IN VITRO INVISTIGATION OF THE HETERODIMERIZATION BETWEEN ANGIOTENSIN II AND THROMBIN RECEPTORS

Isra Mansur Al Zamal

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IN VITRO INVESTIGATION OF THE HETERO DIMERIZATION BETWEEN ANGIOTENSIN II AND THROMBIN RECEPTORS

Isra Mansur Al Zamal

This thesis is submitted in partial fulfilment of the requirements for the degree of Master of Science in Molecular Biology and Biotechnology

Under the Supervision of Dr. Mohammed Akli Ayoub

November 2020
Declaration of Original Work

I, Isra Mansur Al Zamel, the undersigned, a graduate student at the United Arab Emirates University (UAEU), and the author of this thesis entitled “In vitro Investigation of the Heterodimerization between Angiotensin II and Thrombin Receptors”, hereby, solemnly declare that this thesis is my own original research work that has been done and prepared by me under the supervision of Dr. Mohammed Akli Ayoub, in the College of Science at UAEU. This work has not previously been presented or published, or formed the basis for the award of any academic degree, diploma or a similar title at this or any other university. Any materials borrowed from other sources (whether published or unpublished) and relied upon or included in my thesis have been properly cited and acknowledged in accordance with appropriate academic conventions. I further declare that there is no potential conflict of interest with respect to the research, data collection, authorship, presentation and/or publication of this thesis.

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Approval of the Master Thesis

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Abstract

The renin angiotensin system (RAS) through its hormone angiotensin (AngII) and the protease thrombin are two major physiological regulators of vascular and renal functions. RAS is involved in the regulation of blood pressure and water-electrolytes hemostasis, while thrombin has a crucial role in platelets activation and thrombosis. The interplay between these two systems at the physiological and the pathophysiological levels has been documented in many studies. However, such an interplay at the pharmacological level between the AngII and thrombin receptors still has not been explored. Our study aimed to explore the interplay between the AngII type 1 receptor (AT1R) and the thrombin protease-activated receptor (PAR1), which both belong to the G protein-coupled receptors (GPCR) family, using transfected human embryonic kidney (HEK293) cells. For this various bioluminescence resonance energy transfer (BRET) technology-based analytical and biochemical assays were used. BRET saturation and co-immunoprecipitation demonstrated the physical interaction between AT1R and PAR1 indicating their heterodimerization in vitro. Moreover, dose response and real-time kinetic experiments revealed a functional AT1R-PAR1 interaction characterized by a positive allosteric modulation and transactivation of AT1R activity by PAR1 towards its coupling to Gαq protein, inositol phosphate (IP1) pathway, and β-arrestin recruitment. This was contrasted with a trans-inhibition of AT1R internalization as well as its endosomal trafficking upon PAR1 activation. Together, our data demonstrated for the first time the physical and functional interaction between AT1R and PAR1 which may constitute a molecular and mechanistic rationale for the well-known interplay between RAS and thrombin. These findings are of extreme importance in the field of drug discovery. Targeting the pathological conditions involving RAS and thrombin opens promising perspectives for the identification of selective and safer drugs by considering AT1R-PAR1 heterodimers as a potential druggable molecular entity.

Keywords: AngII, thrombin, G protein coupled receptor, AT1R, PAR1, BRET, heterodimerization, allosteric modulation.
دراسة الارتباط والتفاعل ما بين مستقبلات هرمون الأنجيوتينسين 2 وأنزيم الثرومبين

يُعدُ كل من نظام الرنين-أنجيوتينسين عن طريق هرمونه، الأنجيوتينسين 2 (AngII) (و نظام أنزيم الثرومبين (thombin) ذوا أهمية بالغة في الحفاظ على توازن وصحة جهاز القلب والأوعية الدموية ووظائف الكلى. حيث يعمل نظام الرنين-أنجيوتينسين على المحافظة على توازن ضغط الدم والماء والأملاح، بينما يقوم أنزيم الثرومبين بدور أساسي كعامل تخثر ومساعد على تشغيل الصفائح الدموية. العديد من الدراسات ثبت التفاعل ما بين هذين النظامين على مستوى الفسيولوجيا وارتباط كلاهما بالعديد من الأمراض. لكن، حتى الآن لم تتم دراسة هذا التفاعل على مستوى المستقبلات لهذين النظامين. لهذا، سعت دراستنا لفهم إن كان هذا التفاعل بين كل من هذين النظامين يحدث على مستوى المستقبلات الخاصة بهم، مستقبلات هرمون الأنجيوتينسين 2 (AT1R) ومستقبلات أنزيم الثرومبين (PAR1)، والذان ينتميان لعائلة المستقبلات المقترنة ببروتين ج الذي يعَدُّ جينية بحيث تحتوي بروتين ج وذلك باستخدام خلية إنسانية من الكلى الجنينية (HEK293) على هذه المستقبلات. لإتمام هذه الدراسة قمنا باستخدام تقنية بريت (BRET) وتقنيات كيميائية حيوية. حيث أثبتت نتائجنا التقارب الجزيئي بين مستقبلات الأنجيوتينسين 2 ومستقبلات الثرومبين (Co- بإضافة تقنية بريت للتشعُّب (BRET Saturation assay) باستخدام تقنية بريت للتشعُّب (BRET Saturation assay) بالإضافة إلى تقنية التنبيه المناعي (immunoprecipitation) والتي بدورها أكّدت أنهما مترابطان سوياً ويشكلان مركزاً في التجارب المخبرية. إضافة إلى ذلك، كل من تجارب الجرعة والاستجابة (Dose Response) والتجارب الحركية حقيقية الوقت (Real-Time Kinetics)، أثبتت بدورها تفاعل هذا الترابط وظيفياً بالارتباط ببروتين ج (Gaq)، تشبيط مسار إينزيتيول أحادي (IP1)، وتشبيط بروتينات β-أرّستين (β-arrestin2) حيث كان هذا عن طريق تشبيط مستقبلات الأنجيوتينسين 2 بواسطة مستقبلات الثرومبين (Transactivation)، إضافةً لكون مستقبل الثرومبين معتمل تفاعل إيجابي لمستقبلات الأنجيوتينسين. علاوة على ذلك، أظهرت مستقبلات الثرومبين (positive allosteric modulator) الثرومبين تثبيت للإدخال الخلوي لمستقبلات الأنجيوتينسين 2 ومساراتها. دراستنا هذه أثبتت لأول مرة التقارب الجزيئي والتفاعل الوظيفي بين مستقبلات الأنجيوتينسين 2 ومستقبلات الثرومبين، والتي بدورها يمكن أن تكون السبب في التفاعل بين نظام الرنين-أنجيوتينسين والثرومبين. وتعتبر هذه النتائج ذات أهمية كبيرة في مجال علم الأدوية لعلاج الأمراض التي يلعبان فيها هذين النظامين
دوراً في تفاقهما، بهدف الحصول على نتائج مبشرة لاستكشاف أدوية ذات فعالية أكبر وآثار جانبية أقل عن طريق أخذ هذا التفاعل على مستوى المستقبلات وترابطها بعدين الاعتبار.

مفهوم البحث الرئيسية: نظام الرنين-أنجيوتينسين، أنجيوتينسين 2، ثرومبين، المستقبلات المرتبطة ببروتين ج، مستقبلات هرمون الأنجيوتينسين 2، مستقبلات أنزيم الثرومبين، تقنية بريت، الترابط بين المستقبلات، معدل تفارغ إيجابي أنزيم الثرومبين، تقنية بريت. الترابط بين المستقبلات، معدل تفارغ إيجابي.
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Dedication

To my beloved family and friends
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AngII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>AT1R</td>
<td>Angiotensin II type I receptor</td>
</tr>
<tr>
<td>BRET</td>
<td>Bioluminescence resonance energy transfer</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>ECL2</td>
<td>Extracellular loop 2</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FRET</td>
<td>Fluorescence resonance energy transfer</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine exchange factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein coupled receptor</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein coupled receptor kinase</td>
</tr>
<tr>
<td>HEK293</td>
<td>Human embryonic kidney 293 cells</td>
</tr>
<tr>
<td>IP1</td>
<td>Inositol monophosphate</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>PAR1</td>
<td>Protease activated receptor 1</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>RAS</td>
<td>Renin angiotensin system</td>
</tr>
<tr>
<td>VSMC</td>
<td>Vascular smooth muscle cells</td>
</tr>
</tbody>
</table>
Chapter 1: Introduction

1.1 G Protein-Coupled Receptors (GPCRs)

GPCRs are seven-transmembrane receptors that bind to the heterotrimeric G protein and mediate downstream signaling pathways controlling many biological responses [1]. GPCRs have a classical structure composed of seven hydrophobic transmembrane α-helices, extracellular N-terminus, and intracellular C-terminus [2], [3]. They are classified into either five families based on the physiological features and sequence homology named; rhodopsin, adhesion, secretin, glutamate, and frizzled or into four classes; A, B, C, and F. Class A constitutes the largest family thus, carrying out various functions. This family has a common ligand-binding pocket between the helices. Most of these GPCRs have two conserved motifs that are important for protein stabilization and/or G protein activation, NSXXNPXXY in helix VII and a DRY motif (Asp-Arg-Tyr) at the cytoplasmic border of helix III [2], [3]. Class B contains 47 receptors which interact mostly with hormone-like peptides [2], [3]. Class C has 15 GPCRs that have a ‘Venus flytrap’ N terminus composed of two lobes that close around the ligand, and they commonly form dimers. While Class F has 11 receptors that activate Wnt pathway. Around 800 different human genes encode for GPCRs. However, ~300 genes of them encode for orphan GPCRs (with unknown function) [2], [3]. These GPCRs are the receptors for many stimuli such as peptides, ions, hormones, neurotransmitters, lipids, amines, and nucleotides [1], [4]. They represent one of the largest and most diverse group of membrane receptors (~ 4% of the human genome) with incredible array of functions in the human body [1], [4]. GPCRs regulate several physiological responses including but not limited to taste, odor, pain, neurotransmission, blood pressure, coagulation, and immune responses [5], [6]. In addition, GPCRs are involved
in various pathophysiological situations such as cancer, hypertension, diabetes, viral infections, and atherosclerosis [7]–[10]. Therefore, these receptors have a significant contribution in drug discovery and the increased understanding of these receptors has greatly influenced modern medicine, as more than 30% of all drugs in markets nowadays are targeting GPCRs [11], [12].

Figure 1: GPCR activation and signaling.

1.2 GPCR Activation and Regulation

When an agonist binds to its GPCR, it induces conformational changes in the intracellular region of the GPCR, which serves as a binding pocket for the guanine nucleotide exchange factor GEF for G protein binding [2]. Agonist-activated GPCR, facilitates the exchange of GDP (from inactive G protein) to GTP (active G protein), hence the name of G proteins came. Then, the GTP bound α subunit dissociates from the βγ subunits and G protein subunits undergo downstream signaling, each subunit
regulates its effector proteins. As shown in Figure 1, α subunits have four subtypes. Ga\textsubscript{i} and Ga\textsubscript{s} inhibits and stimulates adenylyl cyclase (AC) respectively, Ga\textsubscript{q} activates PLC, and Ga12/13 activates the Rho pathway, while the βγ dimer activates ion channels and PLCβ [2], [4]. This stimulation of G proteins subunits of the first messenger effectors (enzyme or ion channel) mediates the production of second messenger molecules, entry of ions at the plasma membrane, and various kinases activation as shown in Figure 1 [2], [4], [13]. In addition to this classical paradigm of two parts; the GPCR and coupling to its G protein effector, which is oversimplified, many GPCRs can bind to several G proteins and each subunit can activate different signaling pathway. Moreover, GPCRs can also signal independently from G protein (G protein independent signaling) through β-arrestin [2], [14]. These β-arrestin-mediated signaling pathways are engaged in i) protein phosphatase 2 A dephosphorylation of Akt, ii) inhibiting NF-κB, iii) anti-apoptosis, iv) extracellular signal-regulated Kinas ERK-dependent stimulation of protein translation, among others [15].

β-arrestins are multifunctional endocytic adaptors and signal transducer proteins that bind to phosphorylated GPCRs, the phosphorylated GPCR serves as a substrate for β-arrestin (Figure 2) [16]. GPCR kinases (GRK) phosphorylate GPCRs at specific sites in the intracellular region and C-terminus, allowing β-arrestin binding mediating G proteins inactivation/desensitization, GPCRs endocytosis and trafficking, and promoting G protein–independent signaling by scaffolding other signaling proteins [16]. This GRK-arrestin pathway is well known for GPCRs regulation by regulating cellular second messengers production level [17]. Arrestins function is not limited to GPCRs desensitization, they also function as endocytic adaptors, regulating the delivery or removal of receptors from or to the plasma membrane (GPCRs trafficking)
by linking GPCRs to clathrin-coated pits mediating their endosomal trafficking in either early or late endosomes for membrane recycling or degradation, respectively [18]. Moreover, β-arrestins are signal transducers and scaffolding adaptors linking activated GPCRs to various scaffolding signaling pathways such as c-Raf-1 and c-Src that activate MAPK/ERK pathway [16], [17]. To come closer again, MAPK/ERK pathway can be activated dependently and independently from G protein [17]. G protein dependent activation is mediated via G\textsubscript{q}\textsubscript{o}-produced PKC, mediating rapid and transient activation of nuclear ERK, which works on transcription factors controlling cellular proliferation and differentiation. On the other hand, the G protein independent activation of ERK via β-arrestin and other scaffolding proteins including Raf, is slow and persistent and associated with controlling cellular motility, chemotaxis, and apoptosis [17]. The discovery of this role for arrestins as signaling adaptors led to the discovery of the concept of biased agonism. Biased signaling or functional selectivity reflects the ability of a certain receptor to activate one signal transducer over another like β-arrestin over G protein [15], [19]. The importance of biased signaling relies on their crucial role in drug discovery. An example for this is the morphine binding to its receptor, the µ-opioid receptor and signaling through G protein mediates the pain relief effect, while the β-arrestin-dependent signaling apparently causes most of the side effects [19].
1.3 Heterodimerization of GPCRs

Interactions between membrane receptors for intercellular communication have been reported in many studies, mediating many pharmacological, physiological, and pathophysiological roles. This is true for the two major families of receptors, G protein-coupled receptors (GPCRs) and receptor tyrosine kinase (RTKs). These receptors can function not only as monomer, several different signals are mediated by the formation of complexes of dimers; two molecules of the same receptor (homodimer) or two different receptors (heterodimer), as well as high-order oligomers in living cells as a unique way for cellular communications between receptors. Heterodimerization consists of physical and functional interactions between receptors and has been reported for many GPCRs as well as RTKs (Table 1). Ferre et al. defined
a receptor heteromer as “a macromolecular complex composed of at least two (functional) receptor units (protomers) with biochemical properties that are demonstrably different from those of its individual components” [21]. Classically, an allosteric modulator, is a molecule that binds to a site other than the orthostatic binding site, altering its function. However, in the case of GPCRs heterodimerization, one protomer is acting as an allosteric modulator for the second protomer, altering its functionality [14]. This altered functionality includes various consequences on the receptor’s; i) Pharmacology, including altered selectivity to the agonist, antagonist, with co-activation, co-inhibition reflecting the presence of a novel binding pocket in the heterodimer, ii) Transactivation, the ligand-induced conformational change in one protomer receptor, activates the second protomer, iii) Transinhibition, and iv) altered trafficking and internalization compared to the single expression of the receptors [14], [21] (Figure 3). Gomes et al. sorted GPCRs heterodimer formation into three criteria, heterodimer should show: i) Colocalization and have physical interaction, ii) Distinct properties such as unique signaling, and iii) Disruption of the heterodimer, alters its properties [22]. The concept of heterodimerization has developed quickly due to its extreme importance in the field of pharmaceutical manufacturing for the identification of selective compounds with potentially less side-effects, considering the synergistic and antagonistic interactions between drugs, as well as receptor-receptor heterodimerization that may influence the drug-drug interaction at the clinical molecular level [14], [22], [23].
1.4 The Renin-Angiotensin System (RAS) and its Receptors

The renin-Angiotensin system (RAS) is a network of hormones and receptors which have a fundamental role in blood pressure regulation and water-electrolyte homeostasis [25], [26]. The mere use of medications targeting RAS is by itself proof of its significance, including AT1R blockers (ARBs), ACE blockers, and renin inhibitors [25]. Classically, the enzyme renin cleaves the liver-produced angiotensinogen to generate AngI which is then cleaved to AngII by angiotensin converting enzyme 1 (ACE1). Angiotensin II (AngII) acts on two different GPCRs AT1R and AT2R to mediate patho-physiological responses [26]. However, AT1R is regulating most of these responses induced by AngII [27]. AT1R is widely expressed in different tissues in the human body, including vascular tissues, adrenal glands,
adipose tissues, placenta, heart, lungs, and kidneys among others [28]. Ang II through the binding and activation of AT1R, controls many physiological responses including vasoconstriction, proinflammatory, fibrotic, oxidative stress, Alzheimer disease, and insulin resistance among others [27]. On the other hand, AT1R is implicated in many pathophysiological responses such as renal and cardiovascular diseases, fibrosis, aging, and cancer [27], [29]–[31]. AngII binding to AT1R induces conformational changes and activation through Phe$^{8}$(ANG II)/His$^{256}$(AT1R) and Tyr$^{4}$(ANG II)/Asn$^{111}$(AT1R), inducing the coupling to the heterotrimeric G proteins including $G_{aq}$, mediating Phospholipase C activations and production of the second messenger inositol trisphosphate/Ca$^{2+}$ controlling vasoconstriction, aldosterone release and water-salt hemostasis [27], [32]. In addition, AT1R signals through $G_{a12/13}$, and $G_{ai}$ mediating activation of several kinases of serin/threonine, receptor tyrosine kinases, and non-receptor tyrosine kinases such as G12/13 Rho/Rho kinase, extracellular signal-regulated kinases (ERK1/2) and mitogen-activated protein kinase (MAPK) [27], [32] (Figure 4).
1.5 Thrombin and its Receptors

The thrombin system is part of the coagulation cascade with a classical function in the formation of fibrin clots and coagulation hemostasis. Thrombin mediates its effects through multiple protease-activated receptors (PARs) belonging to GPCR family. They are four receptors; PAR1, PAR2, PAR3, and PAR4 [34]. The importance of
PAR1 is considered as a common therapeutic modulator for its crucial role in physiological regulation of vascular tone, platelets activation, and proinflammatory responses [35], [36]. It also contributes to the pathogenesis of multiple diseases such as atherosclerosis, vascular lesions, inflammation, and cancer [34], [35]. PAR1 is expressed in various tissues including heart, spleen, lungs, gall bladder, adrenal gland, brain, and other tissues [37]. It is activated in a unique way; the serine protease thrombin enzyme cleaves the N-terminus of PAR1 at R$_{41}$S$_{42}$, the cleaved N-terminus acts as a tethered ligand that binds to the ECL2, activating PAR1 downstream signaling [34]. Other proteolytic cleavages at different sites of PAR1 are mediated through other proteases such as plasmin, trypsin, factor X$_a$ and VII$_a$ [35]. PAR1 has overlapping downstream signaling depending on the tissue, cell, cleaving protease, as well as soluble ligand binding (such as TFLLRN) [34], [35]. PAR1 signals through the heterotrimeric G-protein including G$_{aq}$, G$_{a12/13}$, and G$_{ai}$, also via the G-protein-independent pathway controlling diverse biological responses (Figure 5).
1.6 The Interplay between RAS and Thrombin

Several studies have demonstrated the physiological and pathological interplay between the Renin Angiotensin System (RAS) and Thrombin system. However, at the pharmacological level of their respective receptors, this is not well explored yet. Both the RAS and thrombin systems regulate mutual physiological responses of vascular development, thrombosis, platelet activation and vascular tone, among others. As well as pathological responses such as cardiovascular diseases, VSMCs chemotaxis and proliferation, inflammation, diabetes, and cancer. Antoniak et al. have documented in their study that, PAR-1 has a role in AngII-mediated pathological cardiac and vascular remodeling and perivascular fibrosis of the heart and aorta [39]. Also, that PAR1 affects AngII-induced inflammatory cytokines and profibrotic genes in aorta, and these
effects were attenuated in PAR1 deficient mice [39]. Also, as thrombosis is a major complication with AngII-induced hypertension, a study by Senchenkova et al. demonstrated a link between these two complications as AngII promotes microvascular thrombosis via the help of thrombin [40]. In vivo studies by Steinberg have documented that activation of PAR1 has two opposing vasoregulatory mechanisms: endothelial-dependent vasorelaxation and endothelium/vascular smooth-muscle-dependent vasoconstriction responses [41]. Another recent study by Hasan et al. supported the previous evidences of the physiological interplay between the two systems, stated that thrombin contributes to atrial endothelial ageing and senescence via AT1R and ACE upregulation contributing to myocardium remodeling [42]. This effect was attenuated by ARB in addition, co-targeting both thrombin and angiotensin is suggested as well for targeting the observed pro-inflammatory, pro-thrombotic, pro-fibrotic and pro-remodeling responses [42]. To add more, Anwar et al.’s study proved that both Ang II and thrombin significantly dropped down IGFBP-4 protein and mRNA levels in VSMCs, and may have a crucial role in normal and pathological vascular growth mediated by Ang II and thrombin via increasing IGF-I availability to bind its receptor [43]. Therefore, all this evidence together supports the possibility of the interaction between AngII and thrombin at the level of their receptors.

In regard to this study of the possibility that AT1R and PAR1 to form heterodimers, Table 1, summaries almost all the reported heterodimers of AT1R as it acts as a signaling hub and it interacts with many GPCRs as well as Tyrosine kinases.
Table 1: The different AT1R-heterodimers described and characterized *in vitro* and/or *in vivo*.

<table>
<thead>
<tr>
<th>Dimer</th>
<th>Present <em>In vitro</em></th>
<th>Pharmacological Effects</th>
<th>Physiological Effects</th>
<th>Major Techniques</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1R-AT1R</td>
<td>Both</td>
<td>Positive allosteric modulation.</td>
<td>Atherogenesis.</td>
<td>Immunoblotting, IHC, BRET, &amp; Ligand dissociation assay.</td>
<td>[44], [45]</td>
</tr>
<tr>
<td>AT1R-AT2R</td>
<td>Both</td>
<td>AT2R antagonizes AT1R function.</td>
<td>Na+ reabsorption &amp; Ca2+ regulation in renal proximal tubule cells.</td>
<td>Co-IP, Confocal microscopy, BRET, &amp; Electrophoretic mobility shift assay.</td>
<td>[46], [47, p. 3], [48, p. 2], [49], [50]</td>
</tr>
<tr>
<td>AT1R-MasR</td>
<td>Both</td>
<td>1. MasR antagonizes AT1R in a ligand independent manner.</td>
<td>1. MAS-knockout mice had enhanced vasoconstriction in the vessels. Also, had AT1R altered signaling in the amygdala. 2. MasR upregulates AT1R through Gq activation of AT1R, but it decreases AT1R signaling.</td>
<td>Co-IP &amp; BRET</td>
<td>[51]–[54]</td>
</tr>
<tr>
<td>AT1R-α2cAR</td>
<td>Both</td>
<td>Dual agonist occupancy resulted in unique Gs activation.</td>
<td>NE hypersecretion and sympathetic nerve hyperactivity <em>in vivo</em>.</td>
<td>FRET, BRET, IF, &amp; Radioligand binding assay.</td>
<td>[60]</td>
</tr>
<tr>
<td>AT1R-α1dAR</td>
<td>both</td>
<td></td>
<td>healthy pregnant rats showed less dimers compared to preeclamptic rats. Thus, this dimerization might be inducing preeclampsia.</td>
<td>Co-IP</td>
<td>[61]</td>
</tr>
<tr>
<td>AT1R-β2AR</td>
<td><em>In vitro</em></td>
<td>AT1R enhances β-arrestin binding to β2AR.</td>
<td></td>
<td>BRET</td>
<td>[62]</td>
</tr>
</tbody>
</table>
Table 1: The different AT1R-heterodimers described and characterized *in vitro* and/or *in vivo* (continued).

<table>
<thead>
<tr>
<th>AT1R-Heterodimer</th>
<th>Condition</th>
<th>Description</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>AT1R-CB1R</td>
<td><em>In vitro</em></td>
<td>1. Potentiation of AT1R signaling &amp; AT1R coupling to multiple G-proteins.</td>
<td>The heteromer affects Ang II-mediated pathophysiological profibrogenic effect via AT1R.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. AT1R induces paracrine transactivation to CB1R.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Antagonizing CB1R, inhibits Ang II-mediated mitogenic signaling and profibrogenic gene expression.</td>
<td></td>
</tr>
<tr>
<td>AT1R-APJ</td>
<td>Both</td>
<td>1. APJ allosterically trans-inhibits AT1R.</td>
<td>1. Apelin antagonizes Ang II-induced hypertension and cardiac fibrosis via inhibiting AT1R signaling.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Diminished IP1 production &amp; β-arrestin recruitment.</td>
<td>2. APJ blocks Ang II pathophysiological effect in vascular diseases through NO production.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3. APJ inhibits the development of Ang II-mediated atherosclerosis.</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4. Apelin has hepatoprotective response against hepatic ischemia reperfusion injury, which is via down regulating hepatic AT1R expression and increased hepatic apelin level.</td>
</tr>
<tr>
<td>AT1R-P2Y6</td>
<td>Both</td>
<td>1. Heteromer formation suppresses β-Arrestin recruitment into AT1R upon Ang II treatment. On the other hand, it enhances G-protein dependent signaling.</td>
<td>AT1R-P2YR heteromers increase in vascular smooth muscles due to the increase in P2YR expression with age. Thus, inducing Ang II-mediated hypertension &amp; vascular remodeling in age-dependent manner.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. UDP (P2Y6 ligand) has no effect on the heteromer.</td>
<td></td>
</tr>
<tr>
<td>AT1R-FP</td>
<td><em>In vitro</em></td>
<td>Both GPCRs allosterically modulate each other’s activity.</td>
<td>Potentiates aortic smooth muscles contraction.</td>
</tr>
<tr>
<td>AT1R-SecR</td>
<td>Both</td>
<td>AT1R plays <em>dual</em> allosteric modulatory function, one is as a negative allosteric modulator when it is inactivated &amp; as a positive allosteric modulator when it is activated &amp; potentiation of cAMP production.</td>
<td>Regulation of drinking behavior in response to hyperosmotic stress by: 1. Vasopressin release to increasing water intake. 2. Ang II-induced aldosterone secretion through Ca+2 mobilization.</td>
</tr>
</tbody>
</table>
Table 1: The different AT1R-heterodimers described and characterized *in vitro* and/or *in vivo* (continued).

| AT1R-EGFR | Both | AT1R transactivates EGFR. | 1. Ang II-mediated pathological remodeling of cardiomyocytes, their growth & cardiac hypertrophy.  
2. AT1R-EGFR crosstalk regulates the development of the renal collecting system. | Co-IP & BRET | [77]-[81] |
| AT1R-CCR2 | Both | Co-treatment with Ang II & CCL2 has a positive allosteric modulation effect on Gi coupling and β-arrestin recruitment (This effect was lost with co-antagonizing the two receptors). | Chronic kidney disease. (blocking both AT1R & CCR2 lessen proteinuria, ischemic brain damage macrophage infiltration, podocyte loss, & crescentic Glomerulonephritis.  
CCR2 deficiency significantly disturbed the formation of Ang II-induced ascending aortic aneurysms, abdominal aortic aneurysms, and atherosclerosis. | BRET | [82]-[84] |
| AT1R-D1R | Both | 1. Losartan acts as a positive allosteric modulator for D1R activation. it also enhanced cAMP production by both GPCRs.  
However, D1R antagonist blocked the antihypertensive effect of Losartan.  
2. Ang II mediated rapid partial internalization of D1R plus blocking D1R signaling. Also, D1R ligand mediated rapid partial internalization of AT1R plus blocking AT1R signaling.  
3. Fenoldopam (D1 like ligand) positively modulates D1R's and negatively AR1R's expression in VSMCs and RPT cells from normotensive rats. However, it has no effect on D1R of hypertensive rat cells and still blocks AT1R. | 1. Regulation Na+ excretion and blood pressure.  
2. Losartan antihypertensive function might be mediated by both blocking AT1R signaling and through enhancing D1R signaling.  
3. Region in carboxyl terminus of D1R (residues 397-416) was found to interact with both AT1R as well as Na+-K+-ATPase. | Co-IP & Glutathione-S-transferase pull-down assays. | [85]-[87] |
| AT1R-D2R | Both | 1. Cross-antagonism of the two GPCRs.  
2. downregulation of AT1R-mediated Ca+2 mobilization co-transfected cells. | Cross-antagonism by ARB blocks D2R signaling causing enhanced D2R-mediated dopaminergic transmission, which control the basal ganglia system of motor control. | BRET, Immunocytochemistry, co-localization & proximity ligation assay. | [88] |
| AT1R-D3   | In vitro | 1. AT1R down regulates D3R expression in RPT cells.  
|          |          | 2. AT1R-D3R interact in RPT cells, but this interaction is impaired in SHR (spontaneous hypertensive rats) compared to WKY. | Co-IP | [89] |
| AT1R-D4   | Both     | D4R receptor down-regulates AT1R expression in WKY RPT cells through ca+2 channels activation. | Regulating Ang II-induced hypertension. | IF & Immunoblotting. | [90] |
| AT1R-D5   | Both     | 1. Negatively regulate the expression of each other.  
|          |          | 2. D5R mediates AT1R degradation through ubiquitin-proteasome pathway. | Regulating Ang II-induced hypertension. | BRET, Co-localization, & Immunoblotting. | [91]–[93] |
| AT1R-μOR  | In vivo  | 1. Decreases Nitric Oxide (NO) production thus elevating blood pressure.  
|          |          | 2. Losartan enhanced the production of NO. | 1. Progressive hypertension.  
|          |          | 2. Blocking AT1R has improvement effect against this heterodimer-mediated response. | IF & Proximity Ligation Assay. | [94] |
| ET1R-AT1R | Both     | 1. Ang II decreases ET1R phosphorylation.  
|          |          | 1. AT1R & ET1R expressed on immune cells enhance chemotaxis and expression of IL-8 & CCL18 upon production of autoantibodies from systemic sclerosis patients.  
|          |          | 2. These Abs enhance severe obliteratorive vasculopathy in graft versus host and systemic sclerosis.  
|          |          | 3. AT1R regulates ET1R expression.  
|          |          | 4. Ang II-mediated hypertension enhances arterioles thrombosis, and this response is mediated by AT2R, AT4R, B1R, and ET1R-mediated signaling. | Co-IP, Co-localization | [95]–[98] |
| AT1R-PRR  | Both     | PRR blockage prevents diabetic nephropathy (including the antifibrotic effect) which is caused by ARBs. | Co-IP, IF & FRET | [99], [100] |
Table 1: The different AT1R-heterodimers described and characterized *in vitro* and/or *in vivo* (continued).

<table>
<thead>
<tr>
<th>AT1R-TPuR</th>
<th><em>In vitro</em></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TPuR upregulates AT1R's function via paracrine transactivation of the TPuR.</td>
<td></td>
<td>Pathogenesis of Ang II-mediated hypertension and cardiac hypertrophy.</td>
<td>BRET &amp; Receptor Selection and Amplification Technology.</td>
<td>[101], [102]</td>
</tr>
</tbody>
</table>
Chapter 2: Hypothesis and Objectives

2.1 Hypothesis

This thesis work hypothesized that the previously mentioned physiological and pathological interplay between RAS and thrombin systems, happens at the level of their receptors AT1R and PAR1, respectively.

Figure 6: The hypothesis of heterodimerization between AT1R and PAR1.

2.2 Objectives

This study aimed to investigate the physical and functional interaction between AT1R and PAR1 in vitro. For this, the two GPCRs were co-expressed in human embryonic kidney (HEK293) cells and bioluminescence resonance energy transfer (BRET) and immunoblotting assays were used:

1- To investigate the physical interaction between AT1R and PAR1 by assessment of PAR1-AT1R molecular proximity using Co-IP and BRET
Saturation Assay.

2- To investigate the functional interactions between AT1R and PAR1 in terms of heterodimerization by investigating; Gq protein activation, β-arrestin recruitment, second messenger production (IP1/3), MAP Kinase, and receptor trafficking/internalization by using Dose response and Kinetics BRET/FRET based technology as well as Western blot.

Figure 7: Objectives of the study.
Chapter 3: Materials and Methods

3.1 Chemicals, Reagents, and Plasmid

PAR1 was purchased from cDNA Resource Center (Bloomsberg, PA, USA), HA-AT1R was kindly provided by Reiter E. (INRA, Nouzilly, France), AT1R-Rluc was a gift from Laporte, S. (McGill University, Montréal, Canada), yPET-β-arrestin 2 was kindly provided by Scott, M. (Cochin Institute, Paris, France), Grb2-Rluc, Grb2-Venus, PAR1-Rluc, and PAR1-Venus were kindly given by Prof. Pfleger KD. (Harry Perkins Institute of Medical Research and UWA, Perth, Australia), and Venus-GaQ, Venus-Kras, and Venus-Rabs that were generously shared by Lambert, N. (Augusta University, GA, USA). AngII, thrombin, and irbesartan were from Sigma (St Louis, MO, USA). SCH79797 was from Tocris (Tocris, Ellisville, MO). The IP1 kit was obtained from Cisbio Bioassays (PerkinElmer, Codelet, France) and the anti-HA tag antibody (ab9110) was purchased from abcam (abcam, MA, USA). Lipofectamine™ 2000 from Invitrogen for transfection and Coelenterazine h the luminescent substrate was purchased from Promega (Promega Corporation, Madison, WI, USA).

3.2 Bacterial Transformation and Plasmid Extraction

The plasmids were received on a paper to be transformed; they were resuspended in 50 μl nuclease free water (NF) for ~ 15 minutes. NEB 5-alpha Competent E. coli (High Efficiency) from NEW ENGLAND BioLabs, was used for transformation according to manufactural instructions. First, the plasmid DNA and cell mixture were flicked several times for mixing. Then, the mixture was incubated for 10 minutes on ice, followed by heat shock at 42°C for 30 seconds, again incubation for 5 minutes on ice took place. Next, 500 μl of room temperature SOC Outgrowth Medium was added to
the DNA-cells mixture and incubated for one hour at 37°C on a shaker (400 rpm). After that, centrifugation at 5000 rpm/5 minutes was carried out and the supernatant was discarded. Then, the Pellet was resuspended in 50-100 µl SOC medium, and streaked on LB Agar plates containing the selective antibiotic (Ampicillin or Kanamycin) with overnight incubation at 37°C. To prepare liquid culture, a single colony from the plate was immersed with 200 mL LB medium containing the selective antibiotic for overnight incubation at 37°C in the shaking incubator. Next day, the transformed bacteria were pelleted by centrifuging the liquid culture at high speed 6000 rpm/15 minutes.

Plasmids were extracted from the bacterial pellets using the Qiagen® Plasmid Maxi kit as per manufacturer’s guidance. Finally, Extracted DNA was resuspended in NF and plasmid concentrations were measured using the NanoDrop 2000 (Thermo Scientific) while Plasmid integrity was assessed by running agarose gel electrophoresis.

3.3 Cell Culture and Transfection

Human embryonic kidney (HEK293) and human epithelial colorectal adenocarcinoma (HT-29) cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplied with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin (Sigma-Aldrich) at 37°C in 5% CO₂.

Transient transfection using HEK293 cells were carried out in 96-well plates with Lipofectamine 2000 by using one third of the 75T 100% confluent flask (~100,000 cells/ well). Briefly, the 96-plates were coated with Poly-L-Lysine for 30 minutes at 37°C, then washed with PBS 3 times. Meanwhile, the transfection mixtures were prepared: 1) plasmid-mix (plasmid mixed with 25 µL Opti-MEM™ media from Gibco
per well), 2) Lipofectamine-mix (0.5 µL of Lipofectamine 2000 mixed with 25 µL Opti-MEM™) each was incubated for 5 minutes at room temperature. Then, the two mixes were combined and incubated for another 20 minutes. Finally, 50 µL of this transfection mix plus 150 µL cells per well were seeded in the 96-well plate. Next day, cells were starved with DMEM-Serum free medium. And after 48 hours transfection, BRET experiments were conducted using the Tristar 2 multilabel plate reader (Berthold, Germany) that allows the sequential integration of light emission detected with two filter settings (480 ± 20 nm and 540 ± 25 nm).

3.4 Transfection Efficacy

Expression of Rluc-tagged proteins was assessed by the addition Coelenterazine h luciferase substrate to measure light emission at 480 nm using the Tristar 2 plate reader. While for the acceptor-tagged proteins (yPET or Venus), was assessed by visual checking using fluorescent microscopy and by reading light emission at 540 nm using the Tristar 2 plate reader.

3.5 BRET and FRET based Assays

Bioluminescence resonance energy transfer (BRET) is a quantitative technology used to study protein-protein interaction in living cells and in real time. It is based on resonance energy transfer from a luminescent energy donor (luciferase-tag) into a fluorescent energy acceptor (fluorescent tag) fusions to the proteins to be studied. If the two tagged proteins are in close proximity within 10 nm (100 Å), upon addition of luciferase substrate, the luciferase will emit light at 480 nm which is within the excitation spectrum of the acceptor, thus allowing emission of the acceptor at 530 nm. The ratio of energy from acceptor’s emission relative to donor’s emission is termed
the BRET signal, or BRET ratio [103] (Figure 8). The fluorescence resonance energy transfer (FRET) differs from BRET with only having a fluorescent donor instead of a luminescent donor. In this study various BRET and FRET-based assays were used to investigate the physical and functional interaction between AT1R and PAR1, including saturation assay, dose response, real-time kinetics, and IP1 measurement.

Figure 8: BRET technology.

3.5.1 BRET Saturation Assay

BRET saturation assay was used to validate the specificity on the interaction between AT1R and PAR1. Cells co-expressing a constant amount of AT1R-Rluc (25 ng) without or with increasing amount of either PAR1-Venus or Grb2-Venus as a negative control (10, 20, 40, 60, 80, 100, 120, 140, 160, 175 ng) were first seeded in 96-well white (for BRET and luminescence measurements) and black (for fluorescence
measurements) plates, washed with 50 μL/well of PBS, and resuspended in 40 μL of PBS. Then, 10 μL of coelenterazine h (2.5 μM) were added and BRET signals were measured. In parallel, the specific luminescence of AT1R-Rluc was measured using a 485 nm emission filter, and the specific fluorescence of PAR1-Venus and Grb2-Venus was quantified using the 485 nm excitation filter and 540 nm emission filter on the Tristar 2 multilabel plate reader. Then, net BRET signals were correlated and plotted over the fluorescence/luminescence ratios (Venus/Rluc ratios).

3.5.2 BRET Dose Response

Dose-response BRET experiments were conducted to assess AngII- as well as thrombin -induced AT1R/Gaq coupling and AT1R/β-arrestin 2 recruitment by transfected HEK293 cells coexpressing AT1R-Rluc (25 ng) with either Venus-Gaq or yPET-β-arrestin 2 (25 ng) in the absence or presence of PAR1 (50 ng), were seeded into 96-well plates. 48 hours post-transfection, cells were starved with serum free medium for at least 3 hours before conducting BRET experiments. Then, cells were washed with 50 μL PBS and 50 μL as total reaction volume per well was used. For AngII dose response assay, 20 μL of the different doses of AngII with 20 μL of PBS or saturating dose of thrombin (1 U/mL). While for thrombin dose response assay, 20 μL of the different doses of thrombin with 20 μL of PBS or sub-saturating dose of AngII (10 nM). Each followed by incubation for 30 minutes at 37°C. Then, 10 μL of coelenterazine h (Promega) was added in a final concentration of 2.5 μM and BRET measurements were carried out using the Tristar 2 multilabel plate reader (Berthold, Germany) that allows the sequential integration of light emission detected with two filter settings (480 ± 20 nm and 540 ± 25 nm).
3.5.3 Real-Time Kinetics BRET

Real-time kinetics experiments were conducted for further investigation of AngII- as well as thrombin -induced AT1R/Gaq coupling and AT1R/β-arrestin 2 recruitment. Transfected HEK293 cells co-expressing AT1R-Rluc (50 ng) with either Venus-Gaq or yPET-β-arrestin 2 (50 ng) in the absence or presence of PAR1 (50 ng) were seeded into 96-well plates. 48 hours post-transfection, cells were starved with serum free medium for at least 3 hours before conducting BRET experiments. Two different kinetics protocol were used. Cells were first washed with 50 µl/well of PBS and resuspended in 40 µl/well of PBS containing coelenterazine h (2.5 µM), and BRET signals were measured in real time for ~5 min to determine the baseline. In the first
direct protocol, after the baseline, 10 µl/well of PBS containing or not, either 10 nM of AngII, thrombin 1 U/mL, or both, was added, and BRET measurements were carried out for 35 minutes. While in the second sequential protocol, after the baseline, BRET signals were measured in real time upon two sequential treatments (T1 then T2). This consists of 10 µl/well of PBS containing or not 10 nM of AngII (T1) for 15 minutes of BRET measurements followed by 10 µl/well of PBS containing or not 1 U/mL of thrombin (T2) for ~45 minutes of BRET measurements.

3.5.4 BRET Titration

BRET titration for AT1R-Rluc/Venus-Gq coupling was conducted to assess the effect of different expression levels of plasmids on AT1R-Gq coupling. For this, HEK293 cells were cotransfected with 25 ng Venus-Gq with different amounts of AT1R-Rluc (10, 25, or 50 ng) in the absence or presence of different amounts of PAR1 (5, 10, 25, 50, or 100 ng), and seeded into 96-well plates. 48 hours post-transfection, cells were starved with serum free media for at least 3 hours before conducting BRET experiments. Then, cells were washed with 50 µL PBS and 50 µL as total reaction volume per well was used. Stimulation with either 10 nM AngII, 1 U/ml thrombin, or both for 30 min at 37°C and addition of 10 µL coelenterazine h (2.5 µM) before BRET signals were measured in live cells.

3.5.5 BRET with Antagonists

HEK293 cells transiently co-expressing AT1R-Rluc (25 ng) with either Venus-Gq or yPET-β-arrestin 2 (25 ng) in the absence or presence of PAR1 (50 ng) were seeded into 96-well plates. 48 hours post-transfection, cells were starved with serum free medium for at least 3 hours before conducting BRET experiments. Then, cells were
washed with 50 µL PBS. First, cells were pretreated or not (control) with 30 µL antagonists; SCH79797 (100 mM) or irbesartan (10 µM) and incubated for 15 minutes at 37°C. Then, 10 µL ligands treatment of either 10 nM AngII, 1 U/mL thrombin, or both were added followed by further incubation for 30 min at 37°C and addition of 10 µL coelenterazine h (2.5 µM) before BRET signals were measured in live cells

3.5.6 IP1 FRET

IP measurement was conducted using the HTFR assay IP-One Tb kit (Cisbio Bioassays, France) according to the manufactural instructions. It is a fluoresce competitive immunoassay based on the principle of FRET that uses selective monoclonal anti-IP1. For this HEK293 cells were transfected to express either AT1R-Rluc, PAR1, or both, and HT-29 cells (with endogenous AT1R and PAR1). Measurement of IP1 accumulation was performed For this, cells were first pre-treated or not (Control) with 30 µl of SCH79797 (100 µM) or irbesartan (10 µM) prepared in the stimulation buffer 1X that contains LiCl₂ to inhibit IP1 degradation, and incubated for 15 min at 37°C. Then, 10 µl of either AngII (10 nM), thrombin (1 U/ml), or both were added followed by further incubation for 30 min at 37°C. For the dose-response experiments, 40 µl of increasing doses of AngII or thrombin in the stimulation buffer 1X were used. The cells were then lysed by adding the supplied assay reagents, and the assay was incubated for 1 h at room temperature. Fluorescence emission was measured at 620 nm and 665 nm, 50 µs after excitation at 340 nm using the Tristar 2 multilabel plate reader (Berthold, Germany).
3.5.7 Trafficking and Internalization

Internalization and endosomal trafficking of AT1R assessed by BRET assays. HEK293 cells transiently coexpressing AT1R-Rluc (25 ng) with either Venus-Kras, Venus-Rab5, or Venus-Rab7 (25 ng) in the absence or presence of PAR1 (50 ng) were used. For BRET measurements, cells were washed with 50 µL PBS, then stimulated or not with either 10 nM of AngII, 1 U/ml of thrombin, or both, for 30 min at 37°C, and addition 10 µL of coelenterazine h (2.5 µM) before BRET signals were measured in live cells.

3.6 Co-immunoprecipitation

HEK293 cells coexpressing or not HA-AT1R with either AT1R-Rluc, PAR1-Rluc, or Grb2-Rluc were used for immunoprecipitation with the anti-HA antibody followed by the measurements of luciferase (Rluc) luminescence directly on the immunoprecipitates. The immunoprecipitation was carried out using the immunoprecipitation (Protein A) assay kit (Roche). Briefly, cells were solubilized in lysis buffer. An equal amount of each protein lysate of Rluc-tagged proteins indicated by their similar luminescence signal (2500000 a.u.) measured at 485 nm was incubated overnight with protein A agarose only for pre-clearing. Precleared lysate (1 ml) was then incubated with the anti-tag HA antibody (3 µg/ml) for 2 h at 4°C, followed by incubation with 25 µl of protein A agarose beads overnight at 4°C. Beads were washed as per the manufacturer’s instructions and the final immunoprecipitates were then resuspended in 50 µl of PBS in 96-well white plate followed by the addition of 10 µl of coelenterazine h (5 µM) as Rluc substrate. The amount of Rluc luminescence in each complex was then measured using a 485 nm emission filter on the Tristar 2 plate reader.
3.7 SDS-PAGE and Western Blot

HT-29 cells (endogenously express AT1R and PAR) along with transfected HEK293 cells with 0.5 µg of either AT1R-Rluc, PAR1-Venus, or both together and seeded onto 6-well plates at a density of 10⁶ cells per well. Cells were starved overnight with DMEM-serum free media. Then, cells were washed with PBS and stimulated or not with either 1 µM, or 10 nM AngII, 1, or 0.1 U/ml thrombin, or both for 5 minutes or 30 minutes at 37°C. After treatment, the cells were washed in cold PBS and lysed with 200 µL/well of ice-cold RIPA lysis buffer (Pierce) supplemented with phenylmethylsulfonyl fluoride (PMSF) (Roche) and protease inhibitors (Sigma-Aldrich) and incubated for 90 minutes at 4°C. Cell lysates were then scraped and collected, followed by centrifugation at 15000 g for 15 minutes at 4°C to remove cell debris. Then, proteins were quantified using BCA Protein Assay Kit (Thermo Fisher Scientific). After that for 10% SDS-PAGE (at 225 V for ~ 1 hour), equal amounts of each protein from each cell lysates sample was mixed with Laemmli buffer (Bio-rad) containing 8% β-mercaptoethanol and heated for 5 minutes at 95°C, and transferred to polyvinylidene fluoride (PVDF) (Bio-Rad) membranes by conventional wet-transfer technique (100 V for 90 minutes). The membranes were incubated for blocking at room temperature for 1 hour in 5% skimmed milk prepared in PBS (Gibco) containing 0.1% Tween 20 (Bio-Rad) (PBST) for 1 hour. And the membranes were incubated overnight at 4°C with primary anti-ERK antibodies; primary mouse monoclonal anti–pERK1/2 (Cell Signaling) (1:2,000 dilution in TBST containing 5% skimmed milk) for the phosphorylated proteins or primary rabbit polyclonal anti-ERK1/2 (1:1,000 dilution in TBST containing 5% bovine serum albumin) for total proteins. Membranes were incubated for 45 minutes with secondary IgG anti-rabbit or anti-mouse
conjugated to horseradish peroxidase (HRP) as per manufactural instructions. Followed by detection of the immunoreactive bands by chemiluminescent substrate (Thermo Fisher Scientific), and HRP was detected by chemiluminescence using the LiCOR C-DiGit Blot Scanner.

3.8 Data Presentation and Statistical Analysis

The BRET values were achieved by the ratio of energy emitted at 540 nm/ energy emitted at 480 nm. Then this BRET ratio was converted into “ligand-induced BRET” signals by subtracting the ratio obtained from vehicle-treated cells from the same ratio obtained from AngII/thrombin-treated cells. Then, the % of responses in the different BRET and IP1 assays were obtained by taking as 100% the maximal AngII/thrombin-induced responses in the control condition. All kinetic and the sigmoidal dose-response curves were fitted to appropriate nonlinear regression equations using GraphPad Prism software (San Diego, CA, USA). Statistical analyses were performed with two-way ANOVA and multiple comparisons test to determine statistical significance between the different conditions relative. ****p-value < 0.0001, ***p-value < 0.001, **p-value < 0.01, * p-value < 0.05, and ns p-value > 0.05.
Chapter 4: Results

4.1 Fluorescence Microscopy

Transfection validation was performed as previously mentioned by reading the expression of Rluc- and Venus- tagged proteins at 480 nm and 540 nm, respectively using the Tristar 2 plate reader. In addition, fluorescent microscopy visualization was performed. As shown in Figure 10, fluorescent tagged proteins were expressed in the cytosol of transfected HEK293 cells, Venus-Gq in Figure 10A and yPET-Arrestin in Figure 10B. Therefore, validating the transfection and expression of these proteins 48 hours post-transfection.

Figure 10: Fluorescence microscopy confirmed the transfection. Visualization of transfected HEK293 cells with AT1R-Rluc/Venus-Gq (A) and AT1R-Rluc/yPET-Arrestin 2 (B) by fluorescence microscope, validating the cytosolic expression of the fluorescent tagged proteins Venus Gq (A) and yPET-Arrestin (B).
4.2 The Physical Interaction between AT1R and PAR1

As mentioned previously, the aim of this study is first to check the physical interaction between AT1R and PAR1 by assessing the molecular proximity between them. For this, BRET saturation assay was performed followed by CO-IP to confirm this physical interaction by forming AT1R-PAR1 heterodimer.

4.2.1 BRET Saturation

BRET saturation assay was used to prove the specificity of the BRET signal between AT1R-Rluc and PAR1-Venus. A fixed amount of the donor AT1R-Rluc (25 ng) was co-expressed with or without different amounts of the acceptors either PAR1-Venus or Venus-Grb2 (the adaptor protein as negative control). Theoretically, the BRET signals should increase with the increased amount of the acceptor until reaching the stage of saturation where all the donors were consumed, and nothing remained to excite the left acceptors. Then, the net BRET ratio is blotted over the acceptor/donor ratio. As shown in Figure 11 A, an increase in BRET signal between AT1R-Rluc and Venus PAR1 was achieved until reaching the saturation stage and it is demonstrated in a hyperbolic (blue curve). However, a linear nonspecific BRET signals were obtained from AT1R-Rluc and Venus-Grb2 interaction (red curve). Thus, confirming the specific molecular proximity between AT1R and PAR1.

4.2.2 Co-immunoprecipitation (CO-IP) Confirmed AT1R-PAR1 Heterodimerization in Vitro

Then, further confirmation of this physical interaction was done by CO-IP experiment. For this, the ability of AT1R to heterodimerize with PAR1 was assessed by transfecting HEK293 to express HA-AT1R with or without either PAR1-Rluc, AT1R-
Rluc as a positive control for HA-AT1R/AT1R-Rluc homodimers, or Grb2-Rluc as a negative control, were used. The HA-AT1R was immunoprecipitated using the anti-HA antibody, followed by the Rluc luminescence measurement of the PAR1-Rluc co-immunoprecipitated with HA-AT1R. As shown in Figure 11 B, for a similar input of Rluc tagged proteins used from all the samples a strong luminescence signal was measured in the immunoprecipitate obtained from HA-AT1R/AT1R-Rluc expressing cells demonstrating their physical interaction and homodimerization as previously reported and validating the assay [44], [104]. Moreover, a weaker but significant Rluc signal was also measured in the immunoprecipitate obtained from HA-AT1R/PAR1-Rluc expressing cells (Figure 11 B). Such a signal was observed neither when PAR1-Rluc was expressed alone without HA-AT1R nor in the immunoprecipitate obtained from HA-AT1R/Grb2-Rluc expressing cells indicating the specificity of the co-immunoprecipitation (Figure 11B). These results indicate that AT1R receptors selectively dimerize with PAR1, but not with Grb2. Together, these data support the presence of a physical interaction between AT1R and PAR1 when co-expressed in HEK293 and the formation of AT1R-PAR1 heterodimer in vitro.
Figure 11: The physical interaction between AT1R and PAR1 studied by BRET saturation in living cells and co-immunoprecipitation: A) BRET saturation assay was used to assess the specificity and molecular proximity between AT1R and PAR1 by using HEK293 cells transfected with constant amount of AT1R-Rluc donor with or without either the acceptor Venus-PAR1 or Venus Grb2. Then, the net BRET values were blotted over the Venus/Rluc ratios. B) HEK293 cells transiently co-expressing HA-AT1R without or with either AT1R-Rluc, PAR1-Rluc, or Grb2-Rluc were used for coimmunoprecipitation using the anti-tag HA antibody. For this, an equal Rluc input was used and the final immunoprecipitates were then used for the quantification of Rluc luminescence of the different Rluc-tagged proteins coimmunoprecipitated or not with HA-AT1R (co-immunoprecipitated Rluc). The BRET saturation data are means ± SEM of three to four independent experiments performed in triplicate. The co-immunoprecipitation data are means ± SEM of three independent measurements. ****p-value < 0.0001 and ns p-value > 0.05.
4.3 The Functional Interaction between AT1R and PAR1

After assessing the physical interaction between AT1R and PAR1, the functional consequences of AT1R-PAR1 heterodimerization have been examined. For this, various biophysical and biochemical techniques were used. BRET and FRET based assays were used to examine G protein coupling to the heterodimer, β-arrestin 2 recruitment, second messengers production, heterodimer’s trafficking and internalization, whereas SDS-PAGE and western blot were used to assess the dimerization effects on the phosphorylation of the key kinases such as the MAP kinases/ERK pathway.

4.3.1 BRET Dose Response Confirmed the Functional Interaction between AngII and Thrombin through AT1R-PAR1 Heterodimer

The effect of thrombin treatment and the transient overexpression of PAR1 on the functional coupling of AT1R with the heterotrimeric G_q protein, and the recruitment β-arrestin 2 was examined. For this, BRET technology using live HEK293 cells co-expressing AT1R-Rluc (as BRET donor) and either Venus-Gq or yPET-β-arrestin 2 (as BRET acceptors) in the absence or presence of PAR1 overexpression and stimulated without or with either AngII, thrombin, or co-stimulated.

Starting with dose-response analysis to assess the specificity of the ligands, AngII and thrombin for binding their respective receptors, AT1R and PAR1 (Figure 12). HEK293 cells expressing either AT1R-Rluc or PAR1-Rluc with Venus-Gq were treated with increased doses of either AngII (Figure 12 A) or thrombin (Figure 12 B). A dose response stimulation was obtained, showing increase in the BRET signals with the increased doses of ligands until reaching saturation. AngII dose response showed a dose dependent BRET signals (Figure 12 A) between AT1R-Rluc/Venus-Gq transfection, but not between PAR1-Rluc/Venus-Gq. Similarly, the thrombin dose
response was between PAR1-Rluc/Venus-Gq, but not AT1R-Rluc/Venus-Gq. Together, these findings confirm the specificity of the receptors to their ligands and their functional activity for Gq coupling in a dose-dependent manner. Moreover, this analysis guided us to determine AngII EC$_{50}$ (10 nM) as well as the E$_{\text{max}}$ of thrombin (1 U/ml) concentrations to be used in all the protocols in this study. Then, AngII dose response analysis was conducted with increasing AngII doses without or with 1 U/ml thrombin in cells expressing AT1R-Rluc with either Venus-Gq (Figure 12 C, D) or yPET-Arrestin 2 (Figure 12 E, F) in the presence (Figure 12 D, F) or absence (Figure 12 C, E) of PAR1 overexpression. Again, here a dose-dependent BRET increase was achieved between the donor and acceptors as was shown in Figure 12 A and B. In the absence of PAR1 overexpression (Figure 12 C, E), when AngII doses were combined with 1 U/ml thrombin stimulation, did not induce a BRET increase, but showed a significant lift-shift of the dose response curves of AngII between AT1R-Rluc/Venus-Gq (Figure 12 C) and AT1R-Rluc/yPET-Arrestin 2 (Figure 12 E) (p-value < 0.0001 or p-value < 0.001). These data revealed a positive allosteric modulation by the 1 U/ml thrombin on AT1R, as the Gq Log EC$_{50}$ values were -7.99 ± 0.08 and -8.82 ± 0.1 (p-value <0.0001, n=4) and for β-arrestin 2 the Log EC$_{50}$ values were -8.32 ± 0.10 and -9.44 ± 0.11 (p-value <0.0001, n=3) in the absence and presence of thrombin, respectively. This positive modulation of AT1R by thrombin might be due to the endogenously expressed PARs in HEK293 cells that has been reported previously [105], [106]. Whereas in the presence of PAR1 overexpression, the co-stimulation with 1 U/ml thrombin combined with AngII doses induced a significant increase in the BRET signals of ~75% with AT1R-Rluc/Venus-Gq/PARA (Figure 12 D) and ~25% with AT1R-Rluc/yPET-Arrestin 2/PARA (Figure 12 F) cells (P-value <0.0001). Excitingly, these data clearly show the positive allosteric effect of thrombin through
its receptor PAR1 to transactivating AT1R and thus, confirming the functional consequences of AT1R-PAR1 heterodimerization mediating Gq coupling and β-Arrestin 2 recruitment in an activated PAR1-dependent manner. Moreover, thrombin dose response was done as shown in Figure 13, for further confirmation of the functional interaction of AT1R-PAR1 heterodimer and the transactivation of AT1R by activated PAR1. HEK293 cells expressing AT1R-Rluc/Venus-Gq/-+PAR1 (Figure 13 A, B) or AT1R-Rluc/yPET-Arrestin 2/-+PAR1 (Figure 13 C, D) were stimulated with increased concentrations of thrombin doses in the presence or absence of 10 nM AngII. As expected, in the absence of PAR1 and 10 nM AngII, no thrombin-induced BRET signal was obtained between AT1R-Rluc/Venus-Gq (Figure 13 A) or AT1R-Rluc/yPET-Arrestin 2 (Figure 13 C). Again, confirming the specificity of thrombin ligand toward its receptor PAR1 but not AT1R. However, in the absence of PAR1 and in the presence of 10 nM AngII combined with the increased doses of thrombin, a marked dose-dependent BRET increase was observed between AT1R-Rluc/Venus-Gq (Figure 13 A) as well as between AT1R-Rluc/yPET-Arrestin 2 (Figure 13 C) with $E_{\text{max}}$ values of 238 to 283 ± 39% and 304 ± 57% (p-value <0.0001, n=4), respectively compared to 100% of AngII single stimulation. This demonstrated the positive allosteric effect of AngII at EC$_{50}$ concentration on the thrombin dose-response curve. While in the presence of PAR1 overexpression (Figure 13 B, D), thrombin alone induced a weak increase in the BRET dose response curve between AT1R-Rluc/Venus-Gq/PAR1 ($E_{\text{max}} = 211 ± 34\%$, p-value <0.0001, n=4) in Figure 13 B and AT1R-Rluc/yPET-Arrestin/PAR1 2 ($E_{\text{max}} = 102 ± 25\%$, p-value <0.0001, n=3). However, when thrombin doses are combined with 10 nM AngII, stronger increase in the dose-dependent BRET signals was shown in cells expressing AT1R-Rluc/Venus-Gq (Figure 13 B) and AT1R-Rluc/yPET-Arrestin 2 (Figure 13 D), with maximum
BRET responses of ($E_{\text{max}} = 342 \pm 33\%$, p-value $<0.0001$, n=4) and ($E_{\text{max}} = 299 \pm 43\%$, p-value $<0.0001$, n=3), respectively compared to 100% of AngII single stimulation. Together, these findings are consistent with the ones in Figure 12, confirming the functional allosteric interaction between AngII and thrombin at the level of their respective receptors heterodimerization to couple Gq and recruit β-arrestin 2, also these data further confirm the specificity of each ligand with its cognate receptor.
Figure 12: AngII dose response analysis to assess AngII-induced AT1R-Gq/Arrestin functional interactions via transactivation. First, the specificity of AT1R and PAR1 towards their ligands was assessed by using HEK293 cells co-expressing either AT1R-Rluc/Venus-Gq (A) or PAR1-Rluc/Venus-Gq (B). Then, the functional consequences of AT1R-PAR1 heterodimerization was assessed by dose response analysis using HEK293 cells expressing AT1R-Rluc with either Venus-Gq (C, D) or yPET-Arrestin 2 (E, F) in the presence (D, F) or absence (C, E) of PAR1 overexpression were treated without or with increased concentration of thrombin doses combined (blue curves) or not (black curves) with 10 nM AngII for 30 minutes at 37°C. BRET signals were measured in live cells. Data are means ± SEM of three to four independent experiments performed in triplicate. The statistical analysis indicates the significance relative to the control curve (the absence of thrombin). ****p-value < 0.0001, ***p-value < 0.001.
Figure 13: Thrombin dose response analysis to assess thrombin-induced AT1R-Gq/Arrestin functional interactions via transactivation. HEK293 cells expressing AT1R-Rluc with either Venus-Gq or yPET-Arrestin 2 in the presence (B, D) or absence (A, C) of PAR1 overexpression were treated without or with increased concentration of thrombin doses combined (blue curves) or not (black curves) with 10 nM AngII for 30 minutes at 37ºC. BRET signals were measured in live cells. Data are means ± SEM of three to four independent experiments performed in triplicate. The statistical analysis indicates the significance relative to the condition in the absence of thrombin for both curves, the control, and the ones in the presence of 10 nM of AngII. ****p-value < 0.0001, ***p-value < 0.001, **p-value < 0.01, *p-value < 0.05.
4.3.2 Real-Time Kinetics Confirmed the Ability of AT1R-PAR1 Heterodimer to Couple Gq and Recruit β-arrestin 2 in a Time Dependent manner

Then, real-time kinetics was performed for further confirmation of the functional consequences of the dimerization between AT1R-PAR1, via conducting two different kinetics protocols, direct (Figure 14) and sequential kinetics (Figure 15) as previously were described. HEK293 cells expressing AT1R-Rluc with either Venus Gq or yPET-Arrestin 2, in the presence or absence of PAR1 overexpression were used. First, the basal activity was determined by reading for ~5 minutes without stimulation. Then for the direct kinetics (Figure 14), reading the baseline was followed or not by the direct addition of either 10 nM AngII, 1 U/ml thrombin, or their combination for ~35 minutes reading. As demonstrated in (Figure 14 A, C), in the absence of PAR1 overexpression, the addition of the single treatment of 10 nM AngII, induced BRET signal between AT1R-Rluc and either Venus-Gq (Figure 14 A) or yPET-Arrestin 2 (Figure 14 C), was observed up to ~35 minutes. This effect was not observed with 1 U/ml thrombin treatment alone. Interestingly, the co-stimulation showed a marked increase in the BRET signal ~200% with Gq and ~220% with β-arrestin 2 compared to 100% single AngII stimulation (P-value <0.0001). Again, this sharp increase might be due to the endogenous expression of PARs in HEK293 cells. Whereas in the presence of PAR1 overexpression (Figure 14 B, D), the single treatment of 10 nM AngII induced ~220% increase in the BRET signal for Gq coupling (Figure 14 B, red curve) and ~246% for β-arrestin 2 recruitment (Figure 14 D, red curve). Also, with the 1 U/ml thrombin, but in a less extent with β-arrestin (Figure 14 D, blue curve). And excitingly, with the co-stimulation, more increase in BRET signals between AT1R-Rluc and either Venus-Gq (Figure 14 B, green curve) or yPET-Arrestin 2 (Figure 14 D, green curve) was observed ~250% (P-value <0.0001). These findings further confirm the functional
consequences of AT1R-PAR1 heterodimerization and its ability to couple to Gq and recruit β-arrestin in time and dose dependent manner.

Then a sequential real-time kinetics was performed (Figure 15), after measuring the baseline level, cells were treated without (vehicle) or with 10 nM AngII (treatment 1 or T1) for ~15 minutes, followed by treating cells or not (vehicle) with 1 U/ml thrombin (Treatment 2 or T2) for ~45 minutes. So, the ligand treatments were added sequentially to see the effect before and after their single and combined stimulation.

This assay demonstrated consistent results with the dose response and the direct kinetics analysis, the 10 nM AngII induced increase in the BRET signal between AT1R-Rluc and both Venus-Gq (Figure 15 A, blue curves) and yPET-Arrestin 2 (Figure 15 C, blue curves). Also, the addition of T2 (1 U/ml thrombin) followed by vehicle, did not induce a BRET signal between them (Figure A, C, red curves). However, T1 addition followed by T2 combination, increased the BRET signal (by 137% with Gq and 125% with β-arrestin 2). Together, these results show that 10 nM AngII was able to induce the coupling of AT1R to Gq as well as to recruit β-arrestin 2, but not the 1 U/ml thrombin. In addition to this, the co-stimulation enhanced this functional activity with the possibility of the presence of other PARs as previously explained. On the other hand, when cells are overexpressing PAR1 with AT1R-Rluc and either Venus-Gq (Figure 15 B) or yPET-Arrestin 2 (Figure 15 D). Here, both T1 (blue curves) and T2 (red curves) when combined with vehicle induced BRET signal increase. But for T2, it showed a weaker response with β-arrestin (Figure 15 D) compared to Gq (Figure 15 B). Attractively, the addition of T1 followed by T2, markedly increased the BRET signal (by ~185% with Gq and 179% with β-arrestin 2). All these data are consistent with the all the previous results and confirming Gq
coupling and β-arrestin 2 recruitment to the AT1R-PAR1 heterodimer formation with the co-stimulation by 10 nM AngII with 1 U/ml thrombin.

Figure 14: Real-time kinetics (Direct protocol) confirmed the functional interaction of AT1R-PAR1 heterodimerization by BRET. HEK293 expressing either AT1R-Rluc/Venus-Gq (A, B) or AT1R-Rluc/yPET-Arrestin 2 (C, D), with (B, D) or without (A, C) PAR1 overexpression were used for real-time BRET kinetic analysis as described in methods. First, the baseline level was determined with basal reading for ~5 min, followed by the direct ligand treatments for ~35 min reading in real-time. BRET signals were in real-time and live cells for 35 min. Data are means ± SEM of 10–11 independent experiments performed in single-point measurements. The statistical analysis indicates the significance relative to the kinetic curves in the presence of 10 nM of AngII. ****p-value < 0.0001.
Figure 15: Real-time kinetics (Sequential protocol) confirmed the functional interaction of AT1R-PAR1 heterodimerization by BRET. HEK293 expressing either AT1R-Rluc/Venus-Gαq (A, B) or AT1R-Rluc/yPET- Arrestin 2 (C, D), with (B, D) or without (A, C) PAR1 overexpression were used for real-time BRET kinetic analysis as described in methods. First, the baseline level was determined with basal reading for ~5 min, followed by T1 (10 nM AngII) or not (vehicle) for ~15 min, and finally T2 (1 U/ml thrombin) with ~45 min reading in real-time. BRET signals were measured in real-time and live cells for 60 min. Data are means ± SEM of eight to nine independent experiments performed in single-point measurements. The statistical analysis indicates the significance relative to the baseline as well as to the kinetic curves in the presence of 10 nM of AngII. ****p-value < 0.0001, ***p-value < 0.001, **p-value < 0.01, *p-value < 0.05.
4.3.3 BRET Titration Assay Demonstrated that AT1R-PAR1 Functional Consequences Depend on the Receptor Expression Level

Then, BRET titration was performed to check if AT1R-PAR1 heterodimerization is affected by the receptor expression level. For this, HEK293 cells co-expressing Venus-Gq with three different amounts of AT1R-Rluc 10, 25, or 50 ng (Figure 16 A, B, and C, respectively) and with or without PAR1 (0, 5, 10, 25, 50, 100 ng) were stimulated without or with 10 nM AngII, 1 U/ml, or their combination. Indeed, in the absence of PAR1 expression, a BRET induced signal between AT1R-Rluc and Venus-Gq was obtained with 10 nM AngII but not with 1 U/ml thrombin and without any significant potentiation with the co-stimulation. Moreover, the 25 ng AT1R-Rluc (Figure 16 B) and 50 ng (Figure 16 C) showed the prettiest response compared to 10 ng (Figure 16 A). Whereas with PAR1 expression, even with the lowest expression amount (5 ng), the positive functional BRET response between AT1R-PAR1 heterodimer and Gq was observed. And 1 U/ml thrombin as well as the co-treatment induced a significant increase in BRET signals with the increased PAR1 levels in the three different figures. Showing that thrombin through its receptor PAR1 positively modulates AT1R and the thrombin-induced AT1R transactivation responses were observed in receptor expression-dependent manner until reaching the saturation level with 25 ng of PAR1. Whereas 50 ng showed the highest BRET increase (340% with 1 U/ml thrombin and 478% with co-treatment) compared to 25 ng PAR1 (294% with 1 U/ml thrombin and 446% with co-treatment) (P-value < 0.0001), indicating the specificity of the BRET signals between AT1R-Rluc and Venus-Gq in PAR1’s presence. So, according to these findings, the BRET experiments were conducted following this expression level of 25 ng AT1R-Rluc with 50 ng PAR1. Moreover, this analysis demonstrated that AT1R-PAR1 functional heterodimerization occurs even at very low expression levels of the
receptors. In addition, transactivation of AT1R by activated PAR1 occurs in a receptor expression-dependent manner until reaching a saturation, reflecting the specificity of the BRET signals of Gq coupling to the heterodimer.
Figure 16: BRET titration analysis. HEK293 cells co-expressing different amounts of AT1R-Rluc 10 ng (A), 25 ng (B), or 50 ng (C) with fixed amount of Venus-Gq, in the absence or presence of different amounts of PAR1 (0, 5, 10, 25, 50 ng), were stimulated or not with either 10 nM AngII, 1 U/ml, or both at 37°C for 30 min. BRET signals were measured. Data are means ± SEM of three independent experiments performed in triplicate. The statistical analysis indicates the significance relative to the response of AngII. ****p-value < 0.0001, ***p-value < 0.001, *p-value < 0.05, and ns p-value > 0.05.
4.3.4 Effect of Receptors Blockage on AT1R-PAR1 Heterodimerization

Next, the effect of blocking the receptors on the functional interaction between AT1R and PAR1 and their dimerization was studied by using their antagonists for further pharmacological investigation on Gq coupling and β-arrestin 2 recruitment. By assessing the effect of the antagonists Irbesartan 10 µM and SCH79797 100 µM as selective inhibitors for AT1R and PAR1, respectively with stimulation by either 10 nM AngII, 1 U/ml thrombin, or both of cells expressing AT1R-Rluc/Venus-Gq/PAR1 (Figure 17 A) and AT1R-Rluc/yPET-Arrestin 2/PAR1 (Figure 17 B). As shown in Figure 17, Irbesartan totally blocked AT1R with both Gq (Figure 17 A) and β-arrestin 2 (Figure 17 B). However, Irbesartan also diminished thrombin signal with Gq (Figure 17 A), but not with β-arrestin. Suggesting that thrombin-mediated AT1R-Rluc/Venus-Gq coupling involves AT1R activation through transactivating it by PAR1, which is consistent with the previous results. Also, SCH79797 significantly showed marked decrease of PAR1 activity represented by diminished thrombin-induced BRET signals with both AT1R-Rluc/Venus-Gq (Figure 17 A) coupling and AT1R-Rluc-yPET-Arrestin 2 recruitment (Figure 17 B) expressing cells. Interestingly, Both Irbesartan and SCH79797 markedly diminished the BRET signal with co-treatment in both AT1R-Rluc with either Venus-Gq or yPET-Arrestin 2 expressing cells. These results display the significance of co-activating both PAR1 and AT1R receptors, inducing the positive allosteric modulation of PAR1 on AT1R through transactivation.
Figure 17: BRET analysis to assess the effect of receptors blockage on AT1R transactivation by PAR1. HEK293 expressing either AT1R-Rluc/Venus-G\(\alpha\)q/PAR1 (A) or AT1R-Rluc/\(\gamma\)PET-\(\beta\)-arrestin 2/PAR1 (B) were first pretreated or not for 15 min at 37\(^\circ\)C with 10 \(\mu\)M Irbesartan or 100 \(\mu\)M SCH79797 antagonists, followed by agonists treatment for 30 min at 37\(^\circ\)C with or without 10 nM AngII, 1 U/ml thrombin, or both. BRET signals were measured in living cells and in real time. Data are means ± SEM of four to six independent experiments performed in triplicates. The statistical analysis indicates the significance relative to the response of AngII or thrombin alone as well as to the condition of the combined treatment in control as indicated. ****\(p\)-value < 0.0001, ***\(p\)-value < 0.001, and ns \(p\)-value > 0.05.
4.3.5 The Effect of AT1R-PAR1 Heterodimerization on Gq/Inositol Phosphate Signaling Pathway

Then, to correlate the effect of AT1R-Rluc/Venus-Gq coupling on its downstream signaling by measuring the production and accumulation of the second messenger IP1, the effect of co-expressing AT1R and PAR1 on the Gq/inositol phosphate pathway in both HEK293 cells transiently expressing the receptors as well as in the human epithelial colorectal adenocarcinoma cells (HT-29) endogenously expressing AT1R and PAR1 [107], [108] was explored. Starting with dose response analysis in HEK293 expressing either AT1R-Rluc (Figure 18 A), or PAR1 (Figure 18 B) to confirm if the previously used 10 nM AngII and 1 U/ml thrombin doses are suitable for this protocol as well. As demonstrated in Figure 18 A and B, the EC50 dose of AngII and the saturating dose of thrombin are consistent with what we got in BRET dose response experiments, also that these concentration of AngII and thrombin were able to activate Gq signaling pathway and promote IP1 production. Then, HEK293 cells expressing either AT1R-Rluc (Figure 18 C), PAR1 (Figure 18 D), or both (Figure 18 E), were used to measure IP1 accumulation. Cells were pretreated or not with either 10 µM Irbesartan or 100 µM SCH79797, followed by ligands treatment without or with 10 nM AngII, 1 U/ml thrombin, or both as shown in Figure 18. Indeed, AT1R-Rluc expressing cells significantly lost the AngII-induced IP1 response with Irbesartan (Figure 18 C), also PAR1 expressing cells lost the thrombin-induced IP1 production when treated with SCH79797 (Figure 18 D) (P-value < 0.0001). However, cells expressing AT1R-Rluc showed IP1 response mediated by thrombin although no PAR1 transfection was there and it was blocked with SCH79797. This is might be due to the endogenous expression of PARs in HEK293 cells. Whereas for PAR1 expressing cells, no AngII-Induced IP1 production was observed at all, reflecting the specificity of
PAR1 again. These findings are consistent with all the previous results and confirming the specificity of the antagonists and receptors, confirming the functional coupling and activation of Gq with AT1R and PAR1 with IP1 production. Interestingly, cells co-expressing both AT1R and PAR1 along with co-activation, had a positive synergetic effect on activating the Gq pathway with an increase in IP1 accumulation levels by ~ 160% (P-value < 0.001). Also, this is consistent with the previous findings and confirming the functional interaction between AT1R and PAR1 when both are activated (Figure 18 E). And both antagonists fully abolished this positive synergetic effect of the co-activation (Figure 18 E, green bars), emphasizing the importance of AT1R and PAR1 activation in the synergetic interaction between AngII and thrombin. In addition to this, with the blockage of one receptor, IP1 production level was not completely inhibited, which might reflect the activation of Gq pathway by AT1R and PAR1 monomers. Finally, HT-29 cells were used to examine AT1R-PAR1 dimerization effect as they originally express these two receptors (Figure 18 F). The obtained data were consistent with the ones observed in HEK293 transfected cells (Figure 18 F). Both AngII and thrombin elicited IP1 production response which was markedly attenuated with Irbesartan and partially with SCH79797. So, the findings with HT-29 cells as well corroborate with the previously obtained data from transfected HEK293 cells, confirming the functional interaction between AT1R and PAR1 when co-activated, even when they are endogenously expressed.
Figure 18: AT1R-PAR1 heterodimerization effect on IP1 production via the Gq signaling pathway assessed via HTFR FRET-based assay IP-One Tb kit (Cisbio Bioassays, France). First, dose response analysis for IP1 production levels was done for HEK293 cells expressing either AT1R-Rluc (A) or PAR1 (B). Then, HEK293 cells expressing either AT1R-Rluc (C), PAR1 (D), or both (E) were pretreated with or without 10 µM AngII blocker or 100 µM thrombin blocker for 15 min at 37°C, followed by ligands stimulation with or without 10 nM AngII, 1 U/ml thrombin, or their combination for 30 min at 37°C to measure IP1 production levels with these different conditions. Finally, HT-29 cells with endogenous AT1R and PAR1 were assessed as well for their ability to activate Gq signaling pathway and synthesize IP1 (F). Data are means ± SEM of three (for dose-response in HEK293 and antagonist data in HT-29) or four (for antagonist data in HEK293) independent experiments performed in triplicate. The statistical analysis indicates the significance relative to the response of AngII or thrombin alone as well as to the condition of the combined treatment in control as indicated. ****p-value < 0.0001, ***p-value < 0.001, **p-value < 0.01, *p-value < 0.05, and ns p-value > 0.05.
4.3.6 PAR1 Trans-inhibited AT1R Trafficking and Internalization

At the end, the regulation of the AT1R-PAR1 heterodimer was analyzed by assessing its endosomal trafficking and internalization in HEK293 cells via BRET. For this, the same previously used conditions were used, cells transiently expressing AT1R-Rluc, with or without PAR1 overexpression, and with either the plasma membrane marker Venus-Kras, or the endosomal markers Venus-Rabs. As demonstrated in Figure 19 A, 10 nM AngII induced decrease in the BRET signal between AT1R-Rluc and Venus-Kras, reflecting AT1R’s internalization. However, this response was not observed with 1 U/ml thrombin stimulation nor the co-stimulation. On the other hand, in the presence of PAR1 overexpression along with the co-stimulation, a sharp decrease by ~60% (p-value < 0.0001, n=3) in the BRET signal was obtained (Figure 19 A). Together, these data clearly demonstrate that PAR1 trans-inhibits AT1R’s internalization when both receptors are activated. Finally, the internalization findings were confirmed by further investigation of the endosomal trafficking using HEK293 cells transiently expressing AT1R-Rluc with either Venus-Rab5 (Figure 19 B) or Venus-Rab5 (Figure 19 C) as endosomal markers to assess the early and late endosomal trafficking, respectively, in the presence or absence of PAR1 overexpression. Similarly, in cells expressing AT1R-Rluc/Venus-Rabs, 10 nM AngII induced AT1R trafficking, but not 1 U/ml thrombin alone (Figure 19 B, C). However, the co-activation diminished its trafficking. Again, this effect might be due to the presence of endogenous PAR1 in HEK293 cells. Excitingly, in the presence of PAR1, 10 nM AngII enhanced the early endosomal trafficking by ~120% with Rab5(Figure 19 B). Interestingly, in the presence of PAR1 plus the co-activation, a significant decrease in the BRET signals was observed with both Rab5 ($E_{\text{max}} = 59 \pm 2\%$, p-value <0.01, n=3) and Rab7 ($E_{\text{max}} = 81 \pm 6\%$, p-value
Confirming that PAR1 negatively modulates the early and late trafficking of AT1R. Together, these results revealed the action of PAR1 as a negative allosteric modulator for AT1R internalization as well as trafficking when the two receptors are activated.

Figure 19: PAR1 negatively modulated AT1R internalization and trafficking. Transient expression of AT1R-Rluc with either Venus-Kras (A), Venus-Rab5 (B), or Venus-Rab7 (C), in the presence or absence of PAR1 overexpression. Cells were treated for 30 min at 37°C with or without 10 nM AngII (blue bars), 1 U/ml thrombin (red bars), or both (green bars). Then BRET signals were measured in live cells. Data are means ± SEM of three independent experiments performed in triplicate. The statistical analysis indicates the significance relative to the condition in the presence of 10 nM of AngII. ****p-value < 0.0001, **p-value < 0.01, and ns p-value > 0.05.
4.3.7 MAPK/ERK Pathway Activation

Then, the ability of AT1R-PAR1 heterodimer to activate the downstream signaling MAPK/ERK pathway (Figures 20, 21, and 22) under the previously used conditions (single versus combined treatment and single expression of AT1R or PAR1 versus their co-expression) was examined. Also, two stimulation times were considered, 5 min (for G protein-dependent ERK1/2) and 30 minutes for (arrestin-dependent ERK1/2) as well as two combinations of AngII and thrombin doses. 1 U/ml thrombin elicited ERK1/2 phosphorylation regardless of the cells used, while 10 nM of AngII showed very weak ERK1/2 phosphorylation in AT1R expressing HEK293 cells (Figure 20 B) whereas the response was more clear in cells co-expressing AT1R-Rluc and PAR1 (Figure 20 C). This was true for both 5 minutes and 30 minutes of stimulation. In the combined AngII/thrombin treatment, it seems like there was a slight potentiation at 5 minutes and a strong reduction at 30 minutes. This observation might be consistent with the inhibition of AT1R internalization upon PAR1 co-expression and activation (Figure 19). In Figure 21, the focus of this study was on HEK293 cells co-expressing AT1R-Rluc and PAR1, and 5 minutes of stimulation using two combinations of AngII and thrombin doses. In Panel A, 10 nM of AngII was combined with 1 U/ml of thrombin and this showed a strong thrombin-mediated ERK1/2 phosphorylation and only weak response with AngII. Their combination tends to elicit a slight potentiation. By contrast, in Panel B, the increase of AngII dose (1 uM) seems to give more ERK1/2 response and its combination with thrombin at a lower dose (0.1 U/ml) promoted an increased ERK1/2 phosphorylation. Finally, ERK1/2 phosphorylation was examined on endogenous receptors in HT-29 cells (Figure 22) and as shown both thrombin (1 U/ml) and AngII (10 nM) showed ERK1/2
phosphorylation and their combination did not have any further effect. Together these preliminary data on ERK1/2 in HEK293 cells suggest a potentiation of ERK1/2 phosphorylation upon combined treatment with AngII and thrombin. However, they did not bring any evidence that this was mediated by AT1R-PAR1 complex or their functional interaction. Further investigation is required to address this aspect of AT1R and PAR1 function and pharmacology. Therefore, these ERK1/2 data are too preliminary and further controls, tools, and investigations are needed to better correlate these findings and BRET data with this important downstream signaling pathway of GPCRs.

Figure 20: Phosphorylation of ERK1/2 in HEK293 cells. HEK293 cells transiently expressing either PAR1 (A) AT1R-Rluc (B), or both (C), were stimulated with AngII (10 nM), thrombin (1 U/ml), or both, for 5 minutes (upper panel) or 30 minutes (lower panel) and then lysed for SDS-PAGE followed by western blot using the mouse anti-phospho-p44/42 (pERK1/2) (Cell Signaling) (1:2000 dilution). The total ERK1/2 was detected using the primary rabbit polyclonal anti-p44/42 ERK1/2 (1:1000 dilution).
Figure 21: Phosphorylation of ERK1/2 in HEK293 cells at two different stimulations. HEK293 cells transiently coexpressing AT1R-Rluc and PAR1 were stimulated for 5 minutes with two different combinations of AngII and thrombin doses: 10 nM of AngII with 1 U/ml of thrombin (A) or 1 µM of AngII with 0.1 U/ml of thrombin (B), as indicated, and then lysed for SDS-PAGE followed by western blot using the mouse anti-phospho-p44/42 (pERK1/2) (Cell Signaling) (1:2000 dilution). The total ERK1/2 was detected using the primary rabbit polyclonal anti-p44/42 ERK1/2 (1:1000 dilution).
Figure 22: Phosphorylation of ERK1/2 in HT-29 cells. HT-29 were stimulated with AngII (10 nM), thrombin (1 U/ml), or both, for 5 minutes and then lysed for SDS-PAGE followed by western blot using the mouse anti-phospho-p44/42 (pERK1/2) (Cell Signaling) (1:2000 dilution). The total ERK1/2 was detected using the primary rabbit polyclonal anti-p44/42 ERK1/2 (1:1000 dilution).
Chapter 5: Discussion and Conclusion

Crosstalk between AngII and thrombin in physiology and pathophysiology has been well documented previously, but not at the pharmacological level and specifically not at the receptor level, AT1R and PAR1, respectively. This thesis study highlighted for the first time the pharmacological interplay between AngII and thrombin and their respective receptors using various resonance energy transfer assays. The findings of this research work clearly demonstrated the physical and functional interaction between AT1R and PAR1 via forming heterodimers in vitro, revealing the positive allostERIC modulation of thrombin through PAR1 on AT1R by transactivating it and transinhibition of its internalization and endosomal trafficking in a co-activation-dependent manner (Figure 23).

Figure 23: AT1R-PAR1 heterodimerization and its functional consequences.
The Co-IP along with the BRET saturation analysis confirmed the physical interaction between AT1R and PAR1 by forming heterodimers *in vitro* as both are in close proximity as they were immunoprecipitated together. On the other hand, the functional consequences of AT1R-PAR1 heterodimerization were proven by dose response and real-time kinetics analysis. This confirmed the specificity of each ligand towards its receptor and the ability of AT1R and PAR1 monomers to activate Gq, mediating the production of the second messenger IP1, as well as their ability to induce β-arrestin 2 recruitment (Figure 12 A, B).

The combined treatment of AngII and thrombin for cells expressing AT1R-Rluc without or with either Gq or β-arrestin 2 and in PAR1’s absence, mediated a left shift in the dose response (Figure 12 C, E) and induced increase in BRET signal with the real-time kinetics (Figure 15 A, C) as well as the IP1 (Figure 18 C) analysis, demonstrating a positive allosteric modulation effect of thrombin on AT1R. Indeed, this effect of thrombin is not through direct binding to AT1R as demonstrated in thrombin doses (Figure 13 A, C) by which cells expressing AT1R-Rluc without PAR1 were treated with thrombin doses did not show any BRET response. Also, this was confirmed in the first dose response analysis (Figure 12 A, B); Ang II dose response was achieved only with AT1R-Rluc expressing cells and similarly thrombin dose response with PAR1-Rluc expressing cells, as well as in the antagonists analysis; when Irbesartan blocked AT1R signal not thrombin-mediated BRET signal (Figure 17). These results build on existing evidence of the endogenous expression of PARs in HEK293 cells [105], [106]. Interestingly, AT1R-PAR1 heterodimerization with the co-stimulation showed significant marked enhanced dose-dependent Gq signal transduction mediating IP1 production (Figure 12 D, 13 B, 17 A, and 18 E) and β-arrestin 2 recruitment (Figure 12 F, 13 D, 17 B, and 18 E) compared to the monomers.
These data reflect positive allosteric modulation of thrombin activated PAR1 by transactivating it (Figure 12 D, F) (Figure 24), as described in the speculative model (Figure 24 C).

In addition, 1 U/ml thrombin alone for cells expressing AT1R and PAR1 with either Gq or β-arrestin 2, induced an increase in the BRET response between AT1R-Rluc and Gq and in a less extent with β-arrestin 2 (Figure 12 D, F, 13 B, D, 15 B, D, and 17). These data might suggest functional selectivity towards β-arrestin 2 over Gq as the preliminary MAPK/ERK findings showed ERK phosphorylation with 30 minutes stimulation (Figure 20 C). Another plausible explanation is that this might be due to conformation of BRET sensors. However, with the co-stimulation both Gq and β-arrestin 2 showed similar responses.

Moreover, these data of the functional consequences of the heterodimerization occur in a dose-dependent manner as well as in a receptor expression level-dependent manner (Figure 16). AT1R-PAR1 heterodimer was able to couple to Gq in all the used expression levels. However, 10 ng AT1R-Rluc (Figure 16 A) demonstrated less positive modulation compared to the 25 and 50 ng (Figure 16 B, C, respectively). Also, this response was seen in an increasing pattern with the increased PAR1 expression. Thus, reflecting the coupling to Gq even at low expression level as well as the importance of the expression level of receptors for transactivating AT1R by PAR1 and thrombin. PAR1 expressed at 50 ng showed the highest BRET response (340% with 1 U/ml thrombin and 478% with co-treatment) compared to 25 ng PAR1 (294% with 1 U/ml thrombin and 446% with co-treatment) (P-value < 0.0001). This might be linked to the different expression levels in the human body in different organs and under
physiological and pathological circumstances such as overexpression in cancers and cardiovascular diseases [27], [29], [31], [34], [35].

Also, BRET analysis with antagonists confirmed the inhibitory activity of each antagonist of its receptor. Irbesartan completely blocked AT1R whereas SCH797979 partially, but significantly diminished PAR1’s activity (Figure 17). Moreover, Irbesartan induced a decrease in the thrombin-mediated Gq coupling and this was not seen with β-arrestin 2. These data suggest that AT1R activation and conformation is needed for thrombin-mediated Gq coupling to AT1R by transactivation via PAR1. Excitingly, both antagonists with the co-stimulation, significantly diminished Gq coupling and β-arrestin 2 recruitment, confirming the importance of co-activating both ATR1 and PAR1 for mediating the transactivation which requires conformational changes in both receptors. Further studies are required to investigate the effect of combining both blockers simultaneously to look for any change in the partial remaining effect with SCH79797 blocking. This is due to the fact that SCH79797 is a selective noncompetitive blocker acting on the ligand binding site, in addition to the fact the PAR1 is activated in a unique way as described previously. These results provide a new insight into the importance of considering this heterodimerization when treating diseases associated with the two physiological systems and might be mediated by the two receptors, as mostly treatment targets only one GPCR blocker [27], [34], [109], [110]. This also has been recommended in the recent study by Hasan et al., which documented the role of thrombin in atrial endothelial ageing and senescence via AT1R, and by using ARBs the response was attenuated [42]. The combination of thrombin and AngII blockers was suggested in their study treating the observed pro-inflammatory, pro-thrombotic, pro-fibrotic and pro-remodeling responses [42].
Additionally, the downstream signaling of Gq-induced IP1 production is mediated by both monomers, AT1R and PAR1 (Figure 18 A, B). So, it was challenging to find a way to determine if the produced IP level is by AT1R, PAR1, AT1R-PAR1 dimer, or even by other components. In general, all the findings are consistent with the previous results, cells expressing AT1R-Rluc activated Gq signal transduction pathway and mediated IP1 production, and this effect was attenuated by Irbesartan (Figure 18 C). However, a response with thrombin as well was obtained as observed previously, with the possibility of other endogenous PARs contribution (Figure 18 C). Similarly, thrombin treated PAR1 expressing cells mediated IP1 production and lost this response with SCH79797 (Figure 18 D). Interestingly, cells expressing both receptors showed remarkable synergetic increase in IP1 production levels by ~160%, and this response was significantly diminished with each antagonist (Figure 18 E, F). However, the inhibition of one receptor did not fully inhibit Gq-mediated production. This finding suggests IP1 production by the monomers.

Surprisingly, PAR1 greatly influenced AT1R’s regulation through transinhibition of its internalization and endosomal trafficking (Figure 19). Altered internalization and endosomal trafficking has been reported in many heterodimerizations (Table 1).

Finally, these preliminary data suggest activation of MAPK pathway by phosphorylation. Two time points were considered, 5 min for G protein and 30 min for β-arrestin 2–mediated MAPK activation. 1 U/ml thrombin induced strong phosphorylation in cells expressing either PAR1, AT1R-Rluc, or both (Figure 20). The 10 nM AngII induced weak phosphorylation in cells expressing AT1R-Rluc, whereas strong activation was observed in cells co-expressing the two receptors. Moreover, co-stimulation in cells co-expressing AT1R-Rluc and PAR1 demonstrated stronger
phosphorylation with 5 in stimulation, while this effect was diminished with 30 min stimulation. This could be due to the finding in Figure 19, where AT1R’s internalization was inhibited by PAR1 when co-stimulated. However, these data did not bring any evidence that this was mediated by AT1R-PAR1 complex or their functional interaction. Further investigation is required to address this aspect of AT1R and PAR1 function and pharmacology. Therefore, it is believed that these ERK1/2 data are too preliminary and further controls, tools, and investigations are needed to better correlate the findings and BRET data with this important downstream signaling pathway of GPCRs. Thus, this part was not included in the revised manuscript.

Further research is needed to establish the opposite configuration for further understanding of this heterodimerization, some data on this are being analyzed now and are all consistent with each other. But unfortunately, due to the limited time they are not shown in this thesis document. Also, investigation AT1R-PAR1 heterodimerization in vivo and its effects as the heterodimer in vitro cannot reach its site of in vivo action, this will pave the way to consider all the findings in drug discovery as well as in understanding the physiological and pathological interplay between RAS and thrombin.
Figure 24: Speculative model of AT1R-PAR1 interplay and its effect on AT1R activity and regulation revealed by BRET. (A) Single AT1R activation, (B) Single PAR1 activation, and (C) Dual activation of AT1R and PAR1 [111].
References


List of Publications
