**Competition Binding Assay.** Radioligand competition binding assay was performed with the radiolabeled  $^{125}\text{I}$ -cyanopindolol (CYP) (PerkinElmer) as previously described for the  $\beta_2$ AR (Ahn et al., 2018). The  $\beta_1$ AR-HDL particles were incubated with 60 pM  $^{125}\text{I}$ -CYP, vehicle (DMSO) or 25  $\mu\text{M}$  Cmpd-6, and a serial dilution of indicated orthosteric ligands in assay buffer (20 mM HEPES pH 7.4, 100 mM NaCl, 0.1% bovine serum albumin, and 1 mM ascorbic acid) at room temperature for 90 minutes. Nonspecific binding was determined in the presence of 20  $\mu\text{M}$  propranolol. The reaction was terminated and harvested by rapid filtration onto 0.3% poly(ethyleneimine)-treated GF/B glass fiber filter paper (Brandel) using a harvester (Brandel).  $^{125}\text{I}$ -CYP on the filter paper was measured by a WIZARD2 2-Detector Gamma Counter (PerkinElmer). Binding data were analyzed in GraphPad Prism using a one-site competition-binding  $\log\text{IC}_{50}$  curve fit with data points weighted equally. Counts per minute values were normalized as percentage of the maximal  $^{125}\text{I}$ -CYP binding level and plotted as mean  $\pm$  S.D.

**GloSensor Assay.** Analysis of cAMP accumulation was performed in human embryonic kidney (HEK) 293 cells transiently transfected with  $\beta_1$ AR and GloSensor constructs. The day before transfection, 5.5 million cells were seeded in one 10-cm tissue culture dish. The cells were transfected with 6  $\mu$ g GloSensor plasmid DNA and 25 ng  $\beta_1$ AR plasmid using FuGene6 (Promega) transfection reagent. After 1 day, 100,000 transfected cells were seeded to each well of 96-well plate in assay media (minimal essential medium with 2% FBS). The next day, the cells were incubated with the GloSensor reagent [Promega; 4% (v/v)] at room temperature for 2 hours; pretreated with 100 nM ICI118,551 to block activation of endogenous  $\beta_2$ AR; and then stimulated with indicated ligands for 5 minutes. Luminescence was detected on a Biotek Neo2 microplate reader.

**EGFR Transactivation Assay.** The level of EGFR transactivation was measured by the endocytosis bioluminescence resonance energy transfer (BRET) assay as previously described (Namkung et al., 2016) with slight modifications. HEK293 cells stably expressing  $\beta_1$ ARs were transfected with 250 ng of EGFR-Renilla luciferase variant 2 (RlucII) and 1  $\mu$ g of recombinant Green Fluorescent Protein (rGFP)-FYVE constructs. The day after transfection, cells were reseeded onto poly(L-ornithine)-coated 96-well plates at ~25,000 cells per well. The next day, cells were pretreated with vehicle (DMSO), 30  $\mu$ M Cmpd-6, or 10  $\mu$ M AG1478 in assay buffer (Hanks' balanced salt solution, 20 mM HEPES and pH 7.4) for 20 minutes at 37°C and then stimulated with ligand at indicated concentration for 25 minutes. The cell-permeable substrate coelenterazine 400a (final concentrations of 5  $\mu$ M) was added ~3–6 minutes before BRET measurements. The BRET measurements were performed with a Neo2 microplate reader (BioTek) with a filter set (center wavelength/bandwidth) of 410/80 nm (donor) and 515/30 nm (acceptor).

**ERK Activation Assay.** HEK293 cells with stable overexpression of FLAG-tagged  $\beta_1$ AR or  $\beta$ -arrestin knockout were described previously. Cells were periodically treated with BM Cyclin (Roche) to avoid mycoplasma contamination. Cells were incubated for 4 hours in serum-free medium supplemented with 0.1% bovine serum albumin, 10 mM HEPES, and 1% penicillin-streptomycin; pretreated with vehicle (DMSO) or 30  $\mu$ M Cmpd-6 for 20 minutes; and stimulated with indicated ligands for 5 minutes. After stimulation, cells were lysed in lysis buffer (20 mM Tris pH 7.4, 137 mM NaCl, 20% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 mM sodium fluoride, 10  $\mu$ g/ml aprotinin, and 5  $\mu$ g/ml leupeptin and phosphatase inhibitors) by rotating for 30 minutes at 4°C. Cell lysate samples were separated by SDS-PAGE, transferred to PVDF Membrane (Bio-Rad), and subjected to immunoblotting with anti-MAPK 1/2 (EMD Millipore) and anti-p44/42 MAPK (Cell Signaling) antibodies. Immunoblots were detected using enhanced chemiluminescence (Thermo Fisher Scientific) and analyzed with ImageJ software. The densitometry values from phosphorylated ERK (pERK) blots were normalized to respective total ERK blots and normalized to the maximum response of control group in each experiment.

**Isoproterenol-Induced Hemodynamic in Mouse Heart.** Eight to 12-week-old C57BL/6J wild-type mice of both sexes were used for this study. Animal experiments carried out for this study were handled according to approved protocols and animal welfare regulations mandated by the Institutional Animal Care and Use Committee of Duke University Medical Center. Mice were treated with vehicle, carvedilol (1, 5, or 20 mg/kg per day), or a combination of carvedilol (1 mg/kg per day) and Cmpd-6 (5 mg/kg per day) for 3 days with Alzet osmotic pump (Durect) implanted into mice subcutaneously. After treatment, mice were anesthetized with ketamine (100 mg/kg) and xylazine (2.5 mg/kg). After bilateral vagotomy, a 1.4 French (0.46 mm) high-fidelity micromanometer catheter (ADInstruments) connected to a pressure transducer (ADInstruments) was inserted into the left ventricle to monitor blood pressure. Basal blood pressure was recorded at steady state after the catheter insertion (2–3 minutes after insertion). Graded doses of isoproterenol were administered at 45-second intervals by intravenous injection through

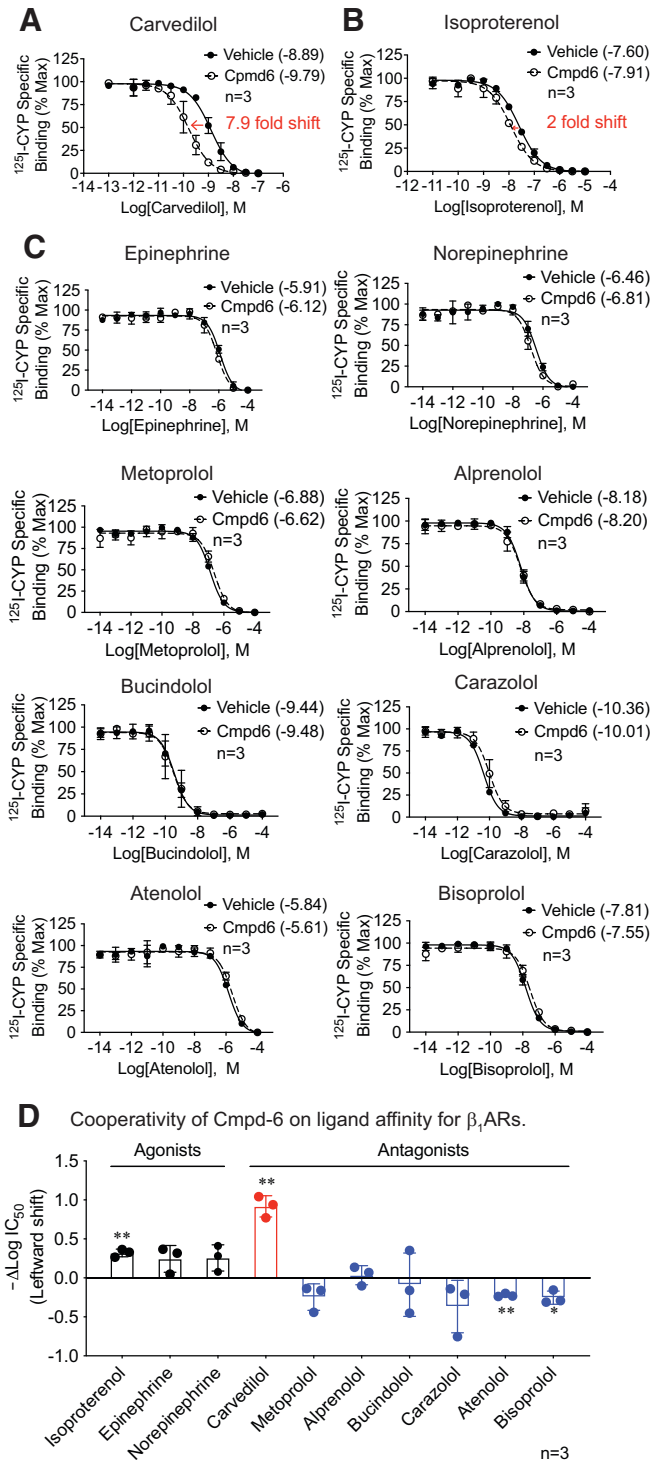
a jugular vein. Blood pressure was monitored continuously and recorded at the steady state (35–45 seconds after each injection). Data analysis was performed using LabChart 8 software (ADInstruments).

**Ischemia/Reperfusion-Induced Cell Apoptosis in Mouse Heart.** Eight to 12-week-old C57BL/6J wild-type mice or 8- to 27-week-old  $\alpha$ -myosin heavy chain ( $\alpha$ MyHC)-Cre:Arrb1<sup>flox/flox</sup>/Arrb2<sup>flox/flox</sup> mice of both sexes were used for this study. Animals were randomly assigned to and the researchers were masked for treatment groups. Alzet osmotic pumps (Durect) were implanted into mice subcutaneously to deliver vehicle (DMSO), Cmpd-6 (5 mg/kg per day), carvedilol (1 mg/kg per day or 20 mg/kg per day), or a combination of Cmpd-6 (5 mg/kg per day) and carvedilol (1 mg/kg per day). After 3 days of treatment, cardiac ischemia was produced by the ligation of left anterior descending coronary artery. After 45 minutes, the ligation was released to allow the blood flow to be restored for 45 minutes. The mice hearts were perfusion-fixed with 4% paraformaldehyde, excised from the body, and placed in 30% sucrose in PBS for 2–4 hours at 4°C. The hearts were then embedded in optimum cutting temperature compound (Sakura Finetek) and snap-frozen in liquid nitrogen. Terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) staining on the cryosections were performed with in situ cell death detection kit (Sigma-Aldrich) according to the manufacturer's protocol. Sections were then mounted in ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). Images were recorded with Zeiss Axio Observer Z1 confocal microscope with 20 $\times$  objective. In each heart, sections from different groups (from base to apex of the tissue, apart by 0.5 mm) were stained, and the group with maximum TUNEL-positive cells were used for quantification. At least nine images from three sections were quantified for each heart. The number of TUNEL-positive cells and the total number of nuclei as determined by 4',6-diamidino-2-phenylindole staining were counted using ImageJ software.

**Statistical Analysis.** Data are expressed as mean  $\pm$  S.D. Statistical comparisons were performed using one-way ANOVA with Tukey correction or two-way repeated-measures ANOVA with Sidak correction for multiple comparison in GraphPad Prism. Differences were considered statistically significant at  $P < 0.05$ .

## Results

**Compound-6 Selectively Potentiates the Binding Affinity of Carvedilol to the  $\beta_1$ AR.** Cmpd-6 is an unbiased positive allosteric modulator for the  $\beta_2$ AR that substantially enhances the affinity of agonists and downstream signaling mediated by either G protein or  $\beta$ -arrestin (Ahn et al., 2018). Carvedilol, a traditional  $\beta$ -blocker widely used in the treatment of cardiac diseases, has also been identified as a  $\beta$ -arrestin-biased agonist for both the  $\beta_1$ AR and the  $\beta_2$ AR (Wisler et al., 2007; Kim et al., 2008). Cmpd-6 potentiates  $\beta$ -arrestin1-induced high-affinity binding of carvedilol to the  $\beta_2$ AR as well as carvedilol-stimulated  $\beta_2$ AR cellular signaling (Pani et al., 2021). To determine the allosteric modulatory activity of Cmpd-6 on the  $\beta_1$ AR, we first tested its effect on the orthosteric binding affinity of a comprehensive ligand panel of agonists and antagonists by performing competition binding assays against radiolabeled antagonist cyanopindolol (<sup>125</sup>I-CYP) binding to purified  $\beta_1$ ARs reconstituted in HDL particles (Whorton et al., 2007). Remarkably, we found that Cmpd-6 leads to ~1-log leftward shift of the carvedilol competition binding curve [Fig. 1A, vehicle: LogIC<sub>50</sub> = -8.89 (-8.98 to -8.80), Cmpd-6: LogIC<sub>50</sub> = -9.79 (-9.91 to -9.67), mean (95% confidence intervals)], indicating that Cmpd-6 strongly potentiates the binding affinity of carvedilol to the  $\beta_1$ AR. Notably, Cmpd-6 minimally enhanced the binding affinity of the full agonist isoproterenol for  $\beta_1$ ARs [Fig. 1B, vehicle:



**Fig. 1.** Cmpd-6 selectively potentiates the binding affinity of carvedilol for the  $\beta_1$ AR. (A) Cmpd-6 led to a leftward shift of the carvedilol competition binding curve against  $^{125}$ I-CYP to the  $\beta_1$ AR, which indicated the increased receptor binding affinity of carvedilol.  $\beta_1$ ARs reconstituted in high-density lipoprotein particles were incubated with vehicle (DMSO) or 25  $\mu$ M Cmpd-6, indicated concentration of carvedilol, and 60 pM  $^{125}$ I-CYP. Values were normalized to the percentage of maximal  $^{125}$ I-CYP binding level. (B) Cmpd-6 modestly enhanced the binding affinity of isoproterenol for the  $\beta_1$ AR by 2-fold. (C) Cmpd-6 had minimal effect on the  $\beta_1$ AR binding affinity of a panel of agonists and antagonists tested. (D) Cmpd-6–led leftward shift of ligands competition binding curves, shown as the difference of log  $IC_{50}$  values of the vehicle- and Cmpd-6–treated groups for each ligand. Cmpd-6 showed strong cooperativity with carvedilol (red bar), a modest PAM activity on full agonists (black bars), and no positive modulation on other antagonists tested (blue bars). Data represent the mean  $\pm$  S.D. for three independent experiments. Competition binding curves were generated with GraphPad Prism. The log  $IC_{50}$  values shown in the legends of (A–C) and represented in (D) were calculated from competition binding curves with GraphPad Prism. In (A–C), statistical comparisons were performed using two-way repeated (related)-measures ANOVA with Sidak correction for multiple comparisons. In (D), two-tailed Student’s *t* tests were performed for each ligand testing the null hypothesis that the shift in Log $IC_{50}$  is 0 (D). \**P* < 0.05 and \*\**P* < 0.01.

Log $IC_{50}$  = -7.60 (-7.65 to -7.54), Cmpd-6: Log $IC_{50}$  = -7.92 (-8.00 to -7.83)], which is in marked contrast to its effect on  $\beta_2$ ARs in which it enhanced isoproterenol binding affinity by 50-fold (Ahn et al., 2018). This marginal effect of Cmpd-6 on isoproterenol affinity for  $\beta_1$ ARs is consistent with previous studies (Ahn et al., 2018; Liu et al., 2019). When tested against a diverse ligand panel, it showed minimal positive cooperativity with the full agonists epinephrine and norepinephrine and no positive cooperativity with any other

antagonist tested (Fig. 1C). Overall, Cmpd-6 showed a subtle increase of agonist binding to the  $\beta_1$ AR, as indicated by a slight leftward shift of competition binding curves and change of  $IC_{50}$  value, and no positive modulation of binding affinity for the five other antagonists tested (Fig. 1, C and D). To compare the Cmpd-6 cooperativity with ligands between  $\beta_1$ ARs and  $\beta_2$ ARs in separate experiments, we determined Cmpd-6–induced affinity shifts of an expanded panel of ligands on the  $\beta_1$ AR (Supplemental Fig. 2A) and plotted against the shifts observed

for the  $\beta_2$ AR shown in Fig. 1B of the accompanying manuscript by Pani et al., 2021 (Supplemental Fig. 2B). Comparison between receptor subtypes highlights that carvedilol is the only ligand among those tested that shows strong cooperativity with Cmpd-6 at both the  $\beta_1$ AR and  $\beta_2$ AR (Supplemental Fig. 2B). The binding affinity of Cmpd-6 to carvedilol-bound  $\beta_1$ ARs can be calculated from the leftward shift in carvedilol binding affinity by Cmpd-6 ( $\Delta\text{LogIC}_{50}$ ) by radioligand competition binding for  $\beta_1$ ARs and  $\beta_2$ ARs. The binding affinity of Cmpd-6 to carvedilol-bound  $\beta_1$ ARs is 17  $\mu\text{M}$  and to carvedilol-bound  $\beta_2$ ARs is 1.2  $\mu\text{M}$  (Pani et al., 2021).

Although traditionally identified and used as a  $\beta$ -blocker, carvedilol has been shown to possess  $\beta$ -arrestin-biased activity by selectively engaging  $\beta$ -arrestins as the transducer in mediating downstream  $\beta$ AR signaling (Wisler et al., 2007; Kim et al., 2008; Wang et al., 2017). Our discovery that Cmpd-6 specifically potentiates the binding affinity of carvedilol to the  $\beta_1$ AR (Fig. 1, A and D) suggests that Cmpd-6 may be a  $\beta$ -arrestin-biased allosteric modulator of the  $\beta_1$ AR.

**Cmpd-6 Shows Positive Cooperativity on  $\beta$ -Arrestin-Mediated but Not Gs Protein-Mediated Signaling Induced by Carvedilol Stimulation of the  $\beta_1$ AR.** To determine the functional significance of the allosteric modulation activity of Cmpd-6 on  $\beta_1$ ARs, we tested the effect of Cmpd-6 on  $\beta_1$ AR-mediated cellular signaling in HEK293 cells. We first monitored Gs protein-mediated cAMP generation in the presence or absence of Cmpd-6. HEK293 cells were transfected with  $\beta_1$ ARs and the luciferase-based cAMP biosensor GloSensor (Fig. 2A). Upon ligand stimulation of the  $\beta_1$ AR, Gs activation of adenylyl cyclase generates cAMP that is detected by a conformational change of GloSensor to produce light (Fig. 2A). We observed that the full agonist isoproterenol robustly induced cAMP production in a dose-dependent manner, whereas the  $\beta$ -arrestin-biased agonist carvedilol induced a low level of cAMP only at high concentrations of ligand (Fig. 2B). This is consistent with a previous study showing that carvedilol displays a very low potency and efficacy with  $\beta_1$ AR-mediated Gs activation in  $\beta_1$ AR-overexpressed cells (Kim et al., 2008). Importantly, Cmpd-6 showed no enhancement of the dose-response curve for either ligand (Fig. 2B), indicating that Cmpd-6 has little or no PAM activity on the Gs-mediated  $\beta_1$ AR signaling.

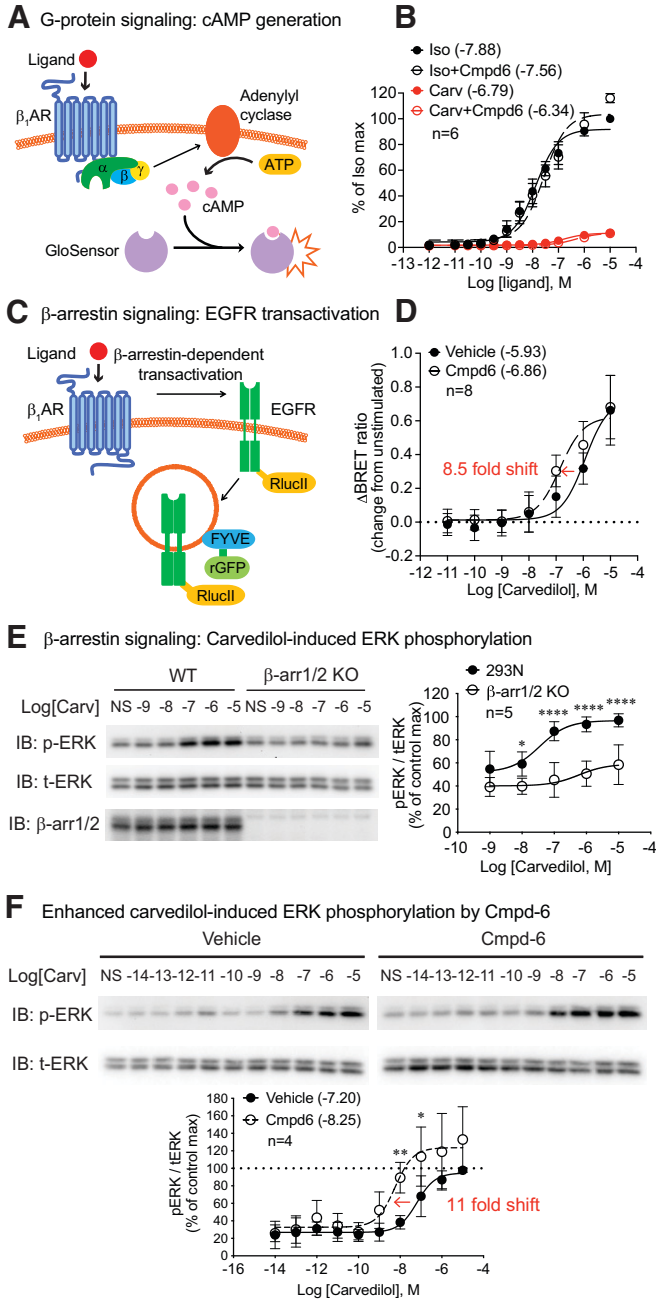
To determine the activity of Cmpd-6 on carvedilol-stimulated  $\beta$ -arrestin-mediated  $\beta_1$ AR signaling, we tested the effect of Cmpd-6 on  $\beta_1$ AR-stimulated EGFR transactivation. Previous studies showed that ligand stimulation of the  $\beta_1$ AR induces G protein-coupled receptor kinase-mediated receptor phosphorylation and subsequent  $\beta$ -arrestin and Src recruitment (Kim et al., 2008). This in turn leads to the activation of matrix metalloproteinases and shedding of heparin-binding EGF that binds to EGFRs promoting its activation and internalization, a process known as EGFR transactivation (Noma et al., 2007; Kim et al., 2008). To allow sensitive and quantitative detection of  $\beta$ -arrestin-mediated EGFR transactivation, we developed a BRET sensor using EGFR-RlucII and the early endosome-targeted FYVE-rGFP (Namkung et al., 2016) to monitor the internalization of EGFRs (Fig. 2C). To assess the fidelity of this biosensor assay system we show that EGF ligand induced a robust change in BRET ratio, which was blocked by the EGFR inhibitor AG1478 (Supplemental Fig. 3A). In  $\beta_1$ AR stably expressing HEK293 cells transfected with EGFR-RlucII and FYVE-rGFP, both

the full agonist isoproterenol and the  $\beta$ -arrestin-biased agonist carvedilol dose-dependently induce EGFR internalization, and the response is diminished by the small interfering RNA knockdown of  $\beta$ -arrestin1/2 (Supplemental Fig. 3B). Cmpd-6 potentiated the carvedilol-induced response as indicated by the leftward shift of carvedilol dose-response curve [Fig. 2D, vehicle:  $\text{logEC}_{50} = -5.93$  ( $-6.28$  to  $-5.61$ ), Cmpd-6:  $\text{logEC}_{50} = -6.86$  ( $-7.19$  to  $-6.46$ )], whereas it showed no PAM activity on the isoproterenol response (Supplemental Fig. 3C).

We next tested Cmpd-6 in  $\beta_1$ AR-mediated ERK activation induced by carvedilol. We have previously shown that carvedilol induces  $\beta_1$ AR-mediated ERK signaling in a  $\beta$ -arrestin-dependent manner (Kim et al., 2008; Wang et al., 2017; Luttrell et al., 2018). Consistent with these earlier studies, we show that carvedilol-induced ERK activation is largely lost in the HEK293 cells in which the genes for both  $\beta$ -arrestin1 and 2 are deleted through CRISPR gene editing (Fig. 2E). The low level of ERK activation in  $\beta$ -arrestin1/2 knockout cells is likely the result of some activation of Gs at high concentrations of carvedilol (Fig. 2B). In marked contrast to the effect observed on Gs activation, Cmpd-6 substantially potentiates carvedilol-induced ERK activation, as indicated by an 11-fold leftward shift in the ERK dose-response curve along with a small increase in the maximal response [Fig. 2F, vehicle:  $\text{logEC}_{50} = -7.20$  ( $-7.44$  to  $-6.98$ ), Cmpd-6:  $\text{logEC}_{50} = -8.25$  ( $-8.68$  to  $-7.85$ )]. Additionally, isoproterenol stimulation showed no cooperativity by Cmpd-6 (Fig. 3A). When expanding our testing to a broad panel of ligands, we found that the effect of Cmpd-6 is highly selective to carvedilol with minimal or no augmentation of ERK phosphorylation in response to stimulation by a comprehensive panel of  $\beta$ AR ligands (Fig. 3). Data of the ERK activation assay are also presented as fold over nonstimulated samples of each treatment (Supplemental Fig. 4).

Interestingly, in our expanded ligand panel on ERK activation, we noticed that a number of other  $\beta$ -blockers robustly induced  $\beta_1$ AR-mediated ERK activation (Fig. 3, E–G). To dissect the mechanism for the various  $\beta$ -blockers on  $\beta_1$ AR signaling, we determined the level of  $\beta$ -arrestin dependence on ERK signaling and Gs activation. In contrast to carvedilol, which activates ERK mainly through  $\beta$ -arrestin (Fig. 2F), the activation induced by alprenolol and carazolol is partially ( $\sim 50\%$ ) dependent on  $\beta$ -arrestin, whereas bucindolol activates ERK independent of  $\beta$ -arrestin (Supplemental Fig. 5, A–C). We then performed GloSensor assays of Gs-mediated cAMP generation, which showed that bucindolol and carazolol induced robust activation of cAMP (40% of the maximal isoproterenol response), whereas alprenolol only modestly induced a response (20% of the maximal isoproterenol response), and metoprolol induced no response (Supplemental Fig. 5D). Importantly, none of the  $\beta$ -blockers tested had positive cooperativity to Cmpd-6 (Supplemental Fig. 5D). These data show that although a number of  $\beta$ -blockers can activate  $\beta_1$ AR signaling, they differentially engage signal transducers to activate downstream pathways as indicated by their differential cooperativity with Cmpd-6, dependence on  $\beta$ -arrestin, and activity on Gs-mediated cAMP production.

Taken together, we show the selective positive cooperativity of Cmpd-6 for the  $\beta_1$ AR with carvedilol with respect to binding affinity and the selective enhancement of carvedilol-stimulated  $\beta$ -arrestin-mediated signaling but not Gs-



**Fig. 2.** Cmpd-6 potentiates carvedilol-stimulated  $\beta$ -arrestin-mediated  $\beta_1$ AR signaling but not Gs protein-mediated  $\beta_1$ AR signaling. (A)  $\beta_1$ AR-mediated Gs activation was monitored with GloSensor cAMP assay (Promega), a luciferase-based biosensor to monitor cAMP level. (B) Cmpd-6 had no effect on isoproterenol- or carvedilol-induced  $\beta_1$ AR-mediated Gs activation. HEK293 cells transiently transfected with  $\beta_1$ ARs and GloSensor were pretreated with vehicle (DMSO) or 30  $\mu$ M Cmpd-6 for 20 minutes with 100 nM ICI118,551, the highly selective  $\beta_2$ AR antagonist, to block the activation of endogenous  $\beta_2$ ARs. Cells were then stimulated with serial doses of isoproterenol or carvedilol for 5 minutes. The luminescence values were normalized to the percentage of maximal isoproterenol-induced level in the vehicle-treated group. (C)  $\beta_1$ AR-mediated EGFR transactivation was determined by monitoring EGFR endocytosis with BRET assay with EGFR\_RlucII and endosomal-located rGFP\_FYVE. (D) Cmpd-6 potentiated carvedilol-stimulated  $\beta_1$ AR-mediated EGFR transactivation, which was indicated by a leftward shift of the dose-response curve. HEK293 cells stably expressing  $\beta_1$ ARs were transfected with EGFR-RlucII and rGFP-FYVE constructs. Cells were pretreated with vehicle (DMSO) or 30  $\mu$ M Cmpd-6 for 20 minutes and then stimulated with indicated concentrations of carvedilol for 25 minutes before BRET measurement. The change in BRET ratio (emission ratio of RlucII to rGFP) is expressed as the difference between ligand-stimulated and unstimulated samples. (E) Carvedilol-induced  $\beta_1$ AR activation of ERK is mediated through  $\beta$ -arrestins. Wild-type (WT) or  $\beta$ -arrestin1/2 knockout ( $\beta$ -arr1/2 KO) HEK293 cells transiently transfected with  $\beta_1$ ARs were stimulated with serial concentrations of carvedilol for 5 minutes. Data are calculated as the ratio of pERK to total ERK (tERK) and then normalized as percentage of the maximum value in the wide-type group. (F) Cmpd-6 led to a leftward shift of carvedilol dose-response curve on  $\beta_1$ AR-mediated ERK activation, indicating its PAM activity on this  $\beta$ -arrestin-dependent signaling. HEK293 cells stably expressing  $\beta_1$ ARs were pretreated with vehicle or 30  $\mu$ M Cmpd-6 for 20 minutes and then stimulated with indicated concentration of carvedilol for 5 minutes. The pERK/tERK ratio is normalized to the maximum value in the vehicle-treated group. Data represent the mean  $\pm$  S.D. for four to eight independent experiments as marked on the figure. Error bars in some data points in (B) are not visualizable because the error bar is shorter than the size of the symbol. Dose-response curves and log EC<sub>50</sub> values shown in legends were generated with GraphPad Prism. Statistical comparisons were performed using two-way repeated (related)-measures ANOVA with Sidak correction for multiple comparisons. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\*\* $P < 0.0001$ .

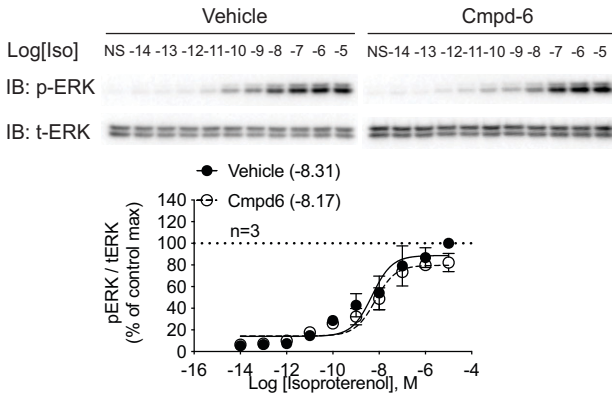
mediated cAMP production. These data support our notion that Cmpd-6 is a  $\beta$ -arrestin-biased PAM of the carvedilol-occupied  $\beta_1$ ARs.

**Cmpd-6 Potentiates  $\beta$ -Arrestin-Dependent Cell Survival by Carvedilol in Response to Ischemia/Reperfusion Myocardial Injury.** Our data from in vitro and cellular assays strongly support that Cmpd-6 selectively cooperates with carvedilol to enhance  $\beta$ -arrestin-biased  $\beta_1$ AR signaling. Previous studies have shown that sustained G protein activation by  $\beta_1$ ARs is associated with deleterious cardiac remodeling, whereas  $\beta$ -arrestin signaling is potentially cardioprotective (Noma et al., 2007; Wang et al., 2018). Although carvedilol, among other  $\beta$ -blockers, is considered a standard therapy in heart failure, a major limitation is that it is often difficult to

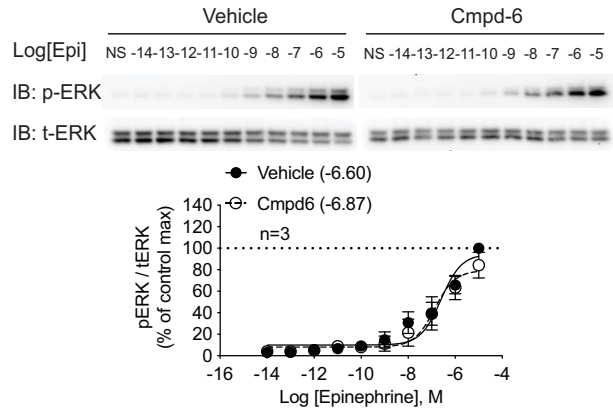
achieve the maximally tolerated dose because of the development of adverse effects (Bhatt et al., 2017). Carvedilol may lead to fatigue and impair functional capacity by virtue of its blockade of the catecholamine-stimulated heart rate response during exercise. We therefore reasoned that a  $\beta$ -arrestin-biased PAM for the  $\beta_1$ AR, such as Cmpd-6, as we identify in this study, could potentially enhance the cardioprotective effect of carvedilol in vivo while minimizing the hemodynamic perturbation.

To test this hypothesis, we first determined the in vivo  $\beta$ -blockade effect of carvedilol on catecholamine-induced cardiac hemodynamics by assessing the response to isoproterenol on heart rate and contractility in mice pretreated with increasing doses of carvedilol. We treated mice with 1, 5, or 20 mg/kg per day carvedilol with Alzet osmotic minipump for

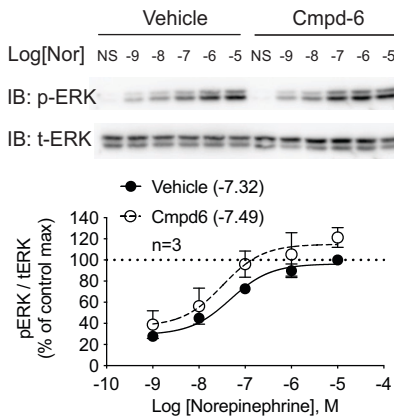
**A** Isoproterenol-induced ERK phosphorylation



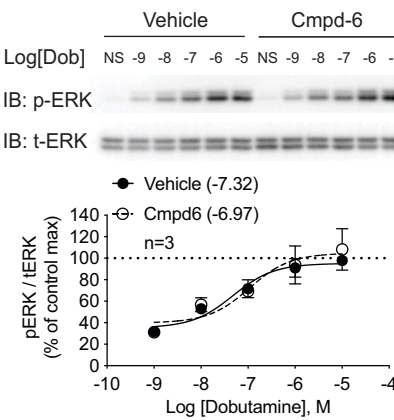
**B** Epinephrine:



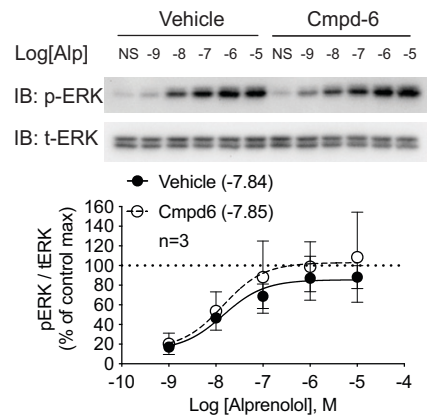
**C** Norepinephrine:



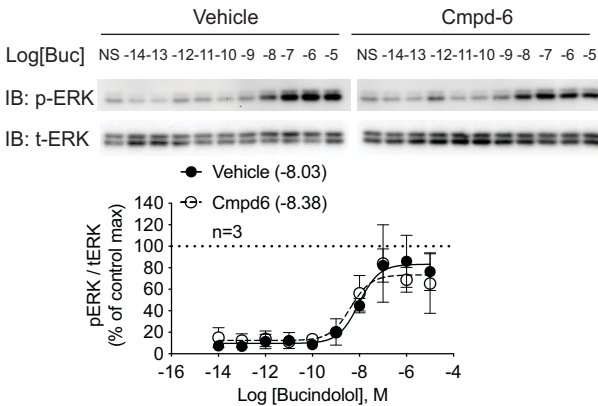
**D** Dobutamine:



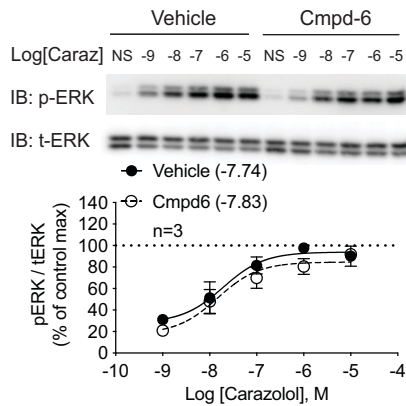
**E** Alprenolol:



**F** Bucindolol:



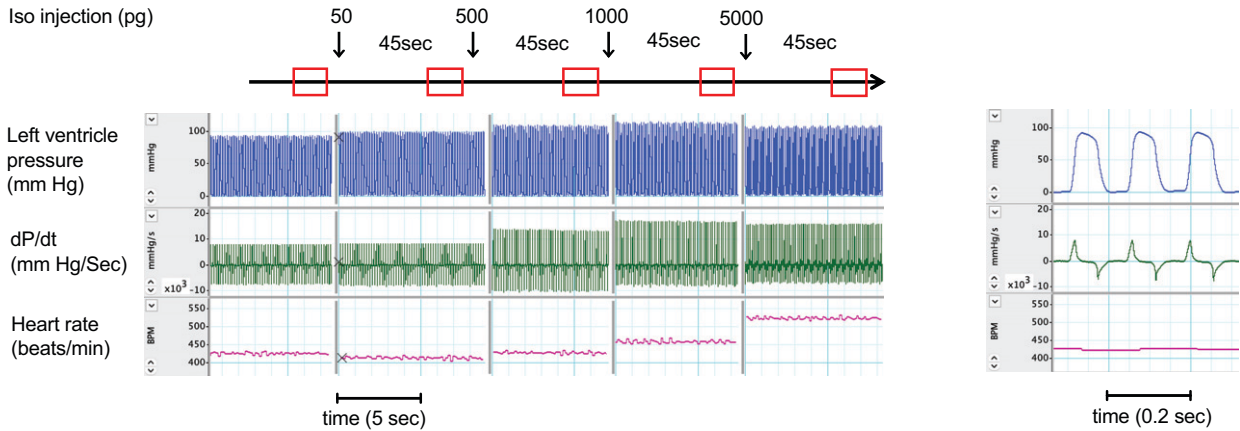
**G** Carazolol:



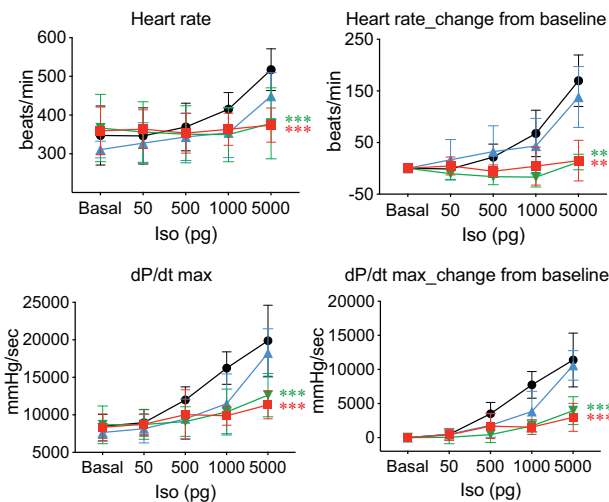
**Fig. 3.** Cmpd-6 shows no PAM activity on  $\beta_1$ AR-mediated ERK stimulated by a comprehensive panel of ligands. HEK293 cells stably expressing  $\beta_1$ ARs were pretreated with vehicle or 30  $\mu$ M Cmpd-6 for 20 minutes and then stimulated with serial concentrations of indicated ligands for 5 minutes. A panel of agonists (A–D) and  $\beta$ -blockers (E–G) dose-dependently induced  $\beta_1$ AR-mediated ERK phosphorylation. Cmpd-6 pretreatment showed no effect on these ligands. Data are presented as pERK/total ERK (tERK) ratios normalized to the maximum value in the vehicle group. Data represent the mean  $\pm$  S.D. for three independent experiments. Dose-response curves and log  $EC_{50}$  values shown in legends were generated with GraphPad Prism. Statistical comparisons were performed using two-way repeated (related)-measures ANOVA with Sidak correction for multiple comparisons and showed no difference between vehicle- and Cmpd-6-treated groups for each ligand. IB, immunoblotting.

3 days and then intravenously injected boluses of increasing doses of isoproterenol while monitoring the hemodynamics with a high-fidelity micromanometer catheter inserted retrograde into the left ventricle (Fig. 4A). Isoproterenol dose-dependently increased cardiac contractility, as indicated by

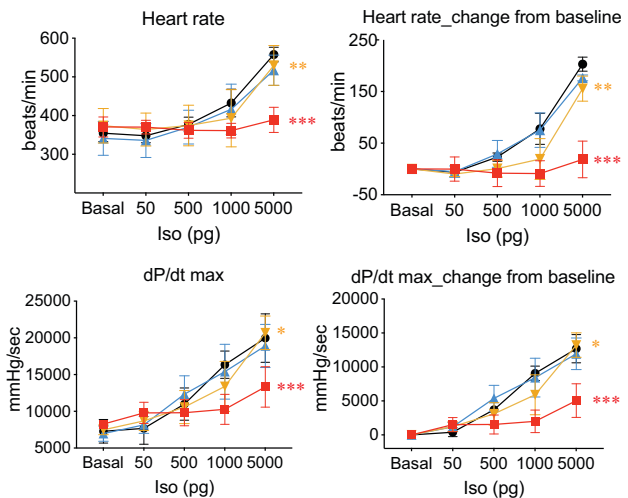
an increase in  $dP/dt$  max as well as heart rate (Fig. 4, A and B). Low dose of carvedilol at 1 mg/kg per day had minimal effect on isoproterenol-induced hemodynamics, whereas higher doses of carvedilol blocked both the heart rate and contractility response (Fig. 4B). Coadministration of Cmpd-6

**A** Isoproterenol-induced hemodynamics.**B** Carvedilol pretreatment

- Vehicle (n=5)
- ▲ Carv, 1 mg/kg/day (n=5)
- ▼ Carv, 5 mg/kg/day (n=5)
- Carv, 20 mg/kg/day (n=5)

**C** Carvedilol/Cmpd-6 pretreatment

- Vehicle (n=4)
- ▲ Carv (1 mg/kg/day) (n=5)
- ▼ Carv (1 mg/kg/day) + Cmpd6 (5 mg/kg/day) (n=5)
- Carv (20 mg/kg/day) (n=5)



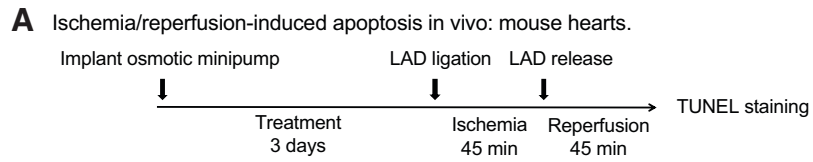
**Fig. 4.** Cmpd-6 modestly enhances the inhibitory effect of carvedilol on catecholamine-induced hemodynamics in mouse hearts. (A) Representative traces of hemodynamic measurements in mouse. A high-fidelity micromanometer catheter connected with a pressure transducer was inserted retrograde into the left ventricle to monitor blood pressure. Serial doses of isoproterenol (50, 500, 1000, and 5000 pg) were injected intravenously at 45-second intervals. Left: hemodynamics were monitored continuously and recorded between 35 and 45 seconds after each injection when steady state was reached. Right: expanded view of cardiac cycles enlarged on x-axis (time). (B) Carvedilol dose-dependently blocks isoproterenol-induced increase in heart rate and contractility (indicated by dP/dt max). Wild-type C57BL/6J mice were treated with vehicle or indicated doses of carvedilol (1, 5, and 20 mg/kg per day) for 3 days with Alzet osmotic pumps before the isoproterenol-induced hemodynamic study. (C) Cmpd-6 led to a modest enhancement of the inhibitory effect of carvedilol. Mice were treated with vehicle, carvedilol (1 or 20 mg/kg per day), or a combination of carvedilol (1 mg/kg per day) and Cmpd-6 (5 mg/kg per day) for 3 days with Alzet osmotic pump. Hemodynamics were monitored and calculated using LabChart8 software. Data represent the mean  $\pm$  S.D. for four or five animals as marked on the figure. Data of each drug-treated group were compared with the vehicle-treated group using two-way repeated (related)-measures ANOVA with Sidak correction for multiple comparisons. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.0001$ , interaction vs. vehicle group.

(5 mg/kg per day) in a  $\sim$ 3:1 molar ratio to carvedilol (1 mg/kg per day) showed only a modest potentiation of the inhibitory effect on heart rate and dP/dt max (Fig. 4C). Importantly, the  $\beta$ -blocker effect of low-dose carvedilol (1 mg/kg per day) with Cmpd-6 is substantially smaller than that of higher doses of carvedilol alone (Fig. 4, B and C), despite the 7.9-fold enhancement on receptor binding affinity of carvedilol in the presence of Cmpd-6 as shown in Fig. 1A. The lack of a rightward shift of the dose-response curve by Cmpd-6 suggests that it has minimal regulatory effects in vivo on  $\beta_1$ AR-mediated Gs signaling.

Based on the above data, we tested whether the positive cooperative effect of Cmpd-6 on low-dose carvedilol could

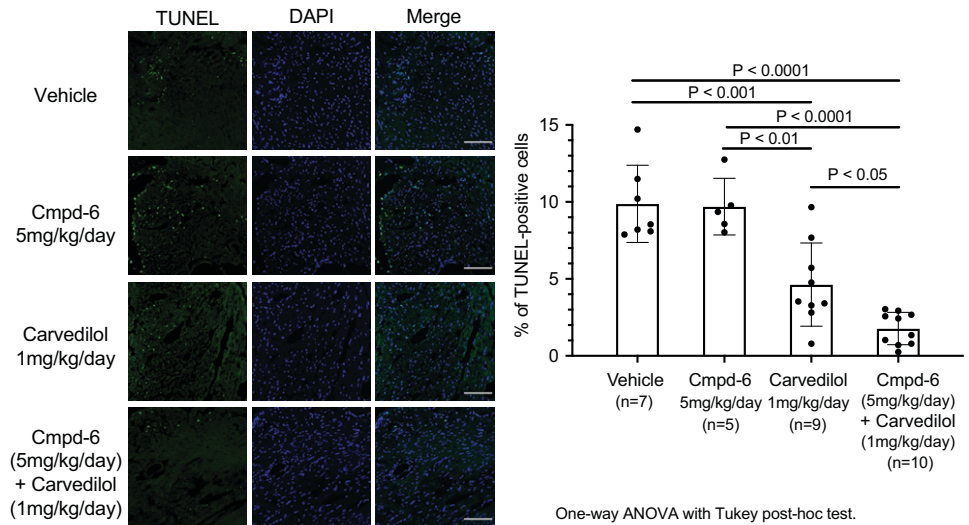
potentiate its action on cell survival in response to ischemia/reperfusion (I/R) cardiac injury. Wild-type mice were treated with vehicle, Cmpd-6 (5 mg/kg per day), carvedilol (1 mg/kg per day), or coadministration of both compounds through Alzet osmotic pump for 3 days to reach the steady state. The left anterior descending coronary artery was occluded for 45 minutes to cause myocardial ischemia and then released for 45 minutes of tissue reperfusion (Fig. 5A). We chose the 1 mg/kg per day dose of carvedilol since this dose was sufficient to provide partial cardioprotection in response to I/R injury (Supplemental Fig. 6A), whereas it only minimally enhanced the  $\beta$ -blockade effect on cardiac hemodynamics (Fig.



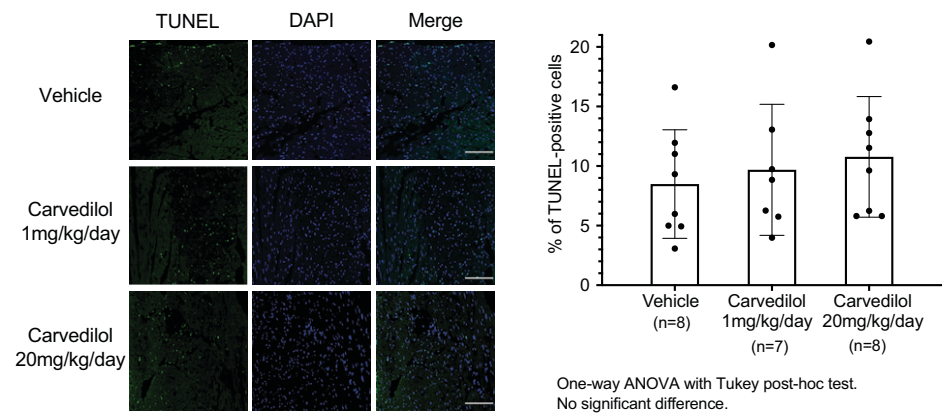


**Fig. 5.** Cmpd-6 potentiates the  $\beta$ -arrestin-dependent in vivo protective effect of carvedilol against ischemia/reperfusion injury-induced myocardium apoptosis. (A) Scheme of the study. Mice were treated with carvedilol and Cmpd-6 individually or in combination for 3 days with Alzet osmotic pumps. The left anterior descending (LAD) coronary artery was ligated for 45 minutes to produce cardiac ischemia and then released to allow blood flow restoration for 45 minutes. Level of apoptosis was assessed with TUNEL staining. (B) Carvedilol diminishes the ischemia/reperfusion-induced apoptosis in hearts, and coadministration of Cmpd-6 enhances this protective effect. Wild-type C57BL/6J mice were treated with vehicle, Cmpd-6 (5 mg/kg per day), carvedilol (1 mg/kg per day), or a combination of both compounds for 3 days with Alzet osmotic pumps before the ischemia/reperfusion procedure was performed. (C) The antiapoptotic effect of carvedilol is abolished in mice with cardiomyocyte-specific deletion of  $\beta$ -arrestins. The  $\beta$ -arrestin1/2 cardiac knockout animals ( $\alpha$ MyHC-Cre:Arrb1<sup>flox/flox</sup>/Arrb2<sup>flox/flox</sup>) were treated with vehicle or carvedilol (1 or 20 mg/kg per day) for 3 days before the ischemia/reperfusion procedure was performed. Data represent the mean  $\pm$  S.D. for 5–10 animals as marked on the figure. Statistical comparisons were performed using one-way ANOVA with Tukey correction for multiple comparisons. DAPI, 4',6-diamidino-2-phenylindole.

**B** Wild type.



**C** Cardiomyocyte specific  $\beta$ -arrestin deletion:  $\alpha$ MyHC-Cre:Arrb1<sup>flox/flox</sup>/Arrb2<sup>flox/flox</sup>



4B). In contrast, although higher doses of carvedilol (5 or 20 mg/kg per day) inhibited I/R-induced apoptosis (Supplemental Fig. 6), carvedilol also showed a high level of competitive antagonism (i.e.,  $\beta$ -blocker activity) to isoproterenol-stimulated, Gs protein-mediated hemodynamics (Fig. 4B).

Cmpd-6 alone has no intrinsic activity on I/R-induced apoptosis as shown by the  $\sim$ 10% TUNEL-positive cells in the ischemic zone in mice pretreated with either vehicle or Cmpd-6 (Fig. 5B). Carvedilol decreased the level of I/R-induced apoptosis compared with vehicle-treated animals to  $\sim$ 5% albeit with considerable variability. In marked contrast, the addition of Cmpd-6 to the same dose of carvedilol substantially and more consistently enhanced the antiapoptotic effect, as indicated by the reduction in the level of TUNEL-positive cells to  $\sim$ 2% with many hearts showing very low levels of injury (Fig. 5B).

To determine whether the antiapoptotic effect of carvedilol is mediated through  $\beta$ -arrestins, we tested carvedilol in mice with cardiomyocyte-specific deletion of  $\beta$ -arrestin1/2 achieved by  $\alpha$ MyHC promoter-driven Cre recombinase ( $\alpha$ MyHC-Cre:Arrb1<sup>flox/flox</sup>/Arrb2<sup>flox/flox</sup>, Supplemental Fig. 6B). Pretreatment with either low- or high-dose carvedilol did not reduce I/R-induced apoptosis in the  $\beta$ -arrestin1/2 knockout animals (Fig. 5C), indicating that the cardioprotective action of carvedilol in vivo is mediated by signals downstream of  $\beta$ -arrestin.

Taken together, these data support our contention that the  $\beta$ -arrestin-biased  $\beta_1$ AR PAM Cmpd-6 enhances the in vivo cardioprotective effects of carvedilol against cardiac injury-induced cell apoptosis and further supports Cmpd-6 as having a potential therapeutic benefit in enhancing the effectiveness of carvedilol under conditions of cardiac injury.

## Discussion

In this study, we show Cmpd-6 is a  $\beta$ -arrestin-biased positive allosteric modulator for  $\beta_1$ ARs occupied with carvedilol. Cmpd-6 was identified as a PAM for the  $\beta_2$ AR that enhances agonist binding affinity and potentiates both the Gs protein- and  $\beta$ -arrestin-dependent signaling. Cmpd-6 was initially shown to cooperate with carvedilol but not other  $\beta$ -blockers on the  $\beta_2$ AR (Pani et al., 2021). This prompted us to assess its properties at the  $\beta_1$ AR. Here we identify that Cmpd-6 selectively enhances the binding affinity of the  $\beta$ -arrestin-biased agonist carvedilol to the  $\beta_1$ AR, whereas it has minimal effects on the affinity of other agonists and antagonists tested. Cmpd-6 also potentiates carvedilol-induced  $\beta$ -arrestin-dependent  $\beta_1$ AR signaling, including EGFR transactivation and ERK activation, whereas it has no effect on Gs protein-activated cAMP generation. In contrast to its minimal effect on the in vivo  $\beta$ -blockade function of carvedilol on catecholamine-induced cardiac hemodynamics, Cmpd-6 augments the cardioprotective roles of carvedilol against myocardial ischemia/reperfusion-induced apoptosis, which we demonstrate is a  $\beta$ -arrestin-dependent process since the antiapoptotic effect of carvedilol is abolished in mice with cardiac-specific deletion of  $\beta$ -arrestin1/2. The highly selective cooperativity of Cmpd-6 with carvedilol on ligand binding affinity to the  $\beta_1$ AR,  $\beta$ -arrestin-biased cellular signaling, and the  $\beta$ -arrestin-dependent antiapoptotic role in vivo suggest that Cmpd-6 is a  $\beta$ -arrestin-biased PAM for the  $\beta_1$ AR and may have therapeutic potential to enhance the clinical effects of carvedilol, which is widely used in the treatment of cardiac diseases.

$\beta_1$ ARs and  $\beta_2$ ARs are the most abundant GPCRs expressed in mammalian hearts and are principal regulators of cardiac pathophysiology (Woo and Xiao, 2012). Prolonged catecholamine stimulation leads to cardiac injury (Haft, 1974) and cardiomyocyte apoptosis (Communal et al., 1999; Noma et al., 2007), and mice with cardiac-specific  $\beta_1$ AR overexpression develop myocyte hypertrophy and cardiac dysfunction (Engelhardt et al., 1999), which indicates that excessive catecholamine activation of  $\beta_1$ ARs is pathogenic to the heart (Yoo et al., 2009). In contrast,  $\beta$ -arrestin-mediated  $\beta_1$ AR signaling appears to provide cardioprotection (Noma et al., 2007). In this study, we show that carvedilol protects hearts from ischemia/reperfusion-induced apoptosis, and Cmpd-6 enhances this antiapoptotic effect. Importantly, we demonstrated that the antiapoptotic effect of carvedilol in cardiomyocyte is at least in part mediated through  $\beta$ -arrestin-driven signaling pathways.

In contrast to the predominant myocyte expression and the proapoptotic effect of the  $\beta_1$ ARs,  $\beta_2$ ARs are primarily expressed on fibroblasts and endothelial cells (Myagmar et al., 2017) and have been shown to be cardioprotective from apoptosis (Communal et al., 1999; Devic et al., 2001; Wang et al., 2018). Based on the predominant expression of  $\beta_1$ ARs in myocytes and the opposing effect of agonist-activated  $\beta_1$ AR and  $\beta_2$ AR on apoptosis, we reason that the protective effects of carvedilol and Cmpd-6 against ischemia/reperfusion-induced apoptosis are most likely to be mediated by the  $\beta_1$ AR. However, a previous study showed that the  $\beta_2$ AR modulator pepducin ICL1-9 reduces I/R-induced cardiomyocyte death in a  $\beta_2$ AR- and  $\beta$ -arrestin-dependent manner (Grisanti et al., 2018). Therefore, it is possible that the enhanced

antiapoptotic effect of carvedilol by Cmpd-6 also involves the  $\beta_2$ AR. The precise dissection of the contribution of receptor subtypes would require testing carvedilol and Cmpd-6 in  $\beta_1$ AR- or  $\beta_2$ AR-knockout animals.

In regard to the mechanism of action for the in vivo cardioprotection by carvedilol, we speculate that the enhancement by Cmpd-6 on carvedilol-stimulated  $\beta$ AR- $\beta$ -arrestin signaling may play an important role.  $\beta_1$ AR-mediated EGFR transactivation is  $\beta$ -arrestin-dependent and confers cardioprotection against myocardial apoptosis induced by chronic catecholamine stimulation (Noma et al., 2007).  $\beta_1$ AR-mediated EGFR transactivation stimulates differential subcellular activation of ERK and Akt (protein kinase B) (Grisanti et al., 2014; Tilley et al., 2009) to modulate caspase 3 activity and apoptotic gene expression (Grisanti et al., 2014). It has also been shown that  $\beta$ -arrestin mediates angiotensin II type 1 receptor-induced EGFR/ERK transactivation and protects mouse hearts against mechanical stress-induced apoptosis (Rakesh et al., 2010). Angiotensin II type 1 receptor stimulation with the  $\beta$ -arrestin-biased agonist SII activates both ERK/p90 ribosomal S6 kinase and phosphoinositide 3-kinase/AKT pathways leading to inactivation of the proapoptotic protein B-cell lymphoma 2-associated agonist of cell death to protect cells from oxidative stress-induced apoptosis (Ahn et al., 2009). Furthermore, the antiapoptotic effects of Cmpd-6 and carvedilol may also be mediated by regulation of microRNA (miR) processing. Carvedilol-stimulated  $\beta_1$ AR-activated  $\beta$ -arrestin1 promotes the processing of a subset of miRs in the mouse heart (Kim et al., 2014), among which miR-125b-5p reduced expression of proapoptotic genes BCL2 antagonist/killer 1 and Kruppel-like factor 13 in cardiomyocytes to protect the mouse heart from ischemic injury (Bayoumi et al., 2018). Finally, the  $\beta$ -arrestin-biased  $\beta_2$ AR modulator pepducin ICL1-9 protects against I/R-induced cardiac injury and cardiomyocyte death through activating the Ras homolog family member A/rhodopsin-associated protein kinase pathway and reducing mitochondrial oxidative stress (Grisanti et al., 2018).

Based on the central role of  $\beta$ ARs in the heart,  $\beta$ -blockers are first-line agents for the treatment of cardiac diseases, such as heart failure (Yancy et al., 2017). However, different  $\beta$ -blockers have variable therapeutic effectiveness in treating heart failure (Bhatt et al., 2017). Advances in understanding the complexities of GPCR biology and detailed dissections of pharmacological actions of  $\beta$ -blockers may assist in understanding their differential efficacy. In this study, we show that the  $\beta$ -blockers alprenolol, carvedilol, bucindolol, and carazolol differentially engage Gs and  $\beta$ -arrestin to activate downstream signaling. We postulate that the unique properties of carvedilol in stimulating  $\beta$ -arrestin-dependent  $\beta_1$ AR signaling may contribute to its potential clinical superiority as suggested by meta-analyses showing that carvedilol has the lowest all-cause mortality among different  $\beta$ -blockers tested in heart failure (Chatterjee et al., 2013; DiNicolantonio et al., 2013).

Although  $\beta$ -blockers are considered standard of therapy in heart failure, the level of patient unresponsiveness to  $\beta$ -blocker treatment and intolerable adverse effects (Bhatt et al., 2017) support a quest to develop novel  $\beta$ AR ligands with improved drug efficacy and safety. Recent developments in identifying allosteric modulators for GPCRs may offer great potential as novel therapeutics since they have the potential for greater receptor subtype specificity, fewer off-

target side effects, and better drug safety profiles than orthosteric ligands (Lane et al., 2017; Wisler et al., 2018).

Cmpd-6 was identified by a DNA-encoded small-molecule library affinity screen for agonist-bound  $\beta_2$ ARs (Ahn et al., 2018). Cmpd-6 potentiates both Gs-mediated cAMP generation and  $\beta$ -arrestin recruitment to  $\beta_2$ ARs with comparative efficacy in HEK293 cells (Ahn et al., 2018), indicating its unbiased PAM activity on  $\beta_2$ ARs. Cmpd-6 also cooperates with the  $\beta$ -arrestin-biased agonist carvedilol but not other  $\beta$ -blockers on  $\beta_2$ ARs (Pani et al., 2021). In contrast to its ~50-fold enhancement of isoproterenol binding affinity for  $\beta_2$ ARs, Cmpd-6 enhances isoproterenol binding affinity for  $\beta_1$ ARs by only 2-fold as shown in this study as well as previous ones (Ahn et al., 2018; Liu et al., 2019). The selectivity of Cmpd-6 on agonist binding affinity for  $\beta_2$ ARs over  $\beta_1$ ARs might be because of the structural differences between the two receptor subtypes in the allosteric binding pocket for Cmpd-6. Mutational studies introducing key  $\beta_2$ AR residues interacting with Cmpd-6 into the corresponding  $\beta_1$ AR sites created gain-of-function mutant  $\beta_1$ ARs, to which Cmpd-6 salvaged its PAM behavior for agonist binding (Liu et al., 2019). Interestingly, in contrast to its marginal effect on agonist binding to  $\beta_1$ ARs, Cmpd-6 enhances the affinity of the antagonist carvedilol to  $\beta_1$ ARs by ~8-fold, leading to a potentiation of  $\beta$ -arrestin signaling. We speculate that this unique property of Cmpd-6 on carvedilol and not any other antagonist tested is likely based on structural determinants whereby Cmpd-6 binding to carvedilol-occupied  $\beta_1$ ARs or  $\beta_2$ ARs stabilizes the receptor to adopt a “ $\beta$ -arrestin” conformation, thereby engaging  $\beta$ -arrestin and not Gs as its preferred signaling transducer.

Our data demonstrate that Cmpd-6 potentiates the  $\beta$ -arrestin-dependent antiapoptotic effects of carvedilol in vivo, whereas it has minimal effect on blocking the physiologic response to catecholamines. Major side effects of carvedilol treatment in patients with heart failure are exertional intolerance, dizziness, and advanced heart block (Krum et al., 1995) due to sympathetic activity attenuation. Accordingly, allosteric modulators, such as Cmpd-6, could provide important therapeutic advantages by enhancing the cardioprotective action of carvedilol but minimizing the adverse effects often found with high-dose carvedilol. In the future, determining the effects of Cmpd-6 in chronic left ventricle dysfunction will be of great interest to further evaluate the therapeutic potential of this compound. Additionally, the structure-activity relationship study of Cmpd-6 may lead to identification of new compounds with desirable receptor subtype selectivity or signaling modulation effects. For instance, a chemically modified analog of Cmpd-6 selectively cooperates with carvedilol on the  $\beta_2$ AR while showing no PAM activity on agonists (Pani et al., 2021), which is similar to the effects of Cmpd-6 on the  $\beta_1$ AR. Based on the differential functional consequences of  $\beta_1$ AR and  $\beta_2$ AR activation in the heart (Wang et al., 2018), further development of Cmpd-6 analogs with high receptor selectivity and selective carvedilol cooperativity may provide additional therapeutic benefits.

In conclusion, we show that Cmpd-6 is a  $\beta$ -arrestin-biased PAM for  $\beta_1$ ARs. It selectively increases the affinity of the  $\beta$ -arrestin-biased agonist carvedilol for  $\beta_1$ ARs and potentiates  $\beta$ -arrestin-mediated signaling stimulated with carvedilol. Importantly, Cmpd-6 enhances the  $\beta$ -arrestin-dependent in vivo effects of carvedilol to protect hearts from ischemia/

reperfusion injury-induced apoptosis. Our data suggest that the development of  $\beta$ -arrestin-biased  $\beta$ AR allosteric modulators may provide a new direction for improving current treatments or developing novel agents for cardiac diseases.

#### Authorship Contributions

*Participated in research design:* Wang, Pani, Gokhan, Xiong, Kahsai, Jiang, Rockman.

*Conducted experiments:* Wang, Pani, Gokhan, Xiong, Kahsai, Jiang.

*Contributed new reagents or analytic tools:* Wang, Pani, Gokhan, Xiong, Kahsai, Jiang.

*Performed data analysis:* Wang, Pani, Gokhan, Xiong, Kahsai, Jiang.

*Wrote or contributed to the writing of the manuscript:* Wang, Pani, Gokhan, Xiong, Kahsai, Jiang, Ahn, Lefkowitz, Rockman.

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