

ORIGINAL ARTICLE

Structure–activity relationship study of angiotensin II analogs in terms of β -arrestin-dependent signaling to aldosterone production

Thairy Reyes Valero¹, Emmanuel Sturchler², Malika Jafferjee¹, Giuseppe Rengo³, Vassiliki Magafa⁴, Paul Cordopatis^{4,†}, Patricia McDonald², Walter J. Koch⁵ & Anastasios Lymperopoulos¹

¹Department of Pharmaceutical Sciences, Laboratory for the Study of Neurohormonal Control of the Circulation, Nova Southeastern University College of Pharmacy, Fort Lauderdale, Florida 33328

²Translational Research Institute, Scripps Florida, Jupiter, Florida 33458

³Salvatore Maugeri Foundation–Scientific Institute of Telesse Terme, Telesse Terme, Italy

⁴Department of Pharmacy, Laboratory of Pharmacognosy & Chemistry of Natural Products, University of Patras, Patras, Greece

⁵Center for Translational Medicine, Temple University, Philadelphia, Pennsylvania 19140

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Correspondence

Anastasios Lymperopoulos, Department of Pharmaceutical Sciences, Nova Southeastern University College of Pharmacy, 3200 S. University Dr., HPD (Terry) Bldg/Room 1338, Fort Lauderdale, FL 33328-2018.
Tel: 954 262 1338; Fax: 954 262 2278;
E-mails: al806@nova.edu;
tasos.lymperopoulos@gmail.com

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[†]Deceased.

Abstract

The known angiotensin II (AngII) physiological effect of aldosterone synthesis and secretion induction, a steroid hormone that contributes to the pathology of postmyocardial infarction (MI) heart failure (HF), is mediated by both G_{q/11} proteins and β -arrestins, both of which couple to the AngII type 1 receptors (AT₁R) of adrenocortical zona glomerulosa (AZG) cells. Over the past several years, AngII analogs with increased selectivity (“bias”) toward β -arrestin-dependent signaling at the AT₁R have been designed and described, starting with SII, the gold-standard β -arrestin-“biased” AngII analog. In this study, we examined the relative potencies of an extensive series of AngII peptide analogs at relative activation of G proteins versus β -arrestins by the AT₁R. The major structural difference of these peptides from SII was their varied substitutions at position 5, rather than position 4 of native AngII. Three of them were found biased for β -arrestin activation and extremely potent at stimulating aldosterone secretion in AZG cells in vitro, much more potent than SII in that regard. Finally, the most potent of these three ([Sar¹, Cys(Et)⁵, Leu⁸]-AngII, CORET) was further examined in post-MI rats progressing to HF and overexpressing adrenal β -arrestin1 in vivo. Consistent with the in vitro studies, CORET was found to exacerbate the post-MI hyperaldosteronism, and, consequently, cardiac function of the post-MI animals in vivo. Finally, our data suggest that increasing the size of position 5 of the AngII peptide sequence results in directly proportional increases in AT₁R-dependent β -arrestin activation. These findings provide important insights for AT₁R pharmacology and future AngII-targeted drug development.

Abbreviations

AngII, angiotensin II; ARB, angiotensin receptor blocker; AT₁R, angiotensin II type 1 receptor; AZG, adrenocortical zona glomerulosa; GPCR, G protein-coupled receptor; GRK, GPCR-kinase; HF, heart failure; MI, myocardial infarction; SAR, structure–activity relationship; StAR, steroidogenic acute regulatory protein.

Introduction

Aldosterone is a cardio-toxic mineralocorticoid hormone, whose circulating levels are significantly elevated in post-myocardial infarction (MI) chronic heart failure (HF), accelerating disease progression (Weber 2001). Its major site of systemic synthesis and secretion is the adrenocortical zona glomerulosa (AZG) cells, which endogenously express AngII type 1 receptors (AT₁Rs) (Rainey *et al.* 2004). Elevated serum K⁺ levels or AngII binding to the AZG AT₁Rs can trigger aldosterone production and release into the systemic circulation (Ganguly and Davis 1994). The AT₁R is primarily a G protein-coupled receptor (GPCR) stimulating aldosterone turnover through G_{q/11} proteins (De Gasparo *et al.* 2000). However, its activation by AngII can also elicit G protein-independent signaling through β -arrestins, scaffolding signal transducers originally discovered as terminators of GPCR signaling (Luttrell and Gesty-Palmer 2010). We have previously shown that AngII-bound AT₁Rs in AZG cells can stimulate aldosterone production via both G proteins and β -arrestin1, and the latter leads to post-MI HF-related hyperaldosteronism (Lymperopoulos *et al.* 2009, 2011). In fact, β -arrestin1 is absolutely crucial for the development of post-MI hyperaldosteronism, since the circulating aldosterone levels of β -arrestin1 knockout mice post-MI appear normal (Bathgate-Siryk *et al.* 2014). What's more, this adrenal β -arrestin1-dependent signaling pathway to aldosterone biosynthesis may underlie the "aldosterone breakthrough" phenomenon observed with the clinical use of angiotensin receptor blockers (ARBs), that is AT₁R-selective antagonist drugs (Bomback and Klemmer 2007; Lymperopoulos *et al.* 2014; Dabul *et al.* 2015).

Although adrenal β -arrestin1 activated by the AT₁R exerts the cardio-toxic effect of hyperaldosteronism, in other tissues, such as the heart itself, it may exert beneficial effects (Lymperopoulos and Bathgate 2013). Therefore, in an effort to come up with better cardiovascular therapeutics, several AngII analog peptides have been developed and characterized over the past few years that presumably only activate β -arrestins at the AT₁R while leaving G proteins unaffected (Violin *et al.* 2013). This was spurred by the discovery of SII ([Sar¹, Ile⁴, Ile⁸]-AngII) as being such a β -arrestin-"biased" analog more than a decade ago (Holloway *et al.* 2002). At present, several other, much more potent than SII, β -arrestin-"biased" analogs have been produced and described, with one of them, TRV120027 ([Sar¹, D-Ala⁸]-AngII), currently under evaluation in early phase clinical trials for acute HF (Violin *et al.* 2010; Soergel *et al.* 2013). In this study, we synthesized and tested a series of such β -arrestin-"biased" AngII peptide analogs by using the

sequence of SII as a template. Thus, compared to the natural octapeptide AngII, we kept the presence of sarcosine (N-methyl-glycine) (instead of aspartate) at position 1 and we also substituted the phenylalanine at position 8 with leucine in all of these compounds. However, we chose to replace the isoleucine of position 5 with varied substitutions, instead of replacing the tyrosine of position 4 (as in SII). Based on *in vitro* assays, one of the analogs turned out to be particularly potent at stimulating β -arrestin-dependent aldosterone production and was further evaluated *in vivo* in post-MI animals progressing to HF. We were also able to make some inferences on the effect of the substitution of Ile⁵ of the AngII peptide sequence on AT₁R- β -arrestin coupling by comparing the *in vitro* potencies of the various analogs we synthesized.

Materials and Methods

Materials

All peptides were custom-made by Pierce Biotechnology (Rockford, IL), except for AngII, SII, CORAML, CORML, and CORET, which were made in Dr. Cordopatis's lab (Univ. of Patras, Patras, Greece). All the peptides used in this study were sequence-verified via mass spectrometry and purified to >95% purity via HPLC.

DiscoverX assay

The PathHunter™ β -Arrestin® assay monitors the activation of a GPCR in a homogenous, nonimaging assay format using a proprietary complementation technology (Fig. S1), developed by DiscoverX (Fremont, CA) (McGuinness *et al.* 2009; Dabul *et al.* 2015). Briefly, the assay utilizes an enzyme fragment complementation reaction with β -galactosidase (β -Gal) as the functional reporter. The enzyme is split into two complementary portions expressed as fusion proteins in the cell. The enzyme acceptor (EA) is fused to β -arrestin and the ProLink donor peptide is fused to the GPCR of interest, in this case, human AT₁R overexpressed in Chinese Hamster Ovary (CHO) cells. Upon GPCR stimulation, β -arrestin is recruited to the receptor for desensitization, bringing the two fragments of β -Gal together and allowing complementation to occur. This generates an active enzyme that can convert a chemiluminescent substrate to generate an output signal detectable on a standard microplate reader.

CellKey assay

This assay utilizes cellular dielectric spectroscopy (CDS) to detect a range of whole-cell responses in a label-free

manner. CellKey is a CDS-based instrument that detects GPCR activation in an automated microplate format (Peters *et al.* 2007; Dabul *et al.* 2015). Agonist and antagonist activity can be accurately quantified under conditions of low receptor expression. In this study, human AT₁R-overexpressing human embryonic kidney (HEK)293 cells were used in the CellKey assay system (see also Fig. S2A–B). Briefly, cells were incubated with the peptide of interest for 15 min at room temperature and the change in impedance was recorded to measure degree of AT₁R activation.

H295R cell culture and transfections

The human AZG cell line H295R was purchased from American Type Culture Collection (Manassas, VA) and cultured as previously described (Lympopoulos *et al.* 2009). Transfection was performed with adenovirus encoding for full length, wild-type rat β -arrestin1, as previously described (Lympopoulos *et al.* 2009). The construction of this adenovirus has also been described previously (Lympopoulos *et al.* 2009).

Plasma and in vitro aldosterone secretion measurements

In vitro aldosterone secretion in the culture medium of H295R cells as well as rat plasma aldosterone levels were determined by EIA (Aldosterone EIA kit, ALPCO Diagnostics, Salem, NH), as described (Lympopoulos *et al.* 2009).

Western blotting

Western blots to assess protein levels of StAR (sc-25806, SantaCruz Biotechnology, Santa Cruz, CA), β -arrestin1/2 (sc-28869, SantaCruz), and GAPDH (MAB374; Chemicon, Temecula, CA) were done using protein extracts from treated H295R cells, as described previously (Lympopoulos *et al.* 2009). Immunoblots were revealed by enhanced chemiluminescence (ECL, Life Technologies, Grand Island, NY) and visualized in the FluorChem E Digital Darkroom (Protein Simple, San Jose, CA) (Salazar *et al.* 2013). Densitometry was performed with the Alpha-View software (Protein Simple, San Jose, CA, USA) in the linear range of signal detection (on nonsaturated bands).

Experimental animals and surgical procedures

The animals in this study were handled according to animal welfare regulations and protocols approved by the authors' Institutional Review (IACUC) Boards and

complied with the ARRIVE guidelines. MI was performed in adult male Sprague–Dawley rats using the cryo-infarct method, as previously described (Lympopoulos *et al.* 2011). Adrenal-specific in vivo gene delivery, at 2 weeks post-MI, was done essentially as described (Lympopoulos *et al.* 2011), via direct delivery of adenovirus encoding full length, wild-type rat β -arrestin1 in both adrenal glands (Lympopoulos *et al.* 2008). The CORET peptide (100 μ g/kg i.p., dose calculated based on EC₅₀ for aldosterone secretion in vitro) or saline was administered daily for seven consecutive days, starting on the day of the adenoviral adrenal-specific gene delivery (2 weeks post-MI). Overexpression of adrenal β -arrestin1 was confirmed via Western blotting and was comparable among animals of the same group (data not shown). All animals were fed exactly the same (low salt) diet throughout the study.

Echocardiography and in vivo hemodynamics

Transthoracic echocardiography was performed with a linear 30-MHz transducer (VeVo 770 High Resolution Imaging System, VisualSonics, Toronto, ON, Canada), as described previously (Lympopoulos *et al.* 2011, 2014). Three independent echocardiographic measurements were taken in both modes. In vivo hemodynamic analysis was also performed as previously described (Lympopoulos *et al.* 2011), with a 1.4-Fr pressure-conductance catheter (Millar Instruments, Houston, TX) to record cardiac hemodynamics and a polyethylene-50 catheter inserted into the right external jugular vein for isoproterenol infusions (maximal dose of isoproterenol: 333 ng/kg of body weight).

Statistical analyses

Data are generally expressed as mean \pm SEM. Unpaired two-tailed Student's *t* test, chi-square test, and one- or two-way ANOVA with Bonferroni or Tukey's tests were generally performed for statistical comparisons, unless otherwise indicated. For multigroup statistical comparisons, Tukey's multiple comparison test using SAS version 8.2 software (Cary, NC, USA) was used (unless otherwise indicated). For all tests, a *P* value of <0.05 was considered to be significant.

Results

CORET potently stimulates β -arrestin coupling to the AT₁R

In a first set of experiments, we synthesized and tested three AngII peptide analogs: CORML: [Met⁵, Leu⁸]-AngII,

CORSML: [Sar¹, Met⁵, Leu⁸]-AngII, and CORET: [Sar¹, Cys(Et)⁵, Leu⁸]-AngII, and tested them in the PathHunter (DiscoverX) and in the CellKey assay systems, that is, in CHO and HEK293 cells, respectively, overexpressing the human AT₁R (see Figs S1 and 2 for more details). CORET was found particularly potent at inducing β -arrestin coupling to the AT₁R, only ~4 times less potent than the natural agonist AngII in this particular assay (Fig. 1, see also Table 1). Importantly, all three analogs were much more (~20–fold) potent than SII, the prototypic β -arrestin-“biased” AngII analog peptide, at stimulating AT₁R- β -arrestin coupling in this assay system (Fig. 1B, see also Table 1). Finally, as shown in Figure 1C, all three compounds, plus the analog [Sar¹, Cys⁵, Leu⁸]-AngII (“Cys”), were less potent than AngII but more potent than SII at β -arrestin stimulation in the CellKey assay system (see also Table 1). As far as efficacy is concerned, all compounds in the CellKey assay showed less maximal effect than AngII but higher than SII (~75% of AngII maximal effect on average vs. only ~40% for SII, Fig. 1C and Table 1). Finally, none of our analogs displayed inverse agonist or allosteric modulator properties at the AT₁R (data not shown).

CORET potently induces aldosterone turnover in AZG cells in vitro

Next, we examined what the above findings mean for the physiological effect of AT₁R-induced, β -arrestin-dependent aldosterone production (Fig. 2A). β -arrestin1 has been found, similarly to G protein-dependent signaling by the activated AT₁R in AZG cells, to stimulate aldosterone synthesis and secretion by upregulating Steroidogenic Acute Regulatory protein (StAR), the enzyme that catalyzes the rate-limiting step of this biosynthetic process (mitochondrial cholesterol uptake) (Fig. 2A) (Rainey et al. 2004; Lymperopoulos et al. 2009). Thus, using the human AZG cell line H295R, transfected to overexpress β -arrestin1 (Lymperopoulos et al. 2009), and in vitro aldosterone secretion as the readout, we found again that all three peptides (CORML, CORSML, CORET) are more potent and efficacious aldosterone secretion inducers than SII in vitro, with CORET being the most potent and efficient out of the three (Table 1 and Fig. 2B). Compared to AngII, the peptides were less potent (Table 1), as expected, since AngII stimulates aldosterone secretion via both G proteins and β -arrestin1, whereas these peptides only via β -arrestin1. Notably, for the very same reason, no effect on aldosterone secretion was seen with any peptide analog (including SII) when endogenous β -arrestin1 was blocked via overexpression of a dominant negative mutant (Fig. S3A–B).

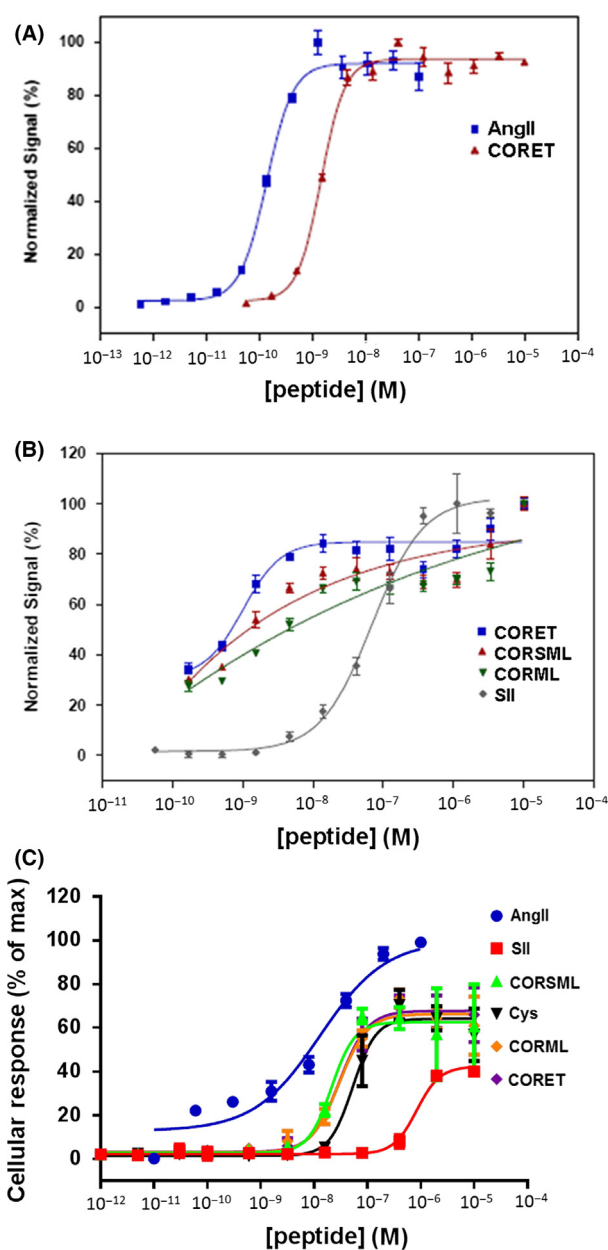


Figure 1. Comparison of potencies of CRSM, CORML, and CORET at stimulating AT₁R- β -arrestin coupling. (A) Comparison of CORET versus AngII (DiscoverX assay). (B) Comparison of CORET, CORSML, and CORML versus SII (DiscoverX assay). (C) Concentration–response curves for AT₁R- β -arrestin binding stimulation, as derived from the CellKey assay (Cys: Sar¹, Cys⁵, Leu⁸-AngII). $n = 5$ independent determinations for each peptide in both assays (two-way ANOVA with Bonferroni test).

Consistent with the findings on aldosterone secretion, all three peptides were again more efficacious stimulators of StAR protein upregulation than SII, with CORET being the most potent of the three again (Fig. 2C–D), indicating that also synthesis of aldosterone is more elevated with

Table 1. Maximal effect (Max. eff., as % of 1 nmol/L AngII \pm SEM) and half-maximal effective concentration ($EC_{50} \pm$ SEM) values for AT_1R -elicited β -arrestin activation in vitro calculated by the CellKey assay system; for AT_1R -elicited β -arrestin activation in vitro calculated by the PathHunter assay system; and for AT_1R -elicited β -arrestin-dependent aldosterone secretion in H295R cells.

	AngII	SII	CORML	CORSML	CORET	PRO	BUT	PEN
EC_{50} (nmol/L) (CellKey)	0.1 \pm 0.06	10 \pm 5.2	0.6 \pm 3.2	0.5 \pm 2.2	0.4 \pm 2.3	6 \pm 4.3	3 \pm 2.3	1 \pm 3.3
Max. eff. (%) (CellKey)	100	40 \pm 6	75 \pm 10	77 \pm 11	81 \pm 4	86 \pm 6	85 \pm 6	80 \pm 5
EC_{50} (nmol/L) (PathHunter)	0.1 \pm 0.04	11 \pm 6.1	0.6 \pm 4.2	0.7 \pm 3.7	0.5 \pm 3.3	4 \pm 3.0	2 \pm 4.4	0.4 \pm 5.3
Max. eff. (%) (PathHunter)	100	42 \pm 7	73 \pm 8	76 \pm 7	82 \pm 5	83 \pm 6	83 \pm 7	80 \pm 7
EC_{50} (nmol/L) (Aldosterone)	6 \pm 4.5	10 ³ \pm 98.3	101 \pm 11.3	95 \pm 12.5	55 \pm 21.2	N.D.	N.D.	N.D.
Max. eff. (%) (Aldosterone)	100	35 \pm 6	55 \pm 9	57 \pm 8	80 \pm 6	N.D.	N.D.	N.D.

$n = 5$ independent determinations per treatment (two-way ANOVA with Tukey's multiple comparison test).

N.D., Not Determined.

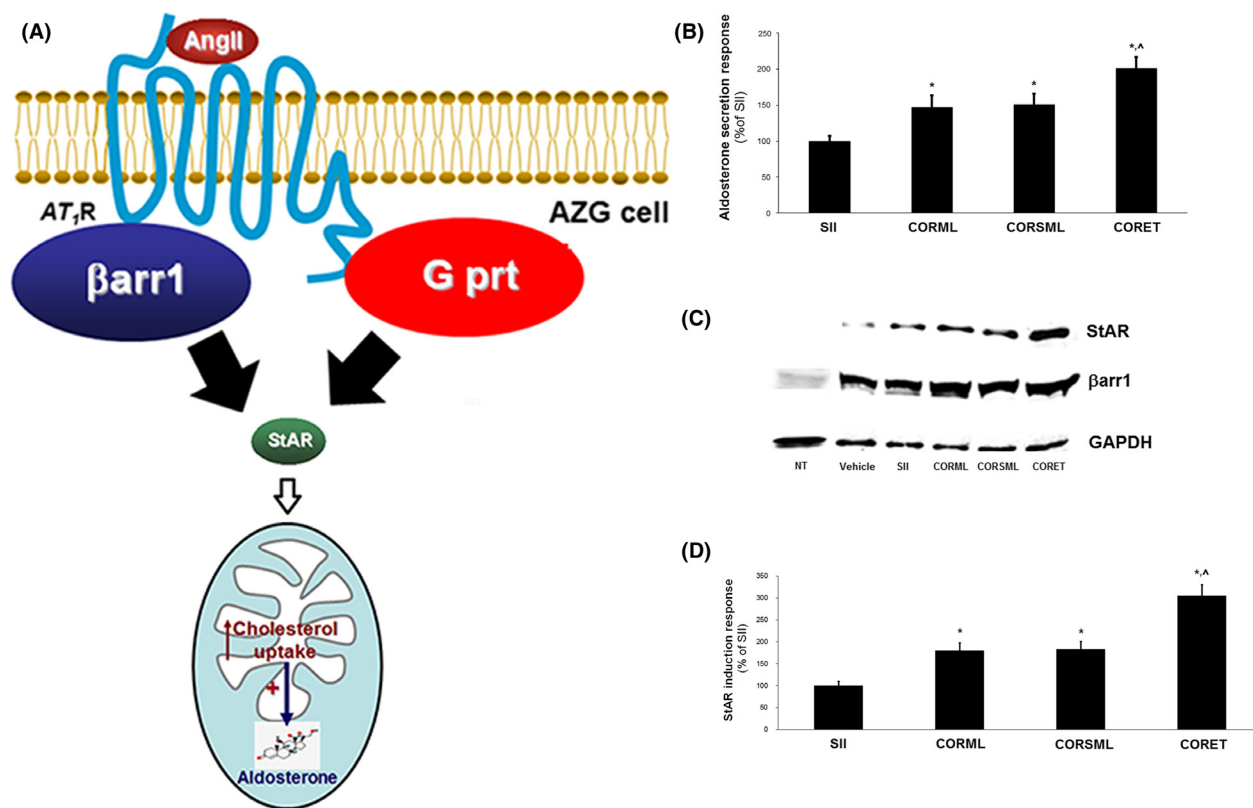


Figure 2. AngII analog peptides and aldosterone turnover in vitro. (A) Schematic representation of the AT_1R -elicited pathways mediated by G proteins (G prt) and β -arrestin1 (β arr1) converging on aldosterone synthesis and secretion in adrenocortical zona glomerulosa (AZG) cells. (B) In vitro aldosterone secretion from H295R cells overexpressing β -arrestin1 and stimulated for 4 h with 100 nmol/L CORML, CORSML, or CORET. Data are presented as % of the secretion induced by 10 μ mol/L SII. * $P < 0.05$, versus SII, ^ $P < 0.05$, versus CORML or CORSML, $n = 5$ independent experiments/treatment. (C, D) Western blotting for StAR protein levels in the same cells treated as in (B). Representative blots of five independent experiments are shown in (C), including blots from nontransfected (NT) H295R cells for β -arrestin1 (β arr1) to confirm its overexpression in the transfected cells, and for GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as loading control, and the StAR protein induction (as % of the SII response), as derived by densitometric quantification, is shown in (D). * $P < 0.05$, versus SII, ^ $P < 0.05$, versus CORML or CORSML, $n = 5$ independent experiments/treatment (two-way ANOVA & chi-square test with Tukey's multiple comparison test).

any of the three peptides (and especially with CORET) than it is with SII. β -arrestin1 overexpression in these cells was confirmed by Western blotting (Fig. 2C) and

again, no effects on StAR protein induction were seen when endogenous β -arrestin1 was blocked (data not shown), as expected.

CORET exacerbates post-MI hyperaldosteronism and cardiac function in vivo

Since CORET was the most potent AngII analog at stimulating β -arrestins and β -arrestin1-dependent aldosterone production in vitro, and adrenal β -arrestin1-dependent hyperaldosteronism plays an important role in experimental post-MI HF progression (Lympelopoulous *et al.* 2011), we next sought to examine the in vivo effects of CORET in post-MI rats progressing to HF and overexpressing β -arrestin1 specifically in their adrenals (Lympelopoulous *et al.* 2011). We treated these animals with daily intraperitoneal injections of CORET (or saline) for seven consecutive days starting at 2 weeks post-MI and at the end of the week-long treatment period (3 weeks post-MI), we measured circulating aldosterone levels and cardiac function. Consistent with a potent β -arrestin stimulation at the adrenal AT₁R in vivo, circulating aldosterone levels of the CORET-treated animals were significantly elevated compared to control, saline-treated post-MI animals (Fig. 3A). Importantly,

this elevation was on top of the hyperaldosteronism normally present in the animals due to their post-MI progression to HF (compare with Sham in Fig. 3A), indicating worsening of the post-MI hyperaldosteronism by the CORET treatment. Finally, this hyperaldosteronism deterioration was reflected on cardiac function of the treated post-MI animals, as both ejection fraction (Fig. 3B) and isoproterenol (a standard cardiac positive inotrope)-stimulated contractility (Fig. 3C) were significantly worsened by CORET, compared to control saline-treated post-MI animals. Taken together, these results strongly suggest that CORET accelerates post-MI HF progression by stimulating adrenal β -arrestin1-mediated aldosterone elevation.

AngII Ile⁵ and AT₁R- β -arrestin coupling

Since the most striking structural difference between SII and CORET is substituted Tyr⁴ versus substituted Ile⁵ of the AngII sequence (respectively), we posited that maybe this is the reason for the enhanced β -arrestin agonism at the AT₁R displayed by CORET. Thus, we focused on the

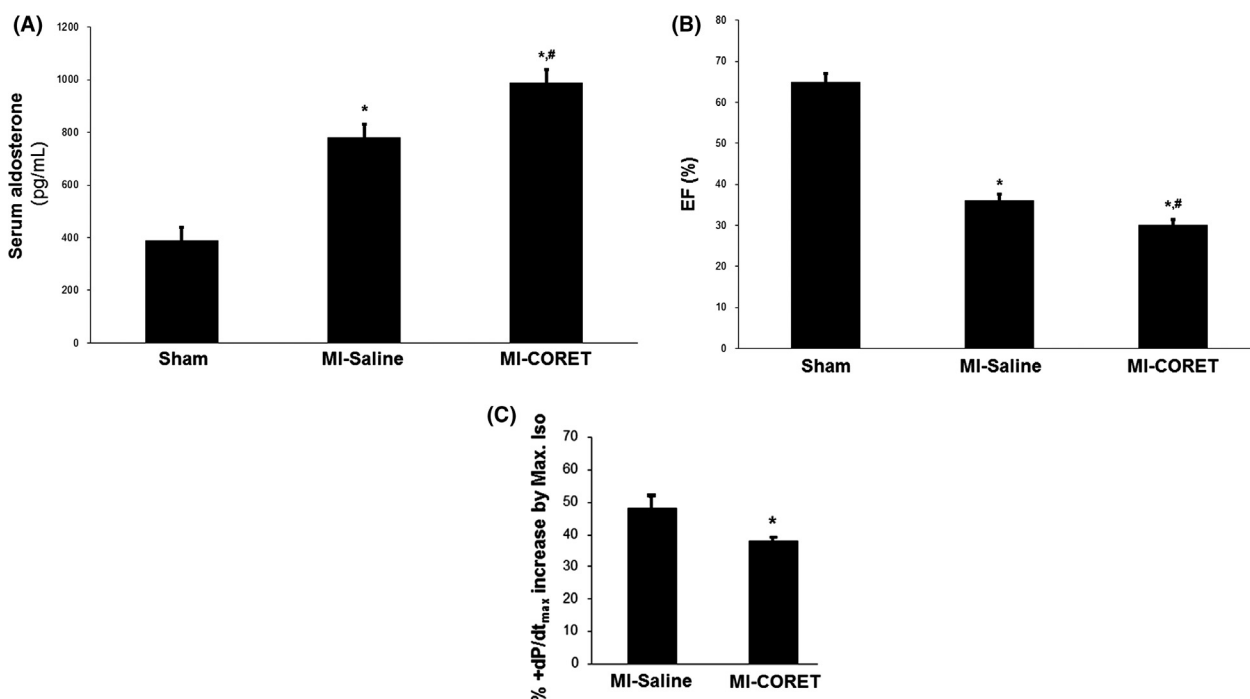


Figure 3. CORET in post-MI HF in vivo. (A) Circulating aldosterone levels in 3-week post-MI rats, overexpressing β -arrestin1 in their adrenals, and treated for seven consecutive days either with saline (MI-Saline) or with daily i.p. injections of CORET. Aldosterone levels in age-matched, sham-operated, healthy, and untreated (and with normal adrenal β -arrestin1 expression levels) animals are also included for comparison. * $P < 0.05$, versus Sham, # $P < 0.05$, versus MI-Saline, $n = 5$ rats/group. (B) Ejection fraction (EF %) of the same rat groups at the end of the 7-day treatment. * $P < 0.05$, versus Sham, # $P < 0.05$, versus MI-Saline, $n = 5$ rats/group. (C) Increases in cardiac contractile function in the post-MI groups at the end of the 7-day treatment, as inferred by the % increase in +dP/dt_{max} that a maximal dose of isoproterenol (Max. Iso) induces. * $P < 0.05$, versus. MI-Saline, $n = 5$ rats/group (two-way ANOVA & chi-square test with Tukey's multiple comparison test).

effect of varying the substitution at this position (Ile⁵) of the AngII sequence on the potency of β -arrestin agonism at the AT₁R. To this end, we synthesized a series of analogs with Cys⁵ substitutions of increasing side-chain size, while keeping the Sar¹ and Leu⁸ substitutions steady (Fig. 4A). We tested all of these compounds in vitro for β -arrestin agonism potency and efficacy at the human AT₁R against the natural (non- β -biased") agonist AngII and the β -arrestin- β -biased" SII, via both the DiscoverRx (Fig. 4B) and the CellKey (Fig. 4C) assay systems. As shown in Table 1, increasing the linear size of the Cys⁵ alkyl side-chain results in a directly proportional increase

in potency (EC₅₀ decrease) for β -arrestin agonism, while maintaining a high level of efficacy (>80% of the AngII's maximal effect). This trend is preserved even in the presence of a residue as "bulky" as Cys(*n*-pentyl) at position 5 of the AngII sequence (Table 1). As far as SII is concerned, consistent with all of our experiments above, it was found much less potent or efficacious than any of the Cys⁵-substituted analogs we tested in this study: ~76 times less potent than AngII versus only ~3–4 times less potency than AngII for all of the compounds; only 40% of AngII's maximal effect versus >80% of AngII's maximal effect for all of the compounds (Table 1). These findings

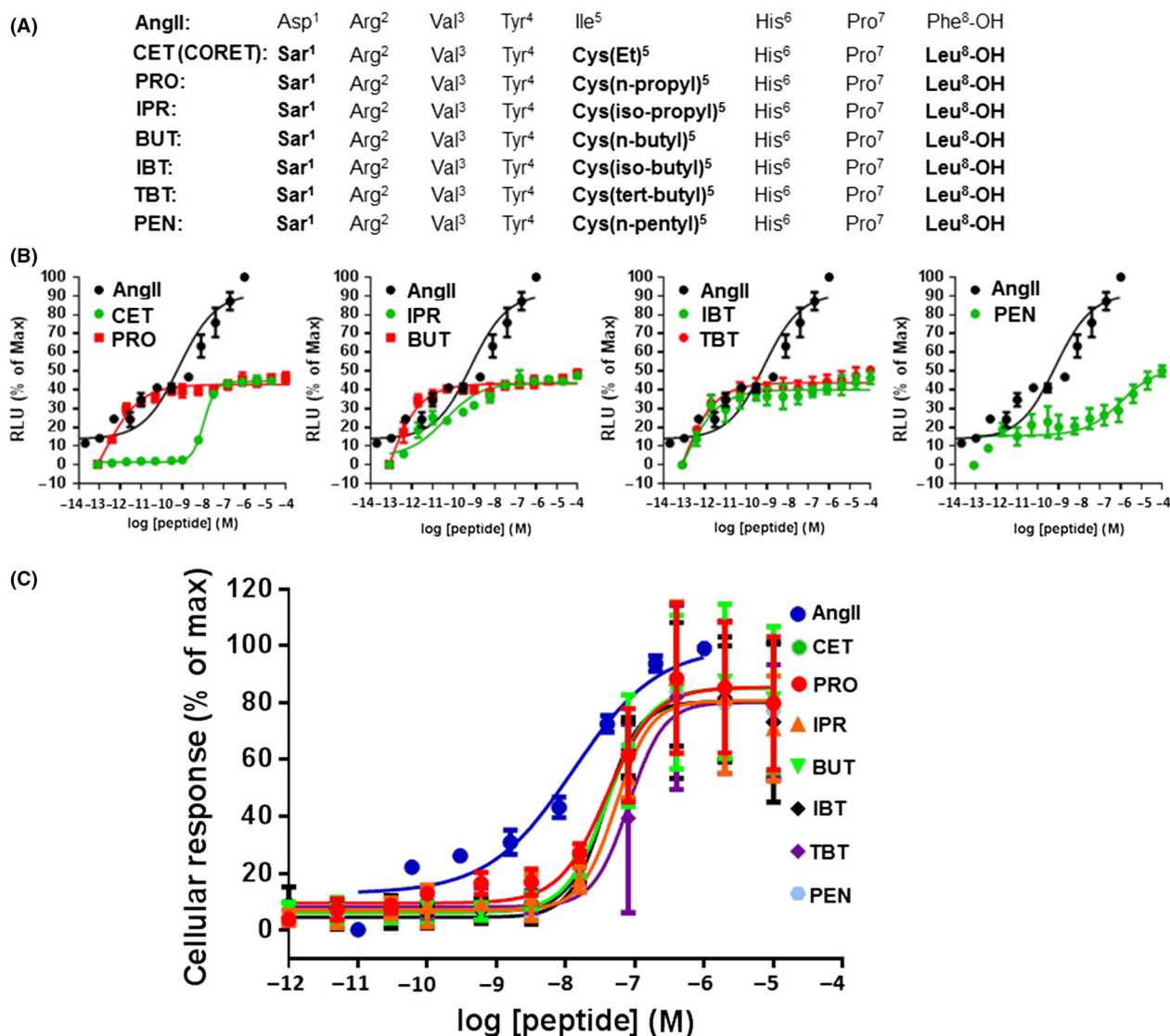


Figure 4. AngII's Ile⁵ substitution and AT₁R-elicited β -arrestin activation. (A) The amino acid sequences of all of the peptides studied. The substitutions differing from AngII are highlighted in bold. (B) Concentration–response curves for stimulation of AT₁R- β -arrestin binding, based on the DiscoverRx assay. (C) Concentration–response curves for stimulation of AT₁R- β -arrestin binding based on the CellKey assay. See also Table 1. $n = 5$ independent determinations for each analog in both assays (two-way ANOVA with Tukey's multiple comparison test).

suggest a profound effect of AngII's Ile⁵ aliphatic side-chain substitution on β -arrestin activation by the AT₁R.

Discussion and Conclusions

In this study, we synthesized a series of β -arrestin-“biased” AngII Ile⁵-substituted peptide analogs and examined their effects on AT₁R- β -arrestin recruitment, as well as their impact on the AT₁R physiological effect of aldosterone production in AZG cells, which is known to be mediated by β -arrestin1 (Lymperopoulos *et al.* 2009). We uncovered two main findings: first, one of these peptides, [Sar¹, Cys(Et)⁵, Leu⁸]-AngII (CORET), is a potent β -arrestin agonist at the human AT₁R, stimulating aldosterone synthesis and secretion *in vitro* more robustly than SII, and it also exacerbates post-MI HF hyperaldosteronism accelerating cardiac performance decline *in vivo*. Since a large number of AngII β -arrestin-“biased” analogs have been developed over the past several years in an effort to come up with safer and more effective cardiovascular therapeutics over the currently available traditional and “unbiased” AT₁R ligands (Violin *et al.* 2013), the possibility of causing excessive aldosterone secretion from the adrenal gland, which can complicate post-MI HF therapy, has to be taken into account when designing such β -arrestin-“biased” AT₁R ligands.

The other major finding of this study is that increasing the size of the alkyl side-chain of Ile⁵ of the AngII peptide sequence results in proportionally more robust β -arrestin recruitment to the AT₁R. A number of previous studies on structure–activity relationships (SAR) of the AngII molecule with regards to AT₁R “bias” toward β -arrestin versus G protein signaling have indicated that replacement of the aromatic side-chain of Phe⁸, absolutely critical for full G_q protein agonism at the AT₁R, is necessary to remove the G protein-activating capacity of the ligand, while maintaining the ability to activate β -arrestins (Miura *et al.* 1999; Nikiforovich *et al.* 2006; Zimmerman *et al.* 2012; Balakumar and Jagadeesh 2014). Additionally, Asp¹ is known to affect only binding (affinity) to the AT₁R and not efficacy and, in fact, substitution with Sar¹ actually enhances receptor binding (Wilkes *et al.* 2002). Finally, Tyr⁴ appears also essential for full G_q protein agonism but does not seem to affect AT₁R phosphorylation (Miura *et al.* 1999; Holloway *et al.* 2002; Nikiforovich *et al.* 2006), a crucial prerequisite event for subsequent β -arrestin binding (Qian *et al.* 2001). Ile⁵ does not seem to affect activity toward G proteins but its side-chain plays a role in maintaining the proper (agonist) conformation of AngII when bound to the AT₁R, that is, it contributes to the proper positioning of the two agonist “switches” (Tyr⁴ & Phe⁸) of the AngII molecule (Tzakos *et al.* 2004;

Nikiforovich *et al.* 2006). With regards to the effects on β -arrestin agonism at the AT₁R, virtually nothing is presently known about the Ile⁵ position; however, it is interesting to note that some of the purported β -arrestin-“biased” analogs with beneficial effects in the heart, currently under investigation for therapy of HF and of dilated cardiomyopathy (DCM), have this Ile⁵ substituted (e.g., to Lys⁵ in TRV120023), whereas others retain it (e.g., TRV120027) (Violin *et al.* 2010). Our present data indicate that this position, although not critical for β -arrestin agonism at the AT₁R like the Phe⁸ position is, can still significantly and positively modulate the potency of the β -arrestin-“biased” AT₁R ligand. Of course, this awaits confirmation in larger SAR studies and with the crystallization of the full AT₁R protein, including the β -arrestin-interacting regions. Unfortunately, the recently solved human AT₁R crystal structure (Zhang *et al.* 2015) was missing the full C-terminal region of the receptor, which the β -arrestins interact with (Balakumar and Jagadeesh 2014), and thus, could not provide any information in that regard. Of course, the process of the AT₁R- β -arrestin interaction and its ultimate effects in the cell is very complex and multifactorial, involving, not only the activating ligand, but also GPCR-kinase (GRK)-dependent receptor phosphorylation (Qian *et al.* 2001), which particular β -arrestin isoform (β -arrestin1 or -2) binds the receptor and the conformational changes the β -arrestin molecule itself undergoes upon binding to the AT₁R (Shukla *et al.* 2008, 2013), and even the duration of this interaction, that is, the stability of the AT₁R- β -arrestin complex in endosomal compartments (Zimmerman *et al.* 2012). Combined differences in all of these molecular determinants are quite likely to be responsible for the fact that biased AT₁R ligand-activated β -arrestin-dependent signaling appears beneficial, at least in the heart, whereas β -arrestins do not seem capable of providing any functional or structural benefit to the heart when they are activated by the AngII-bound AT₁R. Nevertheless, it becomes clear from the above that simply varying the structure of an AT₁R ligand hardly suffices to “bias” the resultant AT₁R- β -arrestin complex toward a specific cellular effect or to even predict the effect on AT₁R-elicited signal transduction.

In conclusion, our present findings suggest that adrenal β -arrestin-dependent aldosterone production is a potential side-effect of AT₁R β -arrestin-“biased” ligands that needs to be taken into account, especially if the ligand is considered a drug candidate for a cardiovascular indication. In addition, we conclude that the substitution of the Ile⁵ position of AngII, so far largely “overlooked” in β -arrestin “bias” studies, may play an important role in stimulating the interaction of AT₁R with β -arrestins. Both of these findings may have important implications for

AngII pharmacology and for future AT₁R-targeted drug development.

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Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Schematic illustration of the DiscoverX PathHunter™ β -Arrestin assay.

Figure S2. Schematic illustration of the CellKey assay protocol employed in this study.

Figure S3. (A) In vitro aldosterone secretion from H295R cells overexpressing a dominant negative mutant β -arrestin1 and stimulated for 4 h with 100 nmol/L CORSML, CORML, or CORET. Data are presented as % of the secretion induced by 10 μ mol/L SII. * $P < 0.05$, versus SII, $^{\wedge}P < 0.05$, versus CORML or CORSML, $n = 5$ independent experiments/treatment. No significant differences were observed among treatments.