Title: Rational Design and Selection of Angiotensinogen-Targeting siRNA Candidates for the Treatment of Hypertension

Abstract: Hypertension is a major risk factor for cardiovascular disease and often requires lifelong treatment, making poor medication adherence a major clinical challenge. RNA interference offers a potential long-acting alternative by suppressing the production of disease-related proteins at the mRNA level. Angiotensinogen (AGT), the liver-derived precursor of the renin-angiotensin-aldosterone system, is a promising target because reducing AGT expression may decrease downstream angiotensin II production and lower blood pressure. In this paper, candidate small interfering RNA sequences targeting human AGT were generated and evaluated using a rational computational screening framework. All possible 21-nucleotide target windows were assessed using sequence-based criteria, including GC content, homopolymer formation, and predicted guide-strand self-pairing. Of 1,411 possible target windows, 178 passed the initial filters, and the highest-ranked candidates had GC contents between 38.1% and 47.6% with no runs of four identical nucleotides. Candidates were prioritized through composite scoring that favored moderate GC content, homopolymer avoidance, and limited predicted intramolecular structure. These results provide a focused set of AGT-targeting siRNA candidates for future evaluation of off-target effects, delivery efficiency, target accessibility, and gene-silencing activity. Because the findings are in silico, experimental validation is required before therapeutic conclusions can be drawn. This framework represents an initial computational step toward developing long-acting RNA interference therapies for hypertension.

Keywords: hypertension, RNA interference, small interfering RNA, angiotensinogen, renin-angiotensin-aldosterone system, computational screening

Introduction

Hypertension is defined as a condition where the force of blood against artery walls is consistently too high, labeled as greater than 140/90 mm Hg. Currently, hypertension is the leading modifiable risk factor for cardiovascular disease and premature death, modifiable meaning that it can be altered or changed. In 2010, a study showed that 31.1% of adults, or 1.39 billion adults worldwide, had hypertension. The leading risk factors of hypertension amount to high sodium intake, obesity, and unhealthy diets[1]. The effects of hypertension are not limited to just health deficits, however, as in 2019 estimated healthcare expenditures due to hypertension were about $219.2 billion, with the number of health care events associated with hypertension at 817.4 million, a majority of those (538.2 million) being prescribed medication visits[2].

One of the ways to stop the formation of angiotensin II, a precursor to hypertension, is an angiotensin-converting enzyme inhibitor (ACE inhibitor). ACE inhibitors block the activation of ACE, which in turn blocks the formation of angiotensin II, which may be abnormally high in many cardiovascular diseases. The generic name for all ACE inhibitors ends with “-pril”; some examples are benazepril, captopril, and enalapril. Currently, ACE inhibitors pose certain limitations[3]. ACE inhibitors are highly effective antihypertensive medication, but their success ultimately depends on their daily use and long-term adherence. Due to the asymptomatic nature of hypertension, patients often disregard the need to utilize their medication daily, leading to an overall increase in health deterioration[4].

In order to reduce the complication of medication nonadherence, RNA interference technology (RNAi) could be utilized. RNA interference is a naturally occurring cellular mechanism that regulates gene expression by degrading specific messenger RNA (mRNA) molecules before they

can be translated into proteins. Small interfering RNAs (siRNAs), which are approximately 21-23 nucleotides in length, are designed to bind to complementary mRNA sequences with a high degree of specificity. Once bound, the RNA-induced silencing complex (RISC) cleaves the targeted mRNA, preventing protein synthesis. Unlike traditional medications that inhibit proteins after they have already been produced, RNAi acts upstream by preventing the protein from being synthesized in the first place. This ability to selectively silence disease-causing genes has led to the development of RNAi therapeutics for several genetic, metabolic, and cardiovascular diseases[5].

Recent advances in RNAi technology have demonstrated significant clinical success, leading to the approval of several RNAi-based therapies by the United States Food and Drug Administration (FDA). These therapies demonstrate that targeted gene silencing can be both effective and clinically safe. The success of these therapies has encouraged researchers to investigate whether the same technology can be applied to more prevalent chronic diseases, including hypertension. Because hypertension often requires lifelong treatment, a therapeutic capable of maintaining blood pressure control for months following a single administration could substantially improve patient adherence while reducing cardiovascular risk associated with inconsistent medication use.

One of the most promising RNAi therapeutics currently under investigation for hypertension is zilebesiran, a small interfering RNA that targets angiotensinogen (AGT), the precursor molecule at the very beginning of the RAAS pathway. Rather than inhibiting angiotensin-converting enzyme as ACE inhibitors do, zilebesiran prevents hepatocytes in the liver from producing angiotensinogen mRNA. Since angiotensinogen is the only precursor from which all downstream angiotensin peptides are formed, reducing its production suppresses activation of the entire RAAS cascade. As a result, less angiotensin II is produced, leading to decreased vasoconstriction, reduced aldosterone secretion, diminished sodium and water retention, and ultimately lower blood pressure. By targeting the most upstream component of the pathway, RNAi has the potential to provide more sustained suppression of RAAS activity than conventional therapies[6].

Effective delivery remains one of the greatest challenges in RNA interference therapeutics because free siRNA molecules are highly unstable and are rapidly degraded by enzymes present in the bloodstream before they can reach their target cells. To overcome this limitation, researchers attach the siRNA to a small carbohydrate-based targeting molecule that is selectively recognized by receptors on liver cells. This allows the therapeutic to be transported directly into hepatocytes, where angiotensinogen is primarily produced, while minimizing uptake by other tissues. Once inside the cell, the siRNA associates with the RNA-induced silencing complex (RISC), a protein complex that recognizes and degrades the target messenger RNA, preventing the production of angiotensinogen. Because a single RISC complex can repeatedly degrade multiple copies of the same mRNA, gene silencing can persist for several months following a single administration[7].

Methods

Within our body, the renin-angiotensin-aldosterone system (RAAS) is one of the key regulators of arterial blood pressure. The pathway utilizes both a change in artery wall rigidity and blood sodium content. In response to decreased blood pressure detected by intrarenal baroreceptors, juxtaglomerular cells are triggered to release renin into the bloodstream. Here, renin converts angiotensinogen, which is continuously produced by the liver, to form angiotensin I, which is

further converted by the angiotensin-converting enzyme (ACE) to form angiotensin II. Angiotensin II triggers multiple functions, including the release of aldosterone from the adrenal gland, which increases sodium and water retention and thereby increases blood pressure, and potent arterial vasoconstriction.

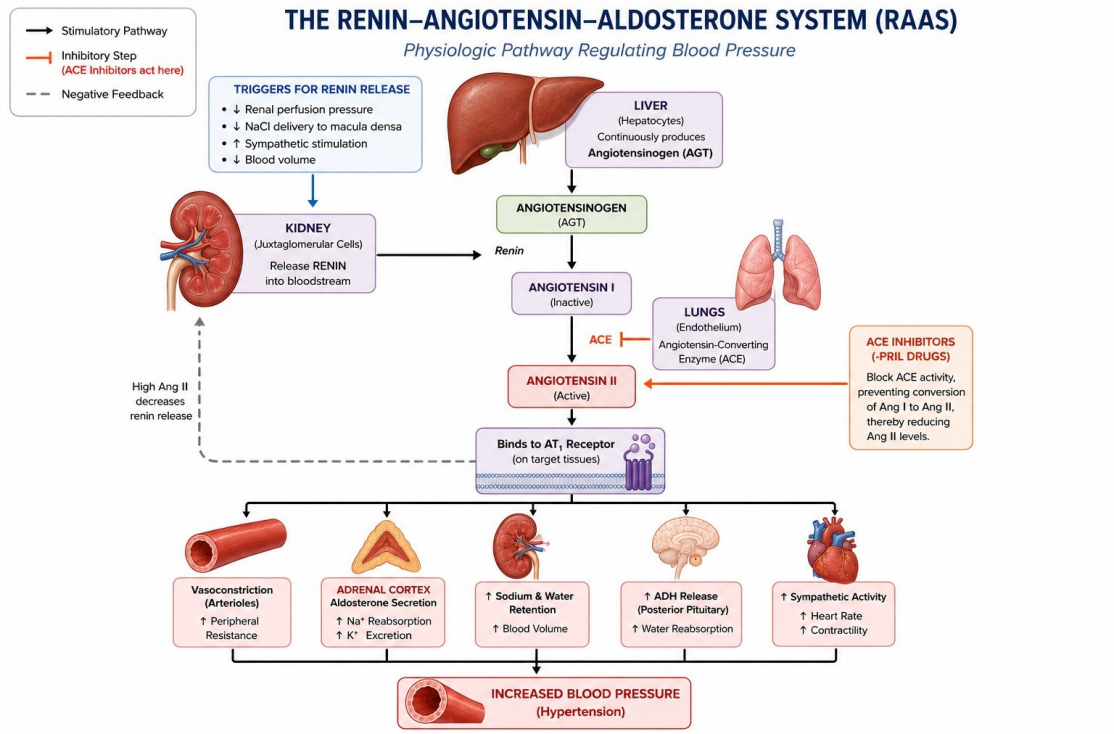

Figure 1 | Overview of the renin-angiotensin-aldosterone system (RAAS) and its role in blood-pressure regulation. The pathway shows renin-mediated conversion of liver-derived angiotensinogen to angiotensin I, ACE-mediated generation of angiotensin II, and downstream effects including vasoconstriction, aldosterone secretion, sodium and water retention, ADH release, and increased sympathetic activity.

As aforementioned, the biggest challenge in using siRNA and the RISC complex to silence genes and treat diseases is the delivery of the siRNA into the intracellular matrix. In order for the siRNA sequence to properly enter the cell without deteriorating from extracellular conditions, lipid nanoparticles, or LNPs, are implemented. LNPs are required for multiple reasons. siRNAs themselves are comparatively large molecules, with 38-50 phosphate groups, and are also highly anionic. This larger size and charge make them unable to cross the phospholipid bilayer effectively compared with small-molecule drugs[8]. LNPs enable the efficient delivery of siRNAs by protecting them from degradation by circulating ribonucleases while simultaneously facilitating their transport across cellular membranes. Encapsulation within LNPs also prolongs circulation time, improves pharmacokinetic stability, and reduces off-target interactions before the siRNA reaches its intended tissue.

The structure of an LNP is carefully engineered to optimize intracellular delivery and typically consists of four primary lipid components: ionizable lipids, phospholipids, cholesterol, and polyethylene glycol (PEG)-lipids. Ionizable lipids play the most critical role by encapsulating the negatively charged siRNA during formulation and promoting its release once inside the target cell. Phospholipids provide structural integrity to the nanoparticle, cholesterol stabilizes the lipid

membrane and enhances particle stability, while PEG-lipids improve circulation time by reducing aggregation and minimizing clearance by the immune system. Following administration, LNPs are internalized into cells through receptor-mediated endocytosis. As the endosome acidifies, the ionizable lipids become protonated, disrupting the endosomal membrane and allowing the encapsulated siRNA to escape into the cytoplasm before degradation can occur. Once released, the siRNA is incorporated into the RNA-induced silencing complex (RISC), where it guides sequence-specific cleavage of complementary messenger RNA, preventing translation of the target protein[9].

Early clinical evidence suggests that inhibiting hepatic angiotensinogen (AGT) with zilebesiran can lower blood pressure for several months after a single subcutaneous dose[5]. In a phase 1 trial published in The New England Journal of Medicine, zilebesiran reduced serum AGT and produced dose-dependent reductions in blood pressure that lasted up to 24 weeks[5]. The phase 2 KARDIA-1 trial found that single doses significantly lowered 24-hour ambulatory systolic blood pressure in patients with mild-to-moderate hypertension[10]. KARDIA-2 showed that zilebesiran could add more reduction in blood pressure when combined with standard drugs such as indapamide, amlodipine, or olmesartan[11]. KARDIA-3 tested higher-risk patients who already took multiple antihypertensive medications and showed a smaller, non-statistically significant reduction in office systolic blood pressure, suggesting that patient selection and background therapy matter[12]. Based on the overall program, the ZENITH phase 3 cardiovascular outcomes trial has begun and will test zilebesiran 300 mg every six months in about 11,000 patients with uncontrolled hypertension and high cardiovascular risk[13].

Results

The RAAS pathway diagram illustrates the position of angiotensinogen in the regulation of blood pressure. Angiotensinogen (AGT) is first produced by hepatocytes within the liver, then cleaved by renin in the bloodstream, produced from juxtaglomerular cells in the kidney, to form angiotensin I. This protein is then cleaved by ACE into angiotensin II. Angiotensin II triggers multiple hypertensive responses, including vasoconstriction, aldosterone secretion, sodium and water retention, antidiuretic hormone release, and increased sympathetic nervous system activity. Due to the sequence of events, RNAi-driven inhibitors are able to limit the formation of angiotensin II and thereby reduce RAAS-controlled responses.

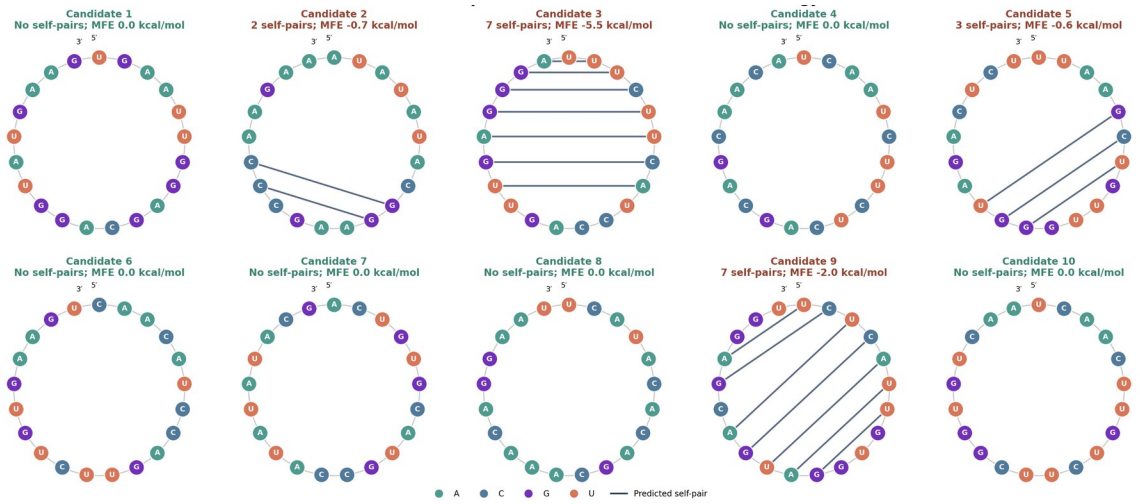

Figure 2 | Predicted guide-strand self-pairing patterns for the top AGT siRNA candidates. Each circle represents one antisense guide RNA sequence, with connecting lines indicating predicted intramolecular base-pairing interactions that could reduce target availability in the computational screen.

The guide-strand self-pairing analysis evaluates the predicted intramolecular structure of the top-ranked AGT siRNA candidates. Each circular diagram represents an antisense guide sequence, with connecting lines indicating predicted internal base-pairing interactions. Extensive self-pairing may reduce the availability of the guide strand for incorporation into the RNA-induced silencing complex or for hybridization with the target AGT mRNA sequence. Therefore, candidates with limited predicted self-pairing are considered more favorable, as their guide strands are more likely to remain accessible for sequence-specific target recognition.

Table 1 | Top-ranked antisense guide RNA candidates targeting human angiotensinogen (AGT).

Rank	Antisense guide RNA (5′-3′)	GC (%)	Homopolymer score	Overall score
1	UGAAUUGGAGCAG GUAUGAAG	42.9	10/10	100.0
2	AUAUAUACGGAAG CCCAAGAA	38.1	10/10	99.6
3	UUUCUUCAUCCAGU UGAGGGA	42.9	10/10	97.4
4	UCAAUCUUCUCAGC AGCAACA	42.9	10/10	96.9
5	UUAAGCUGUUGGG UAGACUCU	42.9	10/10	93.4
6	CAACAUCCAGUUCU GUGAAGU	42.9	10/10	90.4
7	ACUGUGCAUGCCAU AUAUACG	42.9	10/10	89.9
8	UCAUACACAGCAAA CAGGAAU	38.1	10/10	88.6

Rank	Antisense guide RNA (5′-3′)	GC (%)	Homopolymer score	Overall score
9	UCUCAUUGUGGAU GACGAGGU	47.6	10/10	88.3
10	UCAACUUGUCUUCG GUGUCAA	42.9	10/10	86.9

Note: Homopolymer score 10/10 indicates that no run of four identical nucleotides was detected. Overall scores are in silico ranking scores, not experimental efficacy measurements.

The GC-content histogram summarizes the nucleotide composition of all AGT siRNA candidates that passed the computational screening criteria. GC content is an important design parameter because it influences duplex stability, target binding strength, and potential off-target behavior. Candidates with very low GC content may bind the target transcript weakly, whereas candidates with excessive GC content may form overly stable duplexes or increase nonspecific interactions. The concentration of candidates within the preferred GC range supports the suitability of the screened pool for downstream prioritization and experimental validation.

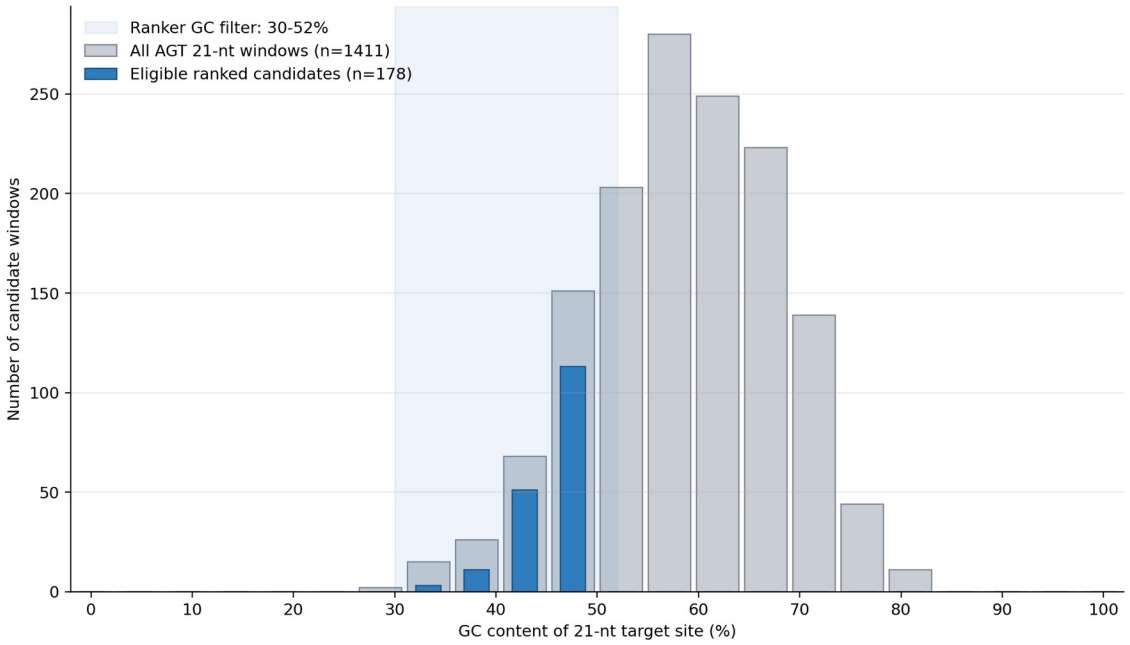

Figure 3 | Distribution of GC content across 21-nt target sites in the AGT coding sequence. Gray bars show all possible AGT 21-nt windows, blue bars show sites passing the initial siRNA candidate filters, and the shaded region indicates the 30-52% GC-content range used for candidate ranking.

Conclusion

This paper presents a rational computational framework for identifying and prioritizing small interfering RNA candidates targeting human angiotensinogen, an upstream regulator of the renin-angiotensin-aldosterone system. By screening all possible 21-nucleotide target windows using sequence-based criteria, including GC content, homopolymer avoidance, and predicted guide-strand self-pairing, I identified a focused set of candidates with properties favorable for further development. The highest-ranked sequences showed moderate GC content, no long homopolymer runs, and relatively limited predicted intramolecular structure, suggesting

improved accessibility for target recognition and incorporation into the RNA-induced silencing complex. However, these rankings represent in silico predictions rather than experimental evidence of gene-silencing efficacy. Future work should evaluate transcriptome-wide off-target effects, thermodynamic strand asymmetry, AGT mRNA target accessibility, transcript-isoform coverage, and population variants within candidate binding sites. Experimental validation in cell-based systems will ultimately be required to measure AGT knockdown, dose response, durability, toxicity, and delivery efficiency. The analysis in this paper provides a systematic starting point for selecting AGT-targeting siRNAs and supports the continued investigation of RNA interference as a potential long-acting strategy for hypertension treatment.

References

1. K. T. Mills, A. Stefanescu, J. He. The global epidemiology of hypertension. Nature Reviews Nephrology. Vol. 16, pg. 223-237, 2020, https://doi.org/10.1038/s41581-019-0244-2.
2. W. J. Elliott. The economic impact of hypertension. The Journal of Clinical Hypertension. Vol. 5, pg. 3-13, 2003, https://doi.org/10.1111/j.1524-6175.2003.02463.x.
3. N. K. Sweitzer. What is an angiotensin converting enzyme inhibitor? Circulation. Vol. 108, pg. e16-e18, 2003, https://doi.org/10.1161/01.CIR.0000075957.16003.07.
4. M. Burnier, B. M. Egan. Adherence in hypertension. Circulation Research. Vol. 124, pg. 1124-1140, 2019, https://doi.org/10.1161/CIRCRESAHA.118.313220.
5. A. S. Desai, D. J. Webb, J. Taubel, S. Casey, Y. Cheng, G. J. Robbie, D. Foster, S. A. Huang, S. Rhyee, M. T. Sweetser, G. L. Bakris. Zilebesiran, an RNA interference therapeutic agent for hypertension. New England Journal of Medicine. Vol. 389, pg. 228-238, 2023, https://doi.org/10.1056/NEJMoa2208391.
6. R. M. Touyz. Silencing angiotensinogen in hypertension. New England Journal of Medicine. Vol. 389, pg. 284-286, 2023, https://doi.org/10.1056/NEJMe2303534.
7. A. S. Menezes Junior, T. H. M. Nogueira, K. B. A. de Lima, H. L. de Oliveira, S. M. Botelho. Exploratory studies on RNAi-based therapies targeting angiotensinogen in hypertension: scoping review. Journal of Personalized Medicine. Vol. 15, pg. 3, 2025, https://doi.org/10.3390/jpm15010003.
8. Y. Dong, D. J. Siegwart, D. G. Anderson. Strategies, design, and chemistry in siRNA delivery systems. Advanced Drug Delivery Reviews. Vol. 144, pg. 133-147, 2019, https://doi.org/10.1016/j.addr.2019.05.004.
9. M. Jeong, Y. Lee, J. Park, H. Jung, H. Lee. Lipid nanoparticles (LNPs) for in vivo RNA delivery and their breakthrough technology for future applications. Advanced Drug Delivery Reviews. Vol. 200, pg. 114990, 2023, https://doi.org/10.1016/j.addr.2023.114990.
10. G. L. Bakris, M. Saxena, A. Gupta, F. Chalhoub, J. Lee, D. Stiglitz, N. Makarova, N. Goyal, W. Guo, D. Zappe, A. S. Desai; KARDIA-1 Study Group. RNA interference with zilebesiran for mild to moderate hypertension: the KARDIA-1 randomized clinical trial. JAMA. Vol. 331, pg. 740-749, 2024, https://doi.org/10.1001/jama.2024.0728.
11. A. S. Desai, A. D. Karns, J. Badariene, A. Aswad, J. M. Neutel, F. Kazi, W. Park, D. Stiglitz, N. Makarova, A. Havasi, D. H. Zappe, M. Saxena; KARDIA-2 Study Group. Add-on treatment with zilebesiran for inadequately controlled hypertension: the KARDIA-2 randomized clinical trial. JAMA. Vol. 334, pg. 46-55, 2025, https://doi.org/10.1001/jama.2025.6681.

12. European Society of Cardiology. KARDIA-3 trial examines blood-pressure lowering effects of zilebesiran in hypertensive patients at high cardiovascular risk. https://www.escardio.org/news/press/press-releases/KARDIA-3-trial-examines-blood-pressure-lowering-effects-of-zilebesiran-in-hypertensive-patients-at-high-cardiovascular-risk/, 2025.
13. Alnylam Pharmaceuticals. Alnylam announces first patient dosed in ZENITH global phase 3 cardiovascular outcomes trial of zilebesiran. https://investors.alnylam.com/press-release?id=29321, 2025.