REGULATION OF ANGIOTENSIN TYPE 1 RECEPTOR FUNCTION BY AN UPSTREAM SHORT OPEN READING FRAME (sORF) IN THE mRNA 5' LEADER SEQUENCE

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ABSTRACT

The angiotensin type 1 receptor (AT$_1$R) plays a critical role in blood pressure control. In this study, we investigated the role of an upstream short open reading frame (sORF) in AT$_{1a}$R signaling. We investigated Angiotensin II (Ang II)- and Sar$_1$Ile$_4$Ile$_8$-Ang II (SII)-induced extracellular signal regulated kinases 1 and 2 (ERK1/2) signaling and its alteration in the presence of an sORF in exon (E) 2 in the AT$_{1a}$R mRNA 5' leader sequence (LS). Human embryonic kidney 293 cells (HEK293) were transfected with WT [E1,2,3-AT$_{1a}$R-EGFP] or MT [E1,2(-108T),3-AT$_{1a}$R-EGFP]. A single nucleotide was mutated at the start codon of the intact sORF (WT) to create a disrupted sORF (MT). Dose response curves for ERK1/2 activation in WT vs MT were significantly different for Ang II (p<0.001, N=3-4) and (SII) (p<0.01, N=3). A time course of Ang II-and SII-induced ERK1/2 activation from 0–80 minutes also showed greater ERK1/2 activation in the MT compared to WT [Ang II: p<0.01; N=3 and SII: p<0.05; N=3]. The rate of internalized AT$_{1a}$R in the vesicles after Ang II (100 nM) treatment was markedly increased in the WT [t$_{1/2}$ in seconds(s): WT, 118 s (n=21) vs MT, 250 s (n=9); p<0.0001]. We also examined the effect of this sORF on apoptosis by flow cytometry. The presence of the sORF (WT) increased the percentage of live EGFP (+) cells (p<0.05, N=3) and reduced the percentage of early apoptotic EGFP(+) cells (p<0.05, N=4). This study shows that the E2 sORF is a selective inhibitor of AT$_{1a}$R-mediated ERK1/2 activation and could be a potential therapeutic for diseases involving AT$_1$R dysfunction by modulating AT$_1$R activity. This study also points out the regulatory role of 5’ LS that is often not present in the expression vectors used to study proteins by transfection or overexpression systems.
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LIST OF ABBREVIATIONS

ACE - Angiotensin converting enzyme

ACE2 - Angiotensin converting enzyme 2

Ang II – Angiotensin II

ANOVA - Analysis of variance

AP - Aminopeptidase

ARAP1 - Angiotensin II type 1 receptor-associated protein 1

ARB - Angiotensin type 1 receptor blockers

AT - Angiotensin receptors

AT\textsubscript{1a}R - Angiotensin type 1a receptor

AT\textsubscript{1}R - Angiotensin type 1 receptor

ATRAP - Angiotensin II type 1 receptor-associated protein

BCA - Bicinchoninic acid assay

CE - Collision energy

CM – Conditioned media

CXP - Cell exit potential

DAG - Diacylglycerol

DMEM - Dulbecco’s modified Eagle’s medium
E - Exon

EGFP – Enhanced green fluorescent protein

EGFR - Epidermal growth factor receptor

ER - Endoplasmic reticulum

ERK1/2 – Extracellular signal regulated kinases 1 and 2

FBS - Fetal Bovine Serum

FLIM/FRET- Fluorescence lifetime imaging-Forster/fluorescence resonance energy transfer

fm- Femto moles

G proteins - GTP-binding proteins

G1 – Growth phase 1

G2 – Growth phase 2

GAPDH - Glyceraldehyde 3-phosphate dehydrogenase

GDP - Guanosine diphosphate

GPCRs – G-protein-coupled receptors

GRK – G protein-coupled receptor kinase

hAT1aR – Human angiotensin type 1a receptor

HEK293 – Human embryonic kidney 293

IP3 - inositol 1,4,5- triphosphate
LAMP-1 - Lysosomal-associated membrane protein 1

LB - Luria Broth

MEF - Mouse embryonic fibroblasts

Mins – Minutes

MRM – Multiple reaction monitoring

MT – Mutant

NLS - Nucleus localization signal

nM – Nano molar

NT – Non-transfected

PBS - Phosphate buffered saline

pERK1/2 – Phospho ERK1/2

PI - Propidium Iodide

PIP₂ - Phosphatidylinositol 4,5-bisphosphate

PKC – Protein kinase C

PLC - Phospholipase C

Q – Quadrant

RAAS - Renin-angiotensin aldosterone system

RAS - Renin-angiotensin system
rAT₁aR – Rat angiotensin type 1a receptor

ROI - Region of Interest

s - Seconds

S.E.M – Standard error of the mean

SEPs – sORF encoded peptides

SII - [Sar₁-Ile⁴-Ile₈]-Ang II

sORF - short open reading frame

WT – Wildtype

µM – Micro molar
CHAPTER 1. INTRODUCTION

1.1 Background

Cardiovascular diseases account for the highest number of deaths as compared to other major communicable and non-communicable diseases. The projected number of deaths by cardiovascular disease is about 24% in 2030, which is much higher than the projected mortality trend by cancer, chronic respiratory diseases, diabetes, tuberculosis, malaria and acquired immune deficiency syndrome [1, 2]. Hypertension is a type of cardiovascular disease, which is a leading cause of health concern worldwide. Ideally normal blood pressure is considered to be 120 mm Hg of systolic pressure and 80 mm Hg of diastolic pressure. Hypertension or high blood pressure is defined as a long term condition with constant elevated blood pressure which is greater than 130 mm Hg of systolic pressure and 80 mm Hg of diastolic pressure [3].

According to National Center for Health Statistics of 2013, about 403,000 males and 398,000 females died of cardiovascular disease in the U.S. Although the prevalence of hypertension increases with age and is found to be similar in males and females, it varies over different races [1]. With the revised guidelines for high blood pressure by American Heart Association, 46% the U.S. population is reported to have hypertension [3]. Hence, hypertension has been a matter of interest to global health care and cost. In 2011-2012, hypertension accounted for 48.6 billion dollars that is projected to increase to 274 billion dollars by 2030 [1].

Since the causes of hypertension can be genetic, metabolic (lipid and glucose metabolism), social (age, housing, education and income) or behavioral (food habits, physical exercise, tobacco/alcohol consumption), it is difficult to control the risk factors for developing high blood pressure. Hypertension is often termed as ‘silent killer’ since people having elevated blood pressure do not show any obvious symptoms and hence these patients often go undiagnosed.
Although, patients with borderline hypertension do not require drug treatment, uncontrolled hypertension can lead to formation of aneurysms and rupturing of blood vessels, impairing functions of vital organs like heart, kidney, eyes and brain. Thus, hypertension may further lead to renal diseases, loss of vision, ultimately heart attack or stroke [2].

1.1.1 Hypertension and Renin-Angiotensin System

Blood pressure control is dependent on three factors namely vascular resistance, cardiac output and intravascular volume. These factors are controlled by vascular smooth muscle constriction and dilation, cardiac contractility and renal sodium homeostasis. Renal sodium excretion and reabsorption as well as vasoconstriction/ vasodilation are two crucial factors regulated by the renin-angiotensin aldosterone system (RAAS). Hypertension is associated with dysregulation of components of RAAS [4]. Until recently, components of RAAS were thought to be involved primarily in cardiovascular homeostasis and extracellular electrolyte balance. Recently, research studies have also revealed a role of RAAS in growth, inflammation, remodeling and development [5]

Renin secreted by the kidneys in response to low blood pressure, converts angiotensinogen (in liver) to the decapeptide angiotensin I. Angiotensin I is further converted to angiotensin II (Ang II) by catalysis via angiotensin converting enzyme (ACE). Ang II is an eight amino acid peptide that binds to angiotensin receptors to response to changes in water and sodium homeostasis. Although these cascade of events involving the formation of Ang II from renin, forms a central part of RAAS, there are other alternatives and inter-connecting events that might take place in the body. Apart from Ang II, other peptides like Ang III, Ang IV or Ang 1-7 are also formed through alternate metabolic pathways of RAAS in the presence of aminopeptidases or angiotensin
converting enzyme 2 (ACE2), respectively. These angiotensin peptides formed in the renin angiotensin system cascade (Figure 1) can interact with angiotensin receptors with varying affinity to elicit response [5, 6].

Decades of research focused on understanding the pathophysiology of hypertension have helped in the development of a number of anti-hypertensive drugs that are now commercially available and widely prescribed for patients. The different classes of anti-hypertensive agents include diuretics, vasodilators, calcium channel blockers, alpha blockers, beta blockers, angiotensin type 1 receptor blockers (ARB), ACE inhibitors, centrally acting drugs, and renin inhibitors. However, a considerable number of patients with hypertension do not have controlled blood pressure due to resistance to treatment, poor patient compliance partially due to adverse side effects, and ineffective drug combinations [7]. Among different classes of anti-hypertensive drugs for treatment of hypertension, ARBs are widely prescribed for patients with hypertension. Although ARBs are one of the well tolerated classes of anti-hypertensive drugs, they are known to cause dizziness, headache, nausea, hyperkalemia, angiodema and cough [4, 8]. Many new treatment options for hypertension have been suggested that have different mechanism of action than the traditional anti-hypertensive drugs on market [4].

1.2 Types of Angiotensin Receptors

Angiotensin receptors (AT) are classified into 4 receptor types namely AT1, AT2, AT4 and Mas (Figure 1) on the basis of several factors including pharmacological differences in ligand binding affinity, signaling mechanisms and their physiological response. Most of the known physiological effects associated with elevated blood pressure are mediated by the interaction of Ang II with angiotensin type 1 receptors [9]. AT1 and AT2 receptors are G protein-coupled
receptors, which bind with similar affinity to Ang II; however, exert opposing effects in response to ligand interaction. The physiological effect of activation of AT₁ receptors is to increase in blood pressure which is counteracted by AT₂ receptor stimulation [10, 11]. AT₄ receptor is shown to have higher affinity to Ang IV than other subtypes of angiotensin receptors. AT₄ was recently found to be an insulin-related aminopeptidase (IRAP) that is known to play a role in functions of central nervous system like memory and cognition [5, 12]. The Mas receptor interacts with Ang 1-7 and this axis results in effects which are similar to AngII-AT₂R i.e vasodilation [5].

The AT₁R-Ang II signaling axis is significant in mediating a number of physiological responses like thirst, fluid and water homeostasis, and maintaining blood pressure. In addition to these, dysregulation of AT₁R-Ang II axis also contributes to many pathophysiological responses like thrombosis, fibrosis, inflammation leading to vascular as well as associated heart and kidney disorders [13]. Hence, it is not surprising that the Renin-Angiotensin system (RAS) is tightly regulated in the body including Ang II and AT₁R, two of the most crucial components of RAS. It is well known that Ang II concentration elsewhere in the body is organ specific, can be variable with sodium concentration in the body, and is differentially regulated. For example, Ang II levels in kidneys are found to be much higher as compared to plasma [14] in male Sprague-Dawley rats [15]. Ang II levels measured by liquid chromatography/ mass spectrometry in kidney cortex were significantly higher in male Zucker obese rats on 2-week high-salt diet as compared to normal-salt diet [16]. Also, on administration of Perindopril for a week to male Sprague-Dawley rats led to decrease in Ang II levels in kidneys but did not change plasma Ang II (measured by radioimmunoassay) [15]. Perindopril operates by inhibiting ACE, thus, the physiological critical tight regulation of Ang II levels in the kidney maintains a constant and appropriate plasma Ang II.
concentration. Since Ang II is critically regulated in the body, small changes to Ang II concentration may have deep impact on physiological effects on the body through the AT₁R [14].

1.2.1 Angiotensin Type 1 Receptor

The interaction of Ang II with AT₁R cause distinct physiological responses in different organs resulting in elevation of blood pressure. Some of the crucial physiological effects include vasoconstriction, release of aldosterone causing increase in reabsorption of water and sodium in kidneys [4, 5, 10], increased sympathetic activity [17], cardiac contractility and remodeling [18]. Apart from these, the AT₁R is responsible for cell growth [10, 11], proliferation, differentiation, [19, 20] and, apoptosis [21] in various model systems. Other than hypertension, AT₁R dysfunction is associated with pathophysiology of many other age-associated diseases like renal diseases [22], type II diabetes [23], metabolic syndrome [24], Alzheimer’s [25], Parkinson’s [21] and cancer [26], to name a few.

AT₁R is encoded by a single gene in humans in contrast to the two subtypes found in rodents namely AT₁aR and AT₁bR. Both these subtypes of AT₁R are widely distributed in blood vessels, heart, kidneys and brain. Of these two subtypes, AT₁aR is found to predominate regulation of blood pressure [27, 28]. Although AT₁a and AT₁b receptors are encoded by different genes and show differential tissue expression; unlike AT₁ and AT₂ receptors, these two isoforms have not shown to be distinct with respect to pharmacology or mechanisms of intracellular signaling [29]. Of these AT₁ receptors, AT₁a receptor is expressed in most tissues whereas AT₁bR which are usually expressed more in pituitary glands, glomeruli and adrenal cortex as compared to AT₁aR. However, the details of precise role of AT₁bR is yet to be investigated [30].
Figure 1: Schematic of Renin-Angiotensin System Cascade (Adapted from [5]).

ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AP, aminopeptidase; AT₁, angiotensin type 1 receptor; AT₂, angiotensin type 2 receptor; AT₄, angiotensin type 4 receptor; Mas, Mas oncogene. Angiotensinogen is cleaved to angiotensin I (Ang I) and Ang I to Ang II by renin and ACE respectively. Ang II can be converted to Angiotensin 1-7 or angiotensin III by ACE2 or AP respectively. Ang II can bind to both AT₁ and AT₂ receptors, Ang III and Ang IV interact with AT₂ and AT₄ receptors whereas angiotensin 1-7 is a ligand for Mas receptor.
The genes encoding rAT\textsubscript{1a}R and rAT\textsubscript{1b}R are comprised of 3 exons whereas hAT\textsubscript{1}R has 4 exons. The coding region for AT\textsubscript{1}R is entirely within exon 3 (E3) and exon 4 (E4) for rodents and humans, respectively. Alternative splicing of rAT\textsubscript{1a}R and rAT\textsubscript{1b}R results in two splice variants (E1,3 and E1,2,3) whereas hAT\textsubscript{1}R has 4 spliced variants (E1,4, E1,2,4, E1,3,4 and E1,2,3,4). The result of this alternative splicing for genes is differential expression of AT\textsubscript{1}R isoforms in different tissues. The ratio of spliced variants of the AT\textsubscript{1}R in rats and humans change with aging and pathological conditions [28]. This suggests that post-transcriptional regulation of the AT\textsubscript{1}R can provide interesting insights on the key players of splicing that control isoforms of AT\textsubscript{1}R transcripts.

1.2.2 Angiotensin Type 1 Receptor, A Member of G-Protein-Coupled Receptor

Superfamily

AT\textsubscript{1}R is a seven transmembrane (serpentine) domain receptor that belongs to the G protein-coupled receptor (GPCR) superfamily. GPCRs are one of the most widely studied class of known receptors and the significance of GPCRs stems from the fact that the large number of members of this superfamily are differentially and redundantly expressed in almost all tissues of the body. GPCRs are involved in diverse physiological functions like chemosensory responses (vision, tastes, smell etc.) as well as response to endogenous molecules (hormones, signaling proteins, neurotransmitters, nucleotides) to mediate complex processes in reproduction, homeostasis, proliferation, metabolism, cognition and behavior [31].

As the name suggests, a characteristic feature of these cell surface GPCRs is the association of the receptor with G proteins (GTP-binding proteins) namely G\alpha, G\beta and G\gamma that are associated with GDP at the carboxyl tail (intracellular) of a GPCR. Ang II stimulation activates the AT\textsubscript{1}R by inducing a conformational change resulting in exchange of GDP (guanosine diphosphate) to GTP.
(guanosine triphosphate) bound to Ga. This exchange leads to dissociation of Ga from Gβγ dimer that can further activate downstream signaling molecules of different pathways. Hydrolysis of GTP to GDP by GTPases inactivates Ga and Gβγ and thus truncates constitutive signaling. Inactivation of GPCRs is caused by association of phosphorylated receptors (mediated by GRKs) with β-arrestin, resulting in internalization of GPCR into endosomal vesicles [32].

AT1R, being a member of class B GPCR has similar affinities for both β-arrestin1 and β-arrestin2 resulting in the formation of receptor- β-arrestin complex into clathrin-coated vesicles [33]. Unlike class A GPCRs, AT1Rs form stable interactions with β-arrestin. The nature of interaction of receptor (transient vs stable interaction) with β-arrestin may affect the rate of recycling of the receptors [34]. Once they are in endocytic vesicles, they can either recycle back on cell surface or they can be targeted to lysosomes for degradation [32].

1.2.3 Angiotensin Type 1a Receptor Signaling

The AT1aR is involved in tissue specific diverse functions that is mediated by coupling of the receptor with different signaling pathways involving Protein Kinase C (PKC) and β-arrestin [17]. Ang II-induced stimulation of AT1aR activates the G protein-dependent as well as independent pathways. As a result of activation of these pathways, extracellular signal regulated kinases 1 and 2 (ERK1/2) are phosphorylated. The traditional GPCR response of AT1aR involves activation of Gq (type of an alpha subunit) that further activates phospholipase C (PLC). Phosphatidylinositol 4,5-bisphosphate (PIP2) in the membrane is cleaved by PLC to secondary messengers - inositol 1,4,5- triphosphate (IP3) and di-acylglycerol (DAG). IP3 releases Ca2+ from endoplasmic reticulum (ER) by binding to calcium channels on ER. DAG at cell membrane along with released Ca2+ ions in the cytosol activate PKC to phosphorylate ERK1/2 [13].
In addition to G protein-dependent pathway, Ang II stimulation activates β-arrestin dependent pathway (presumed to be G protein independent pathway) and growth factor pathways also feeds in AT$_1$R signaling cascade to phosphorylate ERK (Figure 2) [35-37]. In some cell types, on Ang II-induced AT$_1$R activation, other receptors like epidermal growth factor receptor (EGFR) can get activated augmenting the target responses of Ang II. This activation of EGFR which is responsible for cross-talks in a signal transduction network, is termed as transactivation. Whether both G protein and β-arrestin dependent pathways are capable of AT$_1$R-mediated transactivation of EGFR remains elusive [38, 39].

The agonists of AT$_1$R can activate both canonical GPCR pathway and β-arrestin-dependent pathway or can selectively activate one of the two pathways. This selective activation of a subset of a complex signaling network within a cell by a receptor is termed as biased agonism. The key to specificity of a particular biased agonist is stabilization of the activated receptor complex with a particular protein to activate specific downstream cascade [35]. It has been shown that selectively activating a particular AT$_1$R downstream signaling cascade like β-arrestin dependent pathway results in specific and distinct physiological responses [18, 35]. β-arrestin1/2 form a crucial component of GPCR signaling machinery, which were initially thought to be involved in the process of receptor desensitization. However, it is now postulated that AT$_1$R-β-arrestin complex has two simultaneous functions on Ang II stimulation, namely endocytosis and ERK1/2 signaling. Luttrell et al. (2001) [40] showed by confocal microscopy that ERK1/2 colocalized with AT$_1$R-β-arrestin complex in HEK293 cells. β-arrestin also affects the spatial distribution of activated ERK by localizing ERK1/2 in the cytoplasm and thereby decreases the nuclear pool of ERK1/2 [38, 40, 41]. In addition to spatial distribution, β-arrestin can sustain agonist-induced ERK1/2 activation as compared to transient ERK1/2 activation through G protein-
dependent pathway [38, 42]. The role of β-arrestin as a scaffolding protein for ERK1/2 activation and specific targeting of ERK1/2 to subcellular compartments has added to the importance of β-arrestin in AT1R signaling.

Tang et al. (2014) showed that mechanical stress can act as a biased agonist by activating the AT1aR through β-arrestin-dependent pathway leading to ERK1/2 phosphorylation in the absence of Ang II [37]. Other biased agonists for AT1R that selectively activate β-arrestin-dependent pathway are Sar1, Ile4, Ile8 angiotensin II (SII), TRV120023 and TRV120027 [18, 35]. A biased agonist of AT1aR can either stabilize interaction of activated AT1aR-Gq or AT1aR-β-arrestin or may induce a specific conformational change in β-arrestin2 [37]. Wei et al. (2003) showed by immunoblotting for pERK1/2 in HEK293 cells transfected with a mutant receptor which could not couple to G proteins still had ERK1/2 activation. Additionally, β-arrestin2 siRNA completely blocked SII-induced ERK1/2 activation [43]. The concept of biased agonists for AT1R emerged in last two decades and is relatively new addition to AT1R signaling regulation. Biased agonism has been studied using various biased agonists as well as different truncated/mutated AT1Rs to understand the recruitment of β-arrestin, cellular responses and physiological roles. However, the exact molecular mechanism of biased agonism has not been elucidated which limits the use of biased agonists as potential therapeutics [18, 35, 44].

1.3 Angiotensin Type 1a Receptor Trafficking on Agonist Stimulation

The fate of AT1R on agonist stimulation and internalization can be both intracellular as well as extracellular. Endosomes, lysosomes, and nucleus are some of the organelles that AT1R can localize within the cell after internalization (Figure 3)[45-51].
Upon Ang-II stimulation, AT1R activates G protein-dependent signaling through activation of Gq, β-arrestin dependent pathway and EGFR transactivation pathway [35-37]. The secondary messengers of G protein dependent pathway activate EGFR, thereby leading to phosphorylation of ERK1/2. G protein-dependent phosho-ERK1/2 can either move to the nucleus to further phosphorylate nuclear substrates and activate transcription of specific genes whereas, β-arrestin dependent phoshor-ERK1/2 can phosphorylate cellular substrates.[13, 18, 35, 38, 40-42].
1.3.1 Angiotensin Type 1a Receptor Internalization

The presence of AT$_1$R on the cell membrane facilitates this receptor to respond to extracellular ligands or mechanical stress [37]. On agonist stimulation, GPCRs on the cell surface undergo activation to transduce signaling. This activation of the receptor needs a regulatory check to avoid a constitutive target response. This agonist-induced signal transduction can be attenuated by desensitization of the receptor by the process of phosphorylation of receptors, internalization or intracellular sequestration and degradation of receptors. Internalization of cell surface receptors involves a number of proteins facilitating the recruitment of the receptor from the plasma membrane into the cytoplasm. It has been found that serine and threonine phosphorylation sites within the carboxyl tail of AT$_1$R plays an important role in binding of β-arrestin to the receptor, and hence the process of endocytosis [34].

A particular receptor might undergo internalization by one specific mode or might be a combination of different modes of internalization. Three pathways have been widely studied with respect to GPCR internalization depending on whether or not the process of internalization is sensitive to dynamin and β-arrestin. There are reports that suggest that AT$_1$R is internalized via clathrin pits, caveolae as well as dynamin-independent pathway [52, 53]. On Ang II stimulation, conformational change and phosphorylation of receptor are among the first few events leading to AT$_{1a}$R internalization. β-arrestin is one of the crucial molecules involved in the receptor complex that bind to activated AT$_{1a}$R and lead to desensitization and internalization of AT$_{1a}$R. Since β-arrestin binds to clathrin and dominant negative β-arrestin significantly reduced AT$_{1a}$R internalization, investigators concluded that most of the internalization of AT$_{1a}$R is mediated largely by the pathway that it is dependent on β-arrestin [34]
Although AT₁aR undergoes internalization along with Ang II as a receptor-ligand complex, AT₁aR and Ang II are shown to have different trafficking patterns. Colocalization studies with labelled Ang II and markers of lysosomes have revealed that Ang II is delivered to lysosomes; however, the intracellular trafficking of sequestered AT₁aR is much more complex. The process of internalization, degradation and recycling of AT₁aR follows a dynamic equilibrium which is regulated independently of IP₃ and Ca²⁺ signaling pathways [54]. Once activated, AT₁R is internalized with its ligand to either undergo recycling, degradation or trafficking to different cellular compartments of the cell. The process of internalization is usually facilitated by clathrin coated pits which results in the formation of a clathrin vesicle encapsulating the internalized receptor. The trafficking of this clathrin coated vesicles into the cell is mediated by special class of GTP binding proteins termed as rab (Ras superfamily of monomeric G proteins). About 60 rab GTPases can be involved in endosomal trafficking of proteins within a cell. Rab 5a is strongly associated with AT₁aR complexes in endosomes and other endosome markers like rab7 and rab11 are known to be regulating the trafficking of AT₁aR to late endosomes and lysosomes [46].
Ang II interaction with AT₁R leads to both intracellular and extracellular trafficking of the receptor. 1. On Ang-II stimulation, AT₁R is internalized into clathrin–coated vesicles and early endosomes. 2. Early endosomes can either progress to form late endosomes or 3. can recycle AT₁Rs back to cell membrane. 4. Late endosomes can fuse with lysosomes for AT₁R degradation or 5. agonist-induced internalized AT₁R or agonist-independent AT₁R can translocate to the nucleus. 6. Some AT₁Rs can also be sorted and packaged into exosomes by undiscovered mechanisms depicted by ‘?’ [45-51].
1.3.2 Angiotensin Type 1a Receptor Degradation

The AT$_1$R is presumed to be targeted to lysosomes post agonist treatment, however, there is no clear consensus on the time course of AT$_1$R delivery to lysosomes for degradation or its trafficking in a recycling pathway. Although a prolonged agonist treatment (about 3 hours) for AT$_{1a}$R has not shown to target the internalized receptor to lysosomes by confocal microscopy [46], some other studies have reported colocalization of AT$_{1a}$R with lysosomes within 1 hour of agonist stimulation [54] in HEK293 cells. This suggests that the process of targeting a receptor to lysosomes is not a single step transfer but follows a very complex pattern of vesicular transport to early, late and recycling endosomes as well as lysosomes which is regulated by expression of rab proteins and their ability to interact with AT$_{1a}$R [46]. More recently, Li et al. (2010) found the association of AT$_{1a}$R with lysosomal-associated membrane protein 1 (LAMP-1) at 30 mins of agonist stimulation using fluorescence lifetime imaging-Forster/fluorescence resonance energy transfer (FLIM/FRET) microscopy. This suggested that although AT$_{1a}$R can follow quick or slow recycling pathways via rab4 and rab11, there is some part of this receptor that gets degraded in the lysosomes [47].

1.3.3 Other Localization of Angiotensin Type 1a Receptor

Apart from localization of this receptor on cell membrane, in various endosomal compartments and lysosomes, the AT$_{1a}$R stimulated by Ang-II has been shown to translocate to the nucleus in neurons and hepatocytes [51], HEK293 [55] and CHO cells [56]. Yang et al. (1997) showed that agonist-induced translocation of AT$_1$R to the nucleus is MAP kinase-dependent, which is downstream of internalization of AT$_1$R [51]. The AT$_{1a}$R has a nucleus localization signal (NLS) sequence – KKFKR in the eighth helix (NLS in the cytoplasmic tail) that can direct the
delivery of AT$_{1a}$R to the nucleus. The significance of the presence of GPCRs in the nucleus has not been well elucidated; however, the association of GPCRs and other downstream signaling proteins with the nucleoplasm suggests some uncovered mechanisms of GPCR signaling exist [48].

Another interesting aspect of AT$_{1a}$R trafficking is the extracellular fate of internalized AT$_{1a}$R through exosomes. Pironti et al. (2015) demonstrated for the first time that osmotic stress in HEK293 cells and cardiac pressure overload in C57/B6 mice leads to release of exosomes enriched with functional AT$_{1a}$R. This study suggested that the AT$_{1a}$R can be transported via exosomes and taken up by heart and skeletal myocytes. The AT$_{1a}$R transported to target organs respond to Ang II and stimulate intracellular signaling, thereby contributing to the regulation of blood pressure. The packaging of AT$_1$R into exosomes is speculated to be dependent on β-arrestin which suggests that β-arrestin is a major player of intracellular as well as extracellular trafficking of AT$_1$R [49].

1.4 Association and Regulation of Angiotensin Type 1a Receptor Signaling Events

Since ERK1/2 phosphorylation and GPCR internalization are consequences of agonist-induced GPCR stimulation, it was speculated that both these events are inter-related. Some reports suggest that ERK1/2 activation is dependent on GPCR endocytosis while other reports contradict this idea. ERK1/2 phosphorylation and receptor internalization were found to be associated for GPCRs like α–, β– adrenergic receptors (AR), muscarinic and opioid receptors in some cell lines whereas these two GPCR activated downstream events were found to be independent of each other in other cell lines [53]. Pierce et al. (2000) showed that ERK1/2 activation by α$_{2A}$AR and
β<sub>2</sub>AAR stimulation is dependent on clathrin mediated internalization of ARs in COS-7 cells [57]; however, another report by DeGraff et al. (1999) suggested that α<sub>2</sub>AR and β<sub>2</sub>AR internalization is not a pre-requisite for downstream ERK1/2 phosphorylation in COS-1 cells [58].

There are a number of papers that have contributed to the ambiguity of the association of ERK1/2 activation and GPCR internalization suggesting that the mechanism of GPCR trafficking cannot be generalized since it involves a complex network of molecules like G proteins, GRKs, β-arrestins, proteins involved in internalization, transactivation pathway components, etc. The regulation of this process is variable and dependent on various factors like the type of cell, GPCR involved, agonist or the internalization mechanism involved. Apart from these, the experimental approaches used to study mechanism of mitogen-activated protein kinase (MAPK) pathway and receptor internalization can further affect ERK activation or other signaling molecules involved [53, 57, 58]

1.5 Angiotensin Type 1 Receptor Regulatory Proteins

Like most of the receptors, even AT<sub>1</sub>R is regulated at transcription and post-transcriptional levels. These regulatory mechanisms usually control the density of receptors that are expressed on the surface of a cell [28]. In addition to this, regulation of receptor trafficking is directed to fine tune the target responses of ligand receptor interaction. On Ang II-induced internalization of AT<sub>1</sub>aR, the receptor interacts with small G proteins, kinases, phosphatases and other downstream signaling proteins that play a significant role in the process of regulation of AT<sub>1</sub>aR trafficking. Since the formation of clathrin-coated pits followed by internalization, formation of protein complex and activation/attenuation of MAPK pathway are multi-step processes in itself, regulatory
proteins can modulate the overall response of agonist induced GPCR. Apart from β-arrestin and Rab GTPases, there are some other families of GTPases and GPCR interacting proteins that regulate AT₁R trafficking and signaling.

Like other G protein coupled receptors, GRKs are involved in phosphorylation of AT₁R on Ang II stimulation which uncouple AT₁R from G proteins as well as facilitate binding of phosphorylated AT₁R with β-arrestin. Apart from their universal role in desensitization and internalization of receptor, they are now known to have regulatory roles in the selective activation of downstream signaling and AT₁R trafficking [59, 60]. The different isoforms of GRKs namely GRK2, GRK3, GRK5 and GRK6 are involved in modulating Ang-II induced G protein dependent and independent pathways. The phosphorylation of GRK2/3 and GRK5/6 are not only known to be dependent and independent of G proteins respectively, but also have distinct sites of phosphorylation. It has been shown that GRK2 silencing leads to increase in β-arrestin-dependent phospho ERK (pERK) 1/2 whereas GRK5/6 silencing completely abolishes this G protein-independent ERK activation. Moreover, the phosphorylation was seen to be increased on GRK6 in the cells depleted of GRK2. These experiments suggest that GRK2 and GRK5/6 compete to differentially regulate downstream signaling of AT₁R [60]. Although GRK2 and GRK5/6 distinct preferences for downstream signaling pathways, their loss has been associated with a negative effect on cardiac physiology suggesting that interplay of both these GRKs is importance for AT₁R mediated cellular and physiological response [59].

The process of AT₁R internalization, degradation as well as recycling is regulated by interplay of a number of regulatory molecules like the ADP-ribosylation factor 6 (Arf6) [61], rab GTPases [62], RalA [63], AT₁R-associated protein (ATRAP) [64], and AT₁R-associated protein 1 (ARAP1) [65]. Rab GTPases have shown that gene silencing for rab4 and rab11 decreases the
recovery of AT\(_1\)R [62]. The colocalization of AT\(_1\)R with rab4 and rab11 in varying patterns during the course of recycling process suggests that rab4 and rab11 have distinct yet coordinated roles in regulating AT\(_1\)R recycling [62]. Godin et al. (2010) showed by confocal microscopy and BRET that specifically RalA interacts constitutively with AT\(_1\)R but does not affect internalization of AT\(_1\)R in HEK293 cells; however, RalA may play a regulatory role in Ang II-induced IP formation by activating PLC\(\delta\) [63]. ATRAP is a kind of GPCR interacting protein (GIP) that interacts with the carboxyl tail of AT\(_1\)R. ATRAP regulates the process of desensitizing the Ang II-induced AT\(_1\)R in VSMCs and increases the rate of AT\(_1\)R internalization. The inhibition of DNA synthesis on Ang II stimulation in VSMC overexpressing ATRAP further suggests that ATRAP is involved in desensitization of the AT\(_1\)R and inhibits Ang II-mediated proliferation in VSMC [64]. AT\(_1\)R-associated protein 1 (ARAP1), another GIP, binds the AT\(_1\)R between residue 319 and 359 in carboxyl tail of the receptor and does not affect internalization of the AT\(_1\)R but regulates the recycling pathway [65].

1.6 Short Open Reading Frames (sORF) – A Novel Way of Angiotensin Type 1a Receptor Regulation

Major open reading frames (coding region) in the mRNA transcripts are usually know to code for proteins like receptors, hormones, signaling components, growth factors, to name a few. However, there are other smaller stretches of open reading frames present upstream [66] or downstream (3’-UTR) of coding regions. Many of these sORFs encode proteins which are termed as SEPs (sORF encoding polypeptides) found in primitive as well as higher eukaryotes including humans. These sORFs are usually defined to be shorter than 99 codons in length; however, this is not a stringent classification of proteins have coding regions > 99 codons. Although sORFs are
often present in genomes, their short length and non-AUG start codon are two main reasons as a result of which they remained uncovered for a long time. The technological advancements in transcriptomics and peptidomics not only shed light on the presence of sORFs but also revealed that they might code for biologically relevant proteins/peptides (SEPs) [67, 68]. The genes that contain upstream sORFs can have more interesting outcomes of alternative splicing if the exon encoding sORF is spliced for one of the variant, as in the case of AT1Rs [28].

The rAT1aR have one upstream sORF in E2 whereas hAT1aR have 2 sORFs in E2 and 1 sORF in E3 which is in-frame with the coding region. These uORFs have an effect on the translation efficiency of AT1Rs resulting in differential regulation of AT1R expression in the presence and absence of these ORFs. As a result of affecting receptor expression, the level of AT1R response to Ang II is also shown to differ [28]. The sORF in exon 2 of rAT1aR has been found to be in-frame with E3. This sORF is marked by AUG start codon at position -108 to UAA at -87 in 5’LS of rAT1aR. A strong Kozak consensus sequence [69] and lower AT1R density found in E1,2,3 as compared to E1,3 suggests that there is a possibility of this sORF to be translated to a seven amino acid peptide termed as PEP7. An interesting feature of PEP7 is its conservation in rats and mice and its similarity with humans (as depicted by shaded cells across species in Table 1) [Liu, 2014 #104]. Synthetic PEP7 pretreatment in male rats reduces the increases in Ang-II induced arterial mean pressure and also significantly reduced Ang II-induced saline intake but did not affect water intake over time. Furthermore, western blot analysis using HEK293 cells transfected with rE1,2,3 AT1aR showed that pretreatment of PEP7 inhibited Ang II-induced ERK1/2 phosphorylation but did not affect IP3 accumulation [66].
Table 1: Seven Amino Acid Sequence of PEP7.

<table>
<thead>
<tr>
<th>Species</th>
<th>Position # and Amino acid sequence of PEP7</th>
<th>Amino acids</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>#1</td>
<td>#2</td>
</tr>
<tr>
<td>Rat</td>
<td>Methionine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Mouse</td>
<td>Methionine</td>
<td>Alanine</td>
</tr>
<tr>
<td>Human</td>
<td>Methionine</td>
<td>Alanine</td>
</tr>
</tbody>
</table>

The PEP7 sequence in rats, mice and humans is depicted in each row. The conserved amino acids at position # 1, 2, 3, 5 and 6 (methionine, alanine, glycine, lysine and serine, respectively) across all the three species are depicted by grey cells (Adapted from [Liu, 2014 #104]).

1.7 Gaps of Knowledge

About 50 percent of the people having hypertension have uncontrolled blood pressure [70] suggesting that we need better options for treatment of hypertension. Although AT₁R has been studied for many decades, the crystal structure of this receptor was not revealed until 2015 [71]. AT₁R signaling pathways and trafficking is central to the understanding of pathophysiology of hypertension. Non-specific antibodies for AT₁R has further complicated the elucidation of AT₁R signaling and its regulation [72]. The signaling and trafficking patterns underlying AT₁R have not been clearly elucidated. It is important to study AT₁R regulators to improve the prediction for in-vivo effects of AT₁R and develop novel treatment strategies.
CHAPTER 2. HYPOTHESIS

2.1 Thesis Question

Most of the signaling findings on the AT₁R have been focused on the rat AT₁aR. The rat AT₁aR is entirely encoded by E3, however there is an upstream sORF present in E2 of AT₁aR 5’ leader sequence. It has been previously shown that E1,2,3 , an alternatively spliced variant of rat AT₁aR, is less efficiently translated as compared to E1,3 [73]. For decades now, most of the in-vitro studies have focused on the coding region of AT₁aR in exon 3. The significance of the sORF present in the 5’ leader sequence on receptor function, however, has not been adequately studied. Previously, pharmacological effects of synthetic PEP7 have been studied [66]. However, this thesis specifically sheds light on the role of this sORF on agonist induced AT₁aR signal transduction and trafficking in HEK293 cells.

2.2 Hypothesis

Based on the previously published findings on the AT₁aR, we hypothesized that the PEP7 sORF regulates AT₁aR signaling, trafficking and cellular functions like apoptosis and cell cycle.

2.3 Specific Aims

2.3.1 Aim I

To investigate the role of PEP7 sORF in AT₁aR mediated signaling cascade in HEK293 cells.
2.3.2 Aim II

To investigate the role of PEP7 sORF in regulation of agonist stimulated \( \text{AT}_{1a}\)R trafficking in HEK293 cells.

2.3.3 Aim III

To determine if PEP7 sORF affects \textbf{cellular functions} like apoptosis and cell cycle in HEK293 cells.

2.3.4 Aim IV

To determine if PEP7 sORF is translated into a peptide (PEP) in HEK293 cells.
CHAPTER 3. MATERIALS AND METHODS

3.1 Materials for Cell Culture

Dulbecco’s modified Eagle’s medium (DMEM) 1X (#11960-044); Opti-MEM, Reduced Serum Media (#31985-070); Trypsin-EDTA (0.25%), phenol red (#25200-056); Penicillin Streptomycin (Pen/Strep) (#15140-122); L-Glutamine 200mM (100X) (#25030-081) were purchased from Gibco-ThermoFisher Scientific. Fetal Bovine Serum (FBS) (#F2442) was purchased from Sigma-Aldrich.

3.2 Cell Culture

Human embryonic kidney 293 (HEK293) cells obtained from the American Type Culture Collection (ATCC; CRL-1573, LOT 63777489). As mentioned previously in Kadam et al. (2017), HEK293 cells were cultured with complete DMEM [10% FBS, 1% Pen/Strep, 1% Glutamine (1X)] as mentioned previously in Kadam et al, 2017 [74] and maintained at 37°C in humidified air with 5% CO₂. Cells were cultured up to 13 passages for all experiments performed.

3.3 Plasmid Construction and Plasmid Preparation

The start codon of the short open reading frame (sORF) in exon 2 of the rat AT₁₅R transcript was mutated at adenine -108 (A−108 to T−108) to create E1,2(−108T),3-AT₁₅R-EGFP [mutant (MT)] from E1,2,3-AT₁₅R-EGFP [wildtype (WT)] by site-directed mutagenesis and cloned into the pEGFP-N2 plasmid [66]. These constructs (Figure 4) were previously prepared by Dr. Jun Liu in the lab.
Figure 4: Schematic of the Rat AT_{1a}R Gene Construct Cloned into the pEGFP–N2 Plasmid.

AT_{1a}R gene encodes three exons (E) namely E1, E2 and E3. A sORF is encoded by E2 in the 5’ leader sequence (5’LS) upstream of the receptor coding region (CR) and 3’untranslated region (3’UTR). A. Wildtype AT_{1a}R (WT) construct with intact sORF and B. Mutant AT_{1a}R (MT) construct with disrupted sORF. The start codon of the sORF was mutated at adenine −108 (A−108 to T−108) by site-directed mutagenesis.

Transformed bacteria were streaked on agar plates in sterile environment between the burners. The plates were placed upside down in the incubator at 37°C. After 24 hours, the plates were removed and a single isolated colony was picked carefully and added in 20 ml of LB. This suspension was put in rocker at 37°C for 2 hours to grow. 50mg/ml of ampicillin was added after cooling the autoclaved 200 ml of LB in 1L conical flask. 20 ml of LB was then transferred to conical flask and put on the shaker for 16-18 hours at 37°C. The next day, the turbid LB was
transferred to plastic centrifuge bottles and all the bottles were weighed accurately before centrifugation. After spinning for 30 minutes at 5000 rpm, supernatant was discarded and the pellet was resuspended well in 10 ml of P1 buffer. 10 ml of P2 buffer was added to this suspension in corning 50 ml tube and incubated for 5 minutes. 10 ml of cold P3 buffer was added and mixed gently, followed by incubation on ice for 20 minutes. After incubation, suspension was centrifuged for 30 minutes at 12,000 rpm at 4°C.

   The Qiagen-tip 500 column was equilibrated by adding 10 ml Buffer QBT. After the column was completely drained, the supernatant (after decanting and removing bigger chunks) from centrifuged tubes was added onto the column. The column was washed twice with 30 ml Buffer QC. Plasmid DNA was eluted by adding 5 ml Buffer QF and the collected eluate was DNA was precipitated with 10 ml of isopropanol. After centrifugation for 30’ at 12,000 rpm at 4°C. The supernatant was discarded and pellet was resuspended with 5 ml of 70% ethanol, followed by centrifugation for 5 mins at 12,000 rpm at 4°C. The supernatant was discarded and the pellet was air-dried and resuspended in 500 ml Tris-EDTA. This purified plasmid was then transferred to an eppendorf and Nanodrop readings were taken.

3.4 Transient Transfection

   HEK293 cells were plated in 100 mm plates. Before transfection began, the cells were washed with PBS and maintained in Opti-MEM. HEK293 cells were then transfected with the two plasmid constructs at 80-90% confluence using Lipofectamine 2000 (ThermoFisher Scientific; #1168019) incubated for 4.5 – 5.0 hours. After incubation, the media for cells was replaced with complete DMEM and the cells allowed to recover for about 18 hours.
3.5 ERK1/2 Activation Assay

Transfected and non-transfected (NT) HEK293 cells were plated in 6-well plates (1 x 10^6 cells/well) after about 18 hours of transfection. After 12-18 hours, transfected cells in wells were serum starved using serum free DMEM (Life Technologies; #31053-028) for 4 hours and incubated with either Ang II (Sigma-Aldrich; #A9525) or SII (Phoenix Pharmaceuticals; #custom synthesis Lot No. 432951) at desired concentrations for fixed time periods. To perform the inhibitor assay, the cells were pre-treated with PKC inhibitor - Ro 31-8220 (Sigma-Aldrich; #R136) for 15 minutes before Ang II treatment. As soon as the incubation period was completed, the media with agonist was removed and the cells were solubilized by lysis buffer containing 2x Laemmli sample buffer (Bio-Rad; #161-0737) and 2-mercaptoethanol (Sigma-Aldrich; # M7522, 1:20). The plates were then placed on 37°C shaker incubator at 500 rpm for 15 minutes followed by scraping the solubilized cell contents into 1.5 ml Eppendorf tubes. The contents in Eppendorf tubes were and then heated for 15 mins at 95°C before aliquoting and storing at -20°C for western blotting.

3.6 Protein Estimation by Bicinchoninic Acid Assay

100 µl of RIPA lysis and extraction buffer (ThermoFisher Scientific; #89900) was added to each of the wells. The preparation of cell lysate was performed according to the procedure for mono-layer cultured cells recommended by the manufacturer. The preparation of BSA standards and protein estimation of the samples was further estimated by Pierce™ BCA (Bicinchoninic acid assay) Protein Assay Kit (ThermoFisher Scientific; #23225) as per manufacturers protocol.
3.7 Western Blotting

The aliquots were thawed in 37°C water bath for 8 mins. The Eppendorfs were then vortexed and subjected to a quick spin before loading the samples on the gel. Equal amounts of samples were separated on 4-20% Tris-glycine polyacrylamide gels (Bio-Rad; #5671094) in 10x Tris/Glycine/SDS electrophoresis buffer (Bio-Rad; Cat #161-0772, 1:10) Tris/Glycine/SDS electrophoresis buffer for 100 volts for 20 mins initially and then 200 volts for an hour on constant power. The gel was carefully removed and the separated proteins were and transferred to PVDF membranes for immunoblotting using a Trans-blot Turbo transfer system (Serial # 690BR019158, Singapore). Proper protein transfer was confirmed by Pierce™ Reversible Protein Stain Kit for PVDF membranes (ThermoFisher Scientific; #24585). The membrane was blocked using 5% milk in Tris-buffered saline (1X) with 0.05% tween-20 (TBS-t). Phosphorylated ERK1/2 (pERK1/2), non-phosphorylated ERK1/2, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were detected by immunoblotting with anti-phospho-p44/42 MAPK antibody (Cell Signaling Technology®; #9101s, 1:1000), an anti-p44/42 MAPK1/2 (Cell Signaling Technology®; #4695s, 1:1000), and an anti-GAPDH antibody (Cell Signaling Technology®; #5174s, 1:1000) respectively for 16-18 hours. The membranes were quickly rinsed with TBS-t followed by 4 washes with TBS-t for 10 mins each. The membranes were then incubated with Peroxidase-Labeled Antibody to Rabbit IgG (KPL Antibodies; #074-1506, 1:5000) was used as secondary antibody for 1 hour. Chemiluminescent detection was performed by using Clarity™ Western ECL Substrate (Bio-Rad; #170506), and signal on immunoblots was captured by an Amersham Imager and quantified by densitometry using Image J (version 1.51, NIH, Bethesda, MD). The membrane was then washed with TBS-t followed by incubation in Restore™ Western Blot Stripping Buffer (Thermo Fisher Scientific; Cat # 21059) for 30 mins after immunoblotting for pERK1/2 and 50
mins after immunoblotting for non-phosphorylated ERK1/2.

The values for ERK1/2 activation were determined by calculating the ratio of pERK1/2 to non-phosphorylated ERK1/2 and further normalizing this ratio by the GAPDH value for each lane in each experiment. Since western blotting is a semi-quantitative technique and has experiment to experiment variability, we have presented our data as percentage of maximum response within that experiment. The maximum value in each individual run (all samples on that gel) was assigned a 100% and the other values were calculated accordingly to represent y axis of the graphs on the scale of 0-100%.

3.8 Radioligand Binding Assay

Transfected and NT HEK293 cells were plated in 24 well plates and serum starved for 12-16 hours with 400 µl HEPES (25mM) buffered DMEM. Three sets of tubes were labelled to collect free, membrane bound and internalized fractions. Two stock solutions were prepared – namely hot (\(^{125}\text{I}\)-labelled Ang II) and cold (\(^{125}\text{I}\)-labelled Ang II + 10 mM Losartan) to measure non-specific binding. Hot and cold solution was added to wells and incubated for specific time periods on plate incubator (37 °C). The plates were immediately placed on ice to stop the incubation period. The media with \(^{125}\text{I}\)-labelled Ang II was collected with 500 µl PBS wash. The cells were then incubated with acid wash solution (150 mM NaCl/50 mM glacial acetic acid) for 10 min to remove the membrane-bound \(^{125}\text{I}\)-labelled Ang II. This wash solution was then collected to measure acid-released \(^{125}\text{I}\)-labelled Ang II and then the cells were treated with solubilizing buffer (0.5 M NaOH and 0.05% sodium dodecyl sulfate) to collect the internalized \(^{125}\text{I}\)-labelled Ang II (acid-resistant). The radioactivities in acid-
released and acid-resistant fractions were measured by γspectrometry and the percent internalization for each individual point was calculated, after deducting the respective nonspecific value, from the ratio of the acid-resistant binding to the total (acid-resistant plus acid-released) binding.

3.9 Live Cell Imaging

HEK293 cells were seeded in 8 well Lab-Tek II chambered 1.5 German Coverglass System at 50,000 cells/well (Nunc; #155409). The next day, the cells were transfected with either WT or MT construct as described previously [74]. The cells were allowed to recover overnight, followed by serum starvation for 16-18 hours. 100X Ang II stock at 5 µg/µL (4.7793 mM) was prepared in advance. 2.1 µL of this stock was added to 998 µL medium to make stock solution of 10 µM. 3 µL of 10 µM Ang II was added to the well containing 300 µl of serum-free DMEM immediately prior to imaging (final concentration 100 nM).

Preliminary data collected by live cell imaging on Zeiss LSM510 Laser scanning confocal microscope (60X oil immersion lens/ 1.4 N.A. objective) was used to image the transfected HEK293 cells. All images were single pass scanned with the same scan speed, laser intensity, gain and pixel resolution. Images of cells before and after Ang II treatment were taken followed by time lapse imaging of cells after addition of Ang II (final concentration of 100nM). The number of vesicles internalised was calculated using Count Nuclei module of Metamorph and the images were analysed by Metamorph Offline image analysis software. Live cell microscopy was further performed using Nikon TE300 spinning disk microscope (63X oil immersion lens/ 1.4 N.A. objective) equipped with live cell imaging chamber (maintained at 37 °C and 5% CO₂).
For studying the rate of delivery of AT\(_{1a}\)R to lysosomes, 22.5 µL from a 1 µM LysoTracker Red DND-99 (ThermoFischer Scientific; #L7528) stock solution (0.5 µL of 1 mM LysoTracker Red DND-99 diluted in 499.5 µL of serum-free DMEM without phenol red) was added to each well for a final dye concentration of 75 nM. The cells were incubated at 37 °C in a humidified incubator with 5% CO\(_2\) for 25-30 minutes. The dye was removed gently and replaced with 300 µl of fresh serum-free DMEM without phenol red [74]. This set of live cell imaging was captured by Zeiss LSM880 Laser scanning confocal microscope (63X oil immersion lens/ 1.4 N.A. objective). The data analysis was performed by Volocity software.

### 3.10 Immunostaining

In a 12-well plate, clean coverslips were placed in each well. Transfected HEK293 cells were seeded on coverslips and were serum starved the next day. The cells grown on coverslips were incubated with Ang II for desired time and the cells were fixed immediately at the end of incubation. Fixing of cells was performed by quickly aspirating most of the media and flooding the well with 4% paraformaldehyde in phosphate buffered saline (PBS) for 20 mins at room temperature. After 20 mins, most of the paraformaldehyde was removed and PBS was added to fill the wells. Paraffin was used to tightly cover the plates and were stored at 4 °C until ready for immunostaining.

Before beginning the procedure for immunostaining, the cells were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 mins. Each of the coverslips were washed thrice with 1X PBS quickly without allowing the cells to dry out. The frozen goat serum vials were thawed for antibody dilutions for β-arrestin2 (Abcam; #ab54790) and pERK1/2 (Cell Signaling
Technology®; #9101s). The paraffin was stuck to the bench surface by scraping the edges of the film pressing it against the bench. 100 µl of the primary antibody dilution for β-arrestin2 (1:750) and pERK1/2 (1:100) was added on the paraffin (like drop cultures). At this point, each coverslip was carefully picked by the edges (circumference) by the curved forceps and gently inverted on the drop culture with primary antibody such that the cells on the coverslip come in contact with the drop. Following 1 hour of incubation, the primary antibody solution was aspirated and replaced by 100 µl of 1X PBS. PBS washes were pipetted thrice under the coverslip.

Goat anti-Rabbit IgG Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 (ThermoFischer Scientific; #A-11036) and Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 (ThermoFischer Scientific; #A-21052) for detecting pERK1/2 and β-arrestin2. A new paraffin was stuck to the adjacent area on the bench with 100 µl of secondary antibody (1:200) solution as drop culture. The incubation with secondary antibodies and washing was performed as described before. For mounting, each coverslip was inverted on 20 µl Fluoro-gel (Electron Microscopy Sciences; #17985-10) on a glass slide. Excess mounting media was aspirated out (without moving or pressing the coverslip) and allowed to dry. The glass slides were labeled and allowed to dry for 3-4 hours, followed by storing at 4 °C. The preliminary data was collected by imaging coverslips by Zeiss LSM510 laser scanning confocal microscope with 63X oil immersion len/ 1.4 N.A. objective) and analysis was performed by Volocity using Coste’s thresholding of single cells.

For PEP7 antibody (NeoBioLab Rabbit polyclonal Antibody MG-7 #A006922), dilutions of 1:200 and 1:1000 in ICC buffer (PBS with 0.25% gelatin, 2% normal goat serum 0.1% thimerosal, and 0.05% neomycin) were tested to determine working dilutions. The coverslips
seeded with HEK293 cells were incubated with primary antibody as described above for an hour at room temperature, followed by washing and then incubation with Fluoro-labeled goat anti-rabbit IgG. Mounted slides were then viewed by Zeiss LSM510 at 10X to make qualitative observations.

3.11 Imaging

This section is adapted from [74].

The stage of the microscope was adjusted down to avoid damage to the lens during startup of an automated microscope. The microscope power, scanner power, laser power and then laser emission and finally the metal halide lamp for visual observation was turned on. Desired lasers were turned on in the imaging software program (Zeiss or Leica). Imaging parameters like bit depth (12), pinhole (1 Airy disk unit), and appropriate emission wavelengths (eg-499-580 nm for EGFP) were set. While imaging, sequential and bidirectional scanning method was used. The objective was centered and a drop of high viscosity, low autofluorescence immersion oil was carefully added onto the objective. The chamber with the transfected cells or mounted slides were placed over the objective slides/chamber to ensure the bottom is flat and restrained from movement. The objective was moved up until the drop of oil just touches the bottom of the slide/chamber [74].

A field of view with healthy cells (preferably not overlapping cells) with optimal EGFP fluorescence (cells showing GFP fluorescence on the membrane and not saturated with GFP to avoid artifacts during analysis) was selected to image. The cell(s) of interest were focused and centered in the field of view. In confocal fast imaging mode, the Region of Interest (ROI) was further focused and centered using the stage XY control. The cell(s) of interest were confirmed to
be well spread (with its bottom or ventral side juxtaposed closely with the coverslip/chamber) and non-blebbing by focusing up and down through the cell. The bottom of the cell was focused to begin the z-stack and the top of the cell to end the stack (step size of 1.0 µm). The scan speed was set to approximately 1 µs per voxel and select 2 averaging by line. The laser intensity and gain for imaging EGFP and other fluorescent markers were adjusted. The imaging for immunostained/fixed cells was performed by collecting z-stack series of cells of interest. For live cell imaging experiments, the duration of scan and frequency of scanning was selected (every 30s over 25 mins or longer). Ang II was added carefully into the well with minimal disturbance and the imaging was started immediately for 20-25 mins [74].

3.12 Analysis Strategy for Imaging

This section is adapted from [74].

To develop a protocol to obtain quantitative evidence in time and space of receptor internalization and colocalization with lysosomes after treatment with Ang II, Volocity software was used. The captured time-lapse confocal images of transfected HEK293 cells were analyzed in 3D to quantitatively measure intensity of EGFP and other fluorescent markers. We have devised a method for 3D imaging and 3D analysis to thoroughly determine the route of the labelled AT1aR through the cell and to detect each subpopulation of vesicles differing in size and position.

Before performing image analyses, the images were exported in a tiff format along with the RAW, 12-bit data. X, Y, Z voxel dimensions and the z-step size were noted down for later use during image analysis. The first step of analysis is to create an image stack by opening the image in Volocity software (Figure 5). Using "Action/Create New/Images from Selected Sequence"
(Figure 5, arrow #1), a single file image was generated. The number of channels, z-sections, and the time points were entered. The final image stack was checked to make sure that it correctly represents the color channels, z-stack, and time points of the original image [74].

**Figure 5: Overview of Image Quantification Software.**

Key items are indicated by numbers and discussed within the methods text. 1) Action item, 2) Selected image, 3) free-hand ROI tool, 4) Measurements tab, 5) Find Objects, 6) Population 1 Find Objects dialog box, 7) Arrow activating time lapse movie, 8) Measurements item, 9) A second Find Objects dialog box containing a Filter Population command.

The image name (Figure 5, highlighted file name at left, arrow #2) was selected. By right clicking on the image or the image field, "Properties" was selected. In the µm pixel for (X) (Y)
and (Z) size, the correct image properties noted above were entered. For Background correction, the darkest areas in the images where there is no sample were inspected to calculate the mean or maximum brightness of pixels in that region (ignoring any unusually bright pixels that could be spurious fluorophore, a randomly bright pixel, or some other phenomenon). "Actions | Create New/Background Subtraction" was selected. The calculated pixel brightness was (made negative) as the Offset [74].

Sometimes, performing color registration is necessary especially in the case of colocalization like colocalized EGFP-containing vesicles and LysoTracker Red (Figure 6). By switching either the red or green color channel off and on, the need for color registration can be assessed. Visually, if registration is off by even 1 pixel, it is apparent to the eye. By selecting "Actions | Create New | Registration Correction" (Figure 5, arrow #1) a registration correction was generated and the dialog box was used to make adjustments. A new folder item entitled "Registration Correction" was created upon completion [74].

3.12.1 Analysis of AT1aR-EGFP Vesicle and Whole Cell Intensity

This section is adapted from [74].

To create measurement sequence, an image was selected (Figure 5, highlighted file name at left, arrow #2) and a region of interest (ROI) was drawn around a single cell using the freestyle region tool (Figure 5, arrow #3). The Extended Focus mode (2D) was used to observe the cell as the cropping tool only works in 2D visualization. By right clicking the image, "Crop to Selection" was selected and a new image item was generated. Clicking on its name, the "Measurement" tab above (Figure 5, arrow #4) for the cropped cell was selected. To define and measure internalized vesicles for GFP, "Find Objects" under "Finding" (Figure 5, arrow #5) was dragged into sequence
box (Figure 5, arrow #6). "Find Objects" Channel was set to the GFP channel. In settings (Figure 5, wheel icon in dialog box, arrow #6) "Threshold using:" was set to SD and "lower limit" to 6. The "Minimum object size" was set to 0 µm³. The SD lower limit depends on individual experiments. Visual confirmation that the correct objects are thresholded is made by examining different time points (Figure 5, arrow #7). The "Measurement | Feedback Options" dialog box was used to choose the way that selected thresholded objects are visualized (Figure 5, arrow #8).

"Measure" in "Find Objects" dialog box (Figure 5, arrow #6) for all channels were selected. "Intensity and Volume Measurements" was chosen as type of measurements [74].

Images with uncorrected and corrected pixel registration are shown together with insets of several colocalizing vesicles containing AT₁aR-EGFP. The red pixels are shifted 1 pixel to the left in the XY plane of the uncorrected image. Insets show boxed areas at higher magnification. Scale bar = 3.3 µm, voxel dimensions = 0.138 X 0.138 X 1 µm.

Figure 6: Color Registration Correction.
To define the thresholding and filters to identify the whole cell and measure total GFP intensity, the same thresholding steps were repeated using the following parameters: SD 0, "Minimum Object size to 2 µm$^3$ to create a second "Find Objects" dialog box (Figure 5, arrow #9). "Filter Population" under "Filtering" was dragged to the sequence box under population 2 (the second "Find Objects" box, Figure 5, arrow #9) and "Volume (µm$^3 > 100$) was selected. It is important to visually ensure that only one object is identified. The entire cell should be thresholded and all other objects filtered away. Once the measurement has been standardized, the protocol was saved by to reuse ("Restore Protocol"). By selecting "Measurements | Make Measurement Item" (Figure 5, arrow #8) and "All Timepoints", measurements were made. A new worksheet item was be created beneath the image [74].

To Analyze the data, "Analyze" tab was selected. Under "Restrict Analysis to:" desired Population was selected for analysis. Under "Analyze these data:" "Sum" (GFP color channel); under "Summarized by:" "Sum"; and, under "Organize the data by:" and "Row" "Timepoint" was selected. Analysis selections were also saved to be reused. The analysed data was then pasted directly to a blank spreadsheet. The whole cell intensity measurement is included with measurements of each vesicle in the population. Also, after plotting the data, if total GFP fluorescence contained within vesicles decreases then this can be Photobleaching [74].

### 3.12.2 Analysis of AT$_{1a}$R-EGFP in Lysosomes

This section is adapted from [74].

In the Find Objects (Population 1), "Find Objects" Channel was set to the red channel and in wheel icon within that dialog box indicated by arrow #6 (Figure 5) and "Threshold using:" was set to SD and "lower limit" to 6. Set "Minimum object size" to 0.017 µm$^3$. By clicking "Measure"
in "Find "Objects" dialog (Figure 5 arrow # 6), channels and types of measurements were selected as described previously. Typically, "All channels" and "Intensity and Volume Measurements" are selected. For Population 2, settings identical to that used to identify the whole transfected cell expressing AT₁aR-EGFP were used. Lysosomes must only be identified within the cell transfected with EGFP cell and not from nearby, untransfected cells. In this case, cropping the cell at the first stage of analysis is critical. The data was analyzed and exported as mentioned before [74].

3.13 Flow Cytometry

Transfected and NT HEK293 cells were plated in 6-well plates (1 x 10⁶ cells/well) followed by 10-12 hours of serum starvation prior to Ang II stimulation for 0, 24, 48 and 72 hours. The incubation period was stopped by placing the plates on ice. The serum-free media from each well was collected and transferred to tubes followed by quick addition and instantly removing ice-cold trypsin EDTA from each of the wells. The cells after detachment were collected in ice-cold complete DMEM and added to the respective tubes containing serum-free media. The tubes with cell suspension was centrifuged at 1000 rpm for 5 minutes, washed with ice-cold PBS-EDTA (0.5 mM) and centrifuged again. The supernatant was removed and the cells were re-suspended in 2ml of PBS-EDTA and collected in 5ml Round Bottom Tube PS w/Strainer Cap (Falcon # 352235) on ice. These tubes were run on a BD LSRFortessa Cell Analyzer (Cat. # 647177) located in the Lombardi Comprehensive Cancer Center Flow Cytometry and Cell Sorting Shared Resource (Georgetown University Medical Center). Data were collected using FACSDIVA™ software (BD Biosciences). Two sets of samples were prepared for Annexin V-Propidium Iodide (PI) staining for apoptosis and PI staining for cell cycle analysis. The raw data obtained from the core facility was analyzed to obtain percentage of EGFP(+) cells, MFI, percentage of EGFP(+) live, early
apoptotic and late apoptotic cells. Blinded flow cytometry data analysis was performed by FlowJo® software (FLOWJO, LLC; Ashland, OR) by PSK for GFP expression and apoptosis. For detecting EGFP(+) cells, the laser for GFP was used and hence in this section, the software shows GFP instead of EGFP.

Figure 7: Illustration of Thresholding to Identify EGFP- and Fluorescent Dye- Containing Vesicles and EGFP-Whole Cells.

A. Identification of whole cells using EGFP. AT$_{1a}$R-EGFP (EGFP, top row) and thresholded EGFP for the whole cell (ID Cell, bottom row) are shown at 0 and 2.5 min after Ang II addition. White boxes indicate inset areas shown in B. and C. B. AT$_{1a}$R-EGFP are compared at 0 and 2.5 min after Ang II addition in single color images (top row). Vesicles identified by thresholding are shown in the bottom row (ID Vesicles, purple). C. Two-color images (AT$_{1a}$R-EGFP /fluorescent dye) are shown at 0 and 2.5 min post Ang II addition. Lysosomes identified by thresholding are shown by red outlines. Scale bar = 7.0 µm, voxel dimensions = 0.114 X 0.114 X 1.4 µm.
Median Fluorescence Intensity - A region of interest (ROI) was first selected on the basis of two populations seen (Figure 8) on the basis of size and granularity. A histogram of count vs GFP was created for any non-transfected sample and a gate for GFP(+) cells was set right after a single peak observed on the histogram. The same ROI and the gate for GFP(+) cells was reused on all the samples. The percentage of GFP(+) cells and their median fluorescence intensity (MFI) was calculated for each sample.

Percentage of Live, Early apoptotic and Late Apoptotic cells – A ROI and gate GFP(+) for Annexin V-PI stained cells was created as mentioned above. The population of GFP(+) cells were further gated to get the percentage of live and apoptotic cells on the basis of Annexin V-PI staining as shown in a representative gating strategy (Figure 9). As seen in the figure, double negative population in Quadrant (Q) 4 are live cells, Annexin V(+) and PI(-) in Q3 are early apoptotic cells, double positive population in Q2 are late apoptotic cells, and Q1 contains debris or fragments of cells. Percentage of cells in growth phase 1 (G1), growth phase 2 (G2) and synthesis phase (S) – The data for cell cycle was analyzed by the core facility.

3.14 Mass Spectrometry

Mass spectrometry was performed by Georgetown Proteomics Shared Resource. A targeted mass spectrometry method, i.e., multiple reaction monitoring (MRM), was developed for the detection of molecular identity of PEP7. To determine the physiochemical property (e.g., hydrophobicity and retention time) and transitions for (multiple reaction monitoring) MRM measurement, a synthetic standard peptide PEP7 (MAGILSG) was analyzed on a NanoAcquity UPLC (Waters) coupled with a TripleTOF 6600 mass spectrometer (Sciex). For quantification,
PEP7 was analyzed on a NanoAcquity UPLC (Waters) coupled with a triple-quadrupole QTRAP 6500 mass spectrometer (Sciex). Instrument control and data acquisition were performed by Sciex Analyst software (version 1.6.2). For testing whether the PEP7 sORF is translated into a peptide, the presence of PEP7 was tested in the conditioned media (CM), exosome pellet of CM, and in HEK293 cell lysate.

Conditioned Media/ Exosome/ Cell lysate Sample Preparation - After transfection, complete DMEM was added to allow recovery of cells. DMEM was also added to culture dishes (150 mm) treated identically without cells (another negative control). Two plates per time point were seeded for WT, MT, NT and negative control. The media was changed to serum-free growth media (just enough media to cover the cells for conditioning) and allow cells to condition the media for 24 and 48 hours. Media was changed for the control plates as well. 21 and 28 ml of serum-free media was used for 24 hr and 48 hr of conditioning. This media from all of the plates after 24 and 48 hours - 2 plates per timepoints were pooled together (42 ml for 24 hr samples and 58 ml for 48 hr samples) followed by centrifugation at 1000g for 10 min.

After centrifugation of conditioned media, supernatant was carefully removed leaving small quantity of media behind (to make sure there are no cells in the media) and aliquoted immediately in 15 ml tubes. To isolate exosomal fraction, the cleared conditioned media (CM) was spun at 20,000 rpm in an ultracentrifuge using SW40Ti rotor. The contents were transferred (not more than 13 ml) into each Beckman tubes (compatible for high speed). All the tubes with the holders were weighed on weighing machine. Minute volumes in other 5 tubes were adjusted and placed on the rack holders to tighten the screws for each tube and then carefully the tubes were hung on the rotor placed on the stand. The rotor in the centrifuge was placed and the centrifuge door was properly closed. The conditioned media was spun at 20,000 rpm, 16 °C for 45 minutes
in vacum. After the run, the rotor was carefully removed and each tube was removed vertically with minimum disturbance. Each tube was pulled out from the holder using a forcep and then placed on ice gently. Immediately the supernatant was removed leaving behind 500 ul. The pellet (invisible to naked eyes) contains exosomes and the supernatant is the soluble fraction of CM. However, marker for exosomes were not tested to verify the presence of exosomes fraction.

Figure 8: Flow Cytometry Gating Representative Image for Selecting GFP(+) Cells.

Representative Flow cytometric analysis using FlowJo® software was performed by first making an ROI as shown in left panel in both (A) and (B), followed by gate for GFP(+) on A. NT sample. The same ROI and gate for GFP was used on all other B. WT/MT samples.
Figure 9: Representative Gating Strategy for Live and Apoptotic Cells.

Flow cytometric analysis using FlowJo® software was performed by gating the population of GFP(+) cells on a chart for Compensated PI area (Comp –PI-A) vs Compensated AlexaFluor 647 Annexin V (Comp-AlexaFluor-A) to separate population of cells in 4 quadrants (Q) on the basis of staining with PI and Annexin V.

For testing the presence of PEP7 in HEK293 cells, transfected cells were washed with 1X PBS twice, trypsinized after 18 hours of transfection and counted as 2.5 x 10^6 cells per aliquot. Aliquots were washed three times in PBS (1x) and stored as cell pellets at -80°C. Before lysis, 20 µl of isotopic labeled PEP7 (MAGIL^SG; the amino acid L is heavily isotopically labeled with ^13C, U^-13C_6, ^15N.; NewEngland Peptide) was spiked in the sample to be used as a positive control.
(The eppendorfs with cell lysate were vortexed for 1 min followed by quick spin down. The cell suspension was sonicated on ice with a probe-tip sonicator for 30 seconds (with 2 pulses for 15 sec each and a 15 sec rest between each pulse), followed by centrifugation at 13000g for 15 mins and the soup was collected in eppendorfs) The protocol for cell lysis and sample enrichment was optimized and the sample was injected into the nanoUPLC and Q-TRAP6500 system by Georgetown Proteomics Shared Resource.

3.15 Statistics

The data are expressed as means ± standard error of the mean (S.E.M). Using PRISM 8 software, statistical significance of the differences between groups (WT vs MT) were determined by two-way analysis of variance (ANOVA) or t-test unless otherwise indicated in each figure. Differences will be considered significant at p<0.05 (*p < 0.05; **p < 0.01; ***p < 0.001) vs WT.
CHAPTER 4. RESULTS

4.1 Plasmid Preparation Results

Each time plasmid was prepared, the concentration of purified plasmids was determined by Nanodrop. An example of Nanodrop results is depicted in Table 2. The plasmids were further sequenced each time for validation (data not shown).

Table 2: Representative Example of Nanodrop Results.

<table>
<thead>
<tr>
<th>Plasmid Sample Type</th>
<th>Concentration (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. WT #a</td>
<td>882.13</td>
</tr>
<tr>
<td>2. WT #b</td>
<td>944.30</td>
</tr>
<tr>
<td>3. MT #a</td>
<td>909.53</td>
</tr>
<tr>
<td>4. MT #b</td>
<td>1000.97</td>
</tr>
</tbody>
</table>

Two preps #a and #b for WT and MT plasmid concentrations.

4.2 Protein Estimation by Bicinchoninic Acid Assay

To determine variability and protein concentrations with the wells for WT and MT, a standard BCA graph is depicted in Figure 10. Protein estimation was performed for 3 wells of WT and MT to calculate the range of protein in each well for WT vs MT. Using this standard graph, concentration for WT and MT was calculated. The protein concentration was not different for WT vs MT. The coefficient of variation was found to be 6.96% and 9.17% respectively.
Figure 10. Protein Estimation by Bicinchoninic Acid Assay.

A Standard color response curve for A. Bovine Serum Albumin using the standard microplate procedure.
B. Protein concentration in WT and MT wells determined by standard BSA curve; N=1, n=3.
4.3  Effect of PEP7 sORF on Ang II-Induced ERK1/2 Activation

To test the effect of the PEP7 sORF on Ang II-induced phosphorylation of ERK1/2 (pERK1/2), HEK293 cells transfected with the AT1aR-WT and -MT constructs were incubated with increasing doses of Ang II. Ang II-induced ERK1/2 activation was measured at 5 minutes. Ang II-induced ERK1/2 activation was markedly inhibited in the cells transfected with the WT as compared to the MT construct (Figure 11).

Analysis by two-way ANOVA showed an effect of concentration of Ang II (p <0.0001) as well as sORF (p<0.001). The ECmax for both WT and MT was similar; however, the EC50 for WT and MT was found to be 5.42 nM and 0.32 nM respectively. Moreover, t-test showed that MT had significant increase in ERK1/2 activation at 10 nM (p<0.05).

4.4  Effect of PEP7 sORF on the Time Course of Ang II-Induced ERK1/2 Activation

To test the effect of the PEP7 sORF has a differential effect on Ang II-induced ERK1/2 activation with respect to time, transfected cells were incubated with Ang II from 0 to 80 minutes. At 10 nM of Ang II, WT cells showed lower Ang II-induced ERK1/2 activation for the first 20 mins as compared to mutant (Figure 12). Analysis by two-way ANOVA showed an effect of time of Ang II stimulation (p <0.0001) as well as sORF (p<0.01). Halftime (t1/2) calculated by non-linear regression curve (one phase association) revealed t1/2 of 3.7 mins and 2.9 mins for WT and MT respectively.
Figure 11: Dose Response of Ang II-Induced ERK1/2 Activation.

HEK293 cells transfected with WT or MT AT1Rs were incubated with Ang II (0, 0.1, 1 and 10 nM) for 5 min and then ERK1/2 activation was determined by Western blot analysis. A. Western blot image of pERK1/2, ERK1/2, and GAPDH. The blot is representative of four biological replicate experiments. B. Quantitation of the Western blot data by Image J represented as percentage of maximum response as a function of Log [Ang II] nM. Data are expressed as the mean ± S.E.M. ***p<0.001 vs. WT, analyzed by two-way ANOVA, N=3-4.
Figure 12: Time Course of Ang II (10 nM)-Induced ERK1/2 Activation.

HEK293 cells transfected with WT or MT AT$_1$Rs were incubated with 10 nM of Ang II for 0-80 min and then Erk1/2 activation was determined by Western blot analysis. A. Western blot of pERK1/2, ERK1/2, and GAPDH. The blot is representative of three biological replicate experiments. B. Quantitation of the Western blot data by Image J represented as percentage of maximum response as a function of time (minutes). Data are expressed as the mean of % maximum response ± S.E.M. **p<0.01 vs. WT, analyzed by two-way ANOVA, N=3.
Similarly, the effect of the PEP7 sORF on Ang II-induced ERK1/2 activation at 100 nM was also tested (Figure 13). 100 nM of Ang II stimulation showed similar Ang II-induced ERK1/2 activation in WT and MT cells at 5 mins as seen previously from dose response like (Figure 11 B). Analysis by two-way ANOVA showed an effect of time of Ang II stimulation (p <0.0001) on Ang II-induced ERK1/2 but no effect of sORF was observed at 100 nM of Ang II.

4.5 Effect of PEP7 sORF on SII-induced ERK1/2 activation

To study the effect of PEP7 sORF on β-arrestin-dependent ERK1/2 activation, SII was used to predominantly activate the β-arrestin-dependent pathway. Transfected HEK293 cells were stimulated with increasing doses of SII for 5 mins. The cells with PEP7 sORF (WT) construct showed attenuated dose response to SII-induced ERK1/2 activation as compared to MT (Figure 14). Analysis by two-way ANOVA showed an effect of concentration of SII (p <0.0001) as well as sORF (p<0.01). The EC50 for WT and MT was found to be 38.66 µM and 11.19 µM respectively.

4.6 Effect of PEP7 sORF on the time course of SII-induced ERK1/2 activation

To study the time course of SII-induced ERK1/2 activation for 0 to 80 mins, 30 µM of SII was selected based on the Kd for SII (0.3 µM) [75]. WT cells consistently responded with lesser ERK1/2 activation as compared to MT (Figure 15). Analysis by two-way ANOVA showed an effect of time of SII stimulation (p <0.0001) as well as sORF (p<0.05). Halftime (t1/2) calculated by non-linear regression curve (one phase association) revealed t1/2 of 7.6 mins and 4.7 mins for WT and MT respectively.
Figure 13: Time Course of Ang II (100 nM)-Induced ERK1/2 Activation.

HEK293 cells transfected with wildtype (WT) or mutant (MT) AT1aRs were incubated with 100 nM Ang II for 0-80 min and then Erk1/2 activation was determined by Western blot analysis. A. Western blot of pERK1/2, ERK1/2, and GAPDH. The blot is representative of three biological replicate experiments. B. Quantitation of the Western blot data by Image J. Data are expressed as the mean ± S.E.M. *P<0.05 vs. WT, analyzed by two-way ANOVA, N=3.
HEK293 cells transfected with WT or mutant MT AT1aRs were incubated with SII (0, 15, 30 and 60 µM) for 5 min and then ERK1/2 activation was determined by Western blot analysis. A. Western blot of pERK1/2, ERK1/2, and GAPDH. The blot is representative of three biological replicate experiments. B. Quantitation of the Western blot data by Image J as percentage of maximum response. Data are expressed as the mean ± S.E.M. **p<0.01 vs. WT, analyzed by two-way ANOVA, N=3.

Figure 14: Dose Response of SII-Induced ERK1/2 Activation.
Figure 15: Time Course of SII-Induced ERK1/2 Activation.

HEK293 cells transfected with WT or mutant MT AT1aRs were incubated with SII (30 µM) for 0-80 min and then ERK1/2 activation was determined by Western blot analysis. A. Western blot of pERK1/2, ERK1/2, and GAPDH. The blot is representative of three biological replicate experiments. B. Quantitation of the Western blot data by Image J as percentage of maximum response as a function of time (minutes). Data are expressed as the mean ± S.E.M. *p<0.05 vs. WT, analyzed by two-way ANOVA, N=3.
4.7 Effect of PEP7 sORF on Ang II-Induced ERK1/2 Activation with PKC Inhibitor

To study the effect of PEP7 sORF on ERK1/2 activation through PKC pathway, Ro 31-8220 (an inhibitor of PKC [76]) was used at increasing doses prior to Ang II (10 nM) treatment (Figure 16). 1, 0.1, and 10 µM of inhibitor inhibited Ang II-induced ERK1/2 activation as compared to the control, however ERK1/2 activation in WT and MT was similar at each of these doses. Analysis by two-way ANOVA showed an effect of Ro 31-8220 concentration (p<0.0001) but not an effect of sORF.

4.8 Effect of PEP7 sORF on Expression of AT₁αR

Using radioligand binding assay, internalized and membrane specific binding for AT₁αR in WT and MT HEK293 cells were measured. MT had higher membrane and internalized AT₁αR binding across all time points. Analysis by two-way ANOVA showed an effect of Ang II stimulation over time was observed (p<0.0001) and an effect of the PEP7 sORF on the internalized (Figure 17A) and membrane specific binding (p<0.0001) (Figure 17B). Moreover, Sidak’s multiple comparisons test also revealed that MT had significantly higher internalized binding for 10-75 mins and higher membrane binding at 30 mins.

However, the percentage of internalized and membrane binding calculated for each point was similar for MT and the WT (Figure 18).
HEK293 cells transfected with WT or MT AT₁Rs were incubated with Ro 31-8220 (0, 0.1, 1 and 10 µM) for 15 min and then incubated with Ang II (10 nM). ERK1/2 activation was determined by Western blot analysis. A. Western blot of pERK1/2, ERK1/2, and GAPDH. The blot is representative of three biological replicate experiments. B. Quantitation of the Western blot data by Image J as percentage of maximum response. Data are expressed as the mean ± S.E.M. *p<0.05 vs. WT, analyzed by two-way ANOVA, N=3.
Figure 17: $^{125}$I-Ang II Specific Binding Assessed by Radioligand Binding Assays.

HEK293 cells non-transfected (NT), transfected with wildtype (WT), or mutant (MT) AT$_{1a}$R were incubated with $^{125}$I-Ang II for 0-75 mins. $^{125}$I-Ang II specific binding was assessed as A. Intracellular/internalized binding; B. Membrane binding. Data are expressed as mean ± S.E.M, analyzed by two-way ANOVA and Sidak’s multiple comparisons test. * P 0.05 vs. WT (*p < 0.05; **p<0.01; ***p < 0.001; ****p < 0.0001); N=2, n=4.
Figure 18: Percentage of $^{125}\text{I}$-Ang II Binding Assessed by Radioligand Binding Assays.

Time course of A. internalized and B. membrane specific binding in HEK-293 cells transfected with wildtype (WT), or mutant (MT) AT$_1$R. Data are expressed as mean $\pm$ S.E.M, analyzed by two-way ANOVA; * p<0.05 vs. WT; N=2, n=4.
4.9 Effect of PEP7 sORF on Vesicle Number After AT₁aR Internalization

Preliminary data collected on the Zeiss LSM510 Laser scanning confocal microscope showed differences in AT₁aR trafficking in MT as compared to the WT on Ang II (100 nM) stimulation. The white dots in the figure depict AT₁R and as seen from the (Figure 19-20), the puncta formation (white dots) becomes evident 2 minutes after the beginning of time lapse imaging in the WT (Figure 19) sample (time lapse imaging was started approximately 3 minutes after the treatment of Ang II). The puncta formation in the MT (Figure 20) shows puncta formation after 8 minutes of treatment with Ang II (shows 7 minutes from time lapse imaging). The number of vesicles formed after Ang II treatment (normalized to basal) are greater in the presence of the PEP7 sORF (Figure 21).

4.10 Effect of PEP7 sORF on the rate of AT₁aR internalization

To test the effect of the PEP7s ORF on the rate of internalization of AT₁aR, live cell imaging was performed using Nikon TE300 spinning disk confocal microscope. AT₁aR observed by green fluorescence is well diffused in the cytoplasm. Similar to preliminary data, Ang II (100 nM) stimulation leads to internalization of the AT₁aR in the vesicles in both WT and MT over time (p=0.0001) as observed by green puncta. Analysis by two-way ANOVA showed that the PEP7 sORF increased the rate of AT₁aR internalization as compared to the MT (p<0.0001) (Figure 22). The t₁/2 for MT (251 seconds) was almost twice the t₁/2 for WT (118 seconds).
Figure 19: Time Lapse Images of WT HEK293.

Representative time lapse imaging of WT HEK293 cells on Ang II (100 nM) stimulation captured by Zeiss LSM510 Laser scanning confocal microscope; shown as A. images at every 70-71 seconds over 21 minutes and changes observed in AT$_{1a}$R trafficking in WT cells after 2.8 mins B. and 23.8 mins (C) of Ang II treatment.
Figure 20: Time Lapse Images of MT HEK293.

Representative time lapse imaging of MT HEK293 cells on Ang II (100 nM) stimulation Zeiss LSM510 Laser scanning confocal microscope; shown as A. images at every 70-71 seconds over 33 minutes and changes observed in AT$_{1a}$R trafficking in MT cells after B. 1 min and C.33.7 mins of Ang II treatment.
Figure 21: Analysis of Vesicles Number.

On Ang II (100 nM) stimulation, HEK293 cells transfected with WT or MT AT₁aRs were imaged by Zeiss LSM510 Laser scanning confocal microscope. A. the total number of vesicles and B. The ratio for the total number of vesicles formed after Ang II treatment to the basal count. Quantitation of this data was performed by Metamorph Offline image analysis software. Data are expressed as the mean ± S.E.M. *p<0.05 vs. WT, analyzed by unpaired t-test, N=2, n=3-7.

To further test the effect of the PEP7 sORF on the AT₁aR internalization at a lower dose, preliminary data was collected on Lieca SP8 Laser scanning confocal microscope. On Ang II (10 nM) stimulation, AT₁aR was internalized in the vesicles as seen by both live cell (data not shown) and fixed imaging (Figure 23). However, the size of vesicles formed on 10 nM Ang II stimulation was much smaller as compared to 100 nM stimulation (visually compare green puncta in Figure 20A and 21A). Analysis by two-way ANOVA showed an effect of Ang II stimulation (p<0.05) but an effect of PEP7 sORF was not seen on the fraction of the AT₁aR-EGFP in the vesicles.
Figure 22: Analysis of Relative Intensity of AT₁aR in Vesicles.

Live cell Imaging of transfected HEK293 cells captured by Nikon TE300 spinning disk confocal microscope. A. Representative time lapse images of WT and MT HEK293 cells stimulated with Ang II (100 nM) for 0-20 minutes. B. Graphical representation of quantitation of time lapse studies shown in A. Data are expressed as mean ± S.E.M, analyzed by two-way ANOVA. ****p < 0.0001 vs. WT; N=3, n=9-21.
Figure 23: Analysis of Fraction of AT₁aR-EGFP in the Vesicles.

Transfected HEK293 cells were incubated with Ang II (10 nM) and fixed at 0, 30, 60 and 90 mins. The fixed cells were imaged by Leica SP8 Laser scanning confocal microscope. A. Representative images of WT (top panel) and MT (bottom panel) HEK293 cells stimulated with Ang II (10 nM) for 0 and 60 mins. B. Graphical representation of quantitation of fraction of the AT₁aR-EGFP in the vesicles. Data are expressed as mean ± S.E.M, analyzed by two-way ANOVA. *p < 0.05 vs. WT; N=1-2, n=14-23.
4.11 Effect of PEP7 sORF on the Rate of AT_{1a}R Delivery to Lysosomes

To test the effect of the PEP7s ORF on the rate of AT_{1a}R delivery, live cell imaging was performed using Zeiss LSM880 laser scanning confocal microscope. AT_{1a}R tagged to EGFP was seen as green florescence and lysosomes were stained red. On Ang II (100 nM) stimulation, the AT_{1a}R in both WT and MT internalized over time (Figure 24A). The average intensity of EGFP in the lysosomes was quantified over time by Volocity software. Analysis by two-way ANOVA did not reveal an effect of Ang on the receptor delivery to lysosomes, however, WT had higher average intensity of EGFP in the lysosomes as compared to the MT (p<0.01)

4.12 Effect of PEP7 sORF on colocalization of AT_{1a}R with β-arrestin2

To test the effect of the PEP7 sORF on the colocalization of crucial signaling components downstream of the AT_{1a}R, immunostaining for pERK1/2 and β-arrestin2 was performed (Figure 25). Our preliminary data suggests that presence of the PEP7 sORF reduces the Pearson’s correlation of β-arrestin2 with the AT_{1a}R (Figure 25B) at 10 mins of Ang II (100 nM) stimulation (p<0.05).

4.13 Effect of PEP7 sORF on EGFP Expression

To test the effect of PEP7 sORF on EGFP expression, flow cytometry was performed to measure the percentage of transfected cells i.e EGFP(+) cells (Figure 26A,C) as well as the intensity of EGFP expressed by MFI (Figure 26B,D). Analysis by two-way ANOVA showed an effect of time on the percentage of EGFP(+) cells in vehicle treated (p<0.01) (Figure 26A) as well as Ang II treated cells (p<0.0001) (Figure 26C); and on the MFI in both vehicle (Figure 26B) and Ang II treated cells (p<0.0001) (Figure 26D).
Figure 24: Analysis of AT\textsubscript{1a}R Delivery to Lysosomes.

Live cell Imaging of transfected HEK293 cells stained with LysotrackerRed was captured by Zeiss LSM880 Laser scanning confocal microscope. A. Representative time lapse images of HEK293 cells stimulated with Ang II (100 nM) and stained for lysosomes (shown in red). B. Graphical representation of quantitation of time lapse studies shown in A. Data are expressed as mean ± S.E.M, analyzed by two-way ANOVA **p<0.01 vs. WT; N=1, n=5-7.
Figure 25: Immunostaining of pERK1/2 and β-arrestin2.

HEK293 cells transfected WT or MT stimulated with Ang II (100 nM) followed by immunostaining for pERK1/2 and β-arrestin2 were imaged by Zeiss LSM510 laser scanning confocal microscope. Representative images of Ang II-stimulated WT and MT HEK293 cells for 60 minutes in the left panel with colocalization of AT1aR (green) with A. pERK (magenta), B. β-arrestin2 (yellow) and, C. colocalization β-arrestin2 (yellow) and pERK (magenta). Insets for each of the left panel shows arrows pointing at colocalization examples in that image. The right panels B, D and, F shows the quantitation of colocalization shown by insets in A, C and, E respectively. Data are expressed as mean ± S.E.M, analyzed by t-test *p<0.05 vs. WT; N=1, n=3-6.
However there was no effect of PEP7 sORF in the percentage of EGFP(+) cells in WT vs MT in both vehicle and Ang II treated cells (Figure 26A, C). MFI was slightly higher in MT as compared to WT for vehicle treated cells at zero time point (p<0.05) and Ang II treated (p=0.051) (Figure 26B, D).

![Figure 26: Analysis of EGFP Expression by Flow Cytometry.](image)

EGFP expression analysis of HEK293 cells transfected with wildtype (WT) or mutant (MT) EGFP tagged AT₁aRs by flow cytometry. Shown is the effect of PEP7 sORF on the percent of EGFP(+) cells in A. vehicle treated cells and C. Ang II (10 nM) treated cells; as well as on the MFI of EGFP(+) cells in B. vehicle treated cells and D. Ang II (10 nM) treated cells as a function of time determined by flow cytometry. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=3-6.
4.14 Effect of PEP7 sORF on Cell Death

To test the effect of PEP7 sORF on cellular functions like apoptosis (Figure 27-31), flow cytometry was performed using Annexin-V PI staining. Transfected cells were incubated with 10 nM or 100 nM Ang II or vehicle before processing them for flow cytometry. Preliminary data was collected on 100 nM treated cells (Figure 27). An effect of time of Ang II treatment was seen (p<0.0001) on the percent of live, early apoptotic and late apoptotic cells but no effect of the PEP7 sORF was observed. Initially, a shorter Ang II (10 nM) time course (0-12 hours) was studied. The presence of PEP7 sORF did not affect the percentage of live, early apoptotic or late apoptotic EGFP(+) cells as compared to the MT (Figure 28A-C). Analysis by two-way ANOVA showed an effect of Ang II (p<0.0001) on apoptosis but not an effect of sORF.

Furthermore, vehicle controls were also studied with 10 nM of Ang II treated cells to distinguish the effect of time and Ang II treatment. Analysis by two-way ANOVA showed an effect of Ang II (p<0.0001) on vehicles (B), treated (C) as well as normalized groups (A). The presence of PEP7 sORF increased the percentage of live EGFP(+) cells as compared to the MT in Ang II treated as well as normalized groups (Figure 29A) (p<0.05). There were no significant differences observed for EGFP(+) cells normalized to vehicle controls for early and late apoptosis (Figure 30A, 31A) in WT vs MT. We did observe an effect of sORF on Ang-II induced early apoptosis on vehicle and Ang II treated cells (Figure 30B, C). The presence of the PEP7 sORF reduced the percentage of early apoptotic cells as seen by two-way ANOVA on vehicle treated cells (p=0.051) and treated cells (p<0.01).
Figure 27: Apoptosis Analysis on Ang II (100 nM) Stimulation Over 0-72 Hours.

Analysis of the PEP7 sORF modulation of apoptosis in HEK293 cells transfected with wildtype (WT) or mutant (MT) AT$_{1}$aRs on Ang II (100 nM) treatment. Shown is the effect of PEP7 sORF on the percent of A. live EGFP(+) cells, B. early apoptotic EGFP(+) cells and, C. late apoptotic EGFP(+) cells on Ang II (100nm) treatment cells for each time point, determined by flow cytometry. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=1-2.
Figure 28: Apoptosis Analysis on Ang II (10 nM) Stimulation Over 0-12 Hours.

Analysis of the PEP7 sORF modulation of apoptosis at early time points in HEK293 cells transfected with wildtype (WT) or mutant (MT) AT_{1a}Rs. Shown is the effect of PEP7 sORF on the percent of A. live EGFP(+) cells, B. early apoptotic EGFP(+) cells and, C. late apoptotic EGFP(+) cells on Ang II (10nm) treatment cells for each time point, determined by flow cytometry. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=2-5.
Figure 29: Analysis of Live EGFP(+) Cells Over 0-72 Hours.

Analysis of the PEP7 sORF modulation of cell survival in HEK293 cells transfected with wildtype (WT) or mutant (MT) AT1aRs. Shown is the effect of PEP7 sORF on the percent of live EGFP(+) cells in A. Ang II treated group normalized to vehicle controls, B. vehicle treated cells and, C. Ang II (10nm) treated cells for each time point, determined by flow cytometry. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=3-6.
Figure 30: Analysis of Early Apoptotic EGFP(+) Cells Over 0-72 Hours.

Analysis of the PEP7 sORF modulation of early apoptosis in HEK293 cells transfected with wildtype (WT) or mutant (MT) AT$_{1a}$Rs. Shown is the effect of PEP7 sORF on the percent of early apoptotic EGFP(+) cells in A. Ang II treated group normalized to vehicle controls, B. vehicle treated cells and, C. Ang II (10nm) treated cells for each time point, determined by flow cytometry. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA in A-C and additionally Sidak’s multiple comparisons test in B; N=3-6.
Figure 31: Analysis of Late Apoptotic EGFP(+) Cells Over 0-72 Hours.

Analysis of the PEP7 sORF modulation of late apoptosis in HEK293 cells transfected with wildtype (WT) or mutant (MT) AT<sub>1a</sub>Rs. Shown is the effect of PEP7 sORF on the percent of early apoptotic EGFP(+) cells in A. Ang II treated group normalized to vehicle controls, B. vehicle treated cells and, C. Ang II (10nm) treated cells for each time point, determined by flow cytometry. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=3-6.
Additionally, Sidak’s multiple comparison test revealed that WT group had lesser early apoptotic cells as compared to MT in vehicle treated group at 72 hours (p<0.05) (Figure 30B). Analysis by two-way ANOVA showed an effect of Ang II (p<0.0001) on vehicles as well as treated groups for early and late apoptosis. There was no effect of the presence of PEP7 sORF on late apoptosis (Figure 30C).

4.15 Effect of PEP7 sORF on Proliferation

To test the effect of PEP7 sORF on proliferation, cell cycle analysis was performed by flow cytometry. Preliminary data was collected for both 10 nm and 100 nm of Ang II stimulation. Analysis by two-way ANOVA on preliminary data did not show an effect of PEP7 sORF on the percent of cells in G1, G2 or S phase for both doses of Ang II (Figure 32, 33). There was an effect of time on G1 and S phases for both doses (p<0.0001)

To specifically test the effect of Ang II on EGFP(+) cells, the cells were first sorted and then cell cycle analysis was performed along with the vehicle controls. Ang II had an effect over time of stimulation for cells in G1 phase (p<0.05) but there was no effect of the PEP7 sORF difference in the percentage of cells in G1 (Figure 34A), G2 (Figure 34B) and S phase (Figure 34C) normalized to vehicle controls.
Cell cycle analysis of Ang II (10 nM) modulation of HEK293 cells transfected with wildtype (WT) or mutant (MT) AT$_1$aRs. Shown is the effect of Ang II (10 nm) on the percent of A. cells in G1 phase, B. cells in G2 phase and C. cells in S phase determined by flow cytometry as a function of time. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=1-3.
Cell cycle analysis of Ang II (100 nM) modulation of HEK293 cells transfected with wildtype (WT) or mutant (MT) AT$_1$aRs. Shown is the effect of Ang II (100 nM) on the percent of A. cells in G1 phase, B. cells in G2 phase and C. cells in S phase determined by flow cytometry as a function of time. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=1-2.
Figure 34: Cell Cycle Analysis of EGFP(+) Cells on Ang II (10nM) Stimulation Over 0-72 Hours.

Cell cycle analysis of Ang II (10 nM) modulation of HEK293 cells transfected with wildtype (WT) or mutant (MT) AT_{1a}Rs. Shown is the effect of Ang II (10 nM) on the percent of A. EGFP(+) cells in G1 phase, B. EGFP(+) cells in G2 phase and C. EGFP(+) cells in S phase determined by flow cytometry as a function of time. Data are expressed as the mean ± S.E.M; *P < 0.05 vs. WT, analyzed by two-way ANOVA; N=3.
4.16 Detection of PEP7 by Immunostaining

To detect PEP7 in WT sample, immunostaining was performed on transfected HEK293 cells. Two dilutions -1:200 and 1:1000 (Figure 35) were used to collect some preliminary data on Zeiss LSM510 laser scanning microscope at 10X to get qualitative data. The signal for the rat PEP7 antibody was observed in both WT and MT at both the dilutions in our preliminary data.

![Figure 35: Immunostaining for PEP7.](image)

HEK293 cells transfected the WT or MT AT₁₄R followed by immunostaining for PEP7 were imaged by Zeiss LSM510 laser scanning confocal microscope. Representative image for A. WT and B. MT HEK293. Qualitative observations were made on preliminary data; N=1
Detection of PEP7 by Mass Spectrometry in Transfected HEK293 Lysate

By coupling nanoUPLC with advanced mass spectrometry techniques, a method for the measurement of PEP7 was developed by the Proteomics shared resource. Specifically, PEP7 was analyzed by the NanoAcquity UPLC system coupled with a TripleTOF 6600 mass spectrometer, with a typical mass spectrum shown in Figure 36A. As can be seen, a series of fragments of PEP7 were produced with collision induced dissociation (CID) fragmentation.

To get the highest signal of each fragment, the collision energy by using the QTRAP 6500 mass spectrometer was also optimized. Based on the intensity of fragments, two MRM transitions (i.e., 648.3/486.2 and 648.3/573.3) were selected for the identification and quantification of PEP7, with the optimized CE values shown in Table 3. The limit of quantification (LOQ, S/N>=10) of PEP7 measurement was determined to be 1.5 femto mole (fm), with the corresponding MRM spectra shown in Figure 36B.

For the measurement of PEP7 in cell lysates, we used isotopically labeled PEP7 (MAGIL^SG) as internal standard, with the transitions for MRM detection listed in Table 3. To 2.5 x 10^6 HEK293 cells with/without PEP7 sORF, we spiked in the isotopically labeled PEP7 peptide before cell lysis. After thorough optimization and comparison of several peptide extraction, the methanol-precipitation approach was adopted to extract PEP7 peptide. Although the spiked-in isotopically labeled PEP7 peptide was successfully detected, no confident peaks corresponding to endogenous peptide PEP7 were identified, from the PEP7-transfected HEK293 cells. (Figure 39). For all the mass spectrometry data in Figure 37-41, the blue and the red lines on the graphs depict MRM transitions for b5 and b6 ions respectively.
Figure 36: Mass Spectrometry of PEP7.

Mass spectrometry analysis A. Fragmentation of PEP7 by using nanoUPLC-MS/MS. A nanoAcquity UPLC coupled with a Triple TOF 6600 mass spectrometer was used to investigate the fragmentation pattern of PEP7. A series of fragment ions of PEP7 were obtained by the collision induced dissociation (CID) approach. B. Measurement of PEP7 by using nanoUPLC-MRM-MS method. A nanoAcquity UPLC coupled with a triple-quadrupole Q-TRAP 6500 mass spectrometer was used to monitor the fragments of PEP7. Specifically, 4 transitions (i.e., 648.3/260.1, 648.3/345.2, 648.3/373.2, and 648.3/486.3) are illustrated. The limit of quantification (LOQ, S/N>=10) of PEP7 is determined to be 1 pg (i.e., 1.5 fmol).
Table 3: MRM Transitions for PEP7.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Precursor (Q1)</th>
<th>Fragment (Q3)</th>
<th>Ion type</th>
<th>Optimized CE</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGILSG</td>
<td>648.3</td>
<td>486.2</td>
<td>b5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>648.3</td>
<td>573.3</td>
<td>b6</td>
<td>28</td>
</tr>
<tr>
<td>MAGIL^SG</td>
<td>655.3</td>
<td>493.2</td>
<td>b5^</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>655.3</td>
<td>580.3</td>
<td>b6^</td>
<td>28</td>
</tr>
</tbody>
</table>

MRM transitions used for the detection and quantification of PEP7 (MAGILSG) and its isotopic form (MAGIL^SG; in which amino acid L is heavily labeled with $^{13}$C$_6$$^{15}$N).

Furthermore, the presence of PEP7 was also tested in the CM as well as the exosomal fraction obtained from NT, WT and MT HEK293 cells. With the method that was optimized for cell lysate, we did not observe any peak for PEP7 in the CM (Figure 40) and exosomal fraction (Figure 41) in the WT sample for 24 hours.
Figure 37: Detection of PEP7 in the NT Cell Lysate.

Representative mass spectra with (top panel) and without (bottom panel) the addition of isotopic-labeled synthetic PEP7 (MAGI^LSG) using nano UPLC. Typical MRM Mass spectra analysis is shown for NT HEK293 cells.
Figure 38: Detection of PEP7 in the MT Cell Lysate.

Representative mass spectra with (top panel) and without (bottom panel) the addition of isotopic-labeled synthetic PEP7 (MAGI^LSG) using nano UPLC. Typical MRM Mass spectra analysis is shown for MT HEK293 cells.
Figure 39: Detection of PEP7 in the WT Cell Lysate

Representative mass spectra with (top panel) and without (bottom panel) the addition of isotopic-labeled synthetic PEP7 (MAGI^LSG) using nano UPLC. Typical MRM Mass spectra analysis is shown for WT HEK293 cells.
Figure 40: Detection of PEP7 in the WT and MT CM.

Representative mass spectra of media conditioned for 24 hours by A. WT and B. MT HEK293 cells with (top panel) and without (bottom panel) the addition of isotopic-labeled synthetic PEP7 (MAGI^LSG) using nano UPLC.
Figure 41: Detection of PEP7 in the WT and MT Exosomal Fraction.

Representative mass spectra of exosomal fraction from media conditioned for 24 hours by A. WT and B. MT HEK293 cells with (top panel) and without (bottom panel) the addition of isotopic-labeled synthetic PEP7 (MAGI^LSG) using nano UPLC.
CHAPTER 5. DISCUSSION

This dissertation has five major findings on the role of PEP7 sORF in the 5’ leader sequence of the AT₁₆R. Our data shows that the presence of PEP7 sORF attenuated ERK1/2 activation induced by 1) the natural ligand of AT₁R i.e. Ang II, and 2) the partial agonist of AT₁R, SII which acts through the β-arrestin pathway primarily. Our third and fourth major finding suggests that the presence of PEP7 sORF 3) reduces the expression of the AT₁₆R and 4) increases the rate of Ang II (100 nM)-induced internalization. Our fifth major finding, 5) suggests that the PEP7 sORF promotes cell survival in AT₁₆R transfected HEK293 cells. These observations expand upon our previous studies which showed that synthetic PEP7 pretreatment in male rats reduced the increase in Ang II-induced arterial mean pressure and also significantly reduced Ang II-induced saline intake but not water intake over time [66]. In those studies, western blot analysis using HEK293 cells transfected with the highly conserved rat E1,2,3 AT₁₆R showed that pretreatment of HEK293 cells with synthetic PEP7 inhibited Ang II-induced ERK1/2 phosphorylation [66]. In this study, we focus on the PEP7 sORF by in vitro transfection experiments in which the sORF is expressed using cDNA containing all the exons, an approach not previously taken.

Our first major finding shows that the presence of the sORF from exon 2 [WT; E1,2,3-AT₁₆R-EGFP] in transiently transfected HEK293 cells inhibited Ang II-induced ERK1/2 activation like the effect of the synthetic PEP7 peptide application on HEK293 cells in our previous study [66]. A dose dependent Ang II-induced ERK1/2 activation was observed in both WT and MT HEK293 cells. The inhibitory effect of the PEP7 sORF as compared to the MT on Ang II-induced ERK1/2 activation at the dose of 10 nM was not observed at 100 nM. The dose response curve for both WT and MT shows that the presence of PEP7 sORF significantly increased EC50 (Figure 11).
It is important to note that most of the previous studies done on Ang II-induced ERK1/2 activation were studied with 100 nM of Ang II. However, data from dose response curve (Figure 11) suggested an effect of PEP7 sORF on 10nM of Ang II, we have used this dose primarily to study AT1aR signaling. Our time course data suggests that following application of 10 nM of Ang II, the inhibitory effect of PEP7 sORF on phosphorylation of ERK1/2 was not observed after the first 20 min of Ang II (10 nM) treatment in HEK293 cells (Figure 12). Previous studies have demonstrated that the response to Ang II over time can be divided into an early (transient) vs late (sustained) phase based on immunoblotting for Ang II-induced (100 nM) ERK1/2 in HEK293 cells transfected with HA-AT1aR and β-arrestin2 siRNAs [42]. For example, after β-arrestin2 knockdown, Ang II (100 nM) induced-ERK1/2 activation showed a sharp decrease between 2 – 10 mins followed by further dropping to a basal level at 90 mins whereas controls showed peak activation between 2-10 mins followed by about 40% decrease over time until 90 min [42]. Our time course data using 10 nM suggests that Ang II-induced ERK1/2 activation rises sharply over the first 0-10 mins but later tends to decrease slowly until 80 mins. The presence of PEP7 sORF increased t1/2 of Ang II for ERK1/2 activation as compared to the MT (Figure 12). However, the differences between the WT and MT were not different at 100 nM of Ang II. This kind of dose-different effect that we observed in Ang II-induced ERK1/2 activation for 10 and 100 nM (Figure 11-13) could be a result of Ang II saturation at higher dose.

Our data in Figure 12, 13 indicates the same trend (shape of the curve) over time as that reported previously for HEK293 cells [42]. Our data suggests that at 10nM dose, there was an overall effect of Ang II-induced ERK1/2 activation in MT vs WT indicating the dynamics of downstream signaling of AT1R are temporal. It has been postulated for a long time that Ang II-induced ERK1/2 activation is a consequence of G protein- and β-arrestin-dependent signaling in
HEK293 cells [13, 35-37]. The β-arrestin-dependent ERK1/2 activation in HEK293 cells on Ang II (100 nM) stimulation was shown to be a sustained effect over 5-90 mins [42]. We found that early ERK1/2 activation (2.5 mins) was similar for WT and MT at Ang II (10 nM) but late ERK1/2 activation (5 mins onwards) was significantly different. This suggested that β-arrestin-dependent ERK1/2 activation might be attenuated in the case of WT as compared to MT. The G protein- and β-arrestin-dependent pathways have been shown to have distinct downstream effects both at cellular and functional level. For example, TRV120027, a selective agonist of β-arrestin-dependent pathway showed beneficial effects like blood pressure reduction as well as pathway vs the other has gained momentum over last decade [18, 35, 42]. However, dissecting these two pathways to study the significance of each pathway has not been very clear in the literature.

Our second major finding was that the presence of PEP7 sORF also attenuates SII (partial agonist) -induced ERK1/2 activation (Figure 13, 14). SII has been defined as an agonist of the β-arrestin-dependent pathway downstream of the AT1R and been used to specifically study the consequences of activation of β-arrestin-dependent-ERK1/2 signaling [42, 43, 77]. Our previous findings with synthetic PEP7 showed an effect on ERK1/2 activation but not on IP3 accumulation [66] suggesting that PEP7 may interact with the β-arrestin-dependent pathway. In this study, our second major finding was that PEP7 sORF can also attenuate a partial agonist-induced ERK1/2 activation. Mirroring the results using Ang II, the EC50 (Figure 14) and t1/2 of SII for ERK1/2 activation (Figure 15) was significantly increased for WT vs the MT. Thus, the results for Ang II and SII are consistent with a role of the PEP7 sORF in modulating the response to Ang II and SII. The higher activity of AT1aR found in MT can be attenuated in the presence of the PEP7 sORF in WT (Figure 11, 12, 14, and 15).
The PKC inhibitor Ro 31-8220 at 1 μM inhibited about 50% of Ang II (10 nM)-induced ERK1/2 (Figure 16), however, we did not note any differences between the WT and the MT. Our finding corroborates the previously reported inhibition in Ang II-induced ERK1/2 activation (about 60% with 100 nM Ang II) in HEK293 cells using the PKC inhibitor Ro 31-8425 [43]. Our data thus suggests that inhibition of PKC may have also attenuated the ERK1/2 activation through the β-arrestin-dependent pathway under our experimental conditions. It has been predicted previously that G protein and β-arrestin pathways could be dependent on one another if β-arrestin pathway activation predominates after the termination of G proteins [43]. Recently, it has also been postulated that the β-arrestin-dependent pathway may not be completely independent of G-proteins complexes. Grundmann et al. (2018) showed recently in HEK293 cells that knocking out β-arrestin1/2 slightly reduced Ang II (100 nM)- and SII (30 μM)-induced pERK but knocking out Gaq, Gαolf, Gα11, Gas, Gα12 and Gα13 (by CRISPR/CAS9 approach) and Gai/o inhibition by pertussis toxin completely abolished Ang II and SII-induced pERK1/2 [78]. Signaling was completely abolished as measured by whole cell dynamic mass redistribution in the absence of G proteins knockout cells [78]. Their study suggests dependency of AT1R signaling activities on G proteins but not much on β-arrestin1/2 [78]. Thus, it is possible that inhibiting PKC itself blocked the β-arrestin-dependent pathway and hence we might have not seen any difference between the WT and MT ERK1/2 activation.

Moreover, β-arrestin-dependent pathway requires the activity of GRK5 (that phosphorylates the AT1aR to facilitate binding of the β-arrestin1/2 to the AT1aR) which is also inhibited by Ro 31-8220 [59]. Our data from the inhibitor assay in Figure 16 suggests no differences between the WT and MT at 0.1 -10 μM of Ro 31-8220. An explanation of our results in Figure 16 could be desensitization of AT1R that requires PKC activity to promote β-arrestin2
binding to phosphorylated AT$_1$R which further leads to scaffolding for ERK1/2 cascade [79]. We also speculate that inhibition of GRK5 along with PKC limited our ability to distinguish between the ERK1/2 activation for WT and MT.

Our third major finding was that the presence of sORF reduced the expression of AT$_1$aR on the membrane and inside the cell (Figure 17) as measured by radioligand binding assay. Moreover, the intensity of EGFP was also found to be lower in the WT as compared to the MT (Figure 22A, 24A). This finding is in agreement with the known role of sORFs in inhibiting translation of a downstream protein [80]. Also, it has been shown that presence of exon 2 in the AT$_1$aR mRNA suppresses translation of the rAT$_1$aR [81] and the hAT$_1$aR [82, 83].

On Ang-II stimulation, the regulation in the rate of AT$_1$R internalization into vesicles and recycling of AT$_1$R to the cell membrane is still not completely understood. Live cell imaging is one of the best ways to capture the entire process of receptor trafficking in real time. Hence, techniques like immunostaining should be supplemented with live cell imaging experiments to confirm that findings from the trafficking studies are not artifacts (potential effects of fixation on cell volume, and loss/dimming/redistribution of GFP-chimeric receptor)[74]. Our fourth major finding (live cell imaging of transfected HEK293 cells at 100 nM) suggests that the PEP7 sORF increases the rate of internalization (Figure 22A). MT had twice (251 seconds) the t$_{1/2}$ of the WT (118 seconds). We observed a strong effect of PEP7 sORF on the internalization rate at higher dose like 100 nM (Figure 22A). Our preliminary data also suggested increased vesicle number in the WT vs the MT suggesting faster internalization (Figure 19-21). This data suggests that at 100 nM of Ang II, the PEP7 sORF can desensitize the AT$_1$aR faster. As a result of technical limitations, we were unable to quantify smaller vesicles formed on 10 nM of Ang II stimulation for 30 mins during live cell imaging (data not shown). In order to overcome these limitations, we performed
fixed cell imaging to determine the effect of Ang II (10 nM) on the fraction of AT\textsubscript{1a}R internalized in the vesicles over 90 mins. Our data suggests that the fraction of internalized AT\textsubscript{1a}R did not change significantly between the WT and MT for 60 mins. The MT tends to have more internalized AT\textsubscript{1a}R at 90 mins of Ang II (10 nM) stimulation.

We also speculate that the process of internalization is not dependent on ERK1/2 activation. Our data at 100 nM of Ang II stimulation suggests that the PEP7 sORF increases the rate of internalization (Figure 22) but does not affect Ang II-induced ERK1/2 activation (Figure 13). The relationship of AT\textsubscript{1a}R internalization and downstream signaling has not been clearly elucidated. It has been shown that AT\textsubscript{1a}R endocytosis is not associated with Ang II-induced ERK1/2 phosphorylation in C9 [53], HEK293 cells [84], and vascular smooth muscle cells (VSMC) [85]. Since AT\textsubscript{1a}R was shown to mediate internalization mainly via clathrin-coated pits, inhibitors of clathrin-mediated endocytosis (like sucrose, phenylarsine oxide, monodansylecadaverine C [53] and concanavalin A [53, 84, 85]) were used to study the association of Ang II-induced AT\textsubscript{1a}R endocytosis and ERK1/2 phosphorylation. The notion that Ang II-induced ERK1/2 phosphorylation is independent of AT\textsubscript{1a}R internalization was strongly supported by radioligand binding assays using an internalization deficient construct of AT\textsubscript{1a}R. This mutant with a truncated carboxyl terminus was shown to activate ERK1/2 on Ang II in spite of its inability to internalize [53, 84, 85]. Moreover, an AT\textsubscript{1a}R interacting protein termed as ATRAP, which increased the AT\textsubscript{1a}R internalization, did not show any effect on the phosphorylation of ERK1/2 supporting the independence of AT\textsubscript{1}R internalization and ERK activation [86].

Interestingly, our preliminary data on the rate of delivery of the AT\textsubscript{1a}R to lysosomes (Figure 24) did not show an effect of Ang II over time on the amount of the EGFP tagged AT\textsubscript{1a}R in the lysosomes. This experiment is consistent with the hypothesis that the bulk of the internalized
AT$_1$R is targeted to large, perinuclear endosomes. However, WT cells had significantly more internalized AT$_1$R in the lysosomes as compared to the MT. The experiment design used by Dale et al. (2004) that reported no colocalization of AT$_1$R with lysosomes after 180 minutes of agonist stimulation by live cell imaging studying colocalization of GFP-tagged β-arrestin with lysoTracker Red [46]. The presumption that the AT$_1$R-β-arrestin interaction is not dynamic throughout the process of AT$_1$R degradation and recycling might not hold true. Hence, GFP tagged β-arrestin cannot inform clearly about AT$_1$R trafficking for prolonged agonist treatment. Preliminary results in Figure 24 illustrate that over a rather short time course of 25 min, no appreciable change in delivery of AT$_1$R- GFP to the lysosomes occurred. In general, delivery of internalized receptors to lysosomes could occur as early as 15-20 min. Evidence that at least some AT$_1$R arrives in lysosomes was demonstrated by Li et al., who compared cells at 5 min and 30 min after Ang II treatment and found significant colocalization of LAMP-1 at 30 but not 5 min [47].

Our fifth major finding suggests that on Ang II (10nM) treatment, the presence of sORF promotes cell survival (Figure 29) and gives an anti-apoptotic advantage to cells having this sORF (Figure 30B, C) but has no effect on Ang II-induced cell proliferation (Figure 32, 34). The survival advantage to cells conferred by the PEP7 sORF was also found within the vehicle treated groups as well (Figure 30B, C). This suggests that this improved cell survival in the WT cells could also involve other pathways that are Ang II independent. Our preliminary data on 100 nM of Ang II stimulation did not reveal any effect on apoptosis (Figure 27) or cell proliferation (Figure 33).

Previously, following stimulation with Ang II, the G protein-dependent pathway was shown to contribute to cell death whereas β-arrestin-dependent pathway contributes to decreased cell death and improved cell survival [87-90]. Revankar et al. (2004) showed that on Ang II
stimulation, β-arrestin1/2 deficient mouse embryonic fibroblasts (MEF) transfected with AT1aR showed increased dead cells (as measured by phase contrast and confocal microscopy respectively) as compared to the control MEF [89]. Ahn et al. (2009) showed that on H2O2 and etoposide treatment, vascular smooth muscle cells (VSMC) transfected with β-arrestin2 siRNA increased cleaved caspases-3 and DNA fragments by western blotting and DNA fragmentation assay as compared to control VSMC [88]. These two studies suggest that inhibition β-arrestin-dependent pathway leads to increased apoptosis [88, 89]. It has been shown that in case of beta-adrenergic receptor, β-arrestin1 and β-arrestin2 could be involved in anti- and pro-apoptotic pathways respectively [91, 92]. Our results (Figure 29, 30) suggest an anti-apoptotic role of sORF that could also be an effect of opposing actions of β-arrestin1 vs β-arrestin2 on apoptosis. We suspect that this inhibition of early apoptosis might be an effect of downstream crosstalk in the β-arrestin dependent pathway and apoptotic pathways.

Alternatively, the more efficient translation of the AT1aR may lead to greater cell death due to overload of the AT1aR in the cell, non-specific interactions or imbalance in the stoichiometric ratios of protein scaffolds [93]. Although overexpression systems have contributed most of the signaling and trafficking studies, it is important to acknowledge the limitations of transfection-based studies.

As seen from our data and Liu et al. (2014) [66], the presence of sORF in exon 2 or exogenous addition of synthetic PEP7 resulted in selective attenuation of Ang II-mediated ERK1/2 phosphorylation. In addition to this, a strong Kozak consensus sequence and evolutionary conservation in rodents and humans [66, 94], suggests that it could be translated in vitro or in vivo. However, at this point, we couldn’t detect the molecular identify of PEP7 in WT samples with the optimized sample extraction method and the mass spectrometry techniques (Figure 37-41). It is
possible that PEP7 is not translated and the effects of synthetic PEP7 peptide seen previously are strictly pharmacological. It is also possible that we are not able to detect the peptide in transfected cells for technical reasons. Under the conditions in this study, PEP7 may be expressed in transfected cells at a level below the threshold for detection. Alternatively, detection of PEP7 could be masked as a result of a matrix effect seen in mass spectrometry or it is rapidly degraded.
CHAPTER 6. SIGNIFICANCE AND CONCLUSION

Through this study, we have found that PEP7 sORF plays a role in attenuation of Ang II and SII-mediated ERK1/2 activation. The PEP7 sORF could be a crucial regulator to modulate responses to increased circulating Ang II in the body or under conditions where AT₁R is upregulated. For example, Ang II levels in blood are elevated in patients having malignant and renal hypertension [95] and plasma Ang II levels were elevated in male Wistar rats after acute coronary artery ligation as measured by radioimmunoassay [96]. Similarly AT₁R was increased in myofibroblasts of Sprague-Dawley rats after left coronary artery ligation at the site of myocardial infarction as seen by autoradiography [97]. Most importantly, a polymorphism of AT₁R (homozygous for A/C substitution at 1166 position) that is usually associated with increased incidence of essential hypertension patients was shown to be more responsive to Ang II as compared to other phenotypes (AA or AC) by measuring in-vitro vascular function in human internal mammary artery [98]. These previous findings suggest that the pathophysiological responses of AT₁R mediated by Ang II could to be fine-tuned using a PEP7-mimetic in a clinical setting. Many current medications for hypertension have been used for decades and are efficacious as anti-hypertensive agent; however, most of the traditionally used anti-hypertensives either block the angiotensin receptors, ion channels or inhibit other components of the RAAS [7]. Such mechanisms of action potentially inhibit the entire cascade of AT₁R signaling. It is this fine tuning regulation of AT₁R signaling that should be targeted for treating hypertension and other age-associated diseases involving AT₁R dysfunction.

Upstream small ORFs (median length of 22 codons) have been found in plants, mice, fruit flies and humans. These sORFs have translational regulatory roles as well as encode functional peptides in plants and animal kingdom [99, 100]. The rAT₁aR have 2 upstream sORF in E2 whereas
hAT₁₆R have 2 sORFs in E2 and 1 sORF in E3 [28]. These sORFs have an effect on the translation efficiency of AT₁Rs resulting in differential regulation of AT₁R expression in their presence and absence [81]. The percentage of spliced variants of the AT₁R with exon 2 (having the PEP7 sORF) in humans decreases in pathological conditions like failing hearts as compared to normal atria [82]. Most papers studying angiotensin receptor signaling in transfected cells have used a plasmid with primarily the coding region of the receptor within exon 3 that lacks the 5’ leader sequence potentially resulting in translation of the PEP7 sORF. Our results support the notion that the PEP7 sORF plays a role in regulation of AT₁R activity. In future research, our study may serve as caution to investigators who study cell signaling in transfected cells using constructs devoid of the 5' leader sequence.

This dissertation is one of few studies of an sORF playing a role in cellular functions [101, 102]. Some of the characterized ORFs encoding peptides (SEPs) have been shown to play diverse roles in cellular processes in bacteria and flies [99] and about 40% of SEPs in humans have been attributed to 5’ ORFs [103]. We have uncovered a new role of an upstream short open reading frame (7 codons long) that may directly or indirectly play a role in angiotensin receptor function in HEK293 cells. Clinically, GPCRs are one of the most widely studied class of receptors and about 35% of G protein coupled receptors (GPCRs) are targeted by FDA approved drugs [104]. Hence, these findings not only have implications for the regulation of AT₁Rs in physiological and pathological conditions but also for the superfamily of GPCRs, many of which possess sORFs of unknown function in their 5' leader sequence.
REFERENCES


