Novel Protein Kinases Exhibit Distinct Temporal Recruitment Patterns in the Angiotensin II Type 1 Receptor Pathway

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Abstract

Cardiovascular disease remains the leading cause of mortality worldwide, and the Angiotensin II Type 1 Receptor (AT1R) is a major contributor to blood pressure regulation. Current treatment methods that address these diseases involve inhibiting AT1R in its entirety, such as ACE inhibitors or angiotensin receptor blockers (ARBs). Although they inhibit response from AT1R, they cannot distinguish between beneficial signaling and pathological response, resulting in unwanted side effects. In this study, I aimed to identify non-canonical protein kinase interactions with AT1R that could potentially represent new targets for selective modulation of pathways. NanoBiT luciferase complementation assays were used to confirm recruitment of non-GRK kinases to AT1R upon angiotensin II stimulation in HEK293T cells. From an initial screen of over 200 kinases, three candidates were characterized in detail: GSK3β, ZAK, and CKLiK. CKLiK exhibited transient kinetics with early recruitment followed by active dissociation; ZAK demonstrated high variability and no consistent recruitment; and GSK3β displayed gradual, sustained accumulation over 30 minutes. Then, western blot analysis confirmed that AT1R activation regulates GSK3β activity through time-dependent phosphorylation at Ser9, with peak inhibitory phosphorylation occurring at 15 minutes post-stimulation. This inhibitory regulation correlates with the negative recruitment of another enzyme, AKT, which suggests a β-arrestin-mediated scaffolding mechanism involving AKT-dependent GSK3β phosphorylation and PP2A-mediated dephosphorylation. The results demonstrate complexity in AT1R signaling that has not been characterized before, discovering intracellular kinase recruitment as a novel therapeutic target that could have benefits over standard receptor blockers by allowing for selective modulation of pathological pathways and sparing beneficial signaling.

Keywords: AT1R, G protein-coupled receptor, kinase recruitment, biased signaling, NanoBiT, angiotensin II, GSK3β, CKLiK, GRKs, β-arrestin

1. Introduction

Cardiovascular disease remains the leading cause of death globally, with hypertension influencing the lives of over 1.4 billion people worldwide(Mills et al.). Central to the regulation of blood pressure is the Angiotensin II Type I Receptor (AT1R), a G protein-coupled receptor expressed in many organs including the heart, lungs, kidney, and vasculature (Dasgupta and L. Zhang). Activation of the AT1R leads to numerous physiological effects to counteract low blood pressure, most notably increased salt and water retention, and increased vascular tone. Dysregulated activity of this receptor causes hypertension ("high blood pressure"), vascular remodeling, and may contribute to heart failure(Z. Zhang et al.). Given its significant role in cardiovascular pathophysiology, it has been a major therapeutic target for decades, with angiotensin receptor blockers (ARBs) and ACE inhibitors being some of the most widely prescribed medications (Hill and Vaidya). However, some of these drugs affect patients differently and cannot selectively target downstream pathways, which underscores the need for newer therapeutic approaches that target intracellular signaling mechanisms.

AT1R belongs to the G protein-coupled receptors (GPCR) family of receptors, which are the most prevalent cell membrane receptor family in the human body, constituting over 800 different members and being the target of 35% of all FDA-approved clinical drugs(Sriram and Insel). Upon binding of its endogenous ligand angiotensin II (AngII), AT1R activates heterotrimeric G proteins, primarily $G\alpha_{q/11}$, and leads to phospholipase C activation, calcium release, and vasoconstriction (Kamato et al.). Following activation, GPCR kinases (GRKs) phosphorylate intracellular receptor residues, promoting β-arrestin recruitment, which terminates G protein signaling and often leads to receptor internalization (V. V. Gurevich and E. V. Gurevich). This classical desensitization pathway has been well-characterized for AT1R and many other GPCRs. In addition to desensitization, β-arrestin can activate additional signaling pathways. This has led to the concept of biased signaling, in which ligands at the same receptor can preferentially activate different pathways and yield different cellular and physiological outcomes (Wisler et al.).

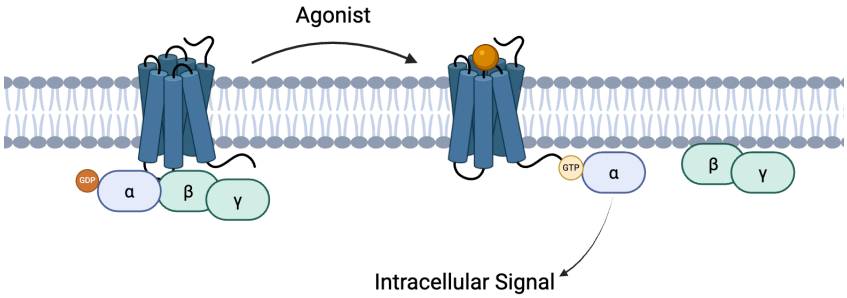

Figure 1: GPCR signaling at the plasma membrane. Agonist binding to the receptor, heterotrimeric G proteins dissociating. GDP being replaced by GTP. G_a causing intracellular signaling. Graph created by the student researcher using BioRender, 2025.

Previous drugs targeting this receptor, specifically ACE inhibitors or AT1R blockers, generally sought to reduce activation of the receptor by AngII (Hill and Vaidya). While effective in many patients, these approaches do not distinguish between beneficial and pathological downstream pathways and cannot address the intracellular mechanisms that may contribute to disease progression without the receptor. Targeting intracellular GPCR interactions offers several potential advantages over traditional ligands that bind to extracellular interactors. The therapeutic promise of biased signaling lies in preserving beneficial signaling while blocking pathological responses. Deducing intracellular mechanisms that are prevalent to interaction with AT1R can be used to find novel targets that may be safer and more efficacious overall.

However, GPCR signaling complexity extends far beyond canonical G protein and GRK/β-arrestin pathways. Studies have shown that numerous non-GRK protein kinases also bind to GPCRs and can play roles beyond desensitization (Nürnberg et al.). These kinases may phosphorylate residues on the C-terminal tail of GPCRs, participate in signal modulation, and contribute to biased signaling where interacting proteins stabilize distinct receptor conformations that activate different downstream pathways. For AT1R specifically, while GRK-mediated regulation has been well-characterized, the full complement of kinase interactors and their functional contributions to AngII signaling remain largely unexplored. This represents a significant knowledge gap, as these interactions may reveal novel regulatory mechanisms and therapeutic targets relevant to cardiovascular disease.

To systematically identify novel kinase interactors of AT1R, a preliminary screen of 200+ protein kinases was performed using NanoBiT luciferase complementation assays, which is a proximity-based technique that detects protein-protein interactions in real-time (Dixon et al.). After being stimulated with AngII, approximately 16 kinases showed variable magnitudes and kinetics of recruitment to AT1R. From this, three non-canonical kinases with diverse biological functions and potential cardiovascular relevance were selected: GSK3β (glycogen synthase kinase 3 beta), ZAK (mitogen-activated protein kinase kinase kinase 20), and CKLiK (Calcium /Calmodulin-Dependent Protein Kinase Type 1D). These kinases were compared with GRK3, a well-studied AT1R regulator that is involved in classical β-arrestin recruitment, which was used as a positive control.

GSK3β is particularly interesting due to its well-known role in cardiovascular physiology. This serine/threonine kinase is involved in the hormonal control of glucose homeostasis and the Wnt signaling pathway, which is involved in regulating glycogen synthase and its downstream actions. In the cardiovascular system, GSK3β activity is dysregulated during heart failure, and its inhibition reduces cardiomyocyte apoptosis and pathological remodeling in animal models (Lal et al.). GSK3β also regulates inflammatory responses in endothelial cells and modulates vascular smooth muscle cell proliferation (Guha et al.). Considering that chronic AT1R activation in involved with pathological cardiac hypertrophy and vascular remodeling, a direct recruitment of GSK3β to AT1R points to the possibility of a more dynamic AT1R-dependent regulation of GSK3β-mediated functions in metabolic and cardiovascular signaling.

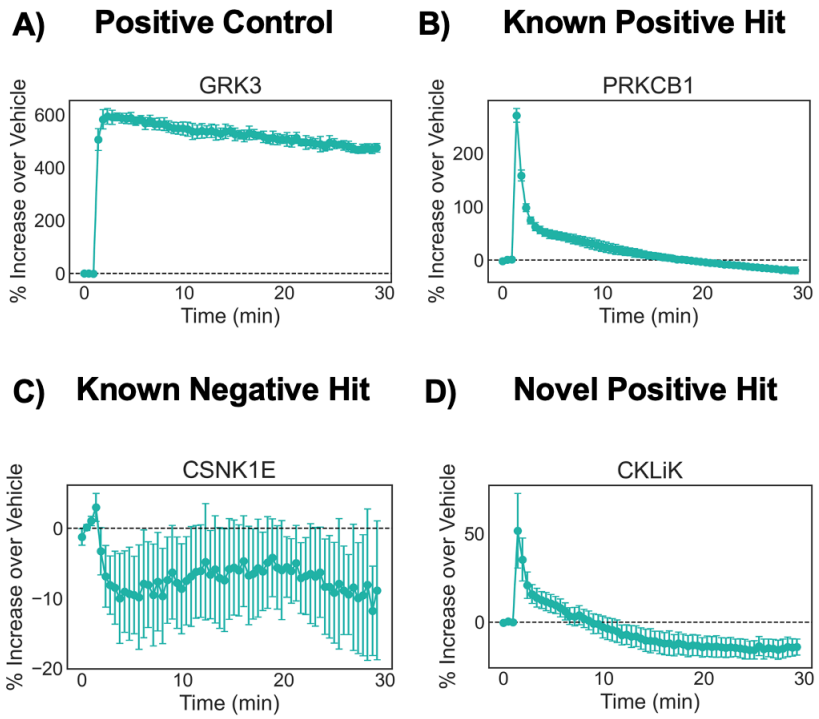

Figure 2: Initial NanoBiT screening identifies candidate kinase interactors with AT1R. Traces from a screen of 200+ kinases in HEK293T cells. A) Positive Control – GRK3 shows rapid, sustained recruitment. B) Known Positive Hit – PRKCB1 has a large initial spike, shows transient interaction. C) Known Negative Hit – CSNK1E remains at baseline with large error bars. D) Novel Positive Hit – CKLiK displays large initial spike. Values normalized to baseline values pre-stimulation. Error bars are ±SEM (n=3). Figure created by Austin Lai, 2025

ZAK is a MAP3K that responds to DNA damage and oxidative stress and has been shown to promote programmed cell death (MAP3K20, NCBI). AngII is known to induce oxidative stress in vascular cells and can promote both proliferation and apoptosis of vascular smooth muscle cells, depending on context, contributing to vascular remodeling in hypertension. A physical interaction between ZAK and AT1R could provide a direct mechanism linking AngII receptor activation to stress response pathways. On the other hand, CKLiK belongs to the calcium/calmodulin-dependent protein kinase 1 family, which is involved in the calcium-dependent protein kinase cascade. Additionally, it has shown involvement with granulocyte regulation and aldosterone synthesis (CAMK1D, NCBI). If proven to directly interact with AT1R, further research can be done on the renin-angiotensin-aldosterone system (RAAS), which has great implications in blood pressure (Fountain et al.).

Taking these observations into account, I hypothesized that non-GRK kinases recruit to AT1R in a

differentially regulated manner that is controlled by the temporal dynamics after stimulation with the ligand. To test this hypothesis, I pursued three different aims: to validate the results of the initial kinase screening, study the time-dependent dynamics of GSK3β recruitment following receptor activation, and propose the regulatory mechanisms that control this interaction over time.

2. Methodology

2.1 NanoBiT Luciferase Complementation Assays

To assay for recruitment of kinases to AT1R, we performed a NanoBiT assay, which is done with two components, a large bit (LgBiT) and a small bit (SmBiT), that are paired to our structures of interest. In our experimentation, we had our kinase of interest paired with our LgBiT, and our receptor, AT1R, paired with the SmBiT. In this assay, when the kinase is recruited to the receptor after activation with AngII, the SmBiT and LgBit will click together, reconstituting a full luciferase enzyme. Specifically, we can discern that if the bits click together, there must be some form of interaction as they will only reconstitute if they are within 10 nm of each other, a negligible distance considering the various other structures present. Figure 3 shows the basic workflow of the NanoBiT assay.

We first transfect our DNA constructs with our LgBiT and SmBiT into HEK293T cells. This cell line, derived from human embryonic kidney cells, does not endogenously express AT1R with the SmBiT required for the assay. In our transfection, we use polyethyleneimine (PEI), a positively charged polymer, to coat our negatively charged DNA constructs, which can then be readily taken up by the plasma membrane, which is also negatively charged. PEI is highly toxic, so confluency of the cell culture must be considered, with 40-60% resulting in optimal transfection efficiency. Low confluence increases toxicity-induced cell death, and high confluence reduces total surface area available to take up PEI.

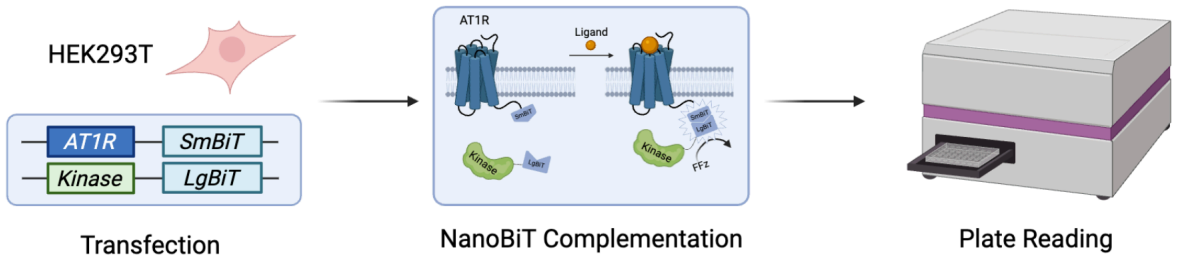

Figure 3: Schematic of the NanoBiT complementation assay. AT1R fused to SmBiT and kinase of interest fused to LgBiT. After stimulation with AngII, kinase recruitment within 10 nm proximity reconstitutes functional nanoluciferase, oxidizing fluorofurimazine (FFz) to emit luminescence. Proximity-based assay allows real-time monitoring of interactions. Image created by the student researcher using BioRender, 2025

In our transfection protocol, we first aspirate and then re-media the cells with warm MEM (Minimum Essential Medium) containing 10% Fetal Bovine Serum (FBS) and 1% Pencillin/Streptomycin (P/S), placing them back in the incubator as we set up the DNA constructs in Eppendorf tubes. We mix OptiMEM, a reduced cell culture medium, with pcDNA, AT1R-SmBiT, and Kinase-LgBiT constructs. We then prepare our PEI/OptiMEM mixture, which acts as a dilution agent for the DNA/OptiMEM. This incubates for 5 minutes at room temperature. Then, we mix the DNA/OptiMEM with the PEI/OptiMEM at a 1:1 volumetric ratio, incubating for 30 minutes and then adding to our freshly remediated cells.

24 hours after transfection, the cells are ready to be plated from the 6-well plate to the 96-well plate, which will be read before and after stimulation with AngII. Before proceeding, it is important to ensure that the cells have grown despite the addition of toxic PEI, and if confluency is higher than before transfection, plating is done. After aspiration of each well, we add 0.5mL PBS to each well to wash out any residual amounts of old MEM, aspirating to make sure the cells are only encountering new media. Then, to lift the cells for transfer to conical tubes, we add 0.5mL of trypsin to each well, which causes the cell colonies to lift off the bottom of each well. After incubation for 3 minutes, 2mL of new MEM with 10% FBS and 1% P/S is added to neutralize the trypsin and decrease cleaving of the residues. Wells are then washed to ensure all major cell groups have lifted and are able to be transferred. After transfer of each well to a different 15mL conical, we centrifuge the tubes at 1000 RPM for 4 minutes. During this time, we prepare the plating media, which contains no FBS but has 1% P/S, and label the 96-well plates that the cells will be plated in. After centrifugation, the media in the conical tubes is aspirated, ensuring that the cell pellet is undisturbed at the bottom. Cells are suspended in plating media. Approximately 100,000 cells per well was seeded. Once plating is done, the plate is placed back in the incubator at 37°C to incubate for 24 hours.

For plate reading, we begin by preparing 3.13μM fluorofurimazine (FFz) in Hank's Balanced Salt solution (HBSS++) supplemented with Ca^{2+}, Mg^{2+}, and 20mM HEPES. In the NanoBiT assay, the FFz is oxidized by the reconstituted NanoLuciferase enzyme, and gives off a quantifiable measure of light, so the amount oxidized is directly proportional to the number of LgBiT and SmBiT that reconstitute. The cells are then taken out of the incubator and the plating media is aspirated. For optimal measurement, a white sticker is added to the bottom of the plate. Then, 80μL of FFz is added to each well, and incubated for 30 minutes.

During this period, the AngII, which is our ligand, is prepared. To perform a dose-response curve for each kinase and map recruitment to the receptor at different doses, we must perform a serial dilution, starting with 5×10^{-6}M as our maximum dose. To get this, 5μL of AngII ligand is diluted in 995μL HBSS++. In a ligand tray, 230μL of AngII, which has been diluted to 5×10^{-6}M is added to the top row. The middle six rows have 180μL HBSS++ added to each well. Then, 20μL is transferred down from each row to the HBSS++, causing a serial dilution of tenfold each time, making the lowest dose 5×10^{-12}M. The last row is vehicle, having no AngII, and serves as a baseline value for comparison. Figure 4 shows the ligand tray in

which the serial dilution was performed. Based on these results, 5×10^{-6}M AngII was used for all time-course experiments.

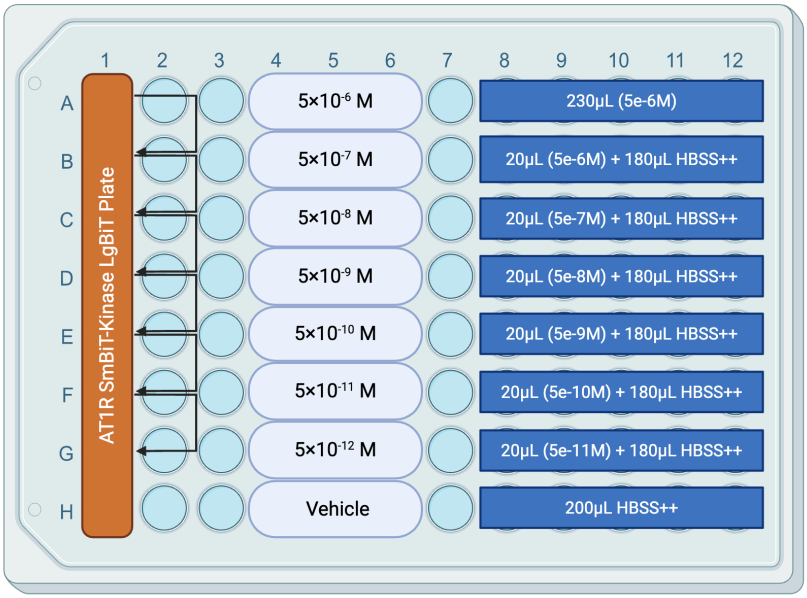

Figure 4: Serial dilution protocol for AngII dose-response assays. Schematic of 10-fold dilution in a 96-well ligand tray. Starting with 5×10^{-6}M AngII in top row (230μL). 20μL transferred downward to achieve final low dose of 5×10^{-12}M. Bottom row is vehicle, with 200μL of HBSS++ only. Enables dose-response curves in NanoBiT assays. Image created by the student researcher using BioRender, 2025.

Before we can add the ligand to our cells, we must prepare the plate reader for prereading, which gives us relative values of luminescence of each kinase construct, helping us to compare the change prior and post-stimulation. The Mithras LB 940 Multimode Microplate Reader by Berthold Technologies and MikroWin software are used to read our plate, providing values for each of the 96 wells. After incubation is complete, the plate is brought to the Berthold and three prereads are taken. Then, rapidly, 20μL of AngII at the serially diluted doses is added to each of the wells of the 96-well plate. 60 post-reads are taken over approximately 30 minutes.

2.2 Starvation, Stimulation, and Lysis of AT1R293 cells

For us to track GSK3β recruitment and phosphorylation temporally after activation, we perform a stimulation protocol in which we first starve our cells, stimulate them with our ligand, and then lyse them to be collected and used in a Western Blot. First, starvation media that the cells will be put in is prepared. 49.25mL of MEM and 1% P/S is mixed with 250μL of 100mM HEPES, which is an organic buffering agent that maintains pH from 6.8 to 8.2, and 500μL of BSA (bovine serum albumin). Then, we must make our RIPA (Radioimmunoprecipitation Assay) buffer, which is a cell lysis buffer that is used to extract proteins from a solution. 2850μL of the RIPA buffer is added to 300μL of PhosStop and 120μL

of cOmplete. PhosStop is a phosphatase inhibitor, which are proteins that work in reversing the effects of kinases, which are undesirable when wanting to determine temporal kinetics. On the other hand, cOmplete is a protease inhibitor that works to eliminate the catalysis of residues that may be involved in GSK3β recruitment.

Because HEK293T cells do not endogenously express AT1R, AT1R293 cells, which stably express AT1R, are used to ensure consistent AT1R expression across replicates. 100 nM AngII was diluted in starvation media. The aspiration of ligand-containing media is done at three different times after initial stimulation with AngII: 5, 15, and 30 minutes. All conditions are collected at the same time by aspiration of media and lysis with 100μL of the RIPA buffer per well. Cells were immediately put on ice to incubate for 15 minutes. Using a cell scraper, each lysate is collected and sonicated 5 seconds per pulse for three pulses with at least 1 minute of rest on ice in between each pulse. The tubes are then centrifuged at 17,000 RPM for 15 minutes, and the supernatant is transferred to a new tube then placed in the freezer at -20°C.

2.3 Western Blot

To measure GSK3β phosphorylation, we performed a Western Blot on each of the lysates collected. SDS samples with normalized protein concentration were made. Each sample was then boiled at 100ºC for 10 minutes, followed by a 1-minute cooling at room temperature and centrifugation to collect all the fluid at the bottom of the tube. We loaded 15μL of ladder, which acts as a reference to help us identify the molecular weight of the protein in kDa after imaging. Each sample is added accordingly. The gel is then run at 90 volts for two hours. Transfer is done using the TransBlot ® Turbo™ transfer system, run at 25 volts and 1.0 amperes for 30 minutes. To prevent non-specific antibody binding, the membrane is blocked with 5% BSA in TBS-Tween for 1 hour on an orbital shaker at room temperature. Afterwards, the 5% BSA solution is removed, and the membrane is incubated with the primary antibody solution overnight at 4C on a shaker. The primary antibody solution was prepared by performing a 1:1000 dilution of antibody that recognizes phospho-GSK3α (Ser21) and phospho-GSK3β (Ser9) and total GSK3β in 5% BSA/TBS-Tween. The primary antibodies specifically recognize and bind to GSK3β proteins on the membrane.

After 24 hours, the primary antibody solution is removed, and the blot is washed with TBS-T on the orbital shaker at room temperature. These remove any primary antibody that could cause background noise. Then, we prepare the secondary antibody, which is 5% milk in TBS-T, by using a Vortex Mixer to mix 2mL dry milk with 40mL of TBS-T. This is then centrifuged at 3000 RPM for 4 minutes. The supernatant is used to dilute secondary antibody at 1:3000 ratio. The membrane is incubated in the secondary antibody for 1 hour, washed with TBS-T. We mixed Pico substrate in a 1:1 ratio and incubated the blot in this solution for 2-3 minutes. During this time, the horseradish peroxidase catalyzed oxidation of the substrate to produce chemiluminescent light. Membranes were imaged using the BioRad ChemiDoc™ Imaging System with exposure times ranging from 10 to 60 seconds to capture both weak and strong signals without saturation.

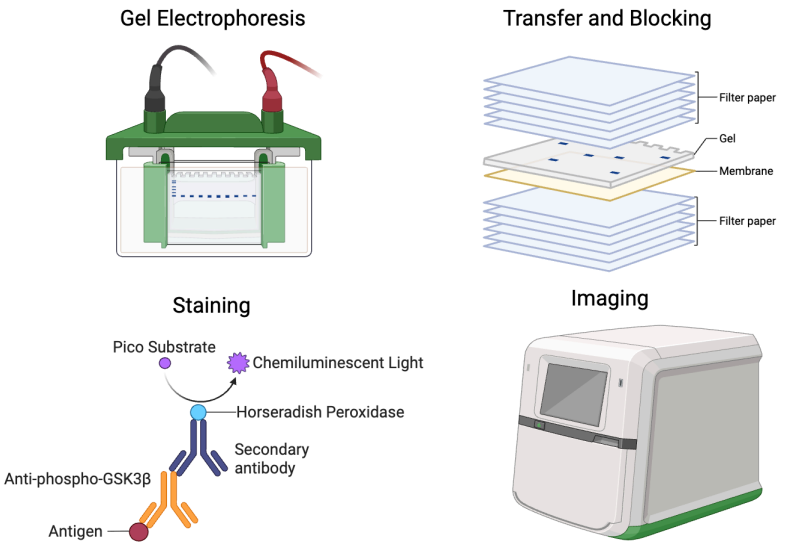

Figure 5: Schematic of the full Western Blot protocol for GSK3β. A) Gel Electrophoresis – Samples in 1x SDS buffer, run at 90 V for 2 hours. B) Blocking – Proteins transferred to PVDF membrane (25 V, 1 A, 30 min), blocked in 5% BSA/TBS-T for 1 hour. C) Staining – Incubated with anti-pSer9-GSK3β primary antibody overnight, then 5% milk in TBS-T. Pico substrate oxidized by HRP to give off light. D) Imaging – Chemiluminescence recorded by imaging system from 10 – 60s. Image created by the student researcher using BioRender, 2025.

2.4 Data Analysis

Data from the plate reader were processed as follows: Pre-reads and post-reads were compiled in Microsoft Excel. Post-read values were normalized to the average of the three pre-reads by dividing each post-read value by the mean pre-read value. The percent luminescence change was then calculated according to the following formula:

$$\% \text{ Change} = \left(\frac{\text{Normalized Post-read} - \text{Vehicle Control}}{\text{Vehicle Control}} \right) \times 100$$

This normalization accounts for variability between wells and enables comparison across all kinases and experimental conditions. All NanoBiT experiments included at least three technical replicates, and data is presented as mean ± SEM. Detailed statistical comparisons are being withheld pending publication of the broader kinase screen data.

To quantify total kinase recruitment over the measured period, area under the curve (AUC) analysis was performed using GraphPad Prism. Time-course plots were also generated in Prism. All results are

presented as mean ± standard error of the mean (SEM).

For western blot analysis, densitometry was performed for the GSK3β Ser9 immunoblot and normalized to GAPDH. Normalized signal is expressed as fold-change over vehicle control. Western blot data shown is representative of two independent experiments; additional replicates are in progress.

3. Results

3.1 CKLiK and GSK3β Recruit Distinctly to the Angiotensin II Type 1 Receptor

To characterize the kinetics of kinase recruitment to AT1R after stimulating with AngII, I performed time-course experiments using the NanoBiT luciferase complementation assays. HEK293T cells overexpressing AT1R-SmBiT and a kinase-LgBiT were stimulated with AngII. The luminescence was continuously monitored for 60 post-reads. Four kinases were examined in detail. One of them, GRK3, is known to phosphorylate AT1R, and served as our positive control.

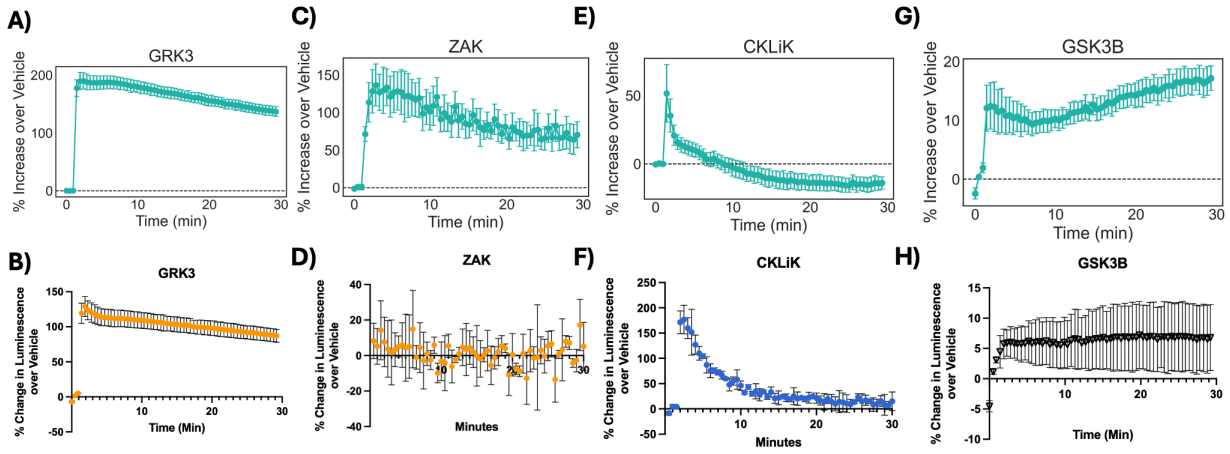

Figure 6: Cells co-expressing AT1R-SmBiT and kinase-LgBiT stimulated with variable amounts of AngII. Luminescence normalized to pre-stimulation baseline values. (A, B): GRK3 (C, D) – ZAK (E, F) – CKLiK (G, H) - GSK3 . Top row shows initial screening data. Bottom row is validation data. Bottom row is Mean ± SEM (n=3). Figure created by the student researcher using GraphPad Prism, 2025.

These kinases showed very different temporal recruitment patterns. GRK3, which is already known to interact, showed rapid and sustained recruitment, with the percent change in luminescence increasing 200% over vehicle within the first minute of stimulation. This remained elevated throughout the 30-minute period. This pattern is consistent with its established role in receptor phosphorylation, G-protein signaling desensitization, and arrestin recruitment.

On the other hand, initial screen completed in the lab showed ZAK had high initial recruitment

over vehicle reaching approximately 150% within 2-3 minutes. However, in subsequent efforts to validate these findings, ZAK showed no significant recruitment. We can conclude that ZAK does not recruit to AT1R under the conditions of stimulation that we experimented.

CKLiK displayed a strong recruitment pattern. After an initial spike to 50-70% over vehicle within the first minute, it was followed by a complete loss of recruitment. Specifically, by 5 minutes after stimulation, luminescence was at baseline, and by 15 minutes, it was completely under baseline at almost -20%. The loss of response throughout suggests that CKLiK is not just dissociated from AT1R but may be actively released as the signal progresses.

Interestingly, GSK3β had slight initial recruitment as compared to GRK3 to about 15% over vehicle, followed by a sustained accumulation throughout the 30 minutes. Throughout the entire postread period, GSK3β remained recruited to AT1R. The gradual nature of this kinase increasing in luminescence is very distinct from the immediate kinetics that are characteristic of GRKs. Given that AT1R at the plasma membrane is rapidly internalized, the sustained recruitment of GSK3β to AT1R may suggest the possibility of a role in mediating AT1R-dependent endosomal signaling.

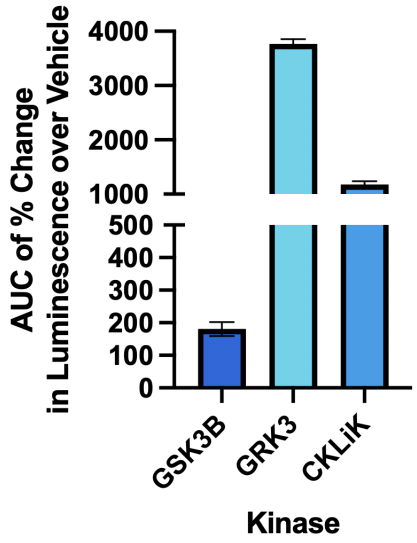

Figure 7: Total recruitment of kinases to AT1R, measured by area under the curve (AUC) of total luminescence. GRK3 (positive control) - 4 times more recruitment than CKLiK. CKLiK – 6 times more recruitment than GSK3 . GSK3 – very little total change in luminescence, suggesting that only few are involved in the gradual accumulation of recruitment. Mean ± SEM (n=3). Graphic generated using GraphPad Prism, 2025

To quantify the total recruitment of the kinase to AT1R over the time-course, an area under the curve (AUC) analysis was performed on the data. Consistent with GRK3's role in GPCR regulation, the positive control exhibited the highest total recruitment, with the total AUC value being approximately 4-

fold higher than CKLiK. On the other hand, CKLiK showed immediate total recruitment. With an AUC approximately 6-fold higher than the GSK3β values, we can see how much the initial spike in the recruitment of CKLiK contributes to the high total luminescence.

3.2 GSK3β Exhibits a Time-Dependent Response to Angiotensin II Stimulation

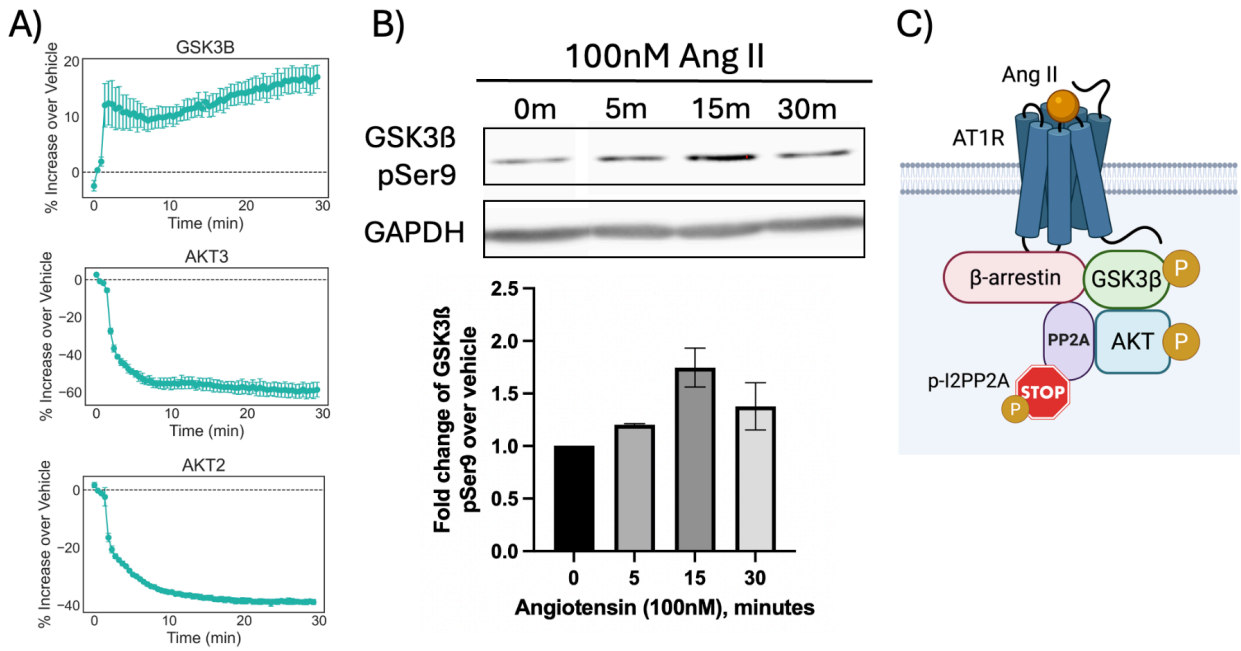

Figure 8: Dynamics of GSK3β and AKT recruitment to AT1R, validated by Western Blot. A) NanoBiT time-course recruitment in HEK293T cells. GSK3β shows gradual accumulation (15% over vehicle by 30 min). AKT3 and AKT2 show no significant association. Error bars: mean ± SEM (n=2). Data from initial kinase screening. Graphs made using GraphPad Prism, 2025. B) Western Blot of phosphor-Ser9-GSK3β (pSer9) and total GSK3β in cells stimulated with AngII for 0, 5, 15, and 30 min. GAPDH used as loading control. Densitometry - pSer9 normalized to total GSK3β. Phosphorylation peaks at 15 min at 1.7-fold of vehicle, declines to 1.3-fold by 30 min. Mean ± SEM (n=3). Graphics made using GraphPad Prism, 2025. C) Proposed Mechanism – AngII results in β-arrestin scaffold and transient AKT activation. This causes Ser9 phosphorylation and inhibits the accumulation of GSK3β. p-I2PP2A inhibits PP2A to sustain phosphorylation of pSer9-GSK3β. Graphic created by the student researcher using BioRender, 2025.

From the sustained recruitment of GSK3β observed in the NanoBiT assays, I validated this interaction biochemically and determine whether AT1R activation regulates GSK3β . The activity of this kinase is negatively regulated by phosphorylation at serine 9 (Ser9), so western blot analysis was performed on lysates stimulated with AngII for 0, 5, 15, and 30 minutes.

The western blot showed a time-dependent increase in GSK3β following AngII stimulation. At 0 minutes, pSer9-GSK3β was barely detectable and had no significant results. 5 minutes after stimulation, however, pSer9-GSK3β values increased, with the highest signal at 15 minutes, followed by a slight decline by 30 minutes. GAPDH, which was our loading constant, remained constant to confirm that equal amounts of protein were loaded. Densitometry analysis in Figure 8B showed that phosphorylation increased by 1.2-fold at 5 minutes, 1.7-fold at 15 minutes, and 1.3-fold at 30 minutes as compared to control. As GSK3β is phosphorylated increasingly at Ser9 as time goes on after stimulation, it can be concluded that agonist binding to AT1R has an inhibitory effect on the function of GSK3β via increased phosphorylation.

GSK3β is known to be a part of an arrestin-mediated signaling complex that includes protein phosphatase 2A (PP2A) and AKT kinase (also known as protein kinase B [PKB]) (Beaulieu et al.). This complex has been a well studied in the context of dopamine D2 receptor (D2R) signaling, another GPCR. In addition, previous work has characterized this in the context of AT1R signaling. To begin to dissect the kinetic recruitment of different members of this complex, we revisited the initial recruitment data for AKT kinases to see how their recruitment correlated with GSK3β. These kinases have been shown to phosphorylate GSK3β at Ser9. Both AKT2 and AKT3 showed no significant recruitment to AT1R.

Figure 8C illustrates the current model of where β-arrestin mediated complex formation with GSK3β, AKT, and PP2A in AT1R signaling. Our data points to multiple interesting questions regarding the subcellular location and timing of AKT recruitment. The sustained increase with the pSer9-GSK3β shows a global reduction of GSK3β activity over time. Given previous data suggesting GSK3β association with β-arrestin, the temporal dynamics of GSK3β recruitment and activity may pose a mechanism of spatial control of signaling. However, more experiments will need to be done to rigorously test this working model.

4. Discussion

Overall, the results not only validated recruitment to the protein kinases but suggested key downstream effects and time-dependent recruitment and phosphorylation. Specifically, GSK3β had sustained recruitment for 30 minutes after stimulation, gradually increasing. Further analysis showed that the phosphorylation of pSer9-GSK3β was also time-dependent, and this coincided with the negative recruitment of known kinases AKT from AT1R. On the other hand, CKLiK recruited robustly after stimulation and rapidly decreased over time, going against the current pattern of GRK3, which has a slower rate of decrease. However, ZAK showed no directional recruitment and had very variable data regardless that it showed initial recruitment in the screen.

4.1 GSK3β Connects Cardiovascular Pathology to AT1R

The gradual recruitment of GSK3β to AT1R represents a non-canonical GPCR interaction pattern. Unlike GRK3, which shows rapid and very immediate recruitment in playing its role in desensitization and

β-arrestin recruitment, GSK3β accumulates over 30 minutes, suggesting that it is likely regulated by more than one pathway or complexes rather than just receptor binding. The temporal delay may be a result of β -arrestin scaffolding, which has shown to result in signaling pathways with the same kinases after internalization (V. V. Gurevich and E. V. Gurevich).

AngII stimulation increases the amount of Ser9 that is phosphorylated on GSK3β, therefore inhibiting its function. GSK3β phosphorylation at Ser9 stops working against cardiac hypertrophy, and therefore, inhibitory phosphorylation of the kinase is shown in weak hearts. Additionally, current research describes active GSK3β to be involved in promoting cardiomyocyte apoptosis, going against its cardioprotective function when inactive (Matsuda et al.). As shown in the results, a 1.7-fold increase in the amount of phosphorylation observed 15 minutes after being treated with AngII shows that AT1R is directly involved in the regulation of GSK3β activity as part of cardiac hypertrophy.

Hypertrophic stimuli that are extracellular, including AngII, work through increasing Ca^{2+} and activating AKT to inhibit GSK3β. I believe that because of β-arrestin scaffolding, this interaction between AT1R and the kinase occurs. Specifically, AKT is transiently in proximity with AT1R where it is activated, shown by its initial association followed by dissociation when AngII stimulates. The activated AKT phosphorylated GSK3β at Ser9, which is demonstrated by the sustained recruitment. The gradual accumulation of GSK3β reflects the multi-step process from activation to phosphorylation.

Another important consideration is the fact that the phosphorylation of Ser9 on GSK3β peaks at 15 minutes and partially declines by the 30-minute mark, despite the continuous presence of AngII. These mechanisms suggest that there must be phosphatase activity to work against AKT-mediated phosphorylation. Specifically, PP2A interacts with and dephosphorylates GSK3β at Ser9, with the activity negatively correlating with the amount of Ser9 phosphorylated. The interaction of PP2A and GSK3β is dynamic, and GSK3β has been shown to complex with PP2A at baseline (Guha et al.).

The possible involvement of PP2A and possibly its regulatory proteins such as I2PP2A, which is an inhibitor of PP2A that is phosphorylated downstream of GPCRs, could have many pathological implications. For example, PP2A is shown to negatively regulate hypertrophic effects in the heart by preventing phosphorylation on histone deacetylase 2 (Yoon et al.). However, this new interaction could lead to development of a novel pipeline for drugs targeting cardiac hypertrophy. Specifically, GPCR agonist activation results in I2PP2A phosphorylation, which consequently inhibits PP2A activity when it binds to it. If AT1R activation is similarly involved with I2PP2A, the mechanism for suppressing PP2A activity immediately after stimulation would result in pSer9-GSK3β accumulating. As the stimulus decreases over time, I2PP2A dephosphorylated and PP2A is released to bring back GSK3β activity.

The AUC analysis for GSK3β provides additional insight into the nature of recruitment. First, GSK3β may interact with AT1R indirectly through scaffolding proteins such as β-arrestin rather than just

direct binding. The overall low luminescence may be due to the intervening protein complexes that interfere with the SmBiT and LgBiT.

4.2 CKLiK Exhibits Transient Recruitment and Actively Dissociates from AT1R

On the other hand, CKLiK had a very distinct biphasic response after stimulation. First, it showed rapid recruitment at about 50-70% above baseline levels within one minute, followed by complete dissociation and decrease of the signal, declining below baseline levels in 15 minutes. The initial spike and change to dissociation from the receptor suggest that CKLiK functions as a transient signaling component that does not interact with kinases that recruit significantly after stimulation, but rather, early in receptor activation.

CKLiK may phosphorylate specific substrates in a brief manner, binding quickly to function and then quickly dissociating. However, since the percent change in luminescence is negative, there is a higher likelihood that it is actively dissociating rather than passively, potentially due to competition with other enzymes to bind or by receptor internalization that physically separates the receptor from CKLiK. As β-arrestin scaffolds form after GRK-mediated receptor phosphorylation, CKLiK may also be displaced because of components that bind at later times.

As CKLiK belongs to the calcium/calmodulin-dependent protein kinase family and is known to be involved in calcium-dependent signaling pathways and aldosterone synthesis, its transient recruitment to AT1R suggests that it serves as an early regulator that connects the receptors signaling path to calcium-dependent transcription downstream or by controlling the production of aldosterone in the RAAS pathway. Therapeutic targets that preserve the activity of CKLiK can therefore potentially be used for lowering blood pressure. As seen, since CKLiK only has a short burst of activity, sustaining it could possibly lead to a longer decrease in blood pressure via increased production of aldosterone.

In contrast, CKLiK had a relatively high AUC, driven by the high transient initial recruitment. This indicates that it likely interacts directly with the receptor or binds to complexes immediately upon receptor activation. The active dissociation of the kinase, therefore, may be due to competitive displacement by β-arrestin or other proteins as the signaling complex develops.

4.3 ZAK Does Not Exhibit Significant Recruitment to AT1R

Despite initial screening results that suggested that ZAK recruitment to AT1R, validation experiments failed to reproduce these findings. ZAK exhibited no consistent recruitment and highly varied across replicates. This discrepancy can be attributed to multiple factors. First, ZAK is a stress-activated MAP3K that responds to DNA damage and oxidative stress (MAP3K20, NCBI). Since our HEK293T cells were actively growing under standard culturing procedures, the conditions do not provide the appropriate conditions for activation of ZAK. It is possible that ZAK recruitment to AT1R occurs only under specific pathological conditions such as oxidative stress, which was not replicated and studied in our procedure. Alternatively, it is also possible that the initial signal reflected transient interactions that were too brief or weak to be

reproducibly detected. Given the high variability and lack of consistent recruitment in validation, I conclude that under the conditions we tested, ZAK does not show any significant recruitment to AT1R. Future studies could mimic appropriate conditions to test ZAK recruitment in different cell types.

4.4 Therapeutic Implications

The collected data identifies novel therapeutic targets that can allow for pathway-selective modulation rather than current treatments that block entire pathways, eliminating beneficial effects along with the unwanted. Current GSK3β inhibitors such as Tideglusib could be repurposed to play a role like AngII, which inhibits GSK3β via phosphorylation of Ser9 (Martínez-González et al.). Similarly, if focusing directly on CKLiK, intracellular interactions may stabilize and lower the production of aldosterone without having overbearing effects on the entire RAAS pathway, disrupting homeostasis. Modulators of PP2A or I2PP2A could temporally control the activity of GSK3β, offering solutions for time-sensitive setbacks. By targeting intracellular kinases rather than entire receptors with ligand binding, new therapeutics can preserve beneficial vasodilation.

4.5 Limitations

All experimental research has limitations that must be acknowledged. This study was conducted in HEK293T cells, which is an immortalized cell line that does not endogenously express AT1R with the LgBiT tagged. Although AT1R293 cells were used for the Western Blot experimentation, these cells are still an artificial system. The overexpression of the SmBiT and LgBiT constructs by transfection could have affected normal protein trafficking. Future studies would need to validate these findings in primary cardiomycytes or vascular smooth muscle cells, which stably express AT1R and the kinases at endogenous levels.

Second, the temporal aspect of our experimentation included only the first 30 minutes post-stimulation. Although this captures early signaling and identified distinct recruitment patterns for kinases, it may have missed later phases of signaling or kinase adaptation that may have occurred hours after. Long-term consequences of kinase recruitment, such as gene expression and cellular adaptation, were not investigated in this study.

Although the NanoBiT assay can test for overall recruitment, it does not confirm direct physical interaction and cannot assess the specific binding sites (Dixon et al.). While Western Blot analysis confirmed that GSK3β is phosphorylated at Ser9, we cannot conclude whether AT1R is a substrate or identify the molecular mechanisms that resulted in recruitment. Co-immunoprecipitation studies could further strengthen these findings.

5. Conclusion & Future Directions

This study shows previously unknown complexity in AT1R signaling through identifying and characterizing the activities of non-canonical protein kinase interactions. CKLiK functions as a transient signaling agent and exhibits rapid recruitment followed by active displacement from AT1R. On the other hand, GSK3β has sustained recruitment accompanied by the regulation via phosphorylation of its Ser9 residue. These distinct temporal patterns indicate that non-GRK kinases may play different roles in AT1R beyond the currently known preset of desensitization, potentially making it possible to regulate specific pathways.

Particularly significant is the interaction between GSK3β and AT1R, given the current roles that GSK3β plays in cardiac hypertrophy, heart failure, and remodeling. The mechanism that AT1R activation leads to inhibition of GSK3β establishes a precedent for future therapeutic targets. As there are GSK3β inhibitors already in clinical trials, they could be repurposed to modulate AT1R signaling, as small molecules targeting intracellular interactions could prove easier to target than current treatments. Specifically, they would give advantages over ARBs by preserving the beneficial and necessary signaling while blocking pathological response.

Future directions should start by validating these interactions with cardiovascular cells that express the proteins at endogenous levels. These can further be extended to chronic disease models, with cells from actual patients being studied to deduce the complex mechanism by which AT1R regulates disease. Characterizing the functional and physiological impacts of the recruitment of a specific kinase on AT1R mediated response such as calcium flux and cellular transcription factors will establish the significance of the kinases in human biology. From this, mapping the molecular mechanisms underlying these interactions will allow us to develop therapeutic options that can provide precision medicine in cardiovascular illnesses.

6. References

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