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Review

New Approaches Targeting the Renin-Angiotensin System: Inhibition of Brain Aminopeptidase A, ACE2 Ubiquitination, and Angiotensinogen

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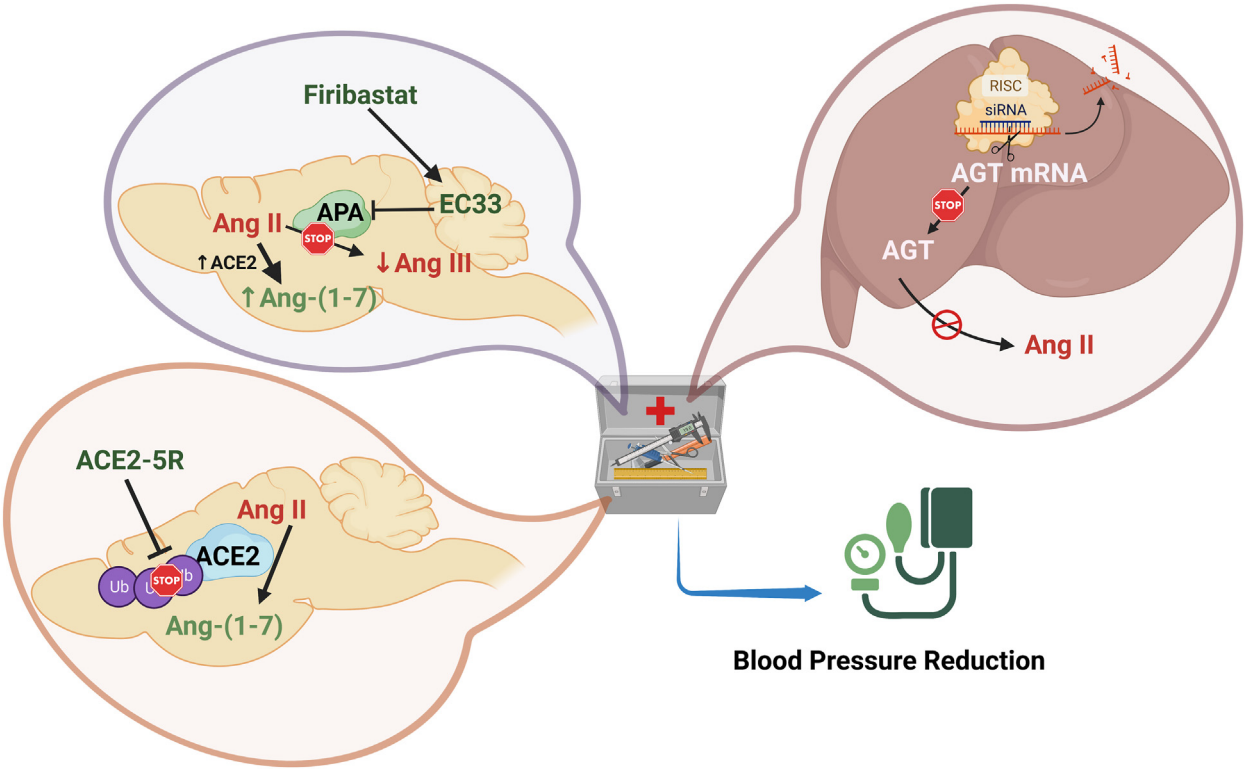

ABSTRACT

Despite the availability of various therapeutic classes of antihypertensive drugs, hypertension remains poorly controlled, in part because of poor adherence. Hence, there is a need for the development of

RÉSUMÉ

En dépit de la disponibilité des diverses classes thérapeutiques d'antihypertenseurs, l'hypertension demeure mal maîtrisée, en partie en raison de la faible adhésion. Par conséquent, il est nécessaire de

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antihypertensive drugs acting on new targets to improve control of blood pressure. This review discusses novel insights (including the data of recent clinical trials) with regard to interference with the renin-angiotensin system, focusing on the enzymes aminopeptidase A and angiotensin-converting enzyme 2 (ACE2) in the brain, as well as the substrate of renin— angiotensinogen—in the liver. It raises the possibility that centrally acting amino peptidase A inhibitors (eg, firibastat), preventing the conversion of angiotensin II to angiotensin III in the brain, might be particularly useful in African Americans and patients with obesity. Firibastat additionally upregulates brain ACE2, allowing the conversion of angiotensin II to its protective metabolite angiotensin-(1-7). Furthermore, antisense oligonucleotides or small interfering ribonucleic acids suppress hepatic angiotensinogen for weeks to months after 1 injection and thus could potentially overcome adherence issues. Finally, interference with ACE2 ubiquitination is emerging as a future option for the treatment of neurogenic hypertension, given that ubiquitination resistance might upregulate ACE2 activity.

mettre au point des antihypertenseurs qui agissent sur de nouvelles cibles pour mieux maîtriser la pression artérielle. La présente revue porte sur de nouvelles connaissances (y compris les données de récents essais cliniques) sur l'interférence avec le système rénine-angiotensine, principalement sur les aminopeptidases A et les enzymes de conversion de l'angiotensine II (ECA2) dans le cerveau, ainsi que sur le substrat de rénine (angiotensinogène) dans le foie. Ceci soulève la possibilité que les inhibiteurs d'aminopeptidase A à action centrale (p. ex. le firibastat), qui empêchent la conversion de l'angiotensine II à l'angiotensine III dans le cerveau, soient particulièrement utiles chez les Afro-Américains. Le firibastat qui régule aussi à la hausse l'ECA2 dans le cerveau contribue à la conversion de l'angiotensine II à son métabolite protecteur, l'angiotensine (1-7). De plus, les oligonucléotides antisens ou les petits acides ribonucléiques interférents contribuent à supprimer l'angiotensinogène d'origine hépatique durant des semaines, voire des mois, après 1 injection et ainsi ils pourraient potentiellement permettre de surmonter les problèmes d'adhésion. Finalement, l'interférence avec l'ubiquitination d'ECA2 se profile comme une option future dans le traitement de l'hypertension neurogène, puisque la résistance à l'ubiquitination régulerait à la hausse l'activité des ECA2.

Hypertension affects one-third of the adult population and is the leading cause of premature death and disability-adjusted life years, being the world's leading risk factor for cardiovascular diseases including myocardial infarction, heart failure, stroke, and end-stage renal disease.[1-3] Despite the availability of various therapeutic classes of antihypertensive drugs, hypertension remains poorly controlled worldwide, and more than 50% of US adults taking antihypertensive medication have blood pressure $\geq$ 130/ 80 mm Hg.[4] This often is caused by nonadherence. Prevalence of hypertension is increasing because of the aging of populations and the obesity epidemic.[5] Therefore, there is still a need for the development of new antihypertensive drugs acting on new targets, with different modes of action, to improve blood pressure control. This review discusses novel insights with regard to interference with the renin-angiotensin system (RAS), focusing on the enzymes aminopeptidase A (APA) and angiotensin-converting enzyme 2 (ACE2), as well as the substrate of renin: angiotensinogen.

Central Aminopeptidase A Inhibition

Brain RAS hyperactivity has been implicated in the development and maintenance of hypertension in several experimental and genetic animal models of hypertension such as spontaneously hypertensive rats (SHR), deoxycorticosterone acetate (DOCA)-salt rats and mice, and transgenic mice overexpressing both the human angiotensinogen and the human renin genes.[6-9] The activity of the systemic RAS is normal in the SHR model and depressed in DOCA-salt rats (characterized by low plasma renin levels and high plasma arginine-vasopressin levels), accounting for the resistance of hypertensive DOCA-salt rats to treatment by systemic RAS blockers.

All components of the RAS, including the precursor and enzymes required for the production and degradation of angiotensins (Angs): Ang I, Ang II, Ang III, Ang IV, and Ang-(1-7) and the specific Ang II receptors type 1 (AT_1) and type 2 (AT_2) as well as the Mas receptor (MasR) have been identified in the brain.[10-12] By analogy with the systemic RAS, brain Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is generated by sequential cleavage of the precursor angiotensinogen by an aspartyl protease, renin (EC 3.4.23.15), producing the decapeptide Ang I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), which is then converted to Ang II by a zinc metalloprotease, angiotensin I-converting enzyme (ACE; EC 3.4.15.1). Subsequently, it was shown that Ang II is converted in the brain into Ang III (Arg-Val-Tyr-Ile-His-Pro-Phe) by APA (EC 3.4.11.7), a 160 kDa homodimeric type II membrane-bound monozinc aminopeptidase, whereas aminopeptidase N (APN; EC 3.4.11.2), another zinc-metalloprotease, metabolizes brain Ang III into Ang IV (Val-Tyr-Ile- His-Pro-Phe).[13] On the other hand, Ang II is converted into Ang-(1-7) (Asp-Arg-Val-Tyr-Ile-His-Pro) by the membrane-bound zinc metalloprotease ACE2 (EC 3.4.17.23), and Ang-(1-7) binds to the MasR with high affinity.[11]

Among the bioactive peptides of the brain RAS, Ang II and Ang III display similar affinities for AT_1 receptors.[14] When injected into the brain, these peptides similarly increase blood pressure and arginine-vasopressin release.[12,13,15] However, because Ang II is converted in vivo into Ang III, the exact nature of the effector peptide of the brain RAS remains unclear. Using in vivo-specific and selective APA and APN inhibitors, EC33 ([S]-3-amino-4-mercaptobutyl sulfonic acid)[16]

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and PC18 ([2S]-2-amino-4-methylsulfonyl butane thiol),[17] respectively, to block the metabolic pathways of brain Ang II and Ang III, central treatment with EC33 blocked the pressor response of intracerebroventricularly applied Ang II in SHR rats, suggesting that the increase in blood pressure requires, in the brain, the conversion of Ang II to Ang III. Moreover, the inhibition of endogenous brain Ang III formation by intracerebroventricular (but not intravenous) injection of EC33 in alert SHR or DOCA-salt rats induced a large dose-dependent decrease in blood pressure.[18,19] Thus, the central blockade by the APA inhibitor of the conversion of brain Ang II to Ang III is responsible for the decrease in blood pressure. Then, endogenous brain Ang III, but not Ang II, exerts a tonic stimulatory effect on the central control of blood pressure in hypertensive rats.[20] This conclusion was strengthened by the significant increase in blood pressure induced by intracerebroventricularly applied PC18.[19] This pressor response is blocked by previous treatment with the AT_1 receptor antagonist losartan (but not with the AT_2 receptor antagonist PD123319), showing that the blockade of the action of APN on brain Ang III metabolism induces an increase in endogenous Ang III levels, resulting in an increase in blood pressure, through interaction with AT_1 but not AT_2 receptors. Finally, the complete inhibition by EC33 of the PC18-induced increase in blood pressure demonstrates the existence of the endogenous enzymatic cascade: Ang II → Ang III → Ang IV under the control of APA and APN, respectively.[19]

Thus, brain APA, the enzyme generating brain Ang III, might be an interesting candidate target for the treatment of hypertension, a conclusion in agreement with the genetic evidence of APA involvement in salt sensitive hypertension.[21] This justified the development of potent and selective APA inhibitors that cross the blood−brain barrier after oral administration as putative centrally acting antihypertensive agents. The central bioavailability of thiol inhibitors of zinc metallopeptidases has been shown to be increased by coupling a neutral endopeptidase inhibitor and an APN inhibitor via a disulfide bridge to produce a prodrug.[22] We applied a similar approach, to develop several orally active APA inhibitor prodrugs.[23-25] The first-in-class prodrug of EC33 (targeting only the S1 subsite of the APA active site) RB150 (4,4-dithio [bis {3S}-3-aminobutyl sulfonic acid]) is composed of 2 molecules of EC33 linked by a disulfide bridge, and was renamed "firibastat" by the World Health Organization. Then the best-in-class prodrugs of NI929 were designed, NI956/QGC006 created by the disulfide bridge-mediated dimerization of NI929 or QGC606 resulting from the coupling of NI929 to L-cysteine through a disulfide bridge. NI929 ([3S, 4S]-3-amino-4-mercapto-6-phenyl-hexane-1-sulfonic acid) is a more potent (K_i = 30 nM: that is, 10-fold more efficient than EC33) and more selective APA inhibitor than EC33 and was designed by incorporating an additional hydrophobic side chain on the EC33 scaffold, allowing NI929 to interact not only with the S1 subsite but also with the S'1 subsite of the APA active site (Fig. 1A).[25,26] The selectivity of firibastat and NI956 toward APA was shown by the lack of affinity of these compounds for other zinc metalloproteases involved in the production or metabolism of vasoactive peptides such as APN, ACE, ACE2, endothelin converting enzyme-1, and neutral endopeptidase 24.11, as well as by the absence of binding of these compounds to either AT_1 and AT_2 receptors or endothelin type A and type B receptors.[25,26]

The thiol group of firibastat, NI956, or QGC606, interacts with the zinc atom located in the APA active site, which is essential for its catalytic activity. Hence, when the thiol group is engaged in a disulfide bridge, they are unable to inhibit APA enzymatic activity. However, the disulfide bridge allows orally administered firibastat, NI956, or QGC606 to cross the intestinal, hepatic, and blood−brain barriers and to enter the brain. In the brain, the disulfide bridge is immediately cleaved by brain reductases to generate 2 active molecules of EC33 or NI929, which inhibit brain APA activity, block the formation of brain Ang III, and decrease blood pressure and arginine-vasopressin release in various experimental models of hypertension.[23-25,27,28]

The decrease in blood pressure induced by firibastat is attributed to 3 different mechanisms: a decrease in arginine-vasopressin release from the posterior pituitary into the bloodstream, leading to an increase in diuresis and reducing extracellular volume; a reduction in sympathetic tone decreasing vascular resistance; and an improvement of the baroreflex function (Fig. 1B).[23,27,29] Orally administered NI956 in hypertensive DOCA-salt rats normalized brain APA hyperactivity and blood pressure for 10 hours after a single dose. This was associated with decreased plasma arginine-vasopressin levels and increased diuresis and natriuresis at a dose 10 times lower than that required for firibastat.[25] NI956 is therefore a best-in-class central acting APA inhibitor prodrug, belonging to the same drug class as firibastat.

Firibastat and NI956 have no effect on blood pressure in normotensive rats, not displaying hyperactivation of brain APA and brain RAS. Thus, firibastat and NI956 act as antihypertensive agents and not as hypotensive agents.

It was also interesting to investigate why the blockade of brain Ang III formation by firibastat, which decreases blood pressure, did not induce brain Ang II accumulation because, if this had been the case, we would have observed a blood pressure increase caused by activation of AT_1 receptors by Ang II. Consequently, this suggests that another metabolic pathway of Ang II must be activated following treatment with firibastat, leading to the conversion of brain Ang II into another Ang fragment with no affinity for AT_1 receptors. To answer this question, firibastat was applied intracerebroventricularly to hypertensive DOCA-salt rats. This not only normalized brain APA hyperactivity but also induced an increase in brain ACE2 activity.[30] Moreover, the decreases in blood pressure and arginine-vasopressin release resulting from brain APA inhibition with firibastat were partially reduced if ACE2 was concomitantly inhibited by MLN4760, a potent ACE2 inhibitor, or if the MasR was blocked by A779, a MasR antagonist.[30] These findings suggest that brain APA inhibition following firibastat treatment in hypertensive DOCA-salt rats not only blocks the formation of brain Ang III, which therefore cannot exert a tonic stimulatory effect on the control of blood pressure, but also leads to the activation of another metabolic pathway of brain Ang II. This concerns conversion of brain Ang II by ACE2 into Ang-(1-7), which, by activating the MasR, partially contributes to the antihypertensive effect of firibastat and its inhibitory effect on arginine-vasopressin release (Fig. 1B). In agreement with these data, chronic intracerebroventricular Ang-(1-7) perfusion was shown to

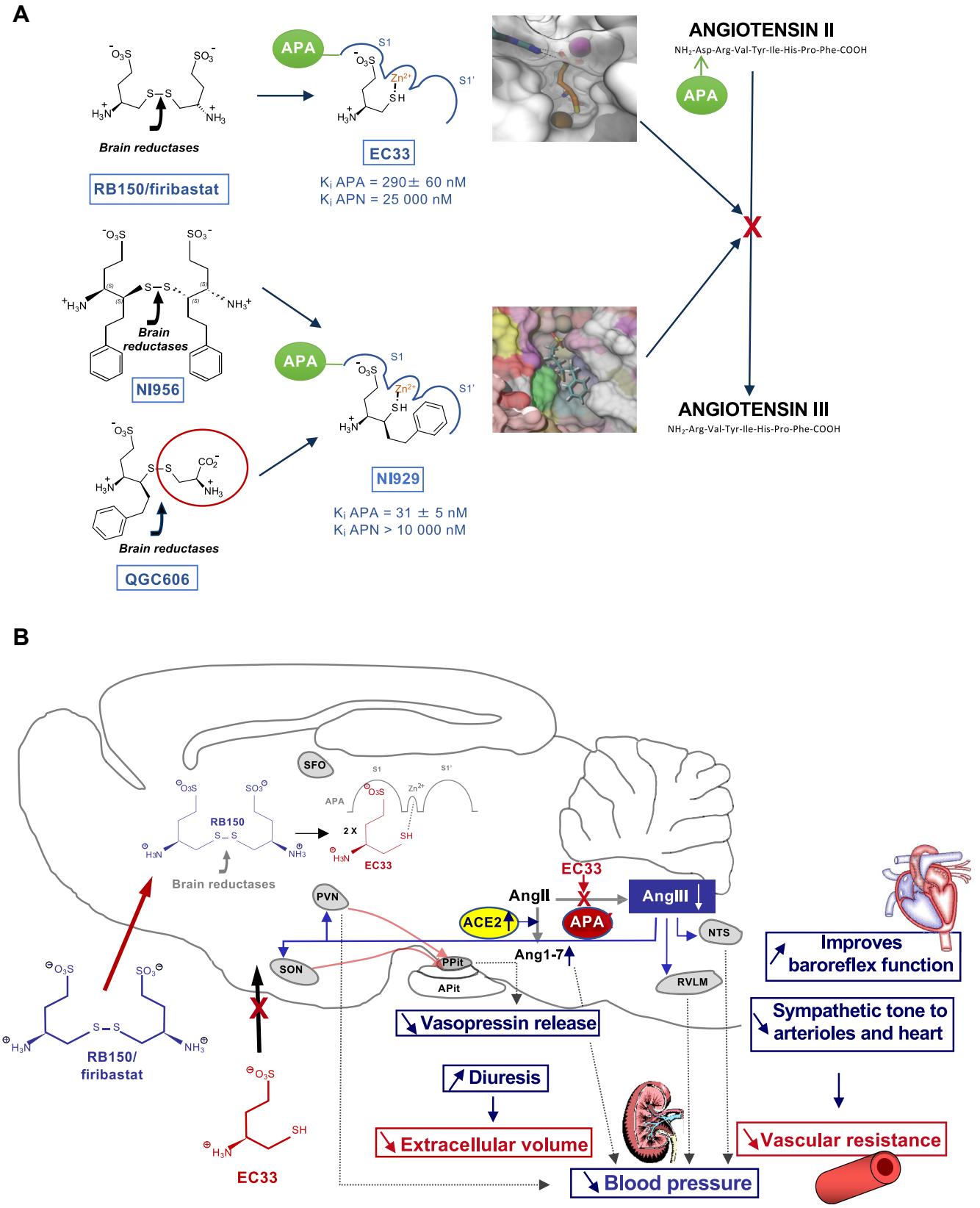

Figure 1. (**A**) Pharmacologic and structural properties of the aminopeptidase A (APA) inhibitors EC33 and NI929, and their prodrugs, RB150/firibastat, NI956, and QGC606.[16,25,26] The S1 subsite is visualized in the 3-dimensional (3D) model of human APA after molecular docking of EC33 (**orange**).[24] Molecular docking of NI929 (**blue**) in the 3D model of human APA is also shown.[25] (**B**) Mode of action of the APA inhibitor prodrug, RB150/firibastat, on the control of blood pressure in hypertensive rats (Modified from Marc et al[27] with permission from Hypertension and

attenuate hypertension in DOCA-salt rats, whereas removing brain ACE2 from the cell membrane facilitated the development of neurogenic hypertension (as discussed under ACE2 ubiquitination).[9,31] One possibility is that Ang II is converted to angiotensin A, which then is metabolized into alamandine, a peptide known to decrease blood pressure and arginine-vasopressin release via activation of the MasR-related G protein-coupled receptor D.[11,32,33]

Together, these data led to the first evaluation of firibastat in humans. Clinical studies in healthy volunteers, in single ascending oral doses (Phase Ia) and multiple oral doses (Phase Ib) have shown that firibastat is clinically and biologically well tolerated up to 750 mg twice daily for 7 days.[34] Two Phase II clinical trials were then conducted. The first in France, Phase IIa of firibastat (NCT02322450) in 34 hypertensive patients (grade I and II).[35] This study showed that firibastat treatment (250 mg twice daily for 1 week, then 500 mg twice daily for 3 weeks) is safe and tends to decrease daytime ambulatory systolic blood pressure (SBP). The effect was not significant, probably because of the small number of patients and the short duration of the treatment. These data were used to guide the design of a large clinical trial (Phase IIb) with firibastat in the United States—**N**ovel **E**valuation **W**ith QGC001 in **H**ypertensive **O**verweight **P**atients of Multiple **E**thnic Origins (NEW-HOPE [NCT03198793])—in 254 overweight or obese hypertensive patients (SBP 145-170 mm Hg), of whom 54% were self-identified African Americans or Hispanics.[36] This study showed that firibastat treatment alone (85% of patients) or combined with a diuretic, hydrochlorothiazide (15% of patients), for 8 weeks (250 mg twice daily orally for 2 weeks, then 500 mg twice daily if automated office blood pressure (AOBP) was $\geq$ 140/90 mm Hg; hydrochlorothiazide 25 mg once daily was added after 1 month if AOBP was $\geq$ 160/110 mm Hg) similarly and significantly lowered systolic AOBP from baseline in patients with obesity, African Americans, and white Americans (by 10.2, 10.5 mm, and 8.9 mm Hg, respectively) in the intention-to-treat population. Diastolic AOBP was also significantly decreased by 4.2 mm Hg. In addition, a significant reduction in daytime ambulatory BP (3.1 ± 3.0 mm Hg for systolic and 1.6 ± 7.8 mm Hg for diastolic, $P = 0.0005$ and $P = 0.003$, respectively) and in 24-hour ambulatory BP was reported after the 8-week treatment. Here it is important to acknowledge that the ambulatory BP technique is challenging in patients with obesity; in fact, in this study, only 181 patients (70%) could have both their baseline and end-of-study ambulatory BP recorded correctly. Most frequent adverse events were headache (4%) and skin reactions (3%). No angioedema was reported. No change in potassium, sodium, and creatinine blood level was observed.

Next, the Phase III clinical trial, **F**iribastat in Treatment-**Res**istant **H**ypertension (FRESH [NCT04277884]),[37] aimed to evaluate the effects and safety of firibastat when given to uncontrolled patients with difficult-to-treat (27%) or resistant (73%) hypertension on top of their chronic antihypertensive therapy. The chronic antihypertensive treatment was either a bitherapy or a triple therapy, including the following antihypertensive drugs: a systemic RAS blocker (either an ACE inhibitor or an angiotensin II type 1 receptor blocker [ARB]), optionally combined with a diuretic; a calcium channel blocker; or a mineralocorticoid receptor antagonist. For treatment-resistant subjects, 1 of the antihypertensive drugs had to be a diuretic. The study was a multicentre (n = 76), randomized, double-blind placebo-controlled Phase III trial, with Dr G. Bakris as the principal investigator, and it was sponsored by Quantum Genomics SA. After a 4-week run-in period, adherent patients who remained uncontrolled (mean systolic daytime ambulatory BP > 135 mm Hg) despite their bi- or triple therapy, were randomized (1:1) to receive firibastat (500 mg twice daily, 255 patients) or placebo (259 patients) in addition to their chronic antihypertensive treatment for 12 weeks. The primary endpoint was the change from baseline in unattended automated office systolic BP. At this step, the mean automated office systolic BP was 146.1 ± 12.1 mm Hg. After 12 weeks of treatment, the addition of firibastat to the bi- or triple therapy did not amplify the drop in office systolic BP induced by the bi- or triple therapy alone. The adjusted change from baseline in unattended office systolic BP was −7.8 mm Hg in the group of patients treated by firibastat and −7.9 mm Hg in the group of patients treated by a matching placebo, with no significant difference between the 2 groups. Results were consistent across all the subgroups (number and class of background antihypertensive drugs, race, sex, body mass index).

It is worth noting that in the Phase IIa study,[35] in the per-protocol population, analysis of changes in daytime ambulatory systolic BP following firibastat treatment, by quartile of baseline systolic BP, showed that firibastat (4 weeks) alone did not change systolic BP in patients with the lowest basal systolic BP values (between 122 and 148 mm Hg), whereas it decreased systolic BP in patients with higher baseline systolic BP (above 148 mm Hg). By comparison, placebo treatment did not induce any change whatever the basal systolic BP value. This is in agreement with experimental models of hypertension showing that firibastat acted as an antihypertensive agent and not as a hypotensive agent. In the Phase IIb clinical trial,[36] this criterion had been taken into account. The patients had an average baseline office systolic BP of 154.0 ±7.3 mm Hg, and firibastat treatment alone (8 weeks) decreased office systolic BP by 9.5 mm Hg. This could perhaps explain in part why in the Phase III FRESH study, firibastat coadministered with a bi- or triple therapy to hypertensive patients with a baseline office systolic BP of 146 mm Hg did not cause an additional drop in office systolic BP compared with bi- or triple therapy alone. On the other hand, we cannot also exclude that the mechanisms of action of the drugs used in the bi- or triple therapy may interfere with or antagonize the

Hmazzou et al,[30] with permission from Clin Sci [Lond]). Angiotensin (Ang) III controls blood pressure via an increase in the release of arginine-vasopressin from the posterior pituitary, activation of sympathetic premotor neuron activity at the level of the rostral ventrolateral medulla (RVLM), and baroreflex inhibition at the level of the solitary tract (NTS).[15] APA converts Ang II into Ang III. Firibastat is composed of 2 EC33 molecules linked by a disulfide bridge. This allows passage of the blood–brain barrier, after which reductases cleave firibastat into 2 EC33 molecules. APA inhibition simultaneously activates ACE2. This enzyme converts Ang II into Ang-(1-7), which activates the Mas receptor, thereby potentially contributing to the blood pressure-lowering effect. APit, anterior pituitary; PPit, posterior pituitary; PVN, paraventricular nucleus; SFO, subfornical organ; SON, supraoptic nucleus. The panels have been generated based upon data in earlier references.[24,25,27]

mechanisms of action of firibastat. In conclusion, adding firibastat as a third- or fourth-line therapy, to induce an additional drop in systolic BP in difficult-to-treat or resistant hypertensive patients with a basal SBP not greater than 146 mm Hg does not appear to be the appropriate strategy.

Considering the mechanism of action of firibastat, this compound administered alone could be at the origin of an alternative class of antihypertensive drugs targeting brain APA for hypertensive patients (including African Americans or patients with obesity) displaying salt sensitivity, low plasma renin levels, high plasma vasopressin levels, sympathetic nervous system overactivity, or reduction in baroreflex sensitivity.[38,39] Yet, this remains to be confirmed in a randomized parallel-group clinical trial. Considering the data of the Phase III study, it would be interesting to now investigate in experimental models of hypertension, the effect and the sequence of administration of firibastat on top of the different bi- or triple therapies, particularly as firibastat, when given together with a diuretic, did lower BP.[36] Finally, another therapeutic application of firibastat was recently reported[24,29,40] in a Phase II study—**Qu**antum Genomics Firibastat **o**r **R**amipril After Ac**u**te **M**yocardial Infarction to Prevent Left Ventricular Dysfunction (QUORUM [NCT03715998])—showing that firibastat treatment (12 weeks) is similar to ramipril for the prevention of left ventricular dysfunction after first acute anterior myocardial infarction. Their safety profiles were also shown to be similar.

ACE2 Ubiquitination

Over the last 2 decades, ACE2 has emerged as a critical member of the compensatory RAS axis, promoting the cleavage of Ang II into the heptapeptide Ang-(1-7). ACE2 expression was found to be widespread through the body including in the heart,[41] vasculature,[42] kidneys,[43] lung,[44] gut,[45] brain,[46] pancreas,[47] liver,[48] and adipose tissue.[49] As a result of reducing Ang II and increasing Ang-(1-7) levels, ACE2 has been shown to be beneficial in hypertension, heart failure, chronic kidney disease, type 2 diabetes, and atherosclerosis. A common feature of these diseases is their association with ACE2 downregulation via mechanisms usually mediated by the AT_1 receptor. Enhanced ACE2 expression or activity by means of gene therapy, recombinant proteins, or activators has been shown to reverse the deleterious phenotype. This is the case for neurogenic hypertension, a disease associated with enhanced sympathetic drive resulting from overactivity of the RAS within key brain regions in the hypothalamus and brainstem.[9,50,51] To better understand the mechanisms involved in the blunted ACE2 compensatory activity in pathologic situations, we and others have focused on ACE2 post-translational modifications. ACE2 shedding was first identified as an AT_1 receptor-mediated mechanism leading to the loss of ACE2 cell surface expression in both an experimental model of salt-sensitive hypertension[9] and in humans.[52] ADAM17, a disintegrin and metalloprotease, also known as tumour necrosis factor-α convertase, is responsible for this process, leading to the release of soluble, active ACE2 in the surrounding milieu. Therefore, in hypertension, type 2 diabetes, and heart failure the rise of soluble ACE2 could be used as a possible biomarker of disease. The second post-translational mechanism identified is associated with ACE2 metabolism within cells. Having observed that Ang II promotes ACE2 internalization and degradation, we speculated that ubiquitination could also contribute to neurogenic hypertension.[53]

Ubiquitination is well known to play a role in cardiovascular diseases. Although we were the first to suggest that ubiquitination of ACE2 could be associated with cardiovascular disorders, including neurogenic hypertension,[53] it was only recently that the first E3 ligase involved in ACE2 ubiquitination was reported.[54] Since then, and because of the immense research efforts invested in understanding the relationship between ACE2 and SARS-CoV-2 infection, several ubiquitination partners have been identified. However, little is known about how they regulate ACE2 expression in cardiovascular diseases and whether their expression is dependent on other members of the RAS.

Ubiquitin is a 76-amino acid polypeptide that binds covalently to lysine residues in substrate proteins to regulate physiological processes, including protein degradation. Usually, monoubiquitinated proteins are targeted to lysosomes for degradation, whereas polyubiquitinated proteins are degraded into proteasomes.[55,56] Ubiquitin is attached under the sequential action of three types of enzymes: ubiquitin activating enzymes (E1), ubiquitin conjugating enzymes (E2) and ubiquitin ligases (E3) and the large array of E3 ligases, 600 in the human genome,[55,56] is responsible for the substrate specificity of ubiquitination. One of the most well-characterized family of E3 ligases is neural precursor cell-expressed developmentally downregulated gene 4 (NEDD4), which has 9 members in mammals.[57] Interestingly, 1 of its members, NEDD4-2, was found to be involved in salt-induced hypertension by impairing renal Na^+ handling.[58] Equally important in cellular homeostasis are the 100 potential deubiquitinases (DUBs) that reversely modify proteins by removing ubiquitin from target proteins. As for E3 ligases, studies have identified a role for DUB in salt-induced hypertension, through the regulation of renal Na^+ channels intracellular trafficking and cellular expression.[59]

The oncoprotein murine double minute 2 (MDM2), known for its negative regulation of p53 in various cancers, was the first E3 ligase shown to promote ACE2 ubiquitination in endothelial cells, a process contributing to pulmonary arterial hypertension (PAH).[54] MDM2 was shown to be overexpressed, whereas ACE2 was decreased in lung samples from patients with PAH and in rodent models of hypoxia-induced PAH. Interestingly, ubiquitination on a specific lysine (K788) in the C-terminal tail of ACE2 appeared to play a key role in its destabilization in mice. Although these data suggest that targeting MDM2 might be beneficial to preserve ACE2 compensatory activity, it should be noted that deletion of MDM2 has been associated with p53-dependent glomerular injury[60] and possibly diabetic nephropathy[61]; therefore, it is unlikely that direct MDM2 targeting could be used therapeutically to preserve ACE2 compensatory activity.

S-phase kinase-associated protein 2 (Skp2) is another E3 ligase whose expression has been associated with several types of cancers. It was reported that benzo(a)pyrene, a polycyclic aromatic hydrocarbon present in cigarette smoke is associated with upregulation of Skp2. Indeed, lung biopsies from smokers were shown to have high levels of Skp2 and low levels of ACE2. Skp2 was found to bind to ACE2 and promote its

ubiquitination,[62] a process that is blocked by the CDK4/6 inhibitor palbociclib.[63] Interestingly, Skp2 is also expressed in vascular injury and has been reported to contribute to vascular remodelling by promoting vascular smooth muscle cell proliferation in a Rac1-dependent manner.[64] However it is unknown whether ACE2 might play a part in this process.

Following the confirmation of ACE2 as a "receptor" for SARS-CoV-2 several groups thought about taking advantage of ubiquitination as a mechanism to downregulate ACE2 from the cell surface and possibly reduce infection. As a result, several E3 ligases, DUB and other agents were shown to interact with ACE2 (Fig. 2). Although some of these potential ACE2 ubiquitination partners have been reported to play a role in cardiovascular diseases, for most of them, it is unclear whether their expression is regulated by RAS components. The interrelationship between ACE2 and these players has not been studied in cardiovascular diseases.

We recently identified NEDD4-2 as a new E3 ligase promoting ACE2 ubiquitination.[65] Using a combination of discovery proteomics and data mining via the UbiBrowser database,[66] we found that ACE2 has 4 putative NEDD4-2 recognizing sites, including 1 located near K788. Lysine to arginine mutations in the C-terminal of ACE2 (ACE2-5R) lead to a 3-fold increase in ACE2 activity and resistance to ACE2 downregulation in vitro. Ang II treatment in HEK293T and human aortic endothelial cells resulted in increased NEDD4-2 expression and ACE2 downregulation, whereas treatment with a NEDD4-2 inactive mutant or NEDD4-2 siRNA preserved ACE2 expression and activity. In mice, Ang II infusion-induced hypertension was associated with NEDD4-2 upregulation in the brain, heart, and kidney in male but not in female mice, whereas ACE2 was concomitantly reduced in those tissues. In addition, NEDD4-2 was also elevated in the left ventricle of hypertensive patients and paralleled by ACE2 downregulation. These data suggest that although ACE2 ubiquitination is dependent on sex differences, it appears to affect many tissues and organs.

We previously reported that ACE2 is expressed on GABAergic but not glutamatergic neurons within the forebrain and contributes to an inhibitory tone responsible for repressing the activity of kidney-projecting glutamatergic presympathetic neurons within the paraventricular nucleus (PVN) of the hypothalamus.[67,68] As ACE2 is downregulated in hypertension, it was postulated that its ubiquitination could contribute to the lack of inhibitory input to PVN presympathetic neurons. To investigate the importance of ACE2 ubiquitination in the development of neurogenic hypertension, an adeno-associated virus encoding ACE2-5R was injected in the bed nucleus of the stria terminalis (BNST), a region known for its GABAergic projections to the PVN.[69] Indeed, photostimulation of GABAergic BNST neurons expressing ACE2 was shown to reduce blood pressure and heart rate, thus confirming the pivotal role of this region in blood pressure regulation.[65] Expression of the ubiquitination-resistant ACE2-5R in the BNST resulted in a reduction of baseline blood pressure and a blunted hypertension in Ang II-infused male mice. Importantly, although the GABAergic inhibitory input to the PVN was blunted in hypertensive mice,[68] it was reinforced in hypertensive mice expressing ACE2-5R in the BNST, suggesting that ubiquitination of ACE2 on GABAergic neurons reduces this inhibitory tone. Protein expression in the BNST confirmed that hypertension was associated with NEDD4-2 (but not MDM2) upregulation and ACE2 downregulation, suggesting that NEDD4-2 could be responsible for ACE2 ubiquitination in Ang II-dependent hypertension. Accordingly, we developed a new working model in which prevention of ACE2 ubiquitination on GABAergic neurons would strengthen the inhibitory input to PVN presympathetic neurons and blunt the development of neurogenic hypertension (Fig. 3).

As for MDM2, direct NEDD4-2 targeting is unlikely to provide a viable solution to preserve ACE2 compensatory activity, considering the several thousands of substrates predicted to be ubiquitinated by this E3 ligase. On the other end, ACE2 ubiquitination itself could be a more promising avenue as Lys→Arg mutations in the C-terminal resulted in a significant increase in activity. Future studies are warranted to determine the impact of ubiquitination-resistant ACE2 expression on physiological functions.

Angiotensinogen Suppression

RAS inhibition invariably results in a so-called RAS escape phenomenon: that is, a rise in renin, thereby potentially increasing Ang II, which may eventually (partially) overcome the effect of RAS blockade. In humans, renin rises of several 100-fold are known to occur.[70] Obviously, without angiotensinogen, a rise in renin would have no consequence. Angiotensinogen is primarily synthesized by the liver, but other tissues—including brain,[71,72] adipose tissue,[73-75] and kidney[76-78]—have been proposed to synthesize angiotensinogen as well. This raises the possibility that angiotensin generation is determined locally in a tissue-specific manner. Yet, studies using liver-specific AGT gene deletion in mice[79] and—more recently—the use of novel hepatocyte-directed ribonucleic (RNA)-based therapies to inhibit the formation of liver-derived angiotensinogen in rats and nonhuman primates[80-83] suggest that most angiotensin in the body is derived from hepatic angiotensinogen.

Liver targeting of angiotensinogen, with either antisense oligonucleotides (ASO) or small interfering ribonucleic acids (siRNA), requires trivalent N-acetylgalactosamine (GalNAc)-conjugation. GalNAc binds to the asialoglycoprotein receptor that is highly expressed on hepatocytes, and this results in rapid endocytosis (Fig. 4).[84] Indeed, GalNAc-conjugation enhances ASO delivery to hepatocytes by ~10-fold vs free ASOs,[85] whereas, in GalNAc-conjugated siRNA-treated animals, hepatic siRNA levels were ~50-fold higher than the levels found in the kidney.[82,83] Given the very long half-life of siRNA, a single siRNA injection has effects that may last 6 months.[86]

The degree of angiotensinogen suppression that can be reached by ASO or siRNA approaches is > 99%.[82,87] Lowering was identical with free and GalNAc-conjugated ASO/siRNA, confirming that circulating angiotensinogen is liver-derived.[88,89] Normally, circulating angiotensinogen levels are in the 1 μmol/L (K_m) range[90]: that is, up to 6 orders of magnitude above the in vivo Ang II levels.[91] This implies that even when lowering angiotensinogen by 99%, its levels are still 4 orders of magnitude above those of Ang II. Thus, substantial angiotensinogen suppression (> 99%) is required truly to lower Ang II. As an alternative, one might add an ARB or ACE inhibitor

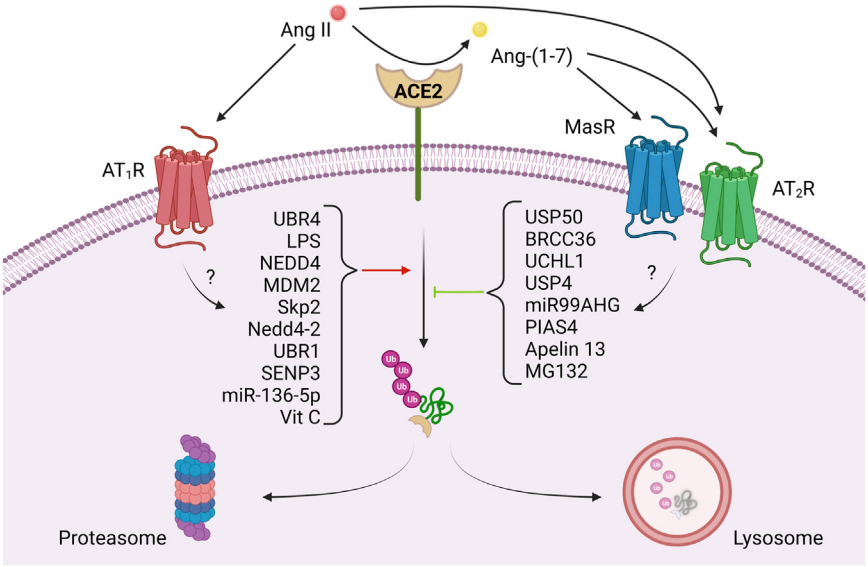

Figure 2. E3 ligases, DUB, and other agents regulating ACE2 ubiquitination. ACE2 ubiquitination leads to internalization and degradation in lysosomes and the proteasome. Several E3 ligases, miRNA, LPS, and vitamin C have been reported to promote ACE2 ubiquitination whereas DUBs, Apelin 13, long noncoding RNA, and MG132 oppose this post-translational modification. With the exception of Nedd4-2, the role of the renin-angiotensin system in these processes has not been elucidated. ACE2, angiotensin-converting enzyme 2; AT_1R, angiotensin II type 1 receptor; AT_2R, angiotensin II type 2 receptor; DUB, deubiquitinase; LPS, lipopolysaccharides; MasR, Mas receptor; Ub, ubiquitin; Vit C, vitamin C.

on top of angiotensinogen siRNA. This would further upregulate renin, thereby accelerating the consumption of any remaining angiotensinogen by renin and leading to the virtual disappearance of Ang II in both blood and tissue.[82]

In SHR, the hypotensive effects of siRNA alone are identical to those of either an ACE inhibitor or an ARB, whereas siRNA plus an ARB resulted in a synergistic drop in blood pressure.[82] Evaluating angiotensinogen suppression in

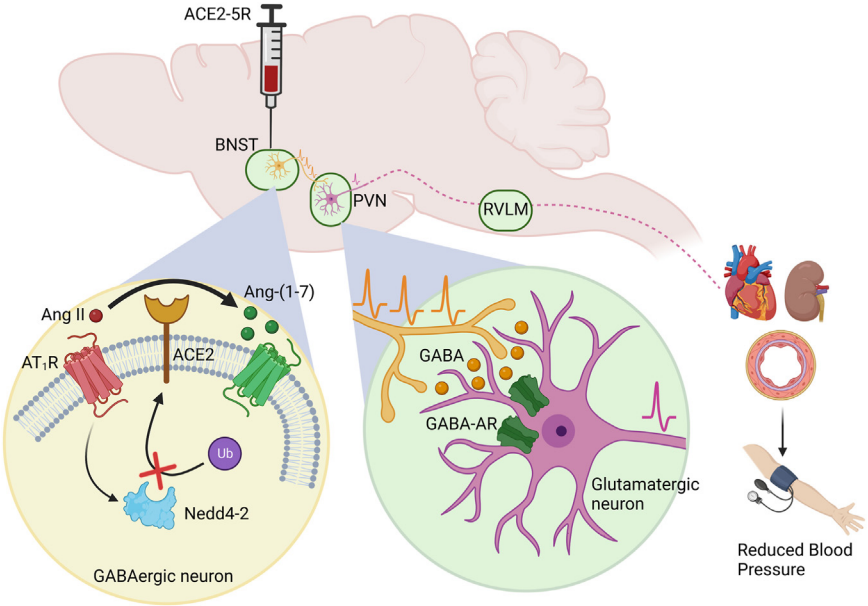

Figure 3. ACE2 ubiquitination in neurogenic hypertension: a working model. ACE2-5R expression in the BNST blocks Ang II-mediated ACE2 ubiquitination by Nedd4-2, thus stabilizing ACE2 compensatory activity in GABAergic neurons and reinforcing the inhibitory tone to PVN glutamatergic neurons. Stimulation of GABA-A receptors in the PVN reduces the excitatory activity of these presympathetic neurons, leading to a reduction of sympathetic drive to end organs, ultimately resulting in a reduction of blood pressure. ACE2-5R, ubiquitination-resistant ACE2; AT_1R, angiotensin II type 1 receptor; BNST, bed nucleus of the stria terminalis; GABA-AR, GABA-A receptor; PVN, paraventricular nucleus; RVLM, rostral ventrolateral medulla; Ub, ubiquitin.

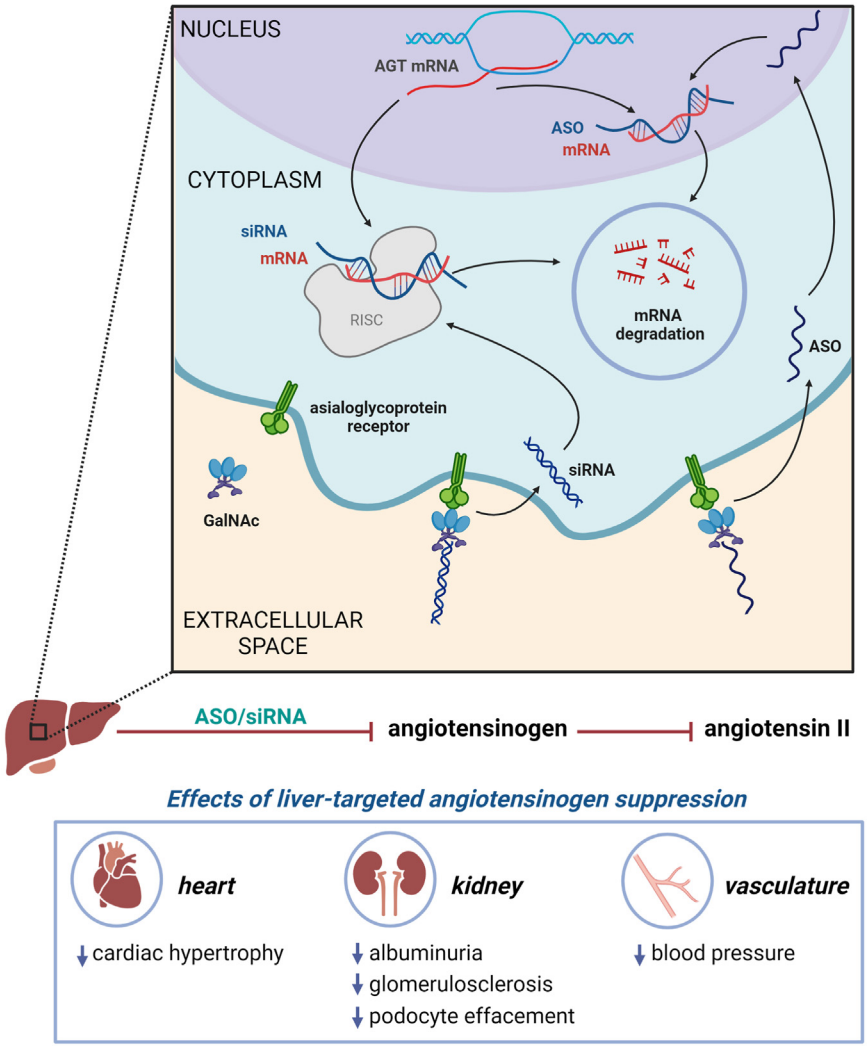

Figure 4. Overview of angiotensinogen (AGT) suppression using antisense oligonucleotides (ASO) or small interference RNA (siRNA). siRNAs, delivered as duplexes, enter the cell and are incorporated into RNAi silencing complex (RISC) in the cytoplasm. The RISC complex with the active guide strand binds the complementary sequence within the target mRNA, resulting in Argonaut 2-mediated cleavage and subsequent angiotensinogen mRNA degradation. Trivalent N-acetylgalactosamine (GalNAc)-conjugation allows high-affinity binding to the asialoglycoprotein receptor. This receptor is highly expressed on hepatocytes and binding results in rapid endocytosis. ASOs are single-stranded oligonucleotides that find their way alone. Liver-targeted angiotensinogen suppression by siRNA or ASO suppresses angiotensin II formation in blood, heart, and kidney, thereby lowering blood pressure and exerting renoprotection and cardioprotection in multiple hypertension-chronic kidney damage models, including the spontaneously hypertensive rat, the 5/6th nephrectomy rat, the deoxycorticosterone acetate-salt rat, and the diabetic TGR(mRen2)27 rat.

low-renin hypertensive models, like the 8% salt-fed SHR,[87] the 5/6th nephrectomy Sprague-Dawley (SD) rat,[81] and the DOCA-salt—treated SD rat,[83] revealed that its modest effects in these models were identical to those of classical RAS blockers: that is, ACE inhibitors and ARBs. In fact, in the case of the DOCA-salt model, no hypotensive effect occurred at all. As discussed under Central APA Inhibition, this is not surprising, as in the DOCA-salt model, systemic renin levels are extremely low.[92]

Importantly, although GalNAc-conjugated angiotensinogen siRNA did not affect renal angiotensinogen mRNA, it did result in an almost complete depletion of renal angiotensinogen in multiple models.[82,93] This strongly supports that renal angiotensinogen is liver derived. Filtered, blood-derived angiotensinogen, such as albumin, is reabsorbed from tubular fluid in the proximal tubules via megalin, a multiligand receptor.[94-96] Disruption of the glomerular filtration barrier increases delivery of angiotensinogen to the tubules and thus enhances generation of kidney Ang II. In fact, when the glomerular barrier was disrupted by inducing podocyte injury, the amount of reabsorbed angiotensinogen was markedly increased concurrently with an increase in urinary angiotensinogen.[79] These data indicate that megalin is an important determinant of generation of renal angiotensin.[97]

Suppressing angiotensinogen in a chronic kidney disease model like the 5/6th nephrectomy rat prevented the development of proteinuria and reduced the occurrence of glomerulosclerosis.[81] Blood pressure and renal Ang II determined

this response. Angiotensinogen ASO decreased kidney size, cyst-volume density, and blood urea levels in mouse models for polycystic kidney disease,[98,99] and this was accompanied by a reduction in fibrosis and inflammation. Similarly, GalNAc-conjugated angiotensinogen siRNA offered renoprotection in diabetic TGR(mRen2)27 rats. These rats overexpress the mouse Ren2 gene and are therefore hypertensive. Streptozotocin-induced diabetes in TGR(mRen2)27 rats resulted in albuminuria, accompanied by glomerulosclerosis and podocyte effacement, with no change in glomerular filtration rate. RAS blockade with an ARB, ACE inhibitor, or angiotensinogen siRNA lowered blood pressure identically, and, when combining these drugs, the effects were—at most—modestly larger. This differs from the synergistic antihypertensive effects of dual RAS blockade observed in the SHR and suggests that the high BP in the TGR(mRen2)27 rat model is the consequence of Ren2 overexpression: that is, it is not caused by the diabetes induction. In other words, as soon as BP is back to normal (by blocking the effects of Ren2 overexpression), more RAS blockade does not yield substantial further lowering of BP. Only angiotensinogen lowered renal Ang I, and all treatments lowered renal Ang II, thereby resulting in comparable lowering of albuminuria. Yet, only angiotensinogen siRNA, with or without an ARB, restored podocyte foot processes and reduced glomerulosclerosis, apparently in a BP-independent manner.

Cardiac hypertrophy often arises as a consequence of high BP. However, as Ang II is an important profibrotic and growth factor, it is also possible that cardiac hypertrophy is caused by local effects of Ang II in a BP-independent manner. Such local formation depends on the uptake of circulating angiotensinogen.[90,100,101] For instance, in DOCA-salt hypertension, angiotensinogen siRNA did not affect BP, yet did reduce the heart weight-to-tibia—length ratio, a marker of cardiac hypertrophy.[83] As this effect was accompanied by the disappearance of cardiac Ang II, this supports the concept that formation of Ang II at cardiac tissue sites depends on hepatic angiotensinogen. Although similar reductions in cardiac Ang II occurred in SHR, 5/6th nephrectomy SD rats and diabetic TGR(mRen2)27 rats, the decrease in cardiac hypertrophy in these models correlated strongly with the decrease in blood pressure, suggesting that it was the consequence of reduction of BP.[81,82,93]

Several Phase I/II angiotensinogen suppression trials are now ongoing, which are expected to be finished over the next 1 to 2 years. Given the liver targeting of the siRNA/ASO approaches, concern has arisen with regard to their liver toxicity or inflammatory and immunologic side effects. However, no such observations were made in patients for inclisiran (a drug using the same GalNAc principle) over a 6-month period.[102] An important question is how far angiotensinogen should go down, considering that too much RAS blockade results in hypotension, hyperkalemia, and kidney dysfunction.[103] After all, a certain degree of RAS activity is required to keep renal function in the normal range,[70] and thus a complete annihilation of the RAS is not desired. Furthermore, although—on the one hand—the long-lasting effects of the ASO/siRNA approach might be considered as an advantage—circumventing adherence problems—it also poses a threat: for instance, in women becoming pregnant during treatment, or in cases of emergency, when severe hypotension occurs, and the RAS is acutely needed. Here, novel tools (REVERSIR, Alnylam Pharaceuticals, Cambridge, Massachusetts, USA) are now being developed, capable of reversing the effect of siRNA.[104] This concerns short, synthetic, high-affinity oligonucleotides, complementary to the siRNA guide strand, which can be targeted to the liver, making use of the same GalNAc approach. Moreover, in rodent models, angiotensinogen siRNA-mediated blood pressure lowering can be rapidly reversed by administration of Ang II or norepinephrine, or gradually reversed by fludrocortisone or high salt intake,[105] suggesting that conventional vasopressors may be an intervention that can address emergency hypotensive episodes.

Conclusions

Novel treatment tools targeting the RAS beyond ACE and the AT_1 receptor are now entering the clinical arena. These might be targeted to specific patient populations (African Americans or patients with obesity: for example, central APA inhibitors), or could be used to overcome adherence problems (long-acting angiotensinogen siRNA). In addition, interference with ACE2 ubiquitination is emerging as a future option for the treatment of neurogenic hypertension. Clinical trials will reveal to what degree these novel drugs might be used individually or on top of existing antihypertensive drugs, including ACE inhibitors and ARBs.

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Ethics Statement

The research reported has adhered to the relevant ethical guidelines.

Patient Consent

The authors confirm that patient consent is not applicable to this article. As no individual personal data or images are provided, patient consent is not required.

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Disclosures

The authors have no conflicts of interest to disclose.

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