Unique Positive Cooperativity Between the β-Arrestin–Biased β-Blocker Carvedilol and a Small Molecule Positive Allosteric Modulator of the β2-Adrenergic Receptor

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ABSTRACT

Among β-blockers that are clinically prescribed for heart failure, carvedilol is a first-choice agent with unique pharmacological properties. Carvedilol is distinct from other β-blockers in its ability to elicit β-arrestin–biased agonism, which has been suggested to underlie its cardioprotective effects. Augmenting the pharmacologic properties of carvedilol thus holds the promise of developing more efficacious and/or biased β-blockers. We recently identified compound-6 (cmpd-6), the first small molecule positive allosteric modulator of the β2-adrenergic receptor (β2AR). Cmpd-6 is positively cooperative with orthosteric agonists at the β2AR and enhances agonist-mediated transducer (G-protein and β-arrestin) signaling in an unbiased manner. Here, we report that cmpd-6, quite unexpectedly, displays strong positive cooperativity only with carvedilol among a panel of structurally diverse β-blockers. Cmpd-6 enhances the binding affinity of carvedilol for the β2AR and augments its ability to competitively antagonize agonist-induced cAMP generation. Cmpd-6 potentiates β-arrestin1− but not Gs-protein–mediated high-affinity binding of carvedilol at the β2AR and β-arrestin–mediated cellular functions in response to carvedilol including extracellular signal-regulated kinase phos-phorylation, receptor endocytosis, and trafficking into lysosomes. Importantly, an analog of cmpd-6 that selectively retains positive cooperativity with carvedilol acts as a negative modulator of agonist-stimulated β2AR signaling. These unprecedented cooperative properties of carvedilol and cmpd-6 have implications for fundamental understanding of G-protein–coupled receptor (GPCR) allosteric modulation, as well as for the development of more effective biased beta blockers and other GPCR therapeutics.

SIGNIFICANCE STATEMENT

This study reports on the small molecule–mediated allosteric modulation of the β-arrestin–biased β-blocker, carvedilol. The small molecule, compound-6 (cmpd-6), displays an exclusive positive cooperativity with carvedilol among other β-blockers and enhances the binding affinity of carvedilol for the β2-adrenergic receptor. Cooperative effects of cmpd-6 augment the β-blockade property of carvedilol while potentiating its β-arrestin–mediated signaling functions. These findings have potential implications in advancing G-protein–coupled receptor allosterly, developing biased therapeutics and remedying cardiovascular ailments.

Introduction

G-protein–coupled receptors (GPCRs), also known as seven-transmembrane receptors, constitute the largest family of transmembrane proteins represented in the human proteome. GPCRs are important and ubiquitous portals of cell signaling that are involved in regulating a myriad of physiologic processes (Lefkowitz, 2007; Rockman et al., 2002). The β2-adrenergic receptor (β2AR) is a widely studied prototypical GPCR, which plays a major role in cardiovascular and pulmonary pathophysiology, along with its closely related β1-adrenergic receptor (β1AR) subtype. Accordingly, drugs that orthostERICALLY target β-adrenergic receptors (β-ARs) (β-agonists and β-blockers) are a current therapeutic mainstay for diseases like asthma and heart failure, respectively (Lohse, 2004; Post et al., 1999; Tilley and Rockman, 2006).
In general, β-blockers tend to simultaneously inhibit G-protein and β-arrestin signaling downstream of activated receptors. An important exception to this pharmacology is the drug carvedilol, a Food and Drug Administration–approved β-blocker used for the treatment of cardiac dysfunctions, including heart failure, hypertension, and postmyocardial infarction (Foody et al., 2002). Unlike other β-blockers that are clinically prescribed, carvedilol retains a unique ability to activate β-arrestin–mediated signaling while still potently blocking G-protein pathways. Categorically, carvedilol is thus considered to be a GPCR β-arrestin–biased ligand, i.e., it can preferentially activate β-arrestin but not G-protein–mediated signaling and thereby elicit a phenomenon referred to as “biased agonism” or “functional selectivity” (Smith et al., 2018; Whalen et al., 2011; Wisler et al., 2014). Of note, clinical studies suggest that carvedilol may be superior to other β-blockers in preventing heart failure exacerbations and improving overall mortality in patients with reduced heart function (Bristow et al., 1996; Colucci et al., 1996; Packer et al., 1996). Although not firmly established, it has been speculated that these cardioprotective effects of carvedilol may be attributable to its unique ability to activate β-arrestin–mediated signaling pathways. The therapeutic implications of biased agonism may be widespread, as biased ligands can refine GPCR functions to regulate only a subset of signaling pathways with desired physiologic outcomes. As such, augmenting the therapeutic profile of carvedilol might lead to the development of improved β-blocker therapeutics potentially even with biased properties.

Allosteric modulators could potentially facilitate the improvement of the pharmacological properties of carvedilol. Compared with orthosteric drugs, allosteric ligands bind at receptor sites that are evolutionarily less conserved and topographically distinct from the endogenous ligand binding site and play critical roles in regulating the functional repertoire of orthosteric ligands. Allosteric ligands mediate their cooperative effects through the selection or stabilization of specific conformational ensembles in the given GPCR bound to an orthosteric ligand (Thal et al., 2018; Wootten et al., 2013). As modulators of orthosteric ligand function, allosteric ligands are broadly classified as either positive allosteric modulators (PAMs), which potentiate agonist responsiveness of a receptor, or negative allosteric modulators (NAMs), which noncompetitively oppose receptor activation by agonists. In addition to their pharmacological function as PAMs or NAMs, allosteric modulators may also engender biased signaling by virtue of differential cooperative interactions with orthosteric ligands for distinct transducer coupling to the receptor (Gentry et al., 2015; Wootten et al., 2018). We recently isolated several small molecule PAMs and NAMs of the β2AR by applying an affinity-based screening strategy on highly diverse DNA-encoded chemical libraries. Of the many small molecule hits obtained from our screens, we identified compound-15 as a lead NAM and compound-6 (cmpd-6) as a lead PAM of the β2AR (Ahn et al., 2017; Ahn et al., 2018). Whereas compound-15 allosterically blocks agonist functions at the β2AR, cmpd-6 conversely enhances agonist, but not antagonist, functions at the receptor (Liu et al., 2017; Liu et al., 2019). Although allosteric ligands continue to evolve as candidates for the development of next-generation therapeutics, the prospect of allosteric modulation of carvedilol function remains unexplored and is an attractive drug discovery avenue. In this study, we present the discovery of an unexpected unique phenomenon of positive cooperativity between the PAM cmpd-6 and the β-arrestin–biased β-blocker carvedilol at the β2AR. The findings reported herein have important implications for understanding the potential relationships between allosteri and biased agonism at GPCRs.

## Materials and Methods

### Reagents
All reagents used in this study are of molecular biology grade. All chemicals and ligands were obtained from Sigma-Aldrich (St. Louis, MO, unless mentioned otherwise. Cmpd-6 (Ahn et al., 2018) and its analogs were synthesized in-house as reported earlier (Liu et al., 2019). Nanobody-6B9 (Nb6B9) (Ring et al., 2013), heterotrimERIC Ga-ζ3/P (Rasmussen et al., 2011b), and a minimal cysteine β-arrestin1 (Shukla et al., 2013) truncated at residue 393 were purified following methods described earlier.

### Cell Culture
HEK293 and U2OS cells (American Type Culture Collection, Manassas, VA) were cultured in standard tissue culture incubator maintained at 37°C and 5% CO2 under humidified condition. HEK293 and U2OS cells were cultured in minimum Eagle’s medium (MEM) or Dulbecco’s modified Eagle’s medium (DMEM), respectively, supplemented with 10% fetal bovine serum and 1× penicillin/streptomycin mix. HEK293 cell lines stably expressing the GloSensor (Promega, Madison, WI) cAMP reporter or the β2AR were maintained as described before. The HEK293 cell line used for total receptor endocytosis was obtained from Eurofins (St. Charles, MO) and maintained according to the manufacturer’s recommendation. Clonal HEK293 cells stably expressing the FLAG-β2AR or FLAG-β2AR–yellow fluorescent protein (YFP) (Han et al., 2012) and U2OS cells stably expressing β2V2R (a chimeric β2AR with C-terminal tail of the vasopressin 2 receptor (V2R)) (Oakley et al., 1999) were generated and maintained under G418 selection. Exp293F suspension cells were cultured as per manufacturer’s instructions (Invitrogen, Wal- tham, MA) in shaker incubator maintained at 37°C and 8% CO2 under humidified condition.

### Receptor Purification and High-Density Lipoprotein Reconsti tutions
Full-length, N-terminal FLAG-tagged wild-type human β2AR was expressed in Sf9 insect cells using recombinant baculovi- rus and purified by n-dodecyl-β-n-maltopyranoside (DDM; Anatrace, Inc., OH) solubilization using anti-FLAG-M1 and alprenolol-ligand affinity chromatography followed by size-exclusion chromatography (SEC) as previously described (Kobilka, 1995). The β2AR with the sortase conjugation site (LPETGHH) inserted after amino acid 365 was expressed in Exp293F suspension cells (Invitrogen) and purified using anti-FLAG-M1 affinity chromatography. Enzymatic (sortase) ligation of a synthetic phosphopeptide (V2Rpp) was done following methods established in previous study (Staus et al., 2018) to generate β2AR-pP. Wild-type human β1AR, with an N-terminal FLAG tag, was expressed in Exp293F cells by transient transfections using ExpiFectamine following manufacturer’s instructions (Invitrogen) and purified using methods established for the β2AR (Choi et al., 2018; Staus et al., 2018). In brief, β1AR-transfected cells were grown for 60 hours in the presence of alprenolol (2 μM), harvested, and solubilized in lysis buffer containing 1% DDM and 0.05% cholesterol hemisuccinate. Clarified lysates were passed through anti-FLAG-M1 affinity column, washed, and eluted in cold elution buffer (20 mM HEPES, pH 7.4, 100 mMNaCl, 0.1% DDM, 0.01% cholesterol hemisuccinate, 5 mM EDTA, 2 μM alprenolol, and 0.2 mg/ml FLAG peptide). Affinity-purified β1AR was further cleaned up by SEC, the monomeric receptor peak was pooled and concentrated, and aliquots (with 20% glycerol) were snap frozen in liquid N2 and stored at −80°C until use. Detergent-free high-density lipoprotein (HDL) particle (also referred as to nanodiscs) reconstitution of respective β-ARs (β1AR, β2AR, and β2AR-pP) were carried out following previously established procedures (Ahn et al., 2018; Staus et al., 2016). Receptor-containing HDLs were isolated from receptor-free HDL particles.
using anti–FLAG-M1 affinity chromatography followed by a further clean-up step by SEC.

Membrane Purification. Membrane preparations from receptor-expressing cells were carried out following previously reported methods (Strachan et al., 2014) with minor modifications. Cells expressing β2AR-YFP or β2VR were harvested in cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 125 mM sucrose, containing 0.2 mM PMSF (phenylmethylsulfonyl fluoride) and EDTA-free protease inhibitor cocktail). Cell suspensions were dounce-homogenized and subjected to differential centrifugation to obtain microsomal crude membrane fractions. Isolated membranes were tryparedata using a syringe with 27-G needle, in cold membrane resuspension buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 12.5 mM MgCl₂, 2 mM EDTA, containing 10% glycerol and EDTA-free protease inhibitor cocktail). Aliquots of homogenously resuspended membranes were snap frozen in liquid N₂ and stored at −80°C until use.

Radioligand Binding. Competition radioligand binding assays were performed at respective β-ARs (β1AR, β2AR, and β2AR-pP) reconstituted into HDL particles (or nanodiscs) or from isolated membrane preparations (β2AR-YFP or β2VR) in assay buffer composed of 20 mM HEPES, pH 7.4, 100 mM NaCl, 0.2 mg/ml bovine serum albumin (BSA), and 0.18 mg/ml ascorbic acid (Ahn et al., 2018; Strachan et al., 2014). [125I]-Cyanopindolol ([125I]-CYP; 2200 Ci/mmol; PerkinElmer, Waltham, MA) was used at 60 pM and was competed with a serially diluted dose of unlabeled ligands without or with cmpd-6 or its analogs (20 μM). Competition bindings at β2AR-pP were carried out in the absence of presence of cmpd-6 (1 μM) along with either Gs-β/δ heterotrimer (GsHet; 5 nM) or β-arrestin1 ([βarr1; 250 nM]. All binding assays were carried out until equilibrium at room temperature in a final reaction volume of 200 μl. Equilibrium binding reactions were harvested onto glass-fiber filters (GF/B) and presoaked with 0.3% (vol/vol) polyethyleneimine in deionized water, using a 96-well Brandel harvester (Brandel, Gaithersburg, MD). The filters were rapidly washed with 10 ml cold wash buffer (20 mM HEPES, pH 7.4, 100 mM NaCl), and the bound [125I]-CYP was measured using a 2470 Wizard² 2-Detector Gamma Counter (PerkinElmer). Competition binding data were analyzed in GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA) using a nonlinear regression curve fit and the one-site-Fit LogIC₅₀ equation to derive the estimates of equilibrium binding constant (Kd) for respective conditions, and normalized cpm values were plotted as means ± S.D. The titration curves representing the change of carvedilol binding affinity with increasing concentrations of PAM (data shown in Figs. 1D and 5D; Supplemental Fig. 2), were fitted using the following equation:

\[
K_{\text{shift}} = \frac{K_T + \beta [\text{PAM}]}{K_T + [\text{PAM}]}
\]

where K-shift indicates the ratio of carvedilol dissociation constants measured in the absence and presence of allosteric modulator, K_T is the dissociation constant of PAM for receptor binding, and β gauges the cooperativity factor between carvedilol and PAM binding. To estimate K_T and β, the data were analyzed by nonlinear regression with a user-defined version of the above equation added to the model library of GraphPad Prism.

[3H]-RS-Cardiviolol ([3H]-Carv, 80 Ci/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) binding was performed by a scintillation proximity assay (SPA) using FLAG-tagged tyramine silicate (Ysi) SPA microbeads (PerkinElmer). Nanodisc-reconstituted β2AR-pP was incubated, in the aforementioned assay buffer, for 90 minutes at room temperature with [3H]-Carv (1 nM) in the presence of DMSO (0.2%) or cmpd-6 (20 μM), GsHet (100 nM), and [βarr1 (1 μM). Non-specific bindings were assessed by using saturating concentration of cold propranolol (20 μM). Receptor was captured on YSi beads via anti-FLAG tagged, and bound radioligand was detected using a Wallac 1450 microBeta scintillation counter (PerkinElmer). Bound specific cpm was expressed as ligand binding in fmol and plotted as bar graphs in GraphPad Prism.

Guanosine 5′-O-3′-[32P]thiotriphosphate ([32P]-GTP; S, 1250 Ci/mmol; PerkinElmer) binding to GhElm was performed using SPA in assay buffer containing 20 mM HEPES, pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 10 μM GTP, 0.2 mg/ml BSA, and 0.18 mg/ml ascorbic acid. Nanodisc-reconstituted β2ARs were incubated with GsHet (100 nM) in the absence (DMSO) or presence of saturating concentration of cmpd-6 (20 μM) or A9 (20 μM), N6BD (1 μM), and stimulated with epinephrine (10 μM) or ICI-118551 (10 μM). [32P]-GTP-S was used at 200 pM, and binding reactions were carried out for 60 minutes at room temperature. Basal [32P]-GTP-S binding to Gs protein was determined in absence of agonist stimulation, and nonspecific binding was determined by including nonradioactive GTP-S (20 μM). After incubations, the β2AR-Gs complexes were captured on YSi beads. Bound radioligand was detected using a Wallac 1450 microBeta scintillation counter (PerkinElmer). Specific [32P]-GTP-S binding for respective conditions was normalized to no ligand stimulation, expressed as fold over basal, and plotted as bar graphs in GraphPad Prism.

Measurements of cAMP Generation. cAMP production was monitored at endogenous β2AR in HEK293 cells stably expressing the plasmid for Gly-Sensor luciferase enzyme (Promega), a chemiluminescence-based cAMP biosensor. Cells were plated in 96-well, white clear-bottom plates at a density of ~80,000 cells per well, and on the following day, chemiluminescence signals generated by the Gly-Sensor luciferase were read using a CLARIOstar microplate reader (BMG Labtech, Cary, NC) as previously described (Ahn et al., 2018). Prior to ligand stimulations, cells were treated with Gly-Sensor reagent and incubated at 27 degrees for 1 hour to allow equilibration. Cells were then treated with either cmpd-6 (20 μM) or a vehicle control (DMSO) diluted in Hanks' balanced salt solution (HBSS) supplemented with 20 mM HEPES, 0.05% BSA, and 3-isobutyl-1-methylxanthine at a final concentration of 100 μM. For cAMP generation by ligands tested in agonist mode, a serial dilution of either epinephrine or carvedilol diluted in HBSS supplemented with 20 mM HEPES was then added to cells, and changes in luminescence were read at various time points ranging from 5 to 35 minutes after addition of orthosteric ligand. To assess blockade of agonist-stimulated cAMP generation, respective β-blockers (carvedilol or metoprolol) were tested in antagonist mode. A serial dilution of these β-blockers was first added to cells and incubated at 27 degrees for ~10 minutes followed by epinephrine at a final concentration of 1 μM diluted in HBSS supplemented with 20 mM HEPES was then added to cells, and changes in luminescence were then read at various time points after stimulation. Concentration–response curves were then generated by plotting normalized chemiluminescence values and were analyzed in GraphPad Prism using nonlinear regression analysis and log (inhibitor) versus response function.

Cell Surface ELISA. U2OS cells stably expressing HA-tagged β2V2R (a chimeric β2AR with C-terminal tail of V2R and β-arrestin2-GFP) (Oakley et al., 1999) were used to assess the extent of receptor internalization in intact cells using cell surface receptor ELISA. Cells were cultured for overnight in 48-well tissue culture plates and subsequently serum-starved overnight in DMEM containing 0.1% IgG- and protease-free BSA (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), 20 mM HEPES, pH 7.4, and 1× GlutaMax (Invitrogen). After serum starvation, cells were stimulated for 16 hours with a serial dilution of respective orthosteric ligands (epinephrine, carvedilol, or ICI-118551) in the absence (DMSO) or presence of cmpd-6 (5 μM). Thereafter, stimulated cells were fixed in freshly prepared 3.6% paraformaldehyde in HBSS for 30 minutes on ice, quenched with Tris-HCl (250 mM) and 0.3% H₂O₂ washed, and blocked with 3% nonfat dry milk in HBSS. Cell–surface β2V2R were labeled using horseradish peroxidase–conjugated anti–HA-tag antibody (Sigma, clone 3F10 used at 1:2500 dilution), developed using 150 μl ultraTM substrate (Thermo Fisher Scientific, Waltham, MA), and quenched with 150 μl acidified HBSS (0.2 N H₂SO₄). A 100

Pani et al. 515
μl aliquot of quenched reaction from each condition was transferred to a 96-well plate for colorimetric readings at 450 nm using a CLARIOstar plate reader (BMG Labtech). Primary assay plates were gently washed with deionized water and stained with 0.2% Janus Green B in HBSS to estimate total cells per well (Raspopin et al., 1999) for data normalizations. After staining, cells were gently washed with deionized water, and accumulated Janus Green B stain was extracted in 300 μl acidified HBSS (0.5 N HCl). A 100 μl aliquot of the extracted whole cell staining from each condition was then transferred to a 96-well plate, and colorimetric readings were taken at 595 nm. Absorbance values for cell surface receptors (450 nm) for each condition were normalized with corresponding whole cell staining (595 nm) and analyzed in GraphPad Prism using nonlinear regression analysis and log (ligand) versus response function to derive estimates of EC50 values for respective ligands in the absence (DMSO) or presence of cmpd-6.

Measurement of β2AR Endocytosis. β2AR endocytosis was monitored in a high-throughput way using a chemiluminescence-based enzyme fragment complementation assay (Eurofins) according to the manufacturer’s recommendations, with minor modifications. HEK293 cells, stably expressing the Enzyme Acceptor–tagged β2AR and endosome-localized ProLink-tethered protein, were plated in 96-well white, clear-bottomed plates at a density of ~80,000 cells per well. On the following day, cells were treated with DMSO or cmpd-6 at 10–30 μM for 10 minutes and then stimulated with a serial dilution of carvedilol for 10 hours to accumulate signals over that time. The extent of β2AR trafficking to endosomes was measured as chemiluminescence signals resulting from the complementation of β-galactosidase fragments (Enzyme Acceptor and ProLink) at endosomes and was detected on a CLARIOstar plate reader (BMG Labtech) using a detection kit (Eurofins).

Extracellular Signal-Regulated Kinase Phosphorylation. HEK293 cells stably expressing the β2AR were plated on 6-well plates at a density to achieve ~50–70% confluency prior to serum starvation on the following day. Serum-free medium was prepared by supplementing MEM with 0.1% BSA, 10 mM HEPES (pH 7.4), and 1x penicillin/streptomycin into standard minimum Eagle’s growth medium. After an overnight serum starvation, cells were pre-treated with cmpd-6 at 5 μM, stimulated with a serial dilution of carvedilol for 5 minutes, and solubilized by directly adding 2x SDS-sample buffer. After sonication with a microtip for 15 seconds, equal volumes of each condition were normalized with corresponding whole cell staining (595 nm) and analyzed in GraphPad Prism using nonlinear regression analysis and log (ligand) versus response function to derive estimates of EC50 values for respective ligands in the absence (DMSO) or presence of cmpd-6.

Results

At the β2AR, Cmpd-6 is Uniquely Cooperative with Carvedilol Among β-Blockers. Cmpd-6 is a recently identified β2AR-specific PAM that selectively shows positive cooperativity with β-agonists but not antagonists at the β2AR (Ahn et al., 2018; Liu et al., 2019). Upon further examination with a structurally and pharmacologically diverse panel of β-AR ligands (Supplemental Fig. 1), we found that cmpd-6 is uniquely, and quite unexpectedly, cooperative with the β-blocker carvedilol (Fig. 1, A and B). Consistent with the previous report (Ahn et al., 2018), cmpd-6 shifts the 125I-CYP displacement binding curve of the full agonist epinephrine to the left by ~2-log to higher affinity [Fig. 1A; epinephrine-DMSO LogIC50 = −6.211, 95% confidence interval (CI) (−6.245 to −6.177); epinephrine + cmpd-6 LogIC50 = −8.254, 95% CI (−8.288 to −8.220)]. As would be anticipated for an antagonist, there was no effect of cmpd-6 on the binding curve of carazolol, a close structural relative of carvedilol that shares the same carbazole head group (Fig. 1, A and B).

However, surprisingly, the competition curve of the antagonist carvedilol was left-shifted by ~1.2 log in presence of cmpd-6 [Fig. 1A; carvedilol-DMSO LogIC50 = −9.062, 95% CI (−9.093 to −9.031); carvedilol + cmpd-6 LogIC50 = −10.13)]. Among a total of 17 β-blockers tested (Fig. 1B) this phenomenon was unique only to carvedilol. Furthermore, as shown in Fig. 1C, across a diverse set of β-agonists, there was a strong positive correlation (R² = 0.83 without carvedilol) between cooperative effects of cmpd-6 with those of another PAM for the β2AR, nanobody-80 (Nb80), which pharmacologically behaves as a Gs mimic (DeVree et al., 2016; Rasmussen et al., 2011a; Stauss et al., 2016). The one glaring exception from this correlation was carvedilol, which further underscores the unique positive cooperativity between cmpd-6 and carvedilol. We next determined the binding affinity of cmpd-6 for the β2AR in the presence of carvedilol in competition ligand binding assays (Fig. 1, D and E). Cmpd-6, in a dose-dependent manner, resulted in progressive left-shifts of 125I-CYP displacement curves by

Camera (Photometrics, Tucson, AZ). β2AR-YFP (green) was illuminated using a 488 nm laser, and stained lysosomes (red) were imaged using 561 nm (for Lysotracker Red) laser. Fluorescent images were captured using both 63x and 100x oil objectives. Captured images were deconvoluted using no neighbor deconvolution to improve signal-to-noise ratio for quantitative analysis. Images were analyzed in 3i’s SlideBook 6 program using the colocalization analysis tool. Background pixels were eliminated using Costes’ automatic thresholding, and pixels with overlapping red and green intensity were counted as colocalated pixels (Costes et al., 2004). Each experimental condition surveyed four to seven independent images with each image containing 15 to 20 cells. Fraction of colocalated pixels were determined for each image by dividing the collocated pixel count by the total number of green (β2AR-YFP) and red (lysosomes) pixels. The resultant fractions of colocalated pixels were normalized to the number of cells within an image to yield the colocalization indices for respective treatments and plotted using GraphPad Prism.

Data Analysis and Statistics. Data analysis and plotting was performed using GraphPad Prism 9.0 and Microsoft Excel. Statistical comparisons were made using unpaired t test, one-way or two-way ANOVA with Bonferroni’s multiple comparisons post hoc tests. Experimental values are expressed as means ± SD. Differences in the mean values were considered to be significant at P < 0.05.

Confocal Imaging. HEK293 cells stably expressing β2AR-YFP were plated on poly-D-lysine–coated glass-bottom dishes (MatTek, Ashland, MA). The next day, cells were serum-starved overnight in MEM supplemented with 20 mM HEPES (pH7.4) and 1 mg/ml BSA. Cells were loaded with Lysotracker Red dye as per manufacturer’s instructions (Invitrogen) for 30 minutes followed by an additional 30 minutes of stimulation with carvedilol (10 nM) or ICI-118551 (10 nM) in the absence (DMSO) or presence of cmpd-6 (5 μM). After ligand stimulations, cells were fixed for 30 minutes at room temperature in a freshly prepared 3.6% paraformaldehyde solution in HBSS, washed, and imaged in FluoBrite DMEM (Invitrogen). Samples were imaged using a Zeiss LSM 710 confocal microscope equipped with the Yokogawa CSU-X1 spinning disc system and an Evolve 512 EMCCD

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carvedilol (Fig. 1D). Titrations of cmpd-6 resulted in nested leftward curve shifts for carvedilol (Fig. 1D) and by plotting the difference in carvedilols’ LogIC$_{50}$ versus dose of cmpd-6 we determined that cmpd-6 binds the receptor with a $1.2 \times 10^{-6}$ M affinity in the presence of carvedilol (Ahn et al., 2018). The allosteric effect of cmpd-6 at the $\beta_1$AR was saturable with a maximal curve shift of $-0.9$ log [Supplemental Fig. 2A; DMSO LogIC$_{50} = -8.849$, 95% CI ($-8.885$ to $-8.813$); cmpd-6 LogIC$_{50} = -9.731$, 95% CI ($-9.770$ to $-9.693$)]. The binding affinity of cmpd-6 was determined to be $1.7 \times 10^{-5}$ M [Supplemental Fig. 2B; cmpd-6 LogK$_T = -4.766$, 95% CI ($-5.146$ to $-3.951$)]. Taken together, these data identify a unique, nonreceptor subtype-specific cooperativity between cmpd-6 and carvedilol among a large number of $\beta$-blockers. A
detailed characterization of the allosteric effects of cmpd-6 on carvedilol-mediated β1AR signaling is reported in the accompanying manuscript by Wang et al. (2021).

**Cmpd-6 Facilitates β-Arrestin1-Induced High-Affinity Binding of Carvedilol to the β2AR.** Carvedilol is a therapeutic β-blocker with a unique bias toward activating β-arrestin signaling. Thus, allosteric modulation of carvedilol function might be of therapeutic importance. Accordingly, we assessed the cooperative effects of cmpd-6 on transducer (Gs or β-arrestin1) coupling to the β2AR. We monitored either carvedilol competition radioligand binding (Fig. 2, A and B) or direct binding of radiolabeled carvedilol (³H-Carv) to the β2AR (Fig. 2C) in the absence (DMSO) or presence of cmpd-6 together with either transducer. To facilitate binding of βarr1 to the receptor, a synthetically phosphorylated C-tail of V2R was ligated in vitro at the C-terminal end of the β2AR (β2V2RpP) using the sortase enzyme as previously described (Staus et al., 2018), and the phospho-peptide ligated receptor was reconstituted into detergent-free HDL particles. Cmpd-6 showed positive cooperativity with βarr1 [Fig. 2A(i); cmpd-6 LogIC50 = –9.554, 95% CI (–9.626 to –9.482); βarr1+cmpd-6 LogIC50 = –10.14, 95% CI (–10.20 to –10.07)] but not with Gs [Fig. 2A(ii); GsHet+cmpd-6 LogIC50 = –9.594, 95% CI (–9.714 to –9.475)] and enhanced the ability of carvedilol [but not carazolol; Fig. 2A(iii)–(iv)] to compete against the radiolabeled tracer ¹²⁵I-CYP binding to the receptor. Remarkably, the combined application of cmpd-6 and βarr1 (but not Gs) resulted in a further high-affinity left-shift of the competition curve compared with that obtained using respective transducers tested individually (Fig. 2B). Additionally, and consistent with the competition binding, we show that cmpd-6 in the presence of βarr1 (but not Gs) substantially increases the direct high-affinity binding of ³H-Carv to the β2AR (Fig. 2C).

**Cmpd-6 Positively Modulates Carvedilol-Stimulated Cellular β2AR Functions.** To determine the cellular implications of the allosteric effect of cmpd-6 on carvedilol-mediated signaling at the β2AR, we performed a series of cell-based assays. We first tested the activation of Gs by monitoring cAMP generation. When used in an agonist mode,}

![Fig. 2. Cmpd-6 and β-arrestin1-mediated high-affinity binding of carvedilol.](#)
carvedilol alone and together with cmpd-6 did not stimulate any detectable levels of cAMP production, unlike the robust response to the agonist epinephrine stimulation, which was augmented by cmpd-6 [Fig. 3A; epinephrine LogEC50 = −7.587, 95% CI (−7.664 to −7.510); epinephrine + cmpd-6 LogEC50 = −8.709, 95% CI (−8.807 to −8.611)]. We next tested carvedilol in an antagonistic mode, essentially evaluating its ability to block the agonist epinephrine-stimulated cAMP responses in comparison with a control ligand, metoprolol, with which cmpd-6 had no positive cooperativity as shown in competition binding (Fig. 1B). Interestingly, in comparison with metoprolol, we found that cmpd-6 substantially augments the blockade of agonist-stimulated cAMP generation by carvedilol [Fig. 3C; + cmpd-6, carvedilol LogKi = −10.33, 95% CI (−10.44 to −10.22); metoprolol LogKi = −7.703, 95% CI (−7.788 to −7.618)]. Compared with metoprolol-mediated inhibition of epinephrine-stimulated cAMP generation, the presence of cmpd-6 remarkably led to ~2350-fold leftward shift of the carvedilol dose-dependent inhibitory curve (Fig. 3C versus ~11-fold shift without cmpd-6, Fig. 3A). Furthermore, this measure of fold-shift in inhibition of cAMP with carvedilol must be underestimated since cmpd-6 also potentiates the epinephrine-stimulated response. These results suggest that the allosteric modulation of carvedilol by cmpd-6 enhances the cellular β-blockade potency of the ligand. We then tested the effect of cmpd-6 on carvedilol-stimulated ERK phosphorylation downstream of the β2AR, which has been shown to be β-arrestin–dependent (Wisler et al., 2007). In HEK cells stably overexpressing the β2AR, cmpd-6 substantially enhanced (by ~5.8-fold) the potency of carvedilol-stimulated ERK phosphorylation [Fig. 3, D and E; DMSO LogEC50 = −7.504; 95% CI (−7.774 to −7.234); + cmpd-6 LogEC50 = −8.268, 95% CI (−8.899 to −7.638)].

**Cmpd-6 Enhances Carvedilol-Stimulated Internalization of the β2AR.** To further evaluate the cellular effects of this unique positive cooperativity, we tested the role of cmpd-6 on carvedilol-mediated receptor internalization, a function attributable to β-arrestins. To this end we employed U2OS and HEK293 cells stably expressing the chimeric β2V2R (Oakley et al., 1999) or the YFP-tagged β2AR, respectively (Han et al., 2012). These recombinant versions of the human β2AR were pharmacologically validated by competition ligand binding using membrane preparations from respective cell lines (Supplemental Fig. 3). Consistent with our in vitro binding data (Fig. 1A), both versions of the β2AR, in cell membrane preps, retained the cooperative effect of cmpd-6 with epinephrine and carvedilol but not with carazolol. The affinity-shifts for carvedilol elicited by cmpd-6 at the β2V2R [Supplemental Fig. 3C; DMSO LogIC50 = −9.019, 95% CI (−9.043 to −8.996); cmpd-6 LogIC50 = −9.959, 95% CI (−9.999 to −9.919)] and β2AR-YFP [Supplemental Fig. 3F; DMSO LogIC50 = −9.086, 95% CI (−9.128 to −9.043); cmpd-6 LogIC50 = −9.919, 95% CI (−9.999 to −9.919)] were determined by two-way ANOVA. ***P < 0.001.

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Fig. 3. Cmpd-6 augments cellular activity of carvedilol at the β2AR. (A) HEK293 cells stably expressing GloSensor were pretreated with either vehicle (DMSO) alone or cmpd-6 (C6) (30 μM) for 15–20 minutes. The extent of cAMP generation by endogenously expressed GloSensor were determined through dividing the p-ERK signal by the t-ERK. Each data point was expressed as percent of the maximal response in the vehicle-treated control cells and represents the mean ± S.D. from five independent experiments. Dose-response curves and EC50 values between vehicle (DMSO)– and cmpd-6–treated samples were obtained by using GraphPad Prism. Statistical significance for the difference in LogKi values (carvedilol versus metoprolol) between vehicle (DMSO)– and cmpd-6–treated curve fits (P < 0.001) was determined by two-way ANOVA. ***P < 0.001.
of carvedilol together with cmpd-6 was significantly greater than that mediated by carvedilol alone. We found that cmpd-6, as with the agonist epinephrine [Fig. 4A; DMSO LogEC50 = −6.901; 95% CI (−7.008 to −6.795); cmpd-6 LogEC50 = −7.916, 95% CI (−8.101 to −7.730)], enhanced the potency (by ~6.8-fold change in EC50) of carvedilol-stimulated endocytosis of the receptor as assessed by cell surface ELISA [Fig. 4B; DMSO LogEC50 = −6.731, 95% CI (−7.018 to −6.445); cmpd-6 LogEC50 = −7.561, 95% CI (−7.764 to −7.358)], as well as by total receptor endocytosis (Supplemental Fig. 4). However, consistent with the binding data (Fig. 1B), no such cooperative effect of cmpd-6 on endocytosis of the receptor was observed in cells treated with the inverse agonist ICI-118551 (Fig. 4C). This further corroborates the unique positive cooperativity of cmpd-6 with the β-blocker carvedilol. Of note, even more effectively than agonists, carvedilol has been reported to target the β2AR to lysosomal compartments (Han et al., 2012). Thus, we tested the cooperative effect of cmpd-6 on the carvedilol-stimulated lysosomal targeting of the β2AR-YFP stably expressed in HEK-293 cells. After ligand stimulation, the colocalization of β2AR-YFP with the lysosomal marker dye (Lysotracker Red) was visualized and quantified (Fig. 4, E and F). Interestingly, compared with DMSO control, cmpd-6 substantially augmented carvedilol-mediated lysosomal targeting of the β2AR. These data further underscore the signaling impact of the cooperative effects of cmpd-6 on carvedilol-mediated cellular functions. In essence, cmpd-6 not only enhances the cellular β-blockade potency of carvedilol but also positively augments β-arrestin–mediated cellular signaling emanating from the carvedilol-occupied β2AR.

Development of a Carvedilol-Specific Allosteric Modulator. Although cmpd-6 potentiates the β-arrestin–biased agonism of carvedilol, it is also a PAM that is positively cooperative with agonists at the β2AR in an unbiased manner (Ahn et al., 2018). We thus set out to test a set of chemically modified analogs of cmpd-6 (A1–A12) with the hopes to identify molecules that would retain the positive allosteric cooperativity with carvedilol while losing the PAM activity with β-agonists. Such modified analogs would not only be of potential therapeutic value but might also pave the way for the development of novel and biased allosteric drugs targeting other GPCRs. Among the several cmpd-6 analogs whose structures we have previously reported (Liu et al., 2019), we identified the analog A9 [Fig. 5A(ii)] to display the desired allosteric cooperative properties. Structurally, A9 differs from its parent cmpd-6 in carrying a terminal amide group at the R2 moiety (Fig. 5B). Although A9 retains its positive cooperativity with carvedilol comparable to the level obtained with the parent cmpd-6, it shows absolutely no PAM activity with agonists including epinephrine (Fig. 5A(ii); Fig. 5C). The allosteric effect of A9 to affinity-shift carvedilol competition curves to the left is saturable and the analog has ~2.9 × 10⁻⁶ M affinity for the carvedilol-bound β2AR [Fig. 5D; A9 LogKT = −5.533, 95% CI (−5.737 to −5.247)], which is comparable to the ~1.2 × 10⁻⁶ M affinity determined for cmpd-6 [Fig. 1E; cmpd-6 LogKT = −5.913, 95% CI (−5.969 to −5.852)]. Interestingly, unlike the PAM activity of cmpd-6 in potentiating β-agonist–stimulated responses, A9 remarkably shows a robust NAM activity for agonist-mediated β2AR functions. Whereas cmpd-6 further augments the activation of heterotrimeric Gs by agonists, A9 markedly blocks the agonist-stimulated activation of Gs both in vitro and in cells, as shown by A9-mediated reduction in 35S-GTP,S binding to Gs in vitro (Fig. 5E). The binding of non-hydrlyizable 35S-GTP,S to Gs is driven by physical coupling of Gs to the β2AR and is increased in response to stimulation by the agonist epinephrine. However, in the presence of the antagonist/inverse agonist ICI-118551, no increase in 35S-GTP,S binding to Gs was observed. Additionally, and as expected, competition with Nb6B9 (an affinity-matured version of the Gs mimic nanobody, Nb80), which shares the transducer binding site (Ring et al., 2013), also reduces 35S-GTP,S binding to Gs (Fig. 5E). Furthermore, in cells, A9 (unlike cmpd-6) also functions as a classic NAM inhibiting agonist isoproterenol-stimulated cAMP generation downstream of the activated β2AR [Fig. 5F; DMSO LogEC50 = −8.073, 95% CI (−8.122 to −8.023); cmpd-6 LogEC50 = −8.914, 95% CI (−9.021 to −8.807); A9 LogEC50 = −7.320, 95% CI (−7.524 to −7.115)].

Discussion
In this study we report on a unique and unexpected pharmacological cooperativity between the recently discovered PAM of the β2AR, cmpd-6, and the Food and Drug Administration–approved β-blocker, carvedilol. Our findings unveil cmpd-6 as a positive allosteric modulator for the pharmacological activity of carvedilol at the β2AR as well as the closely related subtype, β1AR. Remarkably, the cooperativity of cmpd-6 is highly specific to carvedilol among a diverse array of known β-blockers tested in this study. Using orthogonal experimental approaches, both in vitro and in cultured cells, we demonstrate that cmpd-6 augments the binding affinity of carvedilol for both β1AR and β2AR, the potency of carvedilol’s β-blockade activity at the β2AR, and carvedilol-stimulated β-arrestin–mediated β2AR signaling functions such as ERK phosphorylation and receptor trafficking. Notably, we also describe the identification of a cmpd-6 analog, A9, which displays a complete switch in the allosteric properties from a PAM to a classic NAM and yet retains the distinctive positive cooperativity exclusively with carvedilol at the β2AR.

Ligands that bias GPCRs toward preferentially activating either G-protein– or β-arrestin–mediated signaling hold immense therapeutic potential. Carvedilol is unique among β-blockers used in medicine in that it facilitates β-arrestin–biased signaling (unlike other β-blockers) while still blocking the deleterious effects of chronic Gs-mediated cAMP signaling downstream of activated β-adrenergic
receptors (Reiter et al., 2012; Whalen et al., 2011). Indeed, findings from our competition radioligand binding as well as direct binding of $^3$H-carvedilol indicate that cmpd-6 further potentiates the cooperativity between carvedilol and β-arrestin1, but not that with Gs. Previous studies have shown carvedilol stimulation resulting in ERK activation downstream of β1 and β2ARs in a β-arrestin–dependent manner (Luttrell et al., 2018; Wang et al., 2017; Wisler et al., 2007). In the case of the β1AR this is through GPCR-mediated transactivation of the epidermal growth factor receptor (Kim et al., 2008; Noma et al., 2007). This signaling is implicated to be cardioprotective by counteracting G-protein–dependent catecholamine-induced toxicity and apoptotic pathways (Wang et al., 2018). Notably, clinical studies suggest that carvedilol may be superior to other β-blockers (such as metoprolol and propranolol) in preventing heart failure exacerbations and improving overall mortality in patients with reduced heart function. Carvedilol thus continues to be the drug of choice to treat patients with myocardial infarction and heart failure (Bristow et al., 1996; Colucci et al., 1996; Foody et al., 2002). The cardioprotective effects of carvedilol may be attributable to its unique ability to activate β-arrestin–mediated signaling pathways while potently blocking Gs activation (see accompanying manuscript by Wang et al., 2021). Interestingly, our findings show that cmpd-6 potentiates the ability of

**Fig. 4.** Cmpd-6 augments carvedilol-stimulated β2AR internalization. (A–C) Cell surface ELISA showing the cooperative effects of cmpd-6 on loss of cell-surface β2AR after a dose response of epinephrine (A), carvedilol (B), and ICI-118551 (C); normalized mean ± S.D, n = 5. (D) Bar graph showing mean difference in LogEC50 (versus DMSO control) for epinephrine (green, n = 5) and carvedilol (red, n = 5) in presence of cmpd-6. (E) Representative confocal images showing cmpd-6–mediated potentiation of lysosomal targeting of the β2AR upon stimulation with carvedilol (Carv). Scale bar on respective images is 10 μm. (F) Bar graph showing quantification of β2AR-YFP (green) colocalization with lysosomes (red); expressed as colocalization index ± S.D. as described in Materials and Methods. Statistical comparisons were made using one-way ANOVA with Bonferroni’s post hoc tests. **P < 0.01; ***P < 0.001.
Fig. 5. Cmpd-6 analog A9 displays carvedilol-specific cooperativity at the β2AR. (A) Bar graph showing affinity shifts (LogK-shifts) of epinephrine (i) and carvedilol (ii) mediated by cmpd-6 (gray bars) or its analogs (A1–A12, colored bars) compared with DMSO control. (B) Chemical structures of cmpd-6 and its analog A9 highlighting the modified R2 moiety in the analog. (C) Bar graph showing the allosteric cooperative effects of A9 on affinity shifts of a panel of β-ligands (agonists and antagonists). A9 shows positive cooperativity only with carvedilol compared with other ligands tested. Bar graphs in (A) and (C) show mean LogK-shifts ± S.D. obtained from three to five independent experiments. (D) Curve showing affinity shifts in LogIC50 (LogK-Shift) for carvedilol mediated by a dose of A9. Points on the curves represent LogK-Shift values derived from three independent competition binding experiments ± S.D. (E) Bar graph showing binding of 35S-GTPγS to heterotrimeric Gs either under basal, no ligand (NL) condition or after epinephrine stimulation of the β2AR in HDL. Data for respective conditions (n = 4) are normalized to basal (unstimulated) binding of 35S-GTPγS. (F) Curves showing the effect of cmpd-6 and A9 on isoproterenol (ISO)–stimulated cAMP generation. Cells were pretreated for 15–20 minutes with either cmpd-6 or A9 (30 μM) and then stimulated with a dose of ISO. The amount of cAMP production by endogenously expressed β2AR was measured 10 minutes after ISO stimulation. Curve fits were generated in GraphPad Prism with data points obtained from five independent experiments done in duplicate. Each data point was normalized to the maximal level of ISO-induced activity in the vehicle (DMSO) control, expressed as a percentage, and represents mean ± S.D. Statistical comparisons were made using one-way ANOVA with Bonferroni’s post hoc tests. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.
carvedilol, but not that of metoprolol, to block epinephrine-stimulated activation of Gs and cAMP generation. Additionally, cmpd-6 also augments the potency of carvedilol to stimulate β2AR-mediated ERK phosphorylation, which is known to be β-arrin-dependent and involved in cytoprotective signaling. These findings highlight the allosteric potential of cmpd-6 in positively augmenting the desirable signaling properties of carvedilol. Although carvedilol represents a prototypic β-arrin-biased orthosteric drug at the β-ARs, allosteric regulation of its varied signaling functions by cmpd-6 further expands the possibilities of developing improved β-blocker therapeutics even for biased orthosteric ligands. Indeed, by using a murine model of myocardial infarction, Wang et al. (2021) quite remarkably demonstrate the potential clinical implications of the unique positive cooperativity between cmpd-6 and carvedilol.

In addition to desensitizing G-protein-mediated signaling, β-arrin-biases are also known to play a pivotal role in receptor endocytosis. Over the past decade it has become evident that β-arrins are recruited to membrane proteins (GPCRs, receptor tyrosine kinases (RTKs), and even ion channels) where they interact with or serve as scaffolds for components of the cellular endocytic machinery such as clathrin and adaptor protein 2 (AP2). β-arrins thus function as key endocytic adaptors for activated GPCRs to facilitate their internalization resulting in either recycling or lysosomal degradation of the receptors (Freedman and Lefkowitz, 1996; Kovacs et al., 2009; Shenoy and Lefkowitz, 2011). Uniquely, carvedilol (in contrast to other β-blockers) displays pharmaceutical properties that are akin to β-arrin-biases with respect to β-arrin-mediated signaling. Carvedilol stimulation of cells results in G-protein-coupled receptor kinase 6-mediated phosphorylation of the β2AR as well as ubiquitination of the receptor by the E3 ligase, MARCH2 (membrane-associated RING-CH2) (Han et al., 2012). These signaling events have been reported to precede receptor internalization. In primary vascular smooth muscle cells, prolonged carvedilol treatment has been shown to trigger lysosomal trafficking and degradation of the β2AR. Consistent with these studies, we show that cmpd-6 potentiates carvedilol-stimulated loss of cell-surface β2AR leading to endocytosis and trafficking of the receptor to lysosomes. Although the effect of cmpd-6 on the above-noted post-translational modifications of the β2AR was not tested directly, our results on receptor endocytosis and lysosomal trafficking suggest that cmpd-6 enhances these carvedilol-stimulated responses.

Although cmpd-6 was identified to be a highly selective PAM for the β2AR (Ahn et al., 2018), our current findings clearly indicate that the cooperativity between cmpd-6 and carvedilol is also preserved at the β1AR. This property of cmpd-6 deviates from the usual pattern of receptor subtype selectivity that is a hallmark of allosteric modulators. Previous structural work from our group suggests that the binding site of cmpd-6 in the agonist-occupied β2AR is conserved in the β1AR, with different key residues mediating the receptor subtype-specific allosteric effect of cmpd-6 (Liu et al., 2019). In the absence of any structural data on the exact binding site of cmpd-6 to either the carvedilol-bound β1AR or β2AR, it may be speculated that the site could topologically overlap with the binding site of cmpd-6 as that in the agonist-bound β2AR. Although a given GPCR can have multiple, topologically distinct allosteric sites, it is also plausible that the binding site of cmpd-6 resides in a highly conserved structural motif that is common in this receptor family.

An emerging paradigm in GPCR structural biology is that GPCRs exist as ensembles of interconvertible inactive and active states, which are in conformational equilibrium (Hilger et al., 2018; Kobilka and Deupi, 2007; Manglik et al., 2015). Compared with canonical β-blockers, carvedilol has been shown to elicit unique conformational changes in the β2AR. In particular, quantitative proteomic studies (Kahsai et al., 2011) on labeling of solvent accessible reactive lysine and cysteine residues in the β2AR as well as findings from 19F-NMR studies on TM7 dynamics (Liu et al., 2012) suggest a distinct conformational signature of the β2AR when bound to carvedilol. These results also accord with the unique β-arrin-biased agonism of carvedilol compared with other β-blockers (Kim et al., 2020; Wisler et al., 2007), which presumably is displayed only by a minor fraction of the receptor population within the conformational spectrum of carvedilol-bound receptor. Based on the data presented herein, cmpd-6 appears to stabilize a carvedilol-bound distinct conformational signature of the β2AR. Thus, cmpd-6 is a uniquely suited allosteric tool for examining the conformational dynamics of carvedilol-bound β2AR.

Interestingly, our data show that cmpd-6 has absolutely no cooperativity with the antagonist/inverse agonist, carazolol. Although both ligands share an identical carbazole headgroup, carvedilol differs from carazolol in having an extended aliphatic tail terminating with an anisole ring. From previously reported crystal structures of carvedilol- and carazolol-bound β2AR it is clear the carbazole moiety, which is shared by both ligands, occupies the orthosteric site situated deep in the transmembrane core (Bokoch et al., 2010; Cherezov et al., 2007; Ishchenko et al., 2019). However, a striking feature in the structure of carvedilol-bound β2AR is that the extended tail of carvedilol resides at a site distant from the deep orthosteric pocket. Based on this distinct binding modality of carvedilol, it may be hypothesized that the allosteric cooperativity between cmpd-6 and carvedilol is, in part, driven by the tail interactions of carvedilol with the β2AR. To discern the structural mechanism underlying this unique cooperativity, it will be of great interest to obtain atomic-level information on carvedilol-bound β2AR in complex with cmpd-6. Such structural studies will be useful not only for uncovering nuances of GPCR allostery but also for expanding our current understanding of the dynamic nature of GPCR allosteric sites and biased agonism.

The ideal allosteric drug would cooperatively interact with orthosteric ligands to selectively potentiate signaling pathways of therapeutic importance (Christopoulos, 2014; Thal et al., 2018). Although cmpd-6 does augment the putative cardioprotective effects of carvedilol mediated by β-arrin signaling (shown in accompanying manuscript by Wang et al., 2021), it also potentiates agonist-stimulated G-protein signaling at the β2AR. This agonist-mediated signaling of cardiac β-receptors in part underlies the pathophysiology of heart failure. As such, from a potential therapeutic perspective, the dual cooperativity of cmpd-6 with β-agonists and carvedilol at the β2AR is diametrically opposed. Previous structure-activity relationship studies with cmpd-6 led to the synthesis of several chemically modified analogs of the parent compound (Liu et al., 2019). In the current study, we show that the analog A9 has no cooperativity with agonists but still
retains the unique positive allosteric cooperativity exclusively with carvedilol. A9 thereby serves as a small molecule prototype, which displays pharmacologic properties desirable of a potential allosteric therapeutic that together with carvedilol could be advantageous to abate cardiovascular ailments including heart failure.

In summary, our study describes the unique ability of cmcp-6 and its analog A9 to allosterically potentiate the pharmacologic properties of carvedilol, a key cardiovascular therapeutic. The discovery of this unexpected interaction also serves to advance our understanding of GPCR allosteric and biased agonism.

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References

Ahn S, Khasai AW, Pan B, Wang QT, Zhao S, Wall AL, Strachan BT, Stauch B, Han Owl, Bachmann A, Shiraiwa A, Li C, Zategin, N. Westerlund U, Li N, Wang E et al. (2019) Toward G protein-coupled receptor structure-based drug design using X-ray lasers. IUCrF 6:1106–1119.

Khasai AW, Xiao K, Rajagopal S, Ahn S, Chatterjee A, Masoudi A et al. (2016) Allosteric nanobodies reveal the dynamic structure of active β-adrenergic receptors. Nature 535:182–186.

Fondu JM, Farrell MH, and Kramholz HM (2002) Beta-blocker therapy in heart failure: scientific review. JAMA 288:883–889.

Freedman NJ and Lefkowitz RJ (1996) Desensitization of G protein-coupled receptors. Recent Prog Horm Res 51:319–351, discussion 352–353.

Gentsy PR, Sexton PM, and Christopoulos A (2015) Novel allosteric modulators of G protein-coupled receptors. J Bio Chem 290:19478–19488.

Han SO, Xiao K, Kim J, Wu JW, Wisler JW, Nakamura N, Freedman NJ, and Shenyk SK (2012) MAOs induce 5-LOX and lysosomal sorting of carvedilol-bound β2-adrenergic receptors. J Cell Biol 199:817–830.

Hilger D, Masureel M, and Koblika BK (2018) Structure and dynamics of GPCR signaling complexes. Nat Struct Mol Biol 25:4–12.

Ishchenko A, Stauch B, Han OW, Bachmann A, Shiraiwa A, Li C, Zategin, N. Westerlund U, Li N, Wang E et al. (2019) Toward G protein-coupled receptor structure-based drug design using X-ray lasers. IUCrF 6:1106–1119.

Koblika BK (1995) Amino and carboxyl terminal modifications to facilitate the production and purification of a G protein-coupled receptor. Anal Biochem 236:269–271.

Koblika BK and Deupi X (2007) Conformational complexity of G-protein-coupled receptors. Trends Pharmacol Sci 28:397–406.

Kovals JI, Haraba M, Davenport CL, Kim J, and Lefkowitz RJ (2009) Arrestin development: emerging roles for beta-arrestins in developmental signaling pathways. Dev Cell 17:443–458.

Lefkowitz RJ (2007) Seven transmembrane receptors: something old, something new. Annu Rev Med 58:199–212.

Liu JJ, Herst R, Katrich V, Stevens RC, and Wiruth K (2012) Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. Science 335:1106–1110.

Liu X, Ahn S, Khasai AW, Meng KC, Latorraca NR, Pani B, Venkatakrishnan AJ, Masoudi A, Wei WI, Dror BO et al. (2017) Mechanism of intracellular allosteric β2AR antagonist revealed by X-ray crystal structure. Nature 548:480–484.

Liu X, Masoudi A, Khasai AW, Huang LY, Pani B, Stauch DP, Shim BH, Hirata K, Simahal RK, Schwalm AM et al. (2019) Mechanism of β2AR regulation by an intracellular positive allosteric modulator. Science 364:1283–1287.

Lohse MJ (2004) Beta-arrestin polymorphisms and heart failure. Trends Mol Med 10:285–288.

Luttinger LM, Wang J, Ploffe B, Smith JS, Yamani L, Kaur S, Jean-Charles PY, Gauthier L, Mei J, and Pani B et al. (2018) Mechanism of β-arrestin tethering in GPCR signaling elucidated with siRNA and CRISPR-Cas9. Sci Signal 11eaaf76.

Manglik A, Kim TH, Masureel M, Allensbach G, Yang Z, Hiller D, Lorth M, Koblika BK, Thian FS, Hubbell WL et al. (2015) Structural insights into the dynamic process of β2-adrenergic receptor signaling. Cell 161:1101–1111.

Noma T, Lemaire A, Naga Prasad SV, Barki-Harrington L, Tilley DG, Chen J, Le Corre P, Violier P, Jin W, Hsu L et al. (2017) Allosteric nanobodies reveal the dynamic structure of active β-adrenergic receptors. Nature 535:182–186.

Paul SR, Hammond HK, and Insel PA (1999) Beta-adrenergic receptors and receptor signaling. Annu Rev Physiol 61:179–197.

Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Rasmussen SG, DeVree BT, Zou Y, Kruse AC, Chung KY, Kobilka TS, Thian FS, Hubbell WL et al. (2015) Structural insights into the dynamic process of β2-adrenergic receptor signaling. Cell 161:1101–1111.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011a) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011b) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011c) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011d) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011e) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011f) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011g) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Rasmussen SG, De Vree BT, Zou Y, Kruse AC, Chung KY, Koblika KS, Thian FS, Pani B et al. (2011h) Structure of a nanobody-stabilized active state of the β2-adrenergic receptor. Nature 477:549–555.

Simhal RK, Schwalb AM et al. (2019) Mechanism of β-arrestin-biased allosteric modulation of a G protein-coupled receptor. Anal Biochem 535:1–11.

Shenoy SK and Lefkowitz RJ (2014) Adrenaline-activated structure of engineered human β2-adrenoceptor stabilized by an engineered nanobody. Proc Natl Acad Sci USA 111:17245–17243.

Staus DP, Strachan RT, Manglik A, Pani B, Kahsai AW, Kim TH, Wingler LM, Ahn S, Corcoran N, Violier P et al. (2014) Adrenaline-activated structure of an engineered human β2-adrenergic G protein-coupled receptor. Nature 513:182–186.

Uysal S, Huang LY et al. (2013) Structure of active β2-adrenergic receptor. Nature 498:588–592.
Staus DP, Wingler LM, Choi M, Pani B, Manglik A, Kruse AC, and Lefkowitz RJ (2018) Sortase ligation enables homogeneous GPCR phosphorylation to reveal diversity in β-arrestin coupling. Proc Natl Acad Sci USA 115:3834–3839.

Strachan RT, Sun JP, Rominger DH, Violin JD, Ahn S, Rojas Bie Thomsen A, Zhu X, Kleist A, Costa T, and Lefkowitz RJ (2014) Divergent transducer-specific molecular efficacies generate biased agonism at a G protein-coupled receptor (GPCR). J Biol Chem 289:14211–14224.

Thal DM, Glukhova A, Sexton PM, and Christopoulos A (2018) Structural insights into G-protein-coupled receptor allosterity. Nature 559:45–53.

Tilley DG and Rockman HA (2006) Role of beta-adrenergic receptor signaling and desensitization in heart failure: new concepts and prospects for treatment. Expert Rev Cardiovasc Ther 4:417–432.

Wang J, Gareri C, and Rockman HA (2018) G-protein-coupled receptors in heart disease. Circ Res 123:716–735.

Wang J, Hanada K, Staus DP, Makara MA, Dahal GR, Chen Q, Ahles A, Engelhardt S, and Rockman HA (2017) Gαi is required for carvedilol-induced β1 adrenergic receptor β-arrestin biased signaling. Nat Commun 8:1706.

Wang J, Pani B, Gokhan I, Xiong X, Kahsei AW, Jiang H, Ahn S, Lefkowitz RJ, and Rockman HA (2021) β-Arrestin-biased allosteric modulator potentiates carvedilol stimulated β-adrenergic receptor cardioprotection. Mol Pharmacol 100:XX–XX.

Whalen RJ, Rajagopal S, and Lefkowitz RJ (2011) Therapeutic potential of β-arrestin and G protein-biased agonists. Trends Mol Med 17:126–139.

Wisler JW, DeWire SM, Whalen RJ, Violin JD, Drake MT, Ahn S, Shenoy SK, and Lefkowitz RJ (2007) A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling. Proc Natl Acad Sci USA 104:16657–16662.

Wisler JW, Xiao K, Thomsen AR, and Lefkowitz RJ (2014) Recent developments in biased agonism. Curr Opin Cell Biol 27:18–24.

Wootten D, Christopoulos A, Marti-Solano M, Babu MM, and Sexton PM (2018) Mechanisms of signalling and biased agonism in G protein-coupled receptors. Nat Rev Mol Cell Biol 19:638–653.

Wootten D, Christopoulos A, and Sexton PM (2013) Emerging paradigms in GPCR allostery: implications for drug discovery. Nat Rev Drug Discov 12:630–644.

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