The renin–angiotensin system biomolecular cascade: a 2022 update of newer insights and concepts

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A large body of evidence implicates the renin–angiotensin system in the pathogenesis of cardiovascular disease. However, not everyone understands that the magnitude of the risk reduction achieved in clinical trials with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers is only a fraction of the residual risk for cardiovascular events and death. This paper addresses limitations of current therapeutic approaches based on renin–angiotensin system blockade for hypertension and cardiovascular disease by illustrating the complex biochemical physiology and mechanism of classical and alternate angiotensin peptide formation. Emerging evidence of alternate mechanisms that bypass both renin and angiotensin-converting enzyme to produce the angiotensins in tissues and cells is not currently universally recognized. Currently available treatment would benefit from further insights to help fully meet the aims of patient care, and the challenge is to delve more deeply into the renin–angiotensin system cascade, with the aim of enhancing therapeutics for renin–angiotensin system inhibition. This article provides a reappraisal of the renin–angiotensin–aldosterone cascade, highlighting newly elucidated intermediary components and interplay, and their consequent implications and relevance for understanding the long-term contribution of angiotensin II in cardiovascular diseases and their therapy.

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> The aim of science is not to open a door to endless wisdom, but to put a limit to endless error.
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> — Bertolt Brecht, *Life of Galileo*[1]

This review provides a reappraisal of the renin–angiotensin system (RAS) cascade, highlighting newly elucidated intermediary components and their consequent implications and relevance for understanding cardiovascular (CV) disease progression. This topic continues to attract greater attention[2] because of the current overriding importance of angiotensin-converting enzyme (ACE) 2 (ACE2) as the entry vector for coronavirus disease 2019 (COVID-19) infection (see also the article by Hollenberg and Epstein in this supplement[3]). Despite the extensive literature examining the homeostatic regulation performed by the angiotensins, this topic remains fresh and vibrant. Alongside established clinical evidence for the benefit of preventing the production of angiotensin II (Ang II) or blocking access of the hormone to its receptors, evidence is also emerging for alternate mechanisms that bypass both renin and ACE to produce angiotensins. This latter evidence is still not universally accepted,[4] which highlights the fact that more remains to be learned about RAS. In addressing this issue, we acknowledge the benefits that can be achieved with a multifactorial therapeutic approach that includes non-RAS medicines, as exemplified by the enhanced reduction in CV risk reported in the Steno-2 trial[5,6] by combining RAS inhibitors with tight glucose control, aspirin, and and lipid-lowering agents. The limitations of restricting therapy with pharmacologic RAS inhibition in non-albuminuric diabetic kidney disease (DKD) is underscored in a recently published review.[6]

Although the proven therapeutic benefits attained with chemical inhibitors targeting renin, ACE, and Ang II type 1 receptors (ARBs) are generally accepted, clinicians' awareness of the existence of a ***residual risk*** of CV events[7–10] remains low. Given the impressive genetic, molecular, physiological, and clinical evidence for a role of Ang II in the pathogenesis of CV disease,[11,12] the long-term effects of RAS blockade, using direct renin inhibitors, ACE inhibitors, and ARBs, fall short of expectations. In reexamining the magnitude of the risk reduction attained in clinical trials with ACE inhibitors and ARBs, the residual risk of CV events is shown to be several orders of magnitude greater than the relative risk reduction of myocardial infarction, heart failure, or CV

The human angiotensin peptides family

Residue number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
One-letter abbreviation	D	R	V	Y	I	H	P	F	H	L	V	I	H	N	E	S	T	C	E	Q	L	A	K	A	N
Ang-(1-25)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Val	Ile	His	Asn	Glu	Ser	Thr	Cys	Glu	Gln	Leu	Ala	Lys	Ala	Asn
Ang-(1-14)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Val	Ile	His	Asn											
Ang-(1-12)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu	Val	Ile													
Ang-(1-10)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu															
Ang-(1-9)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His																
Ang-(1--8)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe																	
Ang A	Ala	Arg	Val	Tyr	Ile	His	Pro	Phe																	
Ang-(1-7)	Asp	Arg	Val	Tyr	Ile	His	Pro																		
Alamandine	Ala	Arg	Val	Tyr	Ile	His	Pro																		
Ang-(1-5)	Asp	Arg	Val	Tyr	Ile																				
Ang-(1-4)	Asp	Arg	Val	Tyr																					
Ang-(2-10)	Asp	Arg	Val	Tyr	Ile	His	Pro	Phe	His	Leu															
Ang-(2-8)		Arg	Val	Tyr	Ile	His	Pro	Phe																	
Ang-(2-7)		Arg	Val	Tyr	Ile	His	Pro																		
Ang-(3-8)			Val	Tyr	Ile	His	Pro	Phe																	
Ang-(3-7)			Val	Tyr	Ile	His	Pro																		
Ang-(5-7)					Ile	His	Pro																		
Ang-(3-4)			Val	Tyr																					

Figure 1 | Amino acid sequences of the family of angiotensin peptides identified as possessing biological activity. Three-letter abbreviations are given for amino acids. Alamandine is Des[Asp^1]-[Ala^1]-Ang-(1-7). Ala, alanine; Ang, angiotensin; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine. Created with BioRender.com.

death.[13–19] These data are disconcerting, given the strength of the research implicating the RAS in the pathogenesis of hypertension. The non-superiority of RAS inhibitors over other antihypertensive agents remains a source of confusion, as reflected in the recommendations provided by major professional organizations endorsing the benefits of blood pressure lowering *per se* over the range of available antihypertensive agents.[20–22] Yet, a century of rigorous experimental research reveals the diverse nature of the biochemical processes that, within the RAS, lead to the formation of the angiotensins, and the fundamental importance of Ang II as a critical participant in hypertension pathogenicity, adverse cardiac and vascular remodeling, atherogenesis, diabetes, and acute and chronic kidney disease. In the authors' view, the explanation of this dilemma lies in the potential inability of RAS drugs to reach the intracellular sites where Ang II influences CV function,[23–25] owing to poor intracellular penetrability of these inhibitors, activation of alternate Ang II–forming serine endopeptidases with an affinity similar to or even higher than that for ACE,[26,27] or both of these factors combined. We address these issues by providing a detailed new look at the biochemical physiology of the classical and alternate angiotensin peptide–forming mechanisms.

RAS CURRENT BIOCHEMICAL PATHWAYS

The cascade of peptides derived from the angiotensinogen (AGT) protein is illustrated in Figure 1. As stressed by Ryan,[28] linear polypeptides without repeating amino acids have multiple homologs. Ang II has 35 possible lower homologs, and 54 possible homologs can be derived from angiotensin I (Ang I). Hence, viewing the RAS as a linear system in which renin and ACE are the 2 enzymatic steps in the biotransformation of AGT into Ang II is without fundamentals. Over the years, we have been engaged in revising the tenet that the RAS is constituted of a linear biochemical processing path in which the terminal product Ang II is the final biologically active compound.[29–31]

The old and the not so new

The biotransformation mechanisms participating in the generation and metabolism of biologically active angiotensins are illustrated in Figure 2.[32,33] Although the reaction of kidney renin with the plasma AGT remains the crucial step in the initiation of the biochemical cascade, proteolytic enzymes not belonging to the aspartyl protease renin and elaborated by digestive glands were first reported to hydrolyze AGT by Croxatto and Croxatto.[34] From those earlier attempts to identify the nature of non-renin enzymes, only cathepsin G,[35] kallikrein,[36] and tonin[37] remain as enzymes known to belong to the RAS cascade.

Conversion of Ang I. Following the catalytic generation of Ang I by renin, the decapeptide is processed by the somatic active carboxy terminal site of ACE (EC 3.4.15.1) to yield Ang II, and by ACE2 (E.C. 3.4.17.23) to generate angiotensin-(1-9) [Ang-(1-9)].[38] As a dipeptidyl carboxypeptidase, the 2 penultimate amino acids His^9-Leu^{10} of Ang I are cleaved from the C-terminus of Ang I to generate Ang II, and the endopeptidase neprilysin (E.C. 3.4.24.11) cleaves the tripeptide Phe^8-His^9-Leu^{10} of Ang I to yield angiotensin-(1-7) [Ang-(1-7)].[39] Of current significant importance, owing to its participation as the entry vehicle for infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),[40–42] the mono-carboxypeptidase ACE2 cleaves with high affinity the Pro^7-Phe^8 bond of Ang II to generate Ang-(1-7).[43] The first demonstrations of Ang-(1-7)

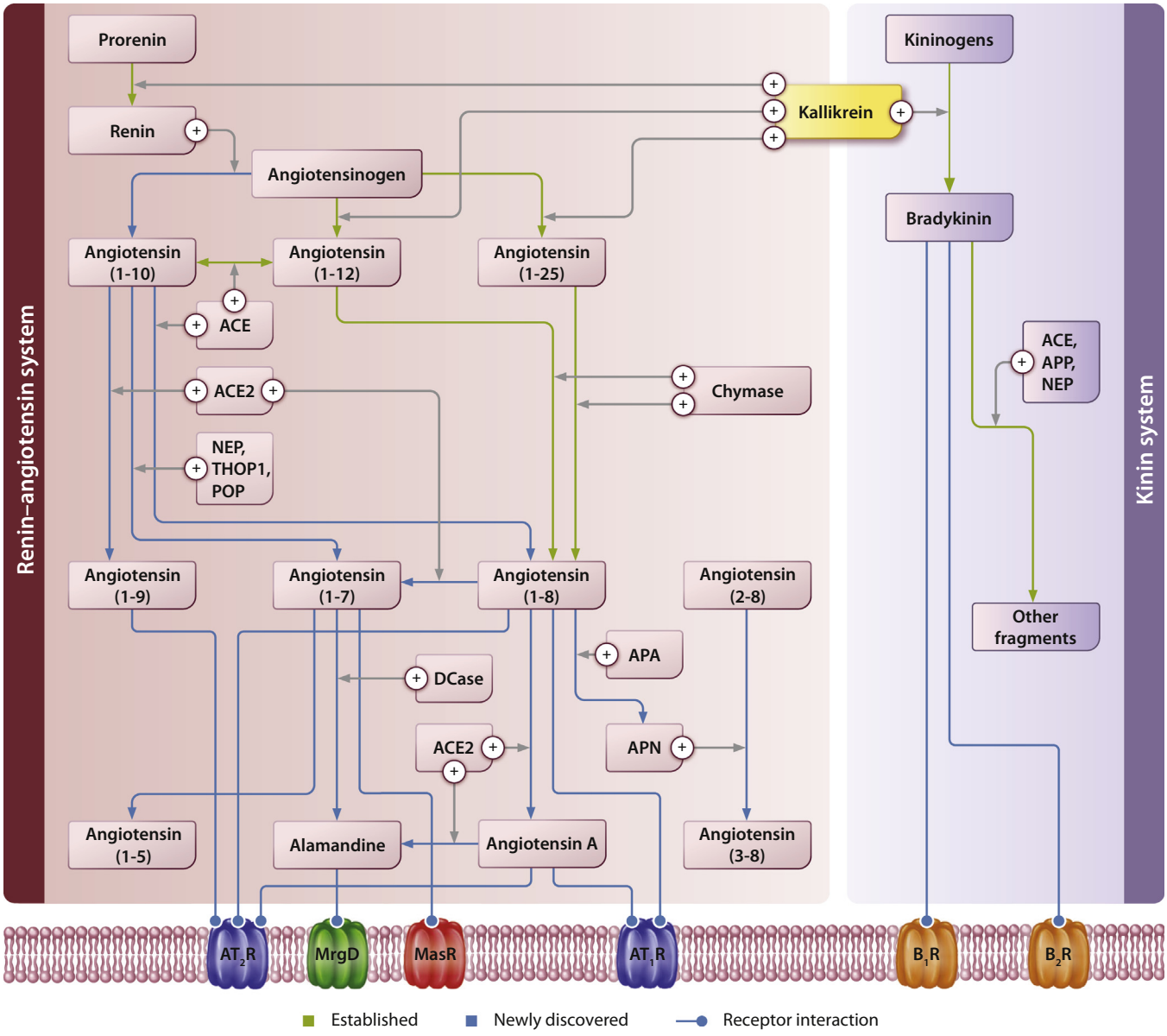

Figure 2 | Schematic representation of angiotensinogen cleaved derivatives by renin and alternate processing by kallikrein or a kallikrein-like enzyme.[32,33] The renin–angiotensin system, along with multiple overlapping systems (the kinin system, the endothelin system, and the natriuretic peptides system) plays a role in the regulation of blood pressure and cardiovascular function through the enzyme-catalyzed formation and degradation of vasoactive peptides and hormones. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ANP, atrial natriuretic peptide; APA, aminopeptidase A; APN, aminopeptidase N; APP, aminopeptidase P; BNP, B-type natriuretic peptide; B1R, Bradykinin receptor B1; B2R, Bradykinin receptor B2; CNP, C-type natriuretic peptide; ETAR, endothelin A receptor; ETBR, endothelin B receptor; NEP, neprilysin; NPR-A, natriuretic peptide receptor-A; NPR-B, natriuretic peptide receptor-B; NPR-C, natriuretic peptide receptor-C; POP, prolyl oligopeptidase; PRCP, prolyl carboxypeptidase; THOP1, thimet oligopeptidase.

vasodilator effects,[44] the presence of low Ang-(1-7) urinary concentrations in primary untreated hypertensive patients,[45] and the report that captopril treatment augmented plasma Ang-(1-7) concentrations in parallel with an antihypertensive effect in hypertensive adults[46] led Ferrario et al.[47] to conclude that Ang-(1-7) may be acting as an endogenous inhibitor of Ang II pathologic actions and that its deficit may facilitate or directly account for the development of primary hypertension. Other investigators now subscribe to this hypothesis.[47]

Release of angiotensin III and IV. Further metabolism of the C-terminus residues of Ang II by aminopeptidases releases the biologically active peptides angiotensin III [Ang-(2-8)] and angiotensin IV [Ang-(3-8)], and ACE further catalyzes Ang-(1-7) into the less-understood functional role of the pentapeptide Asp^1-Arg^2-Val^3-Tyr^4-Ile^5- [Ang-(1-5)]. A dipeptide angiotensin-(3-4) [Ang-(3-4)] is reported to possess strong antihypertensive activity in humans and rats, in part by acting as an allosteric enhancer of renal Ang II type 2 receptor.[48,49] The inclusion of this di-peptide (Val^3-Ty^4) as a genuine member of

the angiotensin family underscores the economy of function that is an inherent property of neuropeptidergic systems.[50] The processing of Ang I and Ang II into the smaller terminally critical active hormones seems to be dictated by tissue-specific compartmentalization of the enzymes,[51] rate of permeability, and diffusion of AGT and Ang I into the organs' interstitial spaces,[52] and the cellular uptake of peptides based on receptor internalization and processing.[23,24,53]

The role of Ang-(1-7). In exploring the role of Ang-(1-7) as an Ang II counterregulatory peptide, Lautner and colleagues[54] isolated a decarboxylated product in which alanine substituted for aspartic acid in position 1 of the Ang-(1-7) sequence. Their study extended the previous discovery of angiotensin A [Des[Asp^1]-[Ala^1]-Ang II] (Figure 1) in human plasma.[55] The decarboxylation process appears to engage a mononuclear leukocyte-derived aspartate decarboxylase.[55] The importance of this parallel processing of Ang II into Des[Asp^1]-[Ala^1]-Ang II and Des[Asp^1]-[Ala^1]-Ang-(1-7) (alamandine) remains to be established, although the decarboxylation of the parent peptides seems to modify the biological actions through decreased dependency of Des [Asp^1]-[Ala^1]-Ang II on Ang II type 1 receptor (AT_1-R) signaling[56] and coupling of alamandine to Mas-related G protein–coupled receptors (MrgD), a subset of relatively promiscuous receptors signaling pruriceptive sensations.[57]

Returning to Ang I, 2 enzymes, other than neprilysin, generate Ang-(1-7). The tissue endopeptidases thimet oligopeptidase (E.C. 3.4.24.15) and prolyl oligopeptidase (E.C. 3.4.21.26)[58–60] generate Ang-(1-7) from Ang I. Thimet oligopeptidase, a thiol-sensitive metalol endopeptidase, engaged in neuropeptide metabolism and inflammation,[61] metabolizes Ang I into Ang-(1-7), and prolyl oligopeptidase can form Ang-(1-7) directly from either Ang I or Ang II.[59,60]

NEW BIOCHEMICAL PATHWAYS FOR THE GENERATION OF ANGIOTENSINS

In the preface of a mostly forgotten 1973 book addressing the heterogeneity of renin and renin substrate, Professor Mohinder P. Sambhi stated the following: "The discovery of multiple forms of renin and renin substrate has generated new directions in research on the biochemistry of the renin-angiotensin system and its functional significance."[62] Almost 50 years later, Sambhi's comments remain true. Renewed interest in the heterogeneity of AGT substrate in mediating acute or chronic changes in RAS activity remains unappreciated, even though the detection of low and high molecular forms of the substrate in the plasma of pregnant women were construed as evidence of the existence of nonhepatic sources for AGT synthesis.[63,64] The potential functionality of heterogenic forms of the AGT protein, first reported by Gordon and Sachin,[65] remained dormant for decades until the identification of proangiotensin-12.[66]

Understanding the role of angiotensin-(1–12)

Proangiotensin-12 was renamed, by us, to the established international nomenclature as angiotensin-(1–12) (Ang-[1–12]; Figure 2). The original studies by Nagata *et al.*[66] were expanded on by pilot observations showing that kallikrein or a kallikrein-like enzyme cleaved Ang-(1-12) from AGT.[42] Renin, on the other hand, shows no catalytic action in the metabolism of AGT into Ang-(1-12) or its degradation into the cleavage of downstream angiotensins.[67] Studies of the fate of Ang-(1-12) revealed that ACE is the primary Ang-(1-12) catalytic enzyme in the circulation of rats,[68] and chymase hydrolyzes Ang-(1-12) in both human and rodent hearts.[69–71] Ang-(1-12)'s functional contribution to blood pressure regulation was shown by blood pressure normalization during cerebroventricular administration of an Ang-(1-12) polyclonal antibody in transgenic rats expressing the *Ren-2* gene,[72] and in studies assessing the effects of local delivery of the substrate in the brainstem region associated with the control of baroreflexes.[73,74] Accumulating evidence indicates that Ang-(1-12) may source Ang II contribution to adverse cardiac and vascular remodeling in hypertension, as increased immunoreactive Ang-(1-12) content was found in the heart of spontaneously hypertensive rats[75] and the blood and heart of transgenic rats expressing the human *AGT* gene.[76,77] Ang-(1-12) participation as a source of Ang II modulation of cardiac function was revealed in studies in which the protein increased the contractile function of WKY cardiomyocytes via augmented intracellular $K^{(+)}$ currents[78] and mobilization of L-type $Ca^{(2+)}$ channels.[79] Consistent with their participation as major sources for tissue generation of angiotensins, high chymase expression and Ang-(1-12) in rat bone marrow $CD68^{(+)}$ myeloid lineage cells were recently demonstrated.[80]

Angiotensin-(1–12): translation of basic science to human studies

Basic research studies were consistent in revealing broad actions of Ang-(1-12) as an Ang II-forming substrate. The successful development of a sensitive radioimmunoassay by Ahmad *et al.*[81] for measurements of human Ang-(1-12) in blood and urine allowed the demonstration of increased plasma Ang-(1-12) in primary hypertensive patients who were naïve[82] or not naïve[2] to antihypertensive therapy. The demonstration of augmented circulating Ang-(1-12) in hypertensive patients suggests a participatory role of this substrate as a hypertension-biomarker with a discriminative value greater than that of circulating AGT and even Ang II.[82]

The range of shorter amino acid sequences of AGT with an apparent high affinity in generating Ang II by chymase (E.C. 3.4.21.39) and ACE was expanded with the additional demonstration of Big angiotensin 25,[83] a 25 amino acid N-terminal protein that is resistant to renin and generates Ang II through the catalytic activity of chymase (Figures 1 and 2). This alternate substrate present in multiple human organs appears to localize specifically to glomerular podocytes and was detected in the urine of patients with diabetes mellitus.[84]

Interwoven with the biochemical physiology of the RAS, the detection of these intermediate sequences of AGT capable of generating Ang II by non-renin and non-ACE-dependent pathways gives new impetus to unveil the mechanisms by

which these substrates may have a preeminent role in tissue production of Ang II, particularly their possible role as an intracellular source for functionally active angiotensins. This interpretation is consistent with a selective increase in chymase activity in cardiac myocytes from ovariectomized spontaneously hypertensive rats.[85] A critical role of chymase as an Ang II–forming pathway is strengthened by a critical role of cardiac chymase in the evolution of experimental volume overload and mitral regurgitation in humans.[86,87] Chymase inhibition augments plasma Ang II concentrations and worsens hypertension in spontaneously hypertensive rats[88] and ameliorates intrarenal renin–angiotensin activity in salt-dependent hypertension.[89]

BIOCHEMICAL PHYSIOLOGY OF THE RAS: RELEVANCE TO CLINICAL PRACTICE

Pioneer efforts to determine the impact of preventing the actions of Ang II in the control of high blood pressure led to the isolation and synthesis of peptide inhibitors of ACE,[90] half a century ago. The evolution of this proof-of-concept clinical finding to the modern pharmacotherapy of CV and renal diseases, and diabetes mellitus, is enthroned as the basic underpinning of contemporary therapeutic regimens and international medical guidelines. The use of ACE inhibitors and ARBs, and to a certain extent the direct renin inhibitor aliskiren fumarate,[91,92] is fundamental to any antihypertensive therapy.[93] Despite their proven benefits, their therapeutic usefulness has several limitations related to limited efficacy, the need of polypharmacy, medication adherence, and side effects. Dzau and Balatbat[94] have underscored the current apathy in identifying new targets for hypertension drug development using molecular and cell-based therapies. Older efforts to explore the use of nonchemical agents in CV disease treatment were abandoned prematurely,[95] owing to the successful results obtained with ACE inhibitors. The status of immunologic approaches for the treatment of high blood pressure is documented below.

Renin inhibition

Early recognition of renin immunogenicity prompted Haber's[96,97] conceptualization of inhibiting renin to prevent Ang II production. Three classes of compounds were visualized—specific antibodies, acid protease inhibitors, and substrate analogs.[98] Focus was placed on exploring several renin-specific antibodies, including the generation of Fab fragments that were documented to induce systemic depressor responses in canines exposed to sodium depletion or acute renal artery constriction.[99] Research included the description of the specificity of renin monoclonal antibodies (mAbs).[99,100] The finding that renin antibodies triggered autoimmune disease in animal models led to cessation of further research into this field.[101]

Gene silencing to inhibit the production of AGT. Research exploring therapeutic interventions upstream from Ang I using novel molecular biological approaches of gene silencing includes strategies directed at blocking the synthesis of hepatic AGT. Mullick *et al.*[102] tested an N-acetylgalactosamine-conjugated AGT antisense oligonucleotide targeting hepatic production of the AGT, and Dutch investigators[103,104] employed small interfering ribonucleic acids (siRNAs) targeting hepatic AGT to achieve similar goals. Dzau and colleagues[105] reported preliminary results of Crispr-Cas9–mediated disruption of AGT in a BRL 3A rat liver cell line and the effective reduction in the systolic blood pressure of prehypertensive and hypertensive spontaneously hypertensive rats, at the American Heart Association (AHA) 2020 meeting. Preclinical data obtained with hepatic AGT suppression generated 2 clinical trials (Table 1[102,106–112]). Ionis Pharmaceuticals, Inc. (Carlsbad, CA) is investigating the tolerability and antihypertensive action of the AGT antisense ONIS-AGT-LRx (AGT antisense oligonucleotide) given subcutaneously once weekly for 12 weeks to primary hypertensive patients (ClinicalTrials.gov Identifier: NCT04714320). Additionally, Alnylam Pharmaceuticals (Cambridge, MA) evaluated the effect of single subcutaneous doses of their AGT-interfering RNA (ALN-AGT01) in hypertensive patients with or without altered salt intake and concomitant treatment with irbesartan (ClinicalTrials.gov Identifier: NCT03934307). The data presented at the 2020 Scientific Sessions of the AHA showed that a 95% reduction in plasma AGT concentrations at week 8 was associated with mean blood pressure reductions of 11 ± 2/8 ± 1 mm Hg.[107] These newer studies build on early original independent basic science studies using antisense oligonucleotide and mAbs to probe the functions of renin and AGT.[113,114]

Although the apparent benefits of AGT inhibition to treat hypertension alone or in reinforcing the therapeutic effects of other antihypertensive agents may be obvious, these interventions assume that the function of AGT is restricted to being a protein substrate for generation of angiotensins. Preliminary data obtained with the ALN-AGT RNA interference combined tolerability with effectiveness[107]; however, targeting AGT gene silencing may lead to unexpected complications over the long term, as *AGT* silencing assumes that no other biological functions are contained within the protein.[115] The human AGT protein is composed of 452 amino acids, of which the N-terminus signal peptide comprises the first 33. The sequence of angiotensins is initiated by Asp^1 at position 34 from the N-terminus Met^1.[116] Thus, the 12 amino acids coding for the current major studied angiotensins constitute 2.65% of the human AGT protein. As recently reported by us,[115] this therapeutic approach assumes that the remaining ~97% of the molecule—des-(Ang I)-AGT[117–119]—has no function. However, Corvol and colleagues[120–123] reported the antiangiogenic actions of des-(Ang I)-AGT and, recently, overexpression of AGT was reported to protect mice from sinusoid remodeling and arterialization in cancerous tissue.[123] Other studies show that AGT acts as an inhibitor of vascular endothelial growth factor cell migration,[120] a modulator of blood–brain barrier permeability,[124] diet-induced obesity, and hepatic steatosis.[125] AGT expression in the renal tubules and its implicated role in the regulation of renal function may be impaired by suppression of the *AGT* gene.[126–128] We suggest that AGT gene silencing may have other consequences, as depletion of the substrate causes dramatic increases in

Table 1 | Summary of currently registered clinical trials aimed at suppressing renin–angiotensin system (RAS) activity

ClinicalTrials.gov identifier	Condition, phase, and sponsor	Study title and intervention	Study design and outcome measures
NCT04083222	• Hypertension • Phase 2, completed • Sponsor: Ionis Pharmaceuticals, Inc.	• A study to assess the safety, tolerability, and efficacy of IONIS-AGT-L_{Rx} • IONIS-AGT-L_{Rx} is a ligand-conjugated AGT antisense oligonucleotide[102,106]	***Study design*** • Double-blind, randomized (2:1), placebo-controlled study in subjects who received once-weekly s.c. IONIS-AGT-L_{Rx} or placebo, with an additional loading dose administered on day 3 • Treatment duration: 8 wk • Post-treatment period: 13 wk ***Outcome measures*** • *Primary*: percentage change in plasma AGT from baseline to study day 57 (study wk 9) compared with placebo • *Secondary*: change in SBP from baseline to each scheduled, post-baseline visit (time frame: baseline up to day 141) • Percentage change in plasma AGT from baseline to each scheduled, postbaseline visit (time frame: baseline up to day 141)
NCT03714776	• Mild hypertension • Phase 2, completed • Sponsor: Ionis Pharmaceuticals, Inc.	• A study to assess the safety, tolerability and efficacy of IONIS-AGT-L_{Rx} in hypertensive subjects with controlled blood pressure	***Study design*** • Subjects randomized (2:1) to receive once-weekly s.c. IONIS-AGT-L_{Rx} or placebo, with an additional loading dose on day 3 • Treatment duration: 6 wk • Post-treatment period: 13 wk ***Outcome measures*** • *Primary*: percentage change in plasma AGT from baseline to study day 43 (wk 7) compared with placebo • *Secondary*: change on in-clinic SBP from baseline to each scheduled, post-baseline visit (time frame: study days 1, 3, 8, 15, 22, 29, 36) • Percentage change in plasma AGT from baseline to each scheduled, post-baseline visit (time frame: study days 1, 3, 8, 15, 22, 29, 36)
NCT03934307	• Hypertension • Phase 1, ongoing • Sponsor: Alnylam Pharmaceuticals	• A study to evaluate ALN-AGT01 in patients with hypertension • ALN-AGT01 is an AGT-interfering RNA[107]	***Study design*** • A 4-part study to evaluate the safety, tolerability, PK, and PD effects of s.c. ALN-AGT01 o Part A: an SAD phase in hypertensive participants o Part B: an SD phase in hypertensive participants with controlled salt intake o Part D: an MD phase in obese hypertensive subjects o Part E: an open-label SD phase with coadministration of irbesartan in hypertensive patients ***Outcome measures*** • *Primary*: number of participants with AEs (time frame: Parts A, B and E, each up to ~12 mo; Part D: up to ~18 mo) • *Secondary*: change from baseline in blood AGT level (time frame: parts A, B and E: each up to ~12 mo; part D: up to ~18 mo) • C_{max} of ALN-AGT01 and of potential metabolites (time frame: parts A, B and E: up to day 15; part D: up to day 99) • AUC of ALN-AGT01 and potential metabolites (time frame: parts A, B and E: up to day 15; part D: up to day 99)
NCT01015703	• Healthy volunteers • Phase 1, completed • Sponsor: BTG International Inc.	• Open-label safety and tolerability study of CoVaccine HT[108] in healthy volunteers	***Study design*** • 5 doses, administered 21 days apart • Subjects withdrew upon experiencing any AE ***Outcome measures*** • *Primary*: safety events, safety/tolerability

(*Continued on following page*)

Table 1 | (Continued) **Summary of currently registered clinical trials aimed at suppressing renin–angiotensin system (RAS) activity**

ClinicalTrials.gov identifier	Condition, phase, and sponsor	Study title and intervention	Study design and outcome measures
NCT00702221	• Hypertension/cardiovascular disease • Phase 2, terminated • Sponsor: BTG International, Inc.	• A study to evaluate safety and efficacy of ATV, which contains the novel adjuvant, CoVaccine HT[109] in subjects with mild-to-moderate hypertension	***Outcome measures*** • *Primary*: change from baseline to wk 8 in mean daytime DBP measured by ABPM
NCT00500786	• Mild-to-moderate essential hypertension • Phase 1/phase 2, completed • Sponsor: Cytos Biotechnology AG	• A study to evaluate safety, tolerability, PD effects, and preliminary evidence for efficacy of the anti-hypertension vaccine CYT006-AngQb	***Study design*** • Multicenter, randomized, placebo-controlled, time-lagged, parallel-group study in healthy and hypertensive subjects to evaluate safety and tolerability of the vaccine CYT006-AngQb[110] • Subjects randomized to arms: 100 μg CYT006-AngQb healthy volunteers; 100 μg CYT006-AngQb hypertensives; 300 μg CYT006-AngQb hypertensives; placebo healthy volunteers; placebo hypertensives ***Objectives*** • *Primary*: to evaluate safety/tolerability of 3 dose regimens of CYT006-AngQb in healthy volunteers and patients with mild-to-moderate essential hypertension • *Secondary*: o Assessment of PD effects and their dose–response (immunogenicity and biomarkers of the RAS) o Exploration of clinical efficacy (effects on SBP and DBP)
NCT00701649	• Mild-to-moderate essential hypertension • Phase 2, completed • Sponsor: Cytos Biotechnology AG	• Study to evaluate safety and tolerability, PD effects, and exploratory efficacy of the CYT006-AngQb vaccine • CYT006-AngQb is a vaccine that induces a B cell–mediated immune response characterized by the generation of specific antibodies (IgG and IgM) against Ang II[110–112]	***Objectives*** • To evaluate safety and tolerability of 5 s.c. injections of 300 μg CYT006-AngQb with Alhydrogel • To assess PD effects, i.e., anti–Ang II immune response and RAS biomarkers • To explore the effect on blood pressure using ABPM
NCT00710372	• Mild and moderate essential hypertension • Phase 2, completed • Sponsor: Cytos Biotechnology AG	• Safety, tolerability, and efficacy of a vaccine against essential hypertension	***Outcome measures*** • *Primary*: AEs (time frame: throughout study until wk 48) • *Secondary*: o Change in daytime, night-time, and 24-hour ABP from baseline (time frame: 24 h) o Anti–Ang II IgG antibody titer (time frame: throughout study until wk 48) o Level of RAS biomarkers (concentrations of plasma renin, Ang II, and aldosterone) (time frame: 24 h)
ACTRN12617001192370	• Essential hypertension • Phase I/IIa	• A phase I/IIa study to assess safety, tolerability, pharmacokinetic, PD effects, and exploratory efficacy of 2 doses of AGMG0201 in patients with essential hypertension	***Study design*** • A dose escalation study of AGMG0201 delivered at 2 doses in up to 24 volunteers o High dose: 0.2 mg plasmid DNA and 0.5 mg Ang II–KLH conjugate o Low dose: 0.2 mg plasmid DNA and 0.25 mg Ang II–KLH conjugate • Each participant will be randomized to receive either the low or high dose of AGMG0201, or placebo (saline) as a single intramuscular injection to the deltoid muscle • A booster vaccination will be administered 30 days after the first vaccination, providing there are no contraindications to a booster dose at visit B0 in accordance with the predefined eligibility criteria

ABPM, ambulatory blood pressure monitoring; AE, adverse events; AGT, angiotensinogen; ALN, Alnylam Pharmaceuticals; Ang II, angiotensin II; ATV, angiotensin therapeutic vaccine; AUC, area under the concentration-time curve; BTG, BTG International Inc.; C_{max}, maximum observed plasma concentration; DBP, diastolic blood pressure; IgG, immunoglobulin G; IgM, immunoglobulin M; KLH, keyhole limpet hemocyanin; PD, pharmacodynamic; PK, pharmacokinetic; SBP, systolic blood pressure; s.c., subcutaneous; SAD, single ascending dose; SD, single dose.

circulating renin, and in principle, the loss of both the pressor [ACE/Ang II/AT_1-R] and depressor [ACE2/Ang-(1-7)/Mas-R axis] component of the system.[129] We think that the potential long-term negative effects of total AGT inhibition are reminiscent of the severe renal dysfunction reported with the combined use of ACE inhibitors and ARBs.[130]

Ang I and Ang II. The promissory value of vaccines in the treatment of cancer, rheumatoid arthritis, and even Alzheimer's disease continues to stimulate research in the development of vaccines targeted to Ang II (Table 1). Ang I vaccines show minimal antihypertensive effects, both in normal and hypertensive subjects.[131] On the other hand, at least 4 clinical trials registered with the National Institutes of Health evaluate the use of Ang II in hypertension (Table 1). Of the completed trials, administration of CYT—6-AngQb (virus-like particles covalently coupled to Ang II) seems to be promising in that it increases anti-Ang II antibody titers, eliminating the morning blood pressure surge, and reducing ambulatory daytime blood pressure at the highest dose tested.[110,132] Unfortunately, additional studies that induced higher anti-Ang II antibody titers failed to duplicate the original findings, and adverse effects related to employed doses led to the termination of another Ang II vaccine incorporating an adjuvant (CoVaccine HT) in a phase 2 randomized, double-blind, placebo-controlled trial (ClinicalTrials.gov Identifier: NCT00702221; Table 1).[108,109]

Ang II type 1 receptors. Preclinical studies suggest that therapeutic efficacy may be achieved with vaccines derived from the second extracellular loop of AT_1-R.[133,134] However, detection of AT_1-R autoantibodies in preeclamptic patients,[135] kidney transplant recipients,[136] and those with primary aldosteronism[137] raises questions as to their clinical potential.

Ang-(1-12). Monoclonal antibody–based treatments of malignancies, familial dyslipidemia, and autoimmune diseases are yielding extraordinary results as new therapeutic strategies.[138,139] Building on these findings, we are examining the efficacy of using the human sequence of Ang-(1-12) [h-Ang-(1-12)] to generate mAbs for the treatment of hypertension and hypertension-related target organ damage. A highly selective mAb has been generated with essentially no cross-reactivity against human AGT or angiotensin peptides. This h-Ang-(1-12)–directed mAb has been injected into the circulation of transgenic hypertensive rats engineered to express the human AGT gene.[77] As illustrated in Figure 3,[76,77] systemic delivery of a single intravenous injection of the h-Ang-(1-12) mAb induced a significant antihypertensive response that was sustained for up to 90 minutes following its administration. These proof-of-concept results are consistent with the demonstration of higher Ang-(1-12) plasma levels in primary hypertensive patients who are naïve or not naïve to antihypertensive medications.[81,82] The new data confirm a real need for reexamining the utility of mAbs for use in treatment strategies against primary hypertension and hypertension-related target organ damage.

The translation of both preclinical and clinical research on vaccines targeting Ang II in the treatment of high blood pressure and hypertension-related target organ damage remains to be crystalized. Of the various approaches described above, AGT gene silencing shows significant therapeutic promise, although the concomitant suppression of des-(Ang I)-AGT functions is a serious concern. These limitations would not influence an approach in which Ang-(1-12) immunoneutralization is targeted, because it will only neutralize the 12–amino acid string of the AGT protein that is the substrate for the generation of the angiotensins.

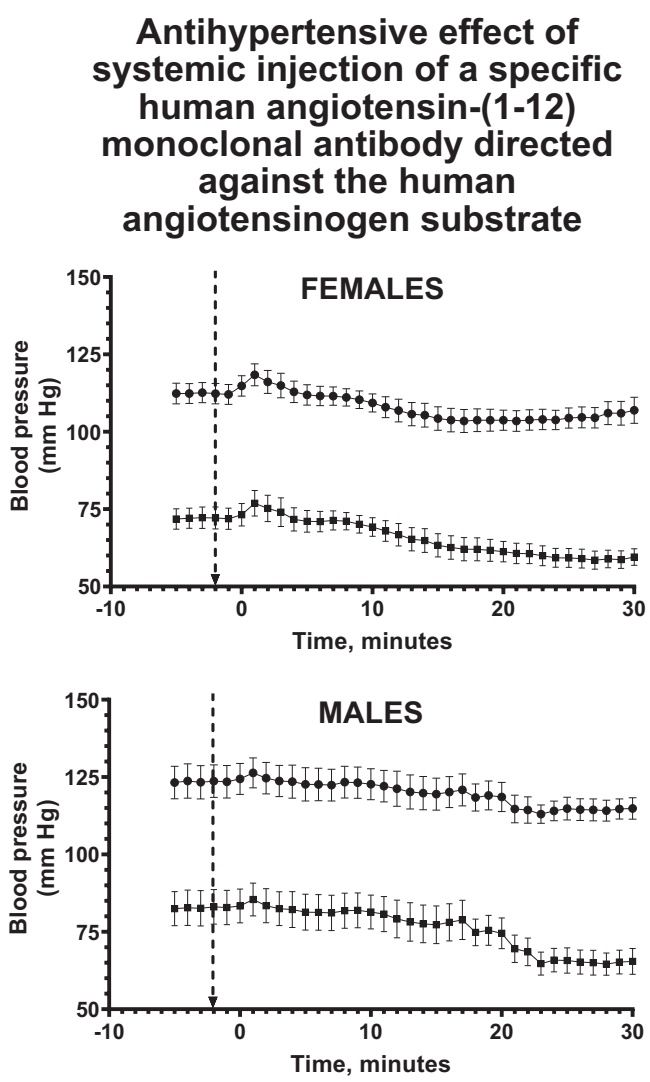

Figure 3 | Time course of the changes in systolic and diastolic blood pressure of anesthetized transgenic hypertensive rats (n = 12) expressing the human angiotensinogen gene in its genome [TGR(hAGT)L1623}][76,77] before and after the intravenous injection of a h-Ang-(1-12) mAb at a dose of 30 mg/kg. Data graphed in Prism version 8 and formatted with BioRender.com. mAb, monoclonal antibody.

COMMENTARY

Increased acceptance of the multifaceted pathways through which the AGT substrate can be metabolized into the active angiotensins within the distinct extracellular and intracellular

compartments of the human body is likely to yield advances in the treatment of human diseases. Randomized clinical trials have cemented the clinical utility of suppressing Ang II formation or binding to AT_1-R receptors in the treatment of heart disease, type 2 diabetes,[15,140] and chronic kidney disease.[141,142] Inconsistencies in the results of randomized controlled trials using RAS, in terms of the presence of a residual risk several orders of magnitude greater than the benefit, need to be more rigorously investigated.[12,13,143] The limitations of current therapeutic approaches to the treatment of hypertensive vascular disease must be appreciated, while alternate mechanisms by which the angiotensins induce pathology are explored. Engineering molecular approaches to increase the anti-Ang II immunogenicity, expanding on our ongoing work using mAbs directed against the human sequence of Ang-(1-12) or even Ang-(1-25) provides a more selective way to suppress Ang II biological actions and avoid the consequences of concomitant suppression of des-(Ang I)-AGT elimination.[115] Further engineering of mAbs into single-domain antibodies (nanobodies)[144,145] will permit intracellular neutralization of angiotensins. Given that these treatments would not require daily administration, the challenge of adherence to medications could also be addressed. We suggest that the approach will yield more specific methods to prevent Ang II from exerting pathologic actions, avoiding the expression or activation of alternate enzymes with catalytic activity against AGT or the intermediate peptides from which Ang II and Ang-(1-7) are generated. Issues related to physician inertia and patients' adherence to therapy will be obviated in large part, as these molecular treatments do not require daily use of medicines. We suggest that such an approach will yield more specific methods to prevent Ang II from exerting pathologic actions, avoiding the activation of alternate enzymes with catalytic activity against AGT or the intermediate peptides from which Ang II and Ang-(1–7) are generated.

CONCLUSIONS

Patient care involves the effective management of both hypertension and cardiometabolic risk factors. Currently available treatment would benefit from further insights to help fully meet the aims of patient care. The challenge is to delve deeper into the RAS cascade, with the aim of enhancing therapeutics for RAS inhibition through recognition of the existence of intermediate alternate mechanisms to produce angiotensins. The presence of renin-independent noncanonical pathways for Ang II production is largely unaffected by agents inhibiting RAS activity. Therefore, it is recommended that future efforts be directed toward development of treatments that can effectively block the synthesis or action of intracellular Ang II through inhibition of the primary intracellular enzymes accounting for Ang II production. Improved drug penetration into cardiac or renal sites of disease, inhibiting chymase, the primary Ang II–forming enzyme in the human heart, or inhibiting Ang-(1-12) as a source for Ang II production may all be more effective strategies to inhibit the system.

DISCLOSURE

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