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Comparative evaluation of biased agonists Sarcosine , d-Alanine -Angiotensin (Ang) II (SD Ang II) and Sarcosine , Isoleucine -Ang II (SI Ang II) and their radioiodinated congeners binding to rat liver membrane AT receptors

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ORIGINAL ARTICLE

Comparative evaluation of biased agonists Sarcosine[1], D-Alanine[8]-Angiotensin (Ang) II (SD Ang II) and Sarcosine[1], Isoleucine[8]-Ang II (SI Ang II) and their radioiodinated congeners binding to rat liver membrane AT_1 receptors

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Abstract

Angiotensin II analogue and β-arrestin biased agonist TRV027 (Sarcosine[1], D-Alanine[8]-Angiotensin (Ang) II; SD Ang II), developed by Trevena, Inc. in the early 2010s, brought hopes of a novel treatment for cardiovascular diseases, due to its ability to simultaneously cause signaling through the β-arrestin signaling pathway, while antagonizing the pathophysiological effects of Ang II mediated by the AT_1 receptor G protein signaling cascades. However, a phase II clinical trial of this agent revealed no significant benefit compared to placebo treatment. Using ^{125}I-Sarcosine[1], Isoleucine[8]-Ang II (^{125}I-SI Ang II) radioligand receptor competition binding assays, we assessed the relative affinity of TRV027 compared to SI Ang II for liver AT_1 receptors. We also compared radioiodinated TRV027 (^{125}I-SD Ang II) binding affinity for liver AT_1 receptors with ^{125}I-SI Ang II. We found that despite its anticipated gain in metabolic stability, TRV027 and ^{125}I-SD Ang II had reduced affinity for the AT_1 receptor compared with SI Ang II and ^{125}I-SI Ang II. Additionally, male–female comparisons showed that females have a higher AT_1 receptor density, potentially attributed to tissue-dependent estrogen and progesterone effects. Peptide drugs have become more popular over the years due to their increased bioavailability, fast onset of action, high specificity, and low toxicity. Even though Trevena®'s biased agonist peptide TRV027 offered greater stability and potency compared to earlier

Abbreviations: ADHF, acute decompensated heart failure; Ala, alanine; AM5, assay medium buffer; Ang, angiotensin; ARBs, angiotensin receptor blockers; Asp, aspartic acid; AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; BSA, bovine serum albumin; eNOS, endothelial nitric oxide synthase; ERK1/2, extracellular signal-regulated kinase; GPCRs, G-protein-coupled receptors; GRKs, G protein receptor kinases; HPLC, high-performance liquid chromatography; Ile, isoleucine; IP_3, inositol trisphosphate; ^{125}I-SD Ang II, ^{125}I-Sarcosine[1], D-Alanine[8]-Angiotensin II; ^{125}I-SI Ang II, ^{125}I-Sarcosine[1], Isoleucine[8]-Angiotensin II; MAPK, mitogen-activated protein kinases; OVX, ovariectomized; Phe, phenylalanine; RAS, renin-angiotensin system; ROS, reactive oxygen species; Sar, sarcosine; SD Ang II, Sarcosine[1], D-Alanine[8]-Ang II; SI Ang II, Sarcosine[1], Isoleucine[8]-Ang II; SII Ang II, Sarcosine[1], Isoleucine[4], Isoleucine[8] Ang II; TEAP, triethylamine phosphate; TRPC3, transient receptor potential cation channel subfamily C3; VSMCs, vascular smooth muscle cells.

AT_1R biased agonists, it failed its phase II clinical trial in 2016. Further refinements to AT_1R biased agonist peptides to improve affinity, as seen with SI Ang II, with better stability and bioavailability, has the potential to achieve the anticipated biased agonism.

KEYWORDS
AT_1R, binding assay, GPCR, D-Alanine^8-Angiotensin II, Sarcosine^1, Isoleucine^8-Angiotensin II, TRV027

1 | INTRODUCTION

According to the World Health Organization (WHO), cardiovascular diseases are the leading cause of death worldwide. Several drugs typically prescribed to treat such diseases target adrenergic and angiotensin (Ang) receptors, both of which are G-protein-coupled receptors (GPCRs)[1] that, upon agonist binding, activate a G protein, initiating classical signal transduction pathways.[2,3] Following dissociation of their G proteins, these receptors undergo phosphorylation of serine and threonine residues on their C-terminal tails catalyzed by G protein receptor kinases (GRKs) and other kinases, leading to the binding of β-arrestin proteins, resulting in receptor desensitization and internalization[3,4] (Figure 1). β-arrestin proteins can act as scaffold proteins, activating G protein-independent signaling pathways.[2-4] The GPCR Ang II type 1 receptor (AT_1R) is highly expressed throughout the cardiovascular system (e.g., vascular smooth muscle, endothelium, heart, kidney). When activated, it promotes intracellular signaling pathways, causing hypertension, endothelial dysfunction, vascular remodeling, myocardial hypertrophy, and cardiac dysfunction.[4,5] When the AT_1R binds β-arrestin proteins,[3] a number of substrates are phosphorylated, in particular, extracellular signal-regulated kinase (ERK1/2),[3,6] leading to cardioprotective effects.[4] Even though classical agonist binding to GPCRs generally activates the G-protein-mediated pathway, some ligands, referred to as "biased agonists," have the ability to activate one versus other signaling pathways coupled to the same receptor,[2,3] as they function as agonists for some receptor functions, but are antagonists or even inverse agonists for others.[7,8]

Years ago, pharmaceutical company Trevena® developed an Ang II analogue octapeptide, Sarcosine^1, D-Alanine^8-Ang II (SD Ang II), identified as TRV120027[9] and TRV027.[2,10,11] Differing from Ang II by two residues: sarcosine (Sar) in the first position and D-Alanine in the eighth, it is classified as an AT_1R β-arrestin biased agonist,[2] antagonizing the classic $G\alpha_{q/11}$ pathway activated by Ang II while simultaneously stimulating β-arrestin recruitment, activating several kinase pathways and endothelial nitric oxide synthase (eNOS) phosphorylation (Figure 2).[4,9,10,11,12,13,14] Due to their ability to block the detrimental effects of the classical G protein pathway on the cardiovascular system, β-arrestin biased agonists, such as SD Ang II, could potentially be used to treat cardiovascular diseases.[15]

In rodents, SD Ang II causes vasodilation (reducing mean arterial pressure), stimulates cardiomyocyte contractility, promotes cardiac function, decreases apoptosis (an effect also seen in vitro[13]) and reduces myocardial oxygen consumption.[4,9,11,12,17] In neonatal mice, SD Ang II elicits a positive inotropic effect, possibly due to activation of L-type Ca^{2+} channels, with no effect on heart rate, reactive oxygen species (ROS) production, or adrenal aldosterone secretion.[4,18] In preclinical models of left heart failure, SD Ang II decreased right atrial and mean arterial pressures, systemic and renal vascular resistances, while increasing cardiac output and renal blood flow.[4] Overall, its effect on blood pressure is similar to that of angiotensin receptor blockers (ARBs). Interestingly, in animal models, unlike ARBs, SD Ang II also improves cardiac performance, preserves cardiac stroke volume, decreases systemic vascular resistance, and improves cardiac output while preserving renal functions.[3]

In 2013, Trevena, Inc. started a clinical trial to test the efficacy of TRV027 in 620 adult patients suffering from acute decompensated heart failure (ADHF). Even though the drug proved to be safe, it was not superior to placebo with respect to the primary and secondary endpoints in the phase IIb trial,[19] leading to trial termination in 2016. Another clinical study was conducted by Soergel et al.[13] to explore TRV027's tolerability, pharmacokinetics, and pharmacodynamics in healthy individuals with sodium intake restriction, which stimulates the renin-angiotensin system (RAS). Participants were given ascending doses of the drug, which was found to have a half-life ranging from 2.4 to 13.2 min. A reduction in blood pressure, more evident in participants with RAS overactivation, was observed. The study showed that TRV027 is safe and well tolerated at doses up to 20-times higher than the 1 μg/kg/min efficacious dose that had been previously estimated in preclinical studies.[13] Recently, however, it was demonstrated that selective β-arrestin 1 activation by TRV027 causes acute catecholamine secretion through coupling with the transient receptor potential cation channel subfamily C3 (TRPC3),[20] which may have contributed to TRV027's failure in phase II clinical trials.

In this study, we attempted to determine whether ^{125}I-Sarcosine^1, D-Alanine^8-Ang II (^{125}I-SD Ang II) and SD Ang II have similar affinity for the AT_1R as ^{125}I-Sarcosine^1, Isoleucine^8-Ang II (^{125}I-SI Ang II) and SI Ang II, another potential biased agonist Ang II analogue that binds with high affinity, respectively.

2 | MATERIALS AND METHODS

2.1 | Animals

The liver tissues used for these studies were derived from 15- to 18-week-old male and female Long-Evans rats (Charles River Laboratories) and 16-week-old female Sprague–Dawley rats (Envigo), euthanized with carbon dioxide or isoflurane anesthesia followed by

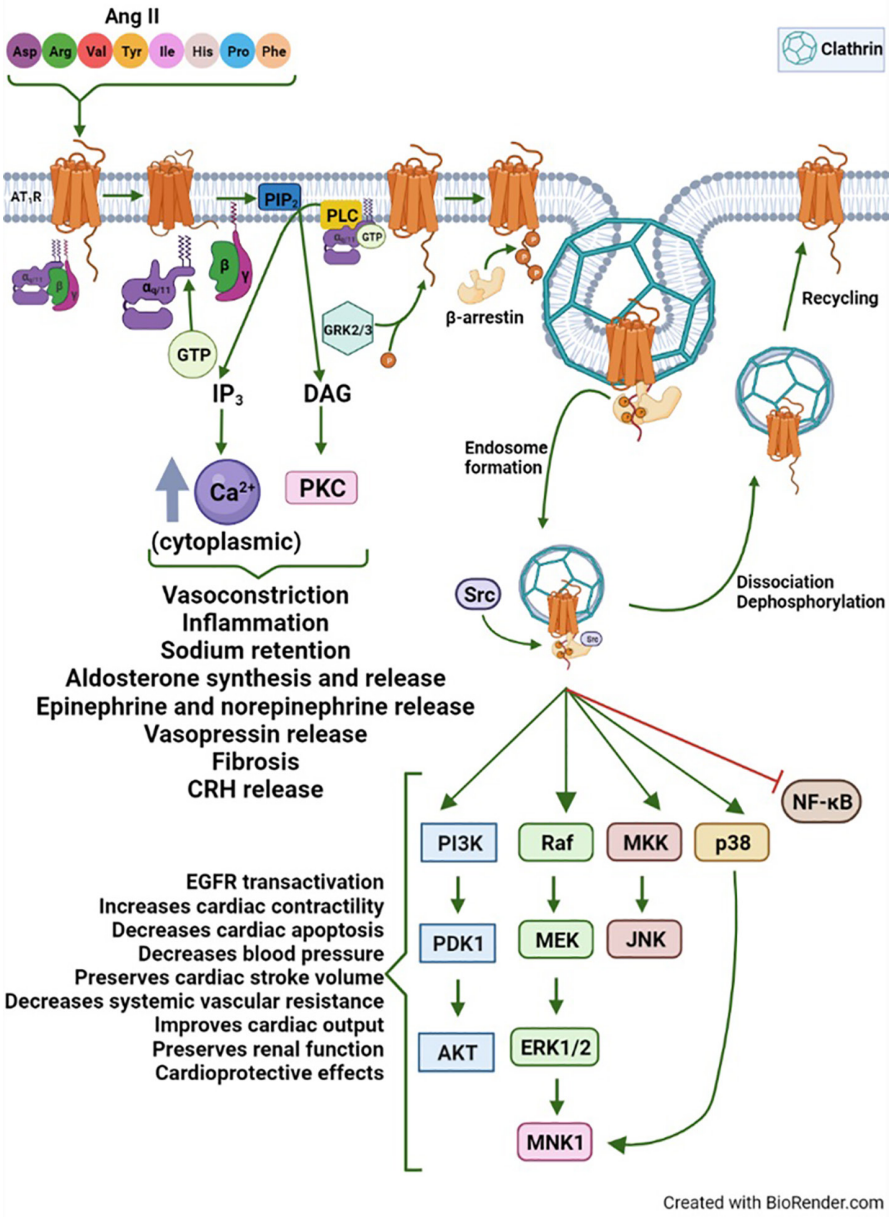

FIGURE 1 Classic Ang II/AT_1R signaling pathway. Binding of Ang II to the extracellular domain of the AT_1R causes G protein to bind to the receptor intracellularly. Hydrolysis of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) by the $\alpha_{q/11}$ subunit activates the protein, resulting in the dissociation of the $\alpha_{q/11}$ from the $\beta\gamma$ subunit. The $\alpha_{q/11}$ subunit is then free to initiate the signaling pathway, activating secondary messengers, and leading to an increase in intracellular calcium and the activation of protein kinase C (PKC). At the AT_1R, G-protein-coupled receptor kinases 2/3 (GRK2/3), phosphorylate its intracellular domain, making it possible for β-arrestin to bind to it, initiating receptor internalization, degradation, and recycling. Clathrin is a structural protein of the plasma membrane that plays a role in creating a "template" of rounded vesicles, such as endosomes, in the cytoplasm to be used in intracellular trafficking. Abbreviations: AKT, Ak strain transforming (also known as protein kinase B); DAG, diacylglycerol; ERK 1/2, extracellular signal-regulated kinase 1/2; GRK 2/3, G-protein-coupled receptor kinase 2/3; GTP, guanosine triphosphate; IP_3, inositol trisphosphate; JNK, c-Jun N-terminal kinase; P, phosphate; PDK1, phosphoinositide-dependent kinase-1; PI3K, phosphoinositide 3-kinase; MEK, mitogen-activated protein kinase; MKK, mitogen-activated protein kinase kinase; MNK1, mitogen-activated protein kinase–interacting kinase 1; NF-κB, nuclear factor kappa B; PIP_2, phospholipase C; PKC, protein kinase C; PLC, phospholipase C; Raf, rapidly accelerated fibrosarcoma; Src, SRC proto-oncogene, non-receptor tyrosine kinase.

exsanguination, respectively. The livers were dissected and stored frozen at −70°C until used for receptor binding assays. Animals were maintained in the Georgetown University and Nova Southeastern University animal facilities in compliance with institutional guidelines and the *Guide for the Care and Use of Laboratory Animals*, National Research Council (NRC) Publication, 2011 edition. All animal protocols were approved by the Georgetown University's (protocol no. 2019-0035) and Nova Southeastern University's (protocol no. 2020.06.

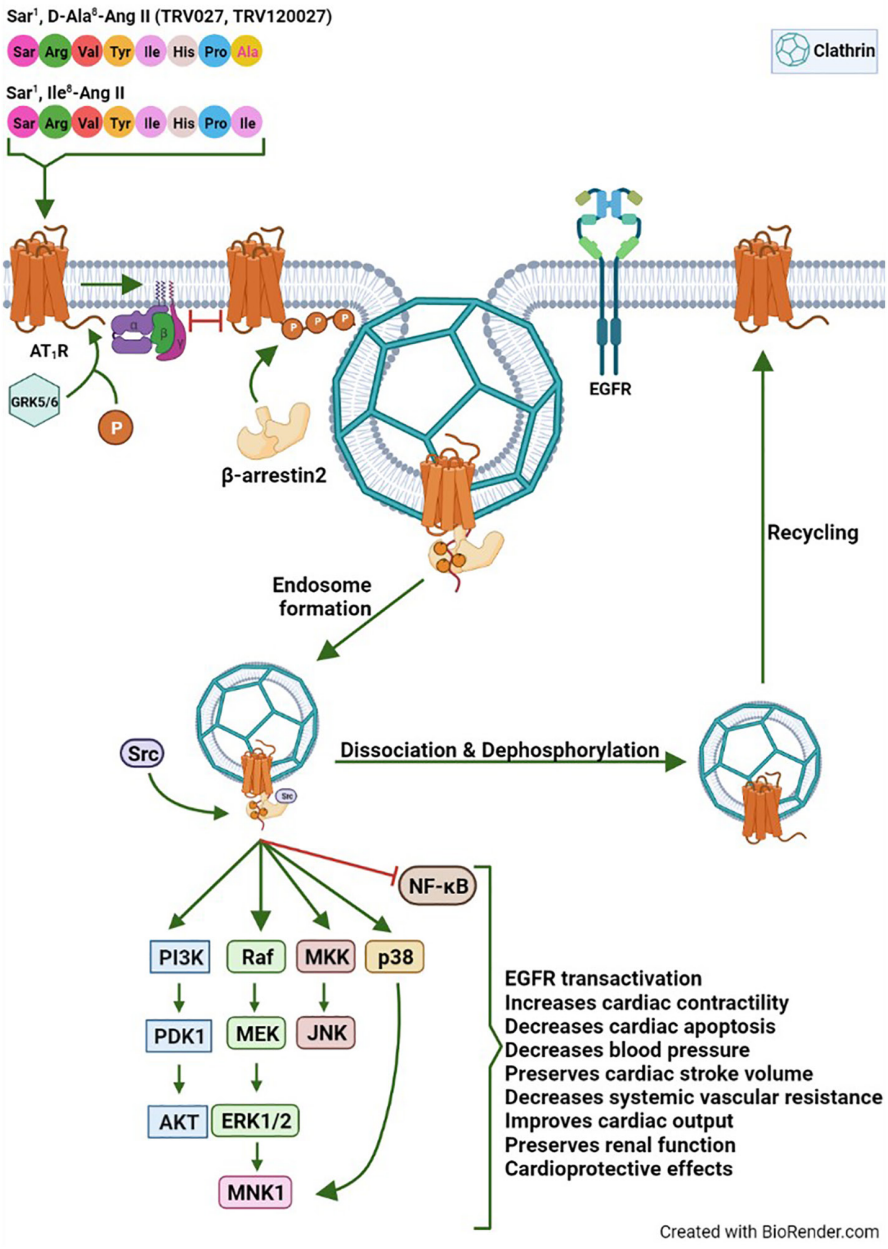

FIGURE 2 SI Ang II and SD Ang II signaling pathway. Binding of biased agonists TRV027 (SD Ang II) or SI Ang II to the extracellular domain of the AT_1R causes the activation of the signaling pathway through β-arrestin2. Before it can bind, the intracellular domain of the receptor needs to be phosphorylated. Studies have shown that binding of these ligands shift the preference toward GRK5/6, as opposed to GRK2/3, which is preferred by Ang II.[16] Upon binding, proto-oncogene tyrosine-protein kinase Src, is recruited to bind to β-arrestin2, initiating a variety of signaling pathways. Abbreviations as in Figure 1.

MH2) Institutional Animal Care and Use Committees, and experiments were carried out according to institutional guidelines. Before euthanization, Sprague–Dawley rats were maintained on a normal 12 h-12 h light–dark cycle and were given ad libitum access to water and Harlan Teklad LM-485 Mouse/Rat Sterilizable Diet (0.3% sodium, 0.8% potassium); Long-Evans rats were maintained on a normal 12 h-12 h light–dark cycle, 5V75 diet (LabDiet) was available ad libitum.

2.2 | Reagents and radioligand preparation

SD Ang II and SI Ang II were radioiodinated using a modification of the chloramine T method[21] developed by Hunter and Greenwood.[22] $Na^{125}I$ was obtained from American Radiolabeled Chemicals or McMaster University (Hamilton, Ontario, Canada). SD Ang II was custom synthesized by ABclonal Technology, SI Ang II was obtained from Phoenix Pharmaceuticals, losartan was kindly provided by Ron Smith (DuPont Merck). The reactants were applied to a high-performance liquid chromatography (HPLC) column (Microsorb-MV 100-5 C18 250 × 4.6 mm, Agilent Technologies) and eluted with 14.5% acetonitrile for SD Ang II or 19% acetonitrile for SI Ang II in 83 mM triethylamine phosphate (TEAP), pH 3.0. This system completely resolved monoradioiodinated SD Ang II and SI Ang II from the uniodinated and diiodinated peptides. After collection of monoradioiodinated SD Ang II and SI Ang II fractions, bovine serum albumin (BSA) was added to achieve a concentration of 2 mg/ml, after which they were frozen at −20°C until use in radioligand-binding assays.

To evaluate receptor binding with a radioiodinated ligand at concentrations in excess of 5 nM (a practical and safe limit to the amount of a pure iodine (I)-125 labeled ligand per 100 μl in an assay tube), the

specific activity of the radioiodinated SD Ang II used in these assays was reduced with a 9:1 stoichiometric ratio of non-radioactive ^{127}I isotope to radioactive ^{125}I isotope. This reduced the specific activity of $^{125/127}$I-SD Ang II from 2175 Ci/mmole to 217.5 Ci/mmole. The concentration of ^{125}I-SD Ang II decreases with a half-life of 60 days according to the decay catastrophe hypothesis,[23] while the concentration of ^{127}I-SD Ang II should remain constant. To account for the changing specific activity of the $^{125/127}$I-SD Ang II, a spreadsheet was developed to calculate the daily change in specific activity based upon the changing ratios of ^{125}I-SD Ang II and ^{127}I-SD Ang II, for example, 60 days after preparation, the specific activity of the 1:9 ratio $^{125/127}$I-Ang 1–7 would be 109 Ci/mmol.

All other reagents were obtained from Fisher Scientific or Sigma Chemical Company and were reagent grade.

2.3 | Saturation binding assays

Frozen rat liver tissues were weighed and mechanically homogenized in a 20 mM hypotonic sodium phosphate buffer (pH 7.2). Homogenates were centrifuged to precipitate the cell membranes, at 48 000 *g* for 20 min at 4 °C. An assay medium buffer (AM5, pH 7.2), composed of 150 mM NaCl, 5 mM disodium EDTA, 0.1 mM bacitracin, and 50 mM dibasic sodium phosphate, was used to resuspend the cell membranes, which were rehomogenized then recentrifuged at same conditions as before. Membranes were resuspended a final time in the assay buffer (20 mg initial weight per ml). Fifty microliter of tissue was incubated for 1 h at approximately 22°C in a volume of 100 µl with ^{125}I-SI Ang II at six concentrations ranging from 0.2 to 4 nM. Nonspecific binding was determined in the presence of 10 µM losartan. A cell harvester (Model M24R, Brandel) was used to separate membrane-bound from unbound radioligand using glass fiber filters (Number 32, Schleicher and Schuell, Keene, NH; or GF/B, Whatman), soaked in a 1 g/L solution of BSA. The bound radioligand retained on the filter disks was assayed in a Cobra II Auto-Gamma Counter. B_{max} (expressed as fmol of radioligand bound per mg initial wet weight) and K_D (expressed as nM) values were determined with the computer program Prism 9.0 (GraphPad Software).

An additional series of radioligand binding assays compared ^{125}I-SD Ang II binding with that of ^{125}I-SI Ang II at concentrations ranging from 0.2 to 5 nM. Under these conditions, ^{125}I-SD Ang II binding was not saturable. To extend the concentration range for ^{125}I-SD Ang II, the ^{125}I was mixed with ^{127}I at a ratio of 1:9, as noted above. The comparative saturation binding assays were rerun with $^{125/127}$I-SD Ang II ranging from 1–30 nM. At this range of concentrations, saturable binding of $^{125/127}$I-SD Ang II could be observed.

2.4 | Competition binding assays

The affinities (IC_{50}) of SD Ang II and SI Ang II for the AT_1R at six concentrations, ranging from 1 nM to 100 µM, were determined using radioligand receptor competition binding assays with ^{125}I-SI Ang II (~0.5 nM) in frozen liver membranes, prepared as described for saturation binding assays. Losartan (100 µM) was used to define specific ^{125}I-SI Ang II binding to the liver AT_1R. Calculation of IC_{50} values was done using Prism 9.0 one-site competition binding algorithm, after which K_i values were derived using the Cheng–Prusoff equation $K_i = IC_{50}/(1 + H/K_D)$ where H is the concentration of ^{125}I-SI Ang II and K_D is the K_D of ^{125}I-SI Ang II, determined by a concomitantly run saturation binding assay.[24]

2.5 | Additional saturation binding assays

To derive K_i values from the IC_{50} values obtained from the competition binding assays, saturation binding assays were run concomitantly. It became apparent that there was a significant difference in B_{max} values between male and female rat livers. To validate this observation, an additional 4 rat livers (2 females and 2 males) were compared for ^{125}I-SI Ang II binding. These data were combined with the saturation binding data run concurrently with the competition binding assays to compare ^{125}I-SI Ang II binding in eight male livers and five female livers.

2.6 | Statistical analyses

In addition to the non-linear regression analyses of B_{max}, K_D, and IC_{50}, groups were also compared by paired and unpaired *t*-tests or by two-way ANOVA. Values are expressed as mean ± SEM. $p \leq .05$ was considered to be statistically significant.

2.7 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[25] and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22.[26]

3 | RESULTS

To assess the potential use of SD Ang II as a radioligand for quantification of Ang II receptor binding, we radiolabeled SD Ang II with ^{125}I and compared its binding to that of ^{125}I-SI Ang II in rat liver membranes, which express high levels of AT_1Rs and negligible levels of angiotensin II type 2 receptors (AT_2Rs).[27] As shown in Figure 3, saturable binding of radioligand ^{125}I-SI Ang II was observed in both male and female rat livers. At concentrations ranging from 0.7 to 3 nM, saturable binding of ^{125}I-SI Ang II presented a K_D of 0.691 nM and a B_{max} of 14.0 fmol in the male rat liver. In the same conditions, a K_D of 0.931 nM and a B_{max} of 31.2 fmol were observed in the female rat liver. A summary of all K_D and B_{max} values obtained can be found

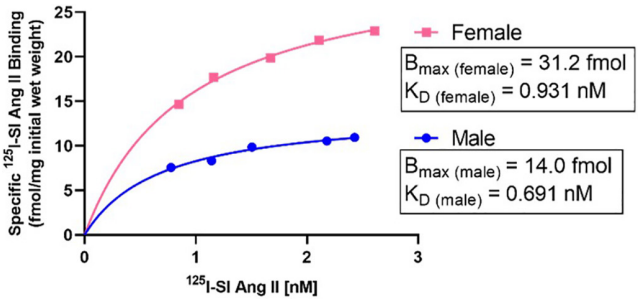

FIGURE 3 ^{125}I-SI Ang II saturation binding assay for female and male rat liver AT_1R. Representative binding in the female and male rat liver indicates saturable binding of ^{125}I-SI Ang II, as analyzed by GraphPad Prism 9.0 using a one site specific binding linear regression. The pink and blue curves are representatives of one of five female and one of eight male rat liver membranes used, respectively.

TABLE 1 Summary of K_D and B_{max} values (±SEM) for the various liver tissues

ID	$K_D \pm$ SEM (nM)	$B_{max} \pm$ SEM (fmol/mg initial wet weight)
Male 1	1.01 ±0.21	17.2 ±1.41
Male 2	0.69 ±0.12	14.0 ±0.73
Male 3	0.54 ±0.19	13.2 ±1.30
Male 4	1.38 ±0.33	14.8 ±1.44
Male 5	0.78 ±0.16	15.6 ±1.12
Male 6	0.62 ±0.17	12.2 ±0.97
Male 7	0.58 ±0.25	9.64 ±7.89
Male 8	0.69 ±0.21	9.13 ±6.89
Female A (estrus)	0.93 ±0.08	31.2 ±0.92
Female B (estrus)	0.42 ±0.13	25.3 ±1.76
Female C (diestrus)	0.60 ±0.11	26.1 ±1.27
Female D (diestrus)	0.73 ±0.50	25.4 ±22.7
Female E (proestrus)	0.69 ±0.35	20.9 ±17.3

Note: Values derived from saturation binding assays performed with ^{125}I-SI Ang II and male ($n = 8$) and female ($n = 5$) rat liver membranes.

in Table 1. Assessment of ^{125}I-SD Ang II at equivalent concentrations failed to show saturable binding (data not shown). Subsequent analysis of ^{125}I-SD Ang II was done using the lower specific activity ($^{125/127}$I-SD Ang II) at concentrations up to 30 nM. With the higher concentration of $^{125/127}$I-SD Ang II, saturable binding was observed in female rat liver membranes ($n = 3$). Figure 4 shows a representative saturation binding comparison for ^{125}I-SI Ang II and $^{125/127}$I-SD Ang II, respectively. The average B_{max} was 28.7 fmol for ^{125}I-SI Ang II and 4.50 fmol for $^{125/127}$I-SD Ang II. The average K_D was 0.483 nM for ^{125}I-SI Ang II and 5.17 nM for $^{125/127}$I-SD Ang II. Paired t-tests revealed a significantly greater B_{max} for ^{125}I-SI Ang II ($p = .015$), and a significantly greater K_D for ^{125}I-SD Ang II ($p = .036$).

To verify that SD Ang II binds to liver membrane AT_1Rs, we compared the ability of SD Ang II with that of SI Ang II to compete for

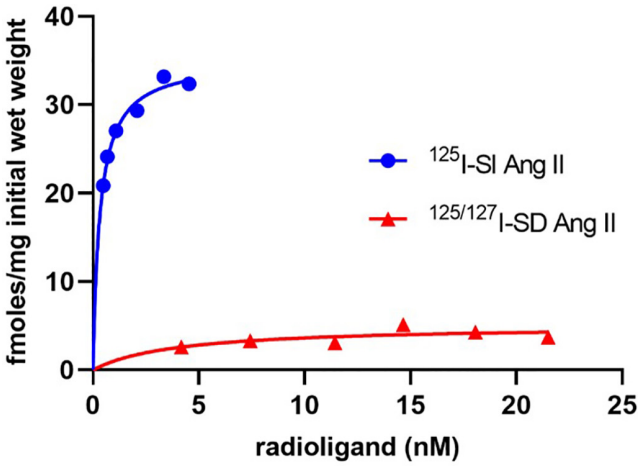

FIGURE 4 ^{125}I-SI Ang II vs. $^{125/127}$I-SD Ang II saturation binding assays in female rat liver membranes. Representative figure of three saturation analyses using female rat liver membrane. B_{max} and K_D values were 35.1 fmol and 0.329 nM, respectively, for ^{125}I-SI Ang II, and 5.09 fmol and 4.00 nM, respectively, for ^{125}I-SD Ang II. Values were obtained by GraphPad Prism 9.0 using a one site specific binding linear regression.

^{125}I-SI Ang II binding. Both peptides inhibited ^{125}I-SI Ang II binding (0.5 nM) to a similar extent. SD Ang II was less potent than SI Ang II when competing for ^{125} I-SI Ang II binding to the liver AT_1Rs in male and female rats (Figure 5).

A two-way ANOVA (SD Ang II vs. SI Ang II and male vs. female) compared the differences observed between competing ligands SD Ang II and SI Ang II. SD Ang II was 15.5 times less potent than SI Ang II ($p < .0001$; Figure 6). There was smaller, males 1.66 times less potent than females, significant difference ($p = .026$). There was also a significant interaction of ligand and sex ($p = .037$, Figure 6).

As shown in Figure 7, unpaired t-tests comparing B_{max} and K_D values in male and female livers demonstrated a significantly greater concentration of AT_1Rs in female livers ($p < .0001$). There was no significant difference in K_D values between sexes ($p = .442$).

4 | DISCUSSION

The AT_1R binding affinity of the novel biased agonist TRV027 (SD Ang II) was substantially less than the more commonly used putative AT_1R biased agonist SI Ang II. When radioiodinated, for use in a radioligand receptor binding assay, ^{125}I-SD Ang II bound to fewer AT_1Rs than ^{125}I-SI Ang II, again with a substantially lower binding affinity (Figures 5 and 6). While SD Ang II was developed to bind to AT_1Rs with higher affinity than the first AT_1R biased agonist; Sarcosine[1], Isoleucine[4], Isoleucine[8] Ang II (SII Ang II), it still has considerably lower affinity for AT_1Rs compared to SI Ang II. This lower affinity might explain TRV027's failure to show therapeutic efficacy in Trevena's 2016 phase IIb clinical trial.[19] The lower B_{max} value for ^{125}I-SD Ang II compared to ^{125}I-SI Ang II may indicate that the AT_1R exists in multiple conformations in the absence of ligand binding that

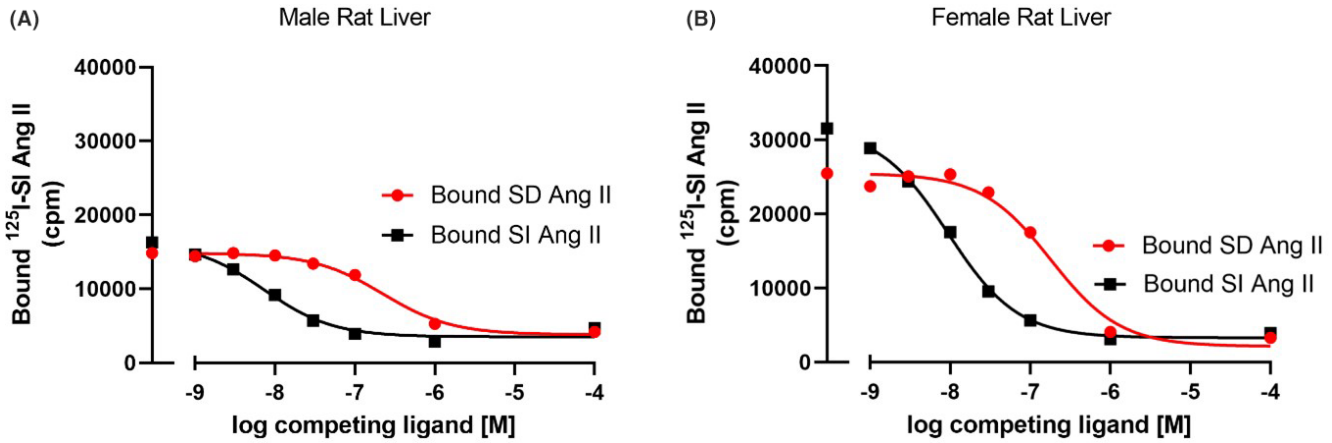

FIGURE 5 Comparison of SD Ang II and SI Ang II competition binding to male and female rat liver AT_1R. Representative competition binding for ^{125}I-SI Ang II by SD Ang II and SI Ang II in the male rat liver ($n = 6$; A) show IC_{50} values of 225 and 7.27 nM, respectively. As for competition binding in the female liver rat ($n = 3$; B), these values are 181 and 9.40 nM, respectively. Analyses were made by GraphPad Prism 9.0 using a one site competition binding algorithm. The values indicated in the y-axis are the amount bound in the absence of any competing ligand.

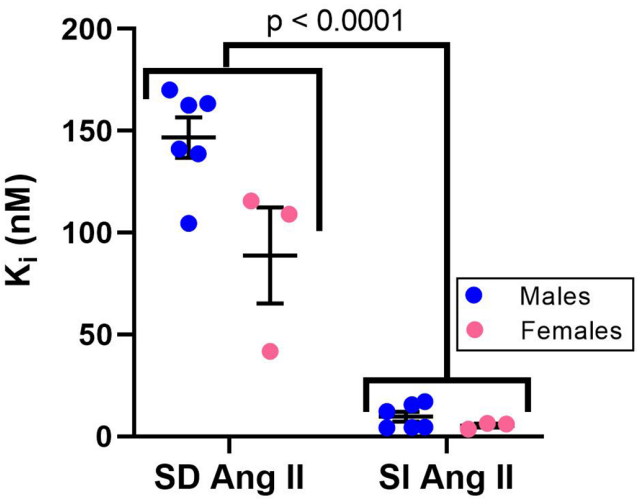

FIGURE 6 Comparison of SD Ang II and SI Ang II K_i values for binding to male and female rat liver membrane AT_1Rs. Comparison by two-way ANOVA showed that there is a significant difference in K_i values between the two competing ligands, $F(1, 7) = 111.6$, $p < .0001$. There was a less significant difference between males ($n = 6$) and females ($n = 3$) ($p = .0258$).

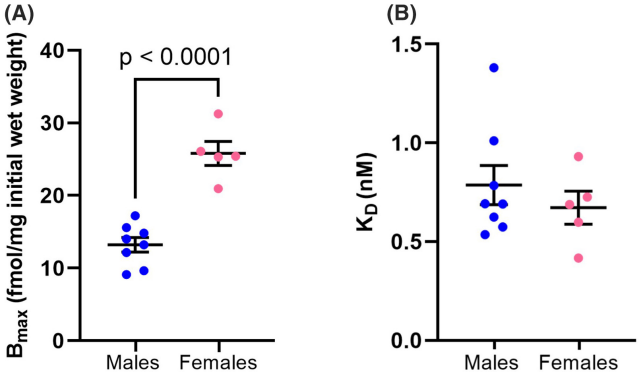

FIGURE 7 Comparison of ^{125}I-SI Ang II binding to AT_1Rs in male and female rat liver membranes. Comparisons done using unpaired *t*-tests showed that (A) There is a significantly greater ($t = 7.01$, $p < .0001$) AT_1R density in females ($n = 5$) compared to males ($n = 8$), and (B) There is no difference in K_D values ($t = 0.800$, $p = .442$).

vary in their ability to bind ^{125}I-SD Ang II, but do not vary in their ability to bind ^{125}I-SI Ang II. Multiple conformations have been shown for $beta_1$ and $beta_2$ adrenergic receptors,[28,29] dopamine D_2 and D_3 receptors[30] and other G-protein-coupled receptors.[29] Another possible explanation for the disparity in Bmax values for SI Ang II and SD Ang II could be differential binding to AT_{1A} and AT_{1B} receptor subtypes. mRNA for both AT_{1A} and AT_{1B}R's are found in approximately equal amounts in female rat livers[31] although this does not necessarily represent AT_1R protein expression. Moreover, the extracellular and transmembrane spanning domain amino acids of these two subtypes have a high similarity with no reports of significantly differential binding of any ligand to these two subtypes. Based upon binding affinity alone, it would appear that SI Ang II would be a superior AT_1R biased agonist than SD Ang II.

Peptide drugs, such as SI Ang II and SD Ang II, typically target extracellular GPCRs, for which it is challenging to synthesize small molecule agonist drugs.[32,33] Current small molecule antihypertensive drugs (losartan and other ARBs) block the AT_1R. However, in the 1970s, the Ang II analogue peptide saralasin ([Sarcosine^1, Alanine^8]-Ang II; Sarenin®) was synthesized to function as an Ang II antagonist. It was a potent competitive antagonist of Ang II in the vasculature, kidneys, adrenals, heart, and central nervous system.[34] The peptide, which had a short half-life, was administered intravenously to identify, and potentially treat, renin-dependent hypertension.[34,35] However, it is no longer marketed. Other peptides, such as SI Ang II and SD Ang II, have additional modifications that impact the molecule's binding affinity and agonistic properties.[36-38]

An evolving issue of having SD Ang II as a drug is that it is a peptide. Because peptides are large molecules with poor oral availability due to poor oral absorption and high rates of first-pass metabolism,[32,39] they have traditionally been administered by injection (intravenously, intramuscularly, subcutaneously) or by other routes (intranasal, buccal, inhalation, transdermal),[39] which decreases patient compliance. Additionally, they have poor membrane permeability due to low lipophilicity and high hydrogen bonding capacity, limiting them primarily to extracellular targets; short half-life (accounting for rapid clearance through enzymatic proteolysis, renal clearance, endocytosis, and/or proteasome degradation), limited stability, and are more expensive and difficult to synthesize.[32,33,39] Consequently, small molecules are usually preferred over peptide drugs, as they are typically cheaper and easier to synthesize, able to permeate through cell membranes, more stable, and orally available.[39] However, over the years peptides have received more attention as potential therapeutics in areas such as cardiovascular diseases, oncology, and metabolic and endocrine disorders.[32,33] The reasoning is that what was previously seen as weaknesses, can actually be beneficial. Peptides have a fast onset of action when administered by injection, causing less side effects due to rapid clearance.[33] Because they mimic naturally occurring signaling molecules in the body, when used as drugs they can mimic natural pathways and trigger/inhibit certain physiological effects.[32] Additionally, they are highly specific and selective, can offer advantages of both small molecules and proteins, have high binding affinity, well-defined mechanisms of action, low toxicity and immunogenicity, and low risk of drug interactions.[39] With the development of novel formulation technologies, such as lipid-based nanocarriers, peptide drugs can form hydrophobic ion pairs with hydrophobic counter ions and have their lipophilic character raised and, therefore, be protected from gastrointestinal peptidases and overcome delivery barriers, for example, enzymatic, sulfhydryl, mucus, and epithelial barriers.[40]

The biased agonistic activity of SD Ang II is associated with modifications in the peptide's amino acid sequence, especially at the eighth position. Studies have shown that residues 1–7 are responsible for the receptor-ligand affinity, specificity, and initiation of signal transduction, while residue 8 determines agonistic responses by influencing receptor conformation.[15,36] Phenylalanine (Phe) 8 is essential for classical agonistic activity of Ang II, as analogues lacking this amino acid failed to trigger classical agonistic responses.[15,41] Substitution of Phe^8 by isoleucine (Ile), moderately reduces G-protein-mediated agonistic activation of inositol trisphosphate (IP_3) signaling, phosphorylation, and mitogen-activated protein kinases (MAPK), whereas a substitution by alanine (Ala) dramatically reduces MAPK activation.[42] Zimmerman et al.[43] showed that non-aromatic amino acid substitutions for Phe^8, such as Ile, which lacked the ability to activate G protein signaling, could instead activate β-arrestin-dependent signaling.[43]

Another substitution seen in SD Ang II is that of aspartic acid (Asp) at position 1 by Sar, which does not interfere with the peptide's agonistic properties, reduces inactivation of the peptide by aminopeptidases, extending its duration of action. However, the biological half-life and the resistance to enzymatic degradation are still short and weak, limiting the therapeutic use of such molecules.[36] Data suggest that the AT_1R recognizes differing epitopes leading to activation of the G protein pathway or the β-arrestin pathway. Which pathway gets activated by a ligand depends on the substitutions present in the molecule, which would induce different conformational changes in the receptor.[15,44,45]

Such modifications might have led Trevena, Inc. to believe that TRV027 (SD Ang II) would be an effective drug. It could be hypothesized that the D-isomer amino acid (D-Ala^8) could cause the molecule to be more resistant to degradation, leading to a longer half-life. D-amino acid-containing peptides are more resistant to proteolytic degradation,[46,47] as they are poor substrates for endogenous proteases,[47] for example, carboxypeptidase Y.[48] When the C-terminus of short peptides is modified with a D-amino acid, the peptide becomes more stable,[47] which could explain the search for an Ang II analogue, different from SI Ang II. Receptors and structural proteins do not typically carry D-amino acids in their natural state, but they have been seen in other drugs such as peptide antibiotics, hormones, neuropeptides, hepatotoxins, and opioids,[49] leading to the presumption that TRV027 could have sustained efficacy.

Given SI Ang II's greater potency, it might possibly be a better biased agonist than SD Ang II. However, the L-Ile substitution in position 8 may be more susceptible to carboxypeptidase metabolism, reducing the half-life of this peptide relative to SD Ang II. An alternative strategy would be to use a non-peptide bond to link the amino acids in positions 7 and 8 of the peptide.

An unanticipated finding was the substantially greater AT_1R expression in the female rat liver. Female liver membranes had approximately twice as high AT_1R density than that of males (Figure 7). This suggests that estrogen and other ovarian steroids, such as progesterone, could possibly promote AT_1R expression. Alternatively, the differences could be associated with sex chromosomal dosage.[50] However, previously published observations of AT_1R binding in the anterior pituitary of ovariectomized (OVX) female rats, indicated that estrogen strongly inhibits AT_1R expression.[51,52] Additionally, the number of AT_1R fluctuated with the stage of the estrous cycle: highest in the diestrus phase, when estrogen levels were low, and lowest in the proestrus phase when estrogen levels were high.[52] In 1998, Nickenig et al.[53] suggested that vascular AT_1R is overexpressed in estrogen-deficient OVX rats and, therefore, estrogen may be a negative modulator of the receptor expression in vascular smooth muscle cells (VSMCs). In a subsequent study, Nickenig et al.[54] reported that 17β-estradiol decreased, while progesterone increased, AT_1R mRNA and protein expression in VSMCs. A study by Krishnamurthi et al.[55] reported, that 17β-estradiol replacement also significantly decreased AT_1R expression in the pituitary and the adrenal of OVX Sprague–Dawley rats, but significantly increased it in the uterus. Subsequent studies suggested that the downregulation of AT_1R in the adrenal is secondary to a reduction in Ang II in the adrenal[56]

and in AT_1R mRNA translation into AT_1R protein.[56,57] Combined, these results suggest that the effects of these hormones, especially estrogen, on the AT_1R are tissue dependent, but further investigation is needed. However, in all cases, their liver membrane AT_1R expression substantially exceeded that of the male's.

5 | CONCLUSION

The development of SD Ang II was based upon a strategy of using a D-substituted amino acid in the omega position of a β-arrestin biased agonist peptide to block its metabolism by carboxypeptidase along with protection from aminopeptidases by the N-methyl substituted terminal amino acid sarcosine substitution. While the Sar^1 substitution does not adversely affect its binding to the AT_1R, this study suggests that the use of D-Ala adversely affects the ability of this Ang II analogue to bind to the AT_1R. Thus, the potential gain in peptide stability is more than offset by its reduced binding affinity for the AT_1R.

Since a specific pharmacophore is needed to bind to the AT_1R to effect a conformational change, allowing it to become phosphorylated by a GRK and leading to β-arrestin binding and signaling, without activating G protein signaling, peptides with an aliphatic amino acid in the omega position fit that criterion for the pharmacophore. However, it would be necessary to substitute non-peptide bonds between the L-amino acids that constitute this pharmacophore to both improve efficacy and increase bioavailability and duration of action.

AUTHOR CONTRIBUTIONS

Participated in research design: Noto, Restrepo, Pang, Stoyell-Conti, West, and Speth. Conducted experiments: Noto, Restrepo, Pang, and Stoyell-Conti. Contributed new reagents or analytical tools: West and Speth. Performed data analysis: Noto, Restrepo, Pang, Stoyell-Conti, and Speth. Wrote or contributed to the writing of the manuscript: Noto, Restrepo, Pang, Stoyell-Conti, West, and Speth.

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DATA AVAILABILITY STATEMENT

Raw data were generated at Nova Southeastern University. Derived data supporting the findings of this study are available from the corresponding author (R.C.S.) upon request.

DISCLOSURE

The authors report no conflicts of interest.

ETHICAL APPROVAL

The studies were carried in compliance with institutional guidelines and the Guide for the Care and Use of Laboratory Animals, National Research Council (NRC) Publication, 2011 edition. All animal protocols were approved by the Georgetown University's (protocol no. 2019-0035) and Nova Southeastern University's (protocol no. 2020.06.MH2) Institutional Animal Care and Use Committees.

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