Biased signaling and allosteric modulation of the Angiotensin II type 1 receptor

By: Stéphanie Clément

Department of Pharmacology and Therapeutics
McGill University, Montreal

December 15th, 2014

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

Abstract

G protein coupled receptors (GPCR) represent over 30% of drug targets and are involved in nearly all physiological and cellular responses. The angiotensin II (AngII) type I receptor (AT1R) is an important member of this receptor family. Its main endogenous ligand is the hormone angiotensin II (AngII), which regulates blood volume and vascular resistance, through the renin-angiotensin system (RAS). This hormone is involved in hypertension and other cardiovascular diseases. A way to improve today's therapeutic approach is to aim for the activation of the beneficial cellular responses without activating the ones responsible for undesirable effects. This type of response can be achieved using ligands that show functional selectivity, also known as biased signaling. This type of ligand imposes distinct conformations to the receptor, therefore promoting selective downstream effector activation. Moreover, our lab has recently shown that functional selectivity was possible using an allosteric modulator (a ligand binding to any site on a GPCR that is topographically distinct from the endogenous binding site). Our group has shown that a peptide mimic of a sequence derived from the second extracellular loop (ECL2) domains of the prostaglandin F2α (PGF2α) receptor (FP) acted as a biased-allosteric modulator of FP.

We hypothesized that the ECL2 of other GPCRs can be used as putative biased-allosteric modulators. To test this, we used the angiotensin II (AngII) type I receptor (AT1R). We have examined the effects of peptides (SC0023/SC0024) derived from AT1R's ECL2 in vascular smooth muscle cells (VSMC).

This was done using techniques including western blots, Time-Resolved Fluorescence Resonance Energy Transfer (TR-FRET) and [^{3}H]-Thymidine incorporation. We show in VSMCs that the SC0023 peptide decreases angiotensin II-induced ERK1/2 activation and

inositol monophosphate (IP1) production, thus acting as a negative allosteric modulator (NAM) on these signaling pathways.

Conversely, SC0024 has no effect on ERK1/2 while acting as a positive allosteric modulator (PAM) on IP1 production. Interestingly, the SC0023 peptide showed no modulatory effect on proliferation in response to angiotensin II, whereas the SC0024 peptide inhibited almost completely this response, hence acting as a NAM on AngII-mediated proliferation. In addition, structure-function studies underscored the importance of three residues (Phe-His-Tyr) of peptide SC0024 for its NAM effect on proliferation. More importantly, these peptides alone showed no effect on any of the pathways studied, thus only working in the presence of agonist, which is characteristic of most allosteric modulators.

These data imply that these two peptides derived from the ECL of AT1R are allosteric modulators with biased signaling properties. Indeed, SC0023 acts as a NAM of ERK1/2 activation and IP1 production, while SC0024 acts as a PAM of IP1 production and a NAM of proliferation. Ultimately, understanding how the ECL2 is allosterically biasing its receptor will allow us to design new and more efficient therapeutics.

Résumé

Les récepteurs couplées aux protéines G (RCPG) sont ciblées par plus de 30% des médicaments présentement sur le marché. Ces récepteurs participent à bon nombre de réponses physiologiques et cellulaires. Les traitements présentement utilisés pourraient être améliorés en utilisant des ligands qui activent sélectivement les réponses cellulaires responsables des effets bénéfiques, sans activer celles responsables des effets secondaires. Ce model d'activation du récepteur est connu sous le nom de *signalisation biaisée* ou *sélectivité fonctionnelle*. Ceci implique que les RCPG peuvent adopter plusieurs conformations différentes pour activer des voies de signalisation précises. Récemment, la sélectivité fonctionnelle fut accomplie par des ligands allostériques, aussi appelés modulateurs allostériques, qui ont la propriété d'occuper d'autres sites que ceux des ligands orthostériques.

Due au fait que la deuxième boucle extracellulaire des RCPG est importante pour la liaison des ligands orthostériques ainsi que l'activation du récepteur, nous avons émit l'hypothèse que celle-lui puisse agir comme un modulateur allostérique biaisé. Nous avons identifié de nouveau ligands allostériques biaisés, qui sont dérivés d'une région extracellulaire du récepteur de l'angiotensine II. Nous avons testé les effets de deux ligands (SC0023 et SC0024) sur des réponses induites par l'angiotensine II. La production d'inositol phosphate (IP1), l'activation de ERK1/2, ainsi que la prolifération ont été testés en présence de ces ligands. Nous avons montré que SC0023 diminue l'activation de ERK1/2 et la production d'IP1 tout en n'ayant aucun effet significatif sur la prolifération. Au contraire, SC0024 augmente la production d'IP1 et bloque presque complètement la prolifération. De plus, des études de structure-fonction ont souligné l'importance de trois résidus (Phe-His-Tyr) de SC0024 pour son effet inhibiteur de sur

la prolifération induite par l'angiotensine. Finalement, aucun des deux peptides n'ont montré d'effet seul sur la signalisation, ce qui est commun pour la plupart des modulateurs allostériques.

Nos résultats suggèrent que ces peptides sont des modulateurs allostériques capables de biaiser la signalisation induite par l'angiotensine II. En effect, SC0023 diminue l'activation de ERK1/2 et la production d'IP1 tandis que SC0024 augmente la production d'IP1 et diminue la prolifération induite par l'angiotensine. Éventuellement, de tels ligands peuvent aider à optimiser les médicaments déjà existants, en réduisant les effets secondaires des thérapies contre les maladies cardiovasculaires.

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Acknowledgements

First, I would like to thank my supervisor, Dr. Stéphane Laporte, who has supported me throughout the course of my Master's degree. Stéphane, you have not only been a great mentor but I believe you have given me the necessary tools in order to succeed in my future career. Your love of science and research is contagious and makes the lab a very pleasant place to work. I consider myself very lucky to have spent the past few years learning from you and I will always cherish this part of my life.

Second, I would like to thank my advisor, Dr. Paul Clarke and my committee members, Dr. Terry Hebert and Dr. Simon Rousseau. Your support and constructive criticism allowed me to grow within the department and continue improving my project. Specifically, thank you Terry for making the past few years as enjoyable as they were. Your enthusiasm and kind nature had a truly positive impact on my experience as a graduate student.

Also, I owe a special thanks to the members of the Laporte lab. Specifically, thank you Dr. Eugénie Goupil, Étienne Khoury, Ljiljana Nikolajev, Dr. Sylvain Armando, Dr. Yoon Namkung, Dr. Olivier Radresa, Sahil Kumar and Dr. Dany Fillion. Eugénie, you were the greatest mentor I could have hoped for and the time you spent introducing me to the lab and explaining several scientific topics has not gone unnoticed- I will always be thankful for that. Étienne and Ljiljana, the last years spent with the both of you have been memorable. Finally, Sylvain and Yoon, thank you for all the conversations and useful tips that helped me grow and improve within the lab. Both on a professional and a personal level, I am grateful to have met all of you. I am leaving the lab with great memories and great friends.

Next, I would like to thank the members of the PPL, which have all contributed to our great work environment. Specifically, Mary Lapenna, without you the past years would not have been the same since you are the heart and soul of the PPL. I also owe a special thank you to Dr. Louise Larose. Louise, you are amongst the ones that have impacted me the most. You are kind, honest and I truly admire you. I will always remember our intense conversations and the mentorship you provided me with.

Finally, I would like to thank the department of pharmacology. More specifically, thank you Tina Tremblay, Hélène Duplessis and Chantal Grignon, you have been very supportive and helpful throughout this whole process.

Author contributions

Chapter 1:

The introduction was based on a review recently published in Frontiers in Endocrinology (Khoury, Clement et al. 2014). I wrote the initial draft of this review and I wrote the final manuscript with Étienne Khoury and Dr. Stéphane Laporte.

Chapter 2:

Figure 1, figure 4A-B and figure 8A were designed with Dr. Eugenie Goupil and then performed by me. Figure 2B-C was designed and conducted by Étienne Khoury. Ljiljana Nikolajev designed figure 9. I designed and performed the rest of the experiments, often with the help of Dr. Stéphane Laporte.

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Abbreviations

GPCR: G protein-coupled receptor

Ang II: Angiotensin II

AT1R: Angiotensin II type 1 receptor

ECL: Extracellular loops (ECL1-3)

ICL: Intracellular loops (ICL1-3)

MAPK: Mitogen-activated protein kinase

ERK1/2: Extracellular signal-regulated kinases 1 and 2

JNK3: c-Jun N-terminal kinase type 3

GTP: Guanosine triphosphate

GDP: Guanosine diphosphate

AC: Adenylyl cyclase

cAMP: cyclic AMP

GRK: GPCR kinases (GRK1-7)

ACE: Angiotensin-converting enzyme

RAAS: Renin-Angiotensin-Aldosterone System

PLC: Phospholipase C

RTK: Receptor tyrosine kinase

EGF: Epidermal growth factor

EGFR: Epidermal growth factor receptor

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor

NAM: Negative allosteric modulator

PAM: Positive allosteric modulator

PGF2α: Prostaglandin F2α

FP: Prostaglandin F2α receptor

MEM: Minimum Essential Media

DMEM: Dulbecco's Modified Eagle Medium

FBS: Fetal bovine serum

PBS: Phosphate-buffered saline

FRET: Fluorescence resonance energy transfer

HEK 293: Human embryonic kidney cells

VSMC: Vascular smooth muscle cells

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

IP: Immunoprecipitation

PAG: Propargylglycine

Bpa: Benzoylphenylalanine

CHAPTER 1:

General introduction and literature review

1.1 G protein-coupled receptors (GPCRs)

1.1.1: History and classification of GPCRs

G protein-coupled receptors (GPCRs) constitute the largest family of cell-surface receptors and are involved in almost all physiological and hormonal responses. Therefore, it is not surprising that they are amongst the best characterized and most targeted in drug discovery programs (Lagerstrom and Schioth 2008). Structurally, they are characterized by seven transmembrane α-helices, which are connected by three extracellular loops (ECL) and three intracellular loops (ICL).

Over time, there has been several classification systems put forth to sort out this GPCR superfamily, which comprises of more than 800 genes encoding for the different receptor. More recently, GPCRs of the human genome were analyzed using the GRAFS (Glutamate, Rhodopsin, Adhesion, Frizzled/Taste2, Secretin) classification system. They used multiple phylogenic analyses, and were able to classify them into five main groups: glutamate (or class C), rhodopsin (or class A), adhesion, frizzled/taste2, and secretin (or class B) (Fredriksson, Lagerstrom et al. 2003). In this thesis, we will focus on the angiotensin II type I receptor, which is classified as a rhodopsin-like or class A receptor.

1.1.2: Heterotrimeric G proteins

As suggested by their name, G protein-coupled receptors transmit signals from outside the cell through their coupling to heterotrimeric G proteins, also known as guanosine nucleotide-binding proteins, which are divided in an alpha, beta and gamma subunits. The alpha subunit (39-46 kDa), which is the GTP-binding subunit, is subdivided into four main groups, namely

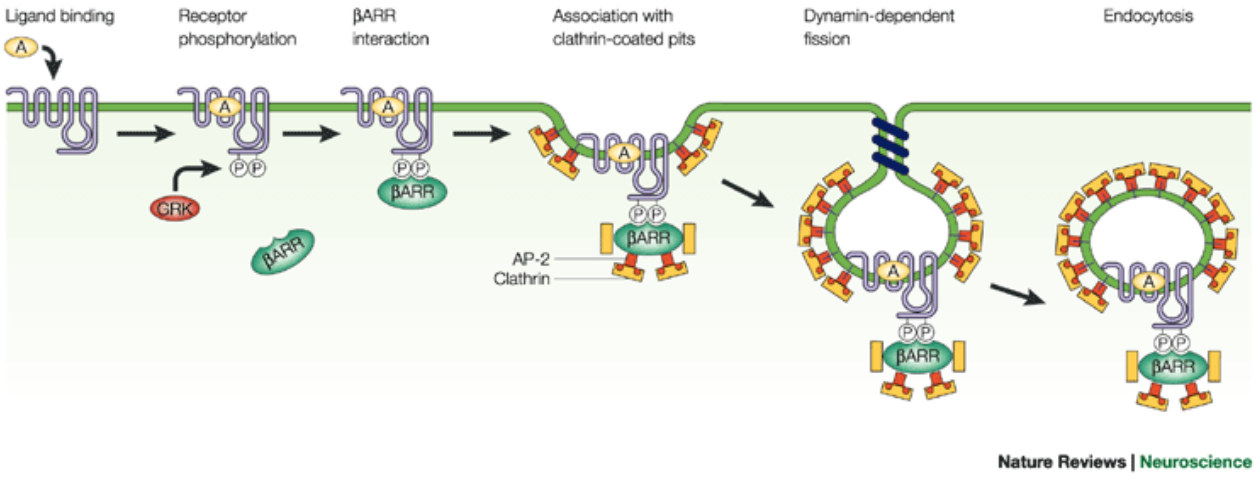
$G\alpha_{s/olf}$, $G\alpha_{i/o/g/z/t}$, $G\alpha_{q/11/14/15/16}$, $G\alpha_{12/13}$ (Hepler and Gilman 1992). In terms of the beta subunit (37 kDa) and gamma subunit (8 kDa), we can count up to seven different Gβ and twelve Gγ genes. A significant part of the GPCR signaling diversity is mainly due to the various possible combinations of Gα and Gβγ subunits that confer differential downstream signals. These proteins function as molecular switches, which are turned on when bound to guanosine triphosphate (GTP) and are turned off when they hydrolyze GTP to guanosine diphosphate (GDP) (Gilman 1987). Upon binding to GTP, the α-subunit presumably dissociate from βγ, which are then able to separately activate different signaling cascades, which will lead to a variety of physiological effects. Amongst the wide array of G protein functions, there is the activation or inhibition of adenylyl cyclase (AC) activity, which is regulated by Gαs and Gαi respectively. They are also directly responsible for the stimulation of phosphoinositide hydrolysis and the regulation of ion channels as well as the activation/inhibition of multiple other downstream effectors. (Gilman 1987).

1.1.3 Receptor desensitization/internalization

There is a well-conserved mechanism by which most GPCRs are desensitized, internalized and then either recycled back to the plasma membrane or degraded. One of the families of proteins that are central to these processes and serves as an adaptor between the desensitization and subsequent internalization are β-arrestins (Fig. 1.1). Once the receptor has been bound by a ligand and the subsequent G protein-dependent signaling cascades have been activated, the receptor has to be desensitized, or in other terms, there needs to be termination of signaling. In order for this to occur, different GPCR kinases (GRKs), phosphorylate the C-terminus of the receptor. There exist seven of these serine/threonine kinases that have been

subdivided into three main subfamilies (Ribas, Penela et al. 2007). The rhodopsin kinase subfamily is comprised of GRK1 and GRK7; the β-adrenergic receptor kinase subfamily includes GRK2 and GRK3 and the GRK4 subfamily consists of the remaining, namely GRK4, GRK5 and GRK6 (Ribas, Penela et al. 2007). Desensitization of the given GPCR will then occur when β-arrestin1 or β-arrestin2 binds to the phosphorylated sites of the receptor. Following receptor desensitization, most GPCRs are internalized into endosomes that will in turn either target the receptor to degradation (downregulation) or the receptor will be recycled back to the plasma membrane. A second important role of the β-arrestin protein family is its involvement in this process of receptor internalization. Indeed, studies have shown that β-arrestins are able to bind to a minimum of two components that are required for clathrin-mediated endocytosis: β2-adaptin, a subunit of the AP-2 complex and clathrin itself (Goodman, Krupnick et al. 1996; Laporte, Oakley et al. 1999). Though this is a relatively common mechanism for endocytosis, conserved amongst many GPCRs, there are clathrin and β-arrestin-independent ways by which some receptors are desensitized and subsequently internalized (Pals-Rylaarsdam, Gurevich et al. 1997; van Koppen and Jakobs 2004; Scarselli and Donaldson 2009).

In addition, it is now common knowledge that β-arrestins are not only important for the processes described above, but are also responsible for activating different signaling pathways such as MAPK activation. In fact, they were shown to activate ERK1/2, p38, as well as c-Jun N-terminal kinase type 3 (JNK3) signaling cascades (Pierce and Lefkowitz 2001; Chavkin, Schattauer et al. 2014). In recent years, their roles as important regulators of cell signaling and various cellular responses are better understood and much attention is being dedicated to their study.



(Pierce and Lefkowitz 2001)

Figure 1.1: β-arrestin-dependent desensitization and internalization of GPCRs

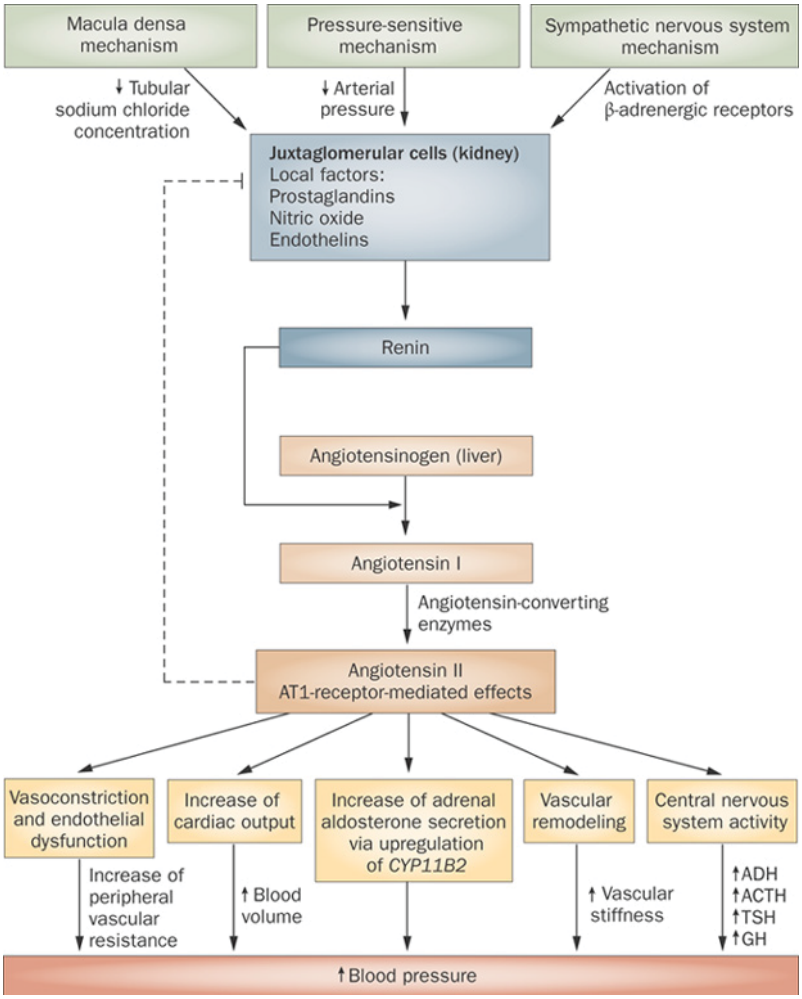
This figure shows a schematic of the events taking place after a ligand binds to its cognate GPCR. It also emphasizes the central role of β-arrestins in initiating receptor desensitization and subsequent internalization via clathrin-coated vesicles (endosomes).

1.2 Angiotensin II

1.2.1 Renin-Angiotensin-Aldosterone System (RAAS)

The Renin-Angiotensin-Aldosterone System (RAAS) is a hormone system that controls blood pressure as well as sodium and water homeostasis (Fig. 1.2) (Tomaschitz, Pilz et al. 2010). The first component of the Renin-Angiotensin-Aldosterone System (RAAS) to be identified, in 1897, was the enzyme renin, which is produced by the kidney (de Gasparo, Catt et al. 2000). Renin is now well characterized as the enzyme that is responsible for the conversion of angiotensinogen, which is released from the liver, to angiotensin I (Weir and Dzau 1999). This first reaction occurs within the circulation and most often in response to an abnormally low arterial blood pressure. Within the lungs, angiotensin I can be further cleaved to angiotensin II, by the angiotensin-converting enzyme (ACE). This octapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) is the active hormone, which is central to the regulation of blood pressure and water balance. More specifically, this hormone regulates blood volume and vascular resistance, making it a key player in cardiovascular homeostasis. This peptide is responsible for various cellular and physiological responses, which in turn regulate cardiovascular and renal functions. Of note, other metabolites of angiotensin II (angiotensin III and IV) possess various biological activity, though they are less potent pressors (de Gasparo, Catt et al. 2000). Angiotensin II was later shown to produce its effect by binding to a cell surface receptor, namely the angiotensin II type I receptor (AT1R), which then activates multiple signaling pathways that are responsible for various cellular and physiological responses. Though this subject will be further discussed in a different section, it is important to note that dysregulation of angiotensin signaling as well as the RAAS has been associated with the development and progression of pathological conditions such as

hypertension, arterial disease, cardiac hypertrophy, heart failure, and diabetic renal disease (Goodfriend, Elliott et al. 1996).



(Tomaschitz, Pilz et al. 2010)

Figure 1.2: The Renin-Angiotensin-Aldosterone System (RAAS)

Several triggers, such as a reduction in blood pressure, can activate this system. First, liver Angiotensinogen can be cleaved and converted into Angiotensin I by the enzyme Renin. Then, Angiotensin I will be cleaved by an Angiotensin Converting enzyme (ACE), which is secreted in the lungs, and be subsequently converted into Angiotensin II. The hormone Angiotensin II will activate a variety of physiological responses, which will in turn increase blood pressure. These responses are mainly known to be mediated by the binding of the hormone to the Angiotensin II type I receptor.

1.2.2 Angiotensin II type I receptor (AT1R)

In 1970, the angiotensin receptor was first identified in the adrenal gland, with an assay using radiolabeled angiotensin (Angiotensin II-I^{125}) (Lin and Goodfriend 1970). This approach utilizes this radioactive probe and was able to show that the hormone was indeed binding to a receptor at the surface of the cells. Only years later, due to the instability and the relatively low abundance of the receptor in different tissues was expression cloning used to clone the AT1 receptors from the bovine adrenal and rat smooth muscle. (de Gasparo, Catt et al. 2000)

There exist different types of the angiotensin receptors (AT1R, AT2R, AT3R, AT4R), which have different cellular distribution and physiological roles. Although most effects of angiotensin II are mediated through its binding to the angiotensin II type 1 receptor (AT1R) (Fig. 1.3), the role of the angiotensin II type 2 receptor (AT2R) has also recently been of interest. Interestingly, the AT2R seems to differ greatly than the AT1R in regards to its cellular and physiological effects, since it was shown to counteract certain AT1R mediated effects. For example, AngII induces antiproliferative effects as well as apoptosis through its binding to the AT2R, while its binding to AT1R is known to induce proliferation and cell survival (de Gasparo, Catt et al. 2000). Though this isoform does indeed mediate different physiological effects, the AT1R has been known as the main target of AngII binding and will be the main focus of the next paragraphs.

The human AT_1R exists as one single large gene that can be alternatively spliced into two different isoforms. Among all cloned mammalian AT1Rs, only mice and rats have two distinct subtypes of the receptor, which could have been due to an event of gene duplication. The rat has genes with lengths of 84kb and 15kb, which correspond to $AT_{1A}R$ and $AT_{1B}R$ respectively. These subtypes are very similar in sequence but mainly differ in their tissue distribution (Burson,

Aguilera et al. 1994). These receptors are crucial and the removal of one or both has had serious negative outcomes. Double knock out (KO) mice that lack both $AT_{1A}R$ and $AT_{1B}R$ were shown to have an abnormal growth rate as well as major impairments in renal structure and function (Oliverio, Kim et al. 1998).

The AT1R is a class A GPCR (Rhodopsin-like receptor), whose structure is characterized by seven transmembrane (TM) domains that are connected by three extracellular loops (ECL) and three intracellular loops (ICL) (Fig. 1.3). Its main endogenous ligand is the peptide hormone angiotensin II. This receptor is expressed in multiple tissues including cardiovascular, neuronal, and endocrine tissues (de Gasparo, Catt et al. 2000). The AT1R has various physiological roles in each of these tissues, a few of which are listed in "Table 1.1". This receptor is known to couple to different G proteins and therefore activate multiple signaling pathways. Though this receptor's coupling to the $G\alpha_{q/11}$ subunit is best characterized, it has also been shown to couple to $G\alpha_{i/o}$ and $G\alpha_{12/13}$ (de Gasparo, Catt et al. 2000).

Among the well-defined signaling pathways downstream from this receptor coupled to $G\alpha_{q/11}$ is the activation of phospholipase C (PLC), which ultimately leads to inositol phosphate (IP) production and calcium release from intracellular stores. This also leads to the activation of protein kinase C, which can activate a cascade of mitogen-activated protein kinases (MAPK), such as ERK1/2. The coupling to $G\alpha_q$ can also lead to the activation of the Rho/ROCK pathway, which is more commonly known to be downstream from $G\alpha_{12/13}$ (Ohtsu, Suzuki et al. 2006). In fact, there is evidence that AT1R can couple to the latter, which can effectively activate the Rho pathway, leading to the reorganization of the cytoskeleton (Seko, Ito et al. 2003; Higuchi, Ohtsu et al. 2007). Interestingly, the Rho/ROCK pathway seems to also be implicated in inflammation, contraction, hypertrophy, migration and vascular remodeling (Ohtsu, Suzuki et al. 2006;

Higuchi, Ohtsu et al. 2007). AT1R has also been shown to couple to $G\alpha_{i/o}$, which leads to the inhibition of adenylyl cyclase (AC) and subsequent decrease in production of cyclic AMP (cAMP) (de Gasparo, Catt et al. 2000).

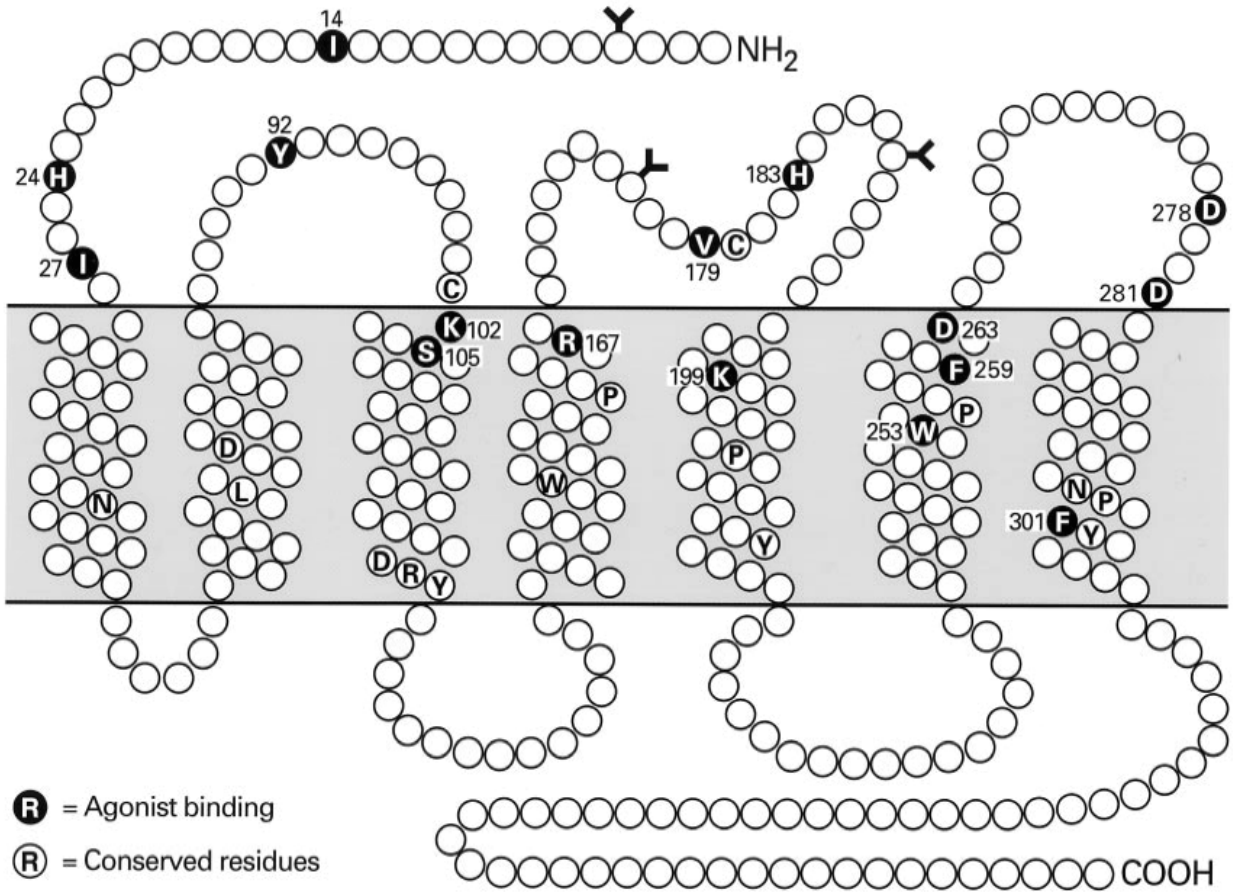
Interestingly, certain effectors such as ERK1/2 can not only be activated through G protein-dependent pathways, but also through G protein-independent pathways such as β-arrestin and transactivation of receptor tyrosine kinases (RTKs) (Shenoy, Drake et al. 2006; Goupil, Wisehart et al. 2012). In fact, in cell types such as vascular smooth muscle cells (VSMC), angiotensin II binding to its receptor can transactivate different growth factor receptors that will then activate various pathways leading to cell proliferation and/or hypertrophy, for instance. Different mechanisms have been proposed in order to explain this phenomenon. First, the activation of AT_1R could lead to clustering and autophosphorylation of receptor tyrosine kinases (RTK). Second, the activation of a tyrosine kinase such as c-src could lead to the phosphorylation of RTKs. And third, in the case of the epidermal growth factor receptor (EGFR), it was shown that heparin binding (HB)-EGF could be generated in response to angiotensin II binding to its receptor. HB-EGF can then activate EGFR and lead to growth responses (Saito and Berk 2001; Saito and Berk 2002). In fact, AT_1R is suspected to not only lead to the transactivation of the EGFR, but also the platelet-derived growth factor receptor (PDGFR) and the insulin-like growth factor 1 receptor (IGF1R) (Ali, Schieffer et al. 1997; de Gasparo, Catt et al. 2000). The transactivation of these RTKs has been proposed to play a role in vascular dysfunction and remodeling (Higuchi, Ohtsu et al. 2007)

	Tissue	Effect	Reference
Angiotensin II type I receptor (AT_1R)	Adrenal gland	Increase in aldosterone secretion	(Laragh, Angers et al. 1960)
	Arteries (Vascular smooth muscle)	Vasoconstriction	(Daemen, Lombardi et al. 1991; de Gasparo, Catt et al. 2000)
		Hypertrophy	
		Proliferation	
	Brain	Thirst	(Saavedra 1992)
		Increase in salt ingestion	
		Facilitates release of catecholamines	
	Heart	Protein synthesis/ growth	(Baker and Aceto 1990; de Gasparo, Catt et al. 2000)
		Hypertrophy	
		Cardiac remodeling	
	Kidney	Decrease in renin release	(Menard, Guyene et al. 1991; Mitchell, Braam et al. 1992)
		Increase in tubular reabsorption of sodium	
		Vasoconstriction	
	Pituitary gland	Release of prolactin, oxytocin and vasopressin	(Saavedra 1992; de Gasparo, Catt et al. 2000)
		Increases the proliferation of mammatrophs	

(Adapted from (Goodfriend, Elliott et al. 1996))

Table 1.1: Angiotensin II (AngII) actions

Angiotensin II, which is responsible for most effects mediated by the AT1R, plays a key role in various tissues. AngII is an important endocrine, paracrine and autocrine hormone, which is central to the normal functioning of the RAAS as well as the pathogenesis of different conditions. Some of its main actions on the adrenal gland, pituitary gland, kidney, heart, arteries and brain are enumerated above.



(de Gasparo, Catt et al. 2000)

Figure 1.3: The Angiotensin II type I receptor (AT1R)

The AT1R is a class A GPCR consisting of seven transmembrane (TM) domains connected by three extracellular loops (ECL) and three intracellular loops (ICL). Here are shown the amino acids that are proven to be important for angiotensin II binding. Most of these residues are situated in the extracellular region (N-terminus, ECL1-2-3), while the remaining are present within the transmembrane (TM) domains.

1.3 Biased and allosteric regulation of GPCRs

1.3.1 Activation models of GPCRs

Over the years, different models have been put forth in order to explain how GPCRs are activated. First, there was the classical two-state model, which assumed that these receptors could exist in only two distinct conformations, namely "on" or "off". Different ligands were thought to shift this equilibrium from one state to the other. Based on this model, the properties of ligands were classified as agonists, antagonists, and inverse agonists. However, multiple studies have shown that this first model was not fully capturing how GPCRs truly function. A new alternative multi-state model was then proposed, where GPCRs can adopt multiple conformations that are responsible for the multiple possible signaling signatures (Fig. 1.4). Consequently, a new class of ligands was described based on this model, which was referred to as biased ligands. These ligands have the ability to bind to the orthosteric binding site of the receptor and differentially affect its coupling to different G proteins. The use of such ligands has the advantage of having the capacity to target the beneficial cellular responses without activating the ones responsible for undesirable effects. This type of signaling is referred to as functional selectivity or biased signaling. Over the recent years, biased ligands have been shown to bias the signaling between different G proteins and/or between G proteins and β-arrestins. However, this type of signaling is not only restricted to G proteins and β-arrestins as it has also been described between other signaling effectors. In addition, there is another class of ligands, namely allosteric ligands or modulators, which also have the ability to bias signaling. Allosteric modulators include ions, ligands, small/large molecules and protein complexes that can bind to any site on a GPCR that is topographically distinct from the endogenous binding site. Moreover, an evolving

concept also suggests that receptor domains, which participate in ligand and/or effector binding, can also be targeted to bias signaling.

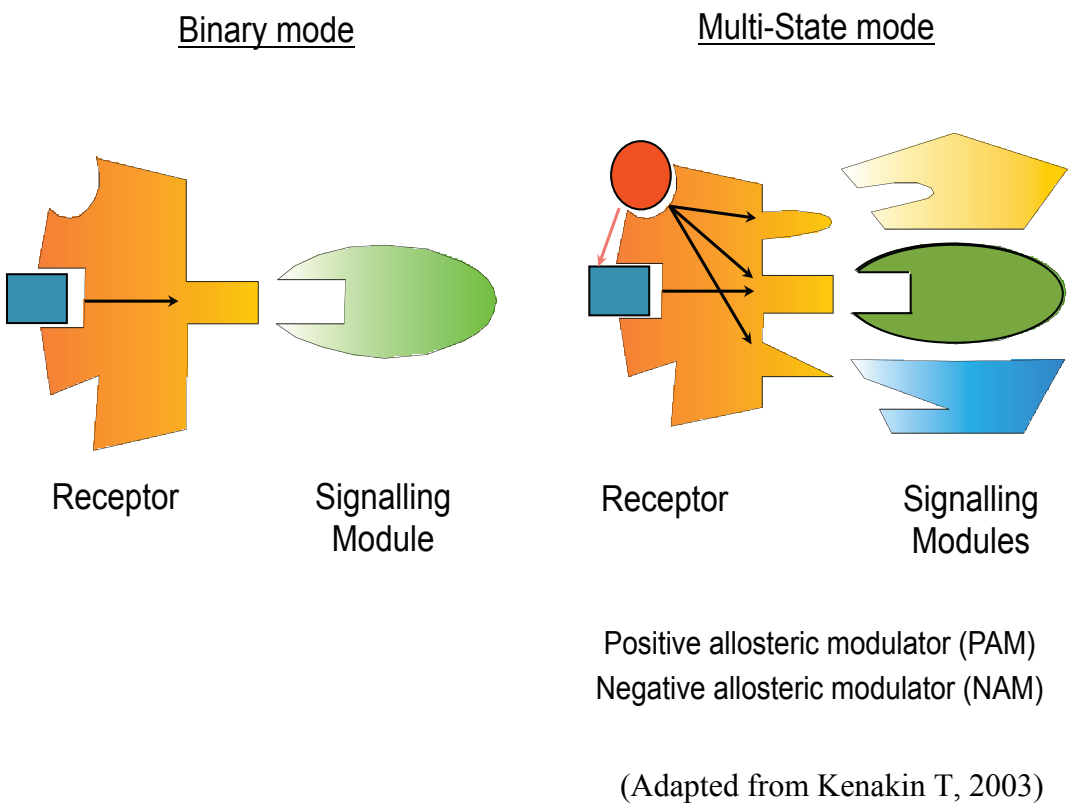


Figure 1.4: GPCR activation models

An early binary model described a very simple representation of GPCR activation. This model assumed that the endogenous orthosteric ligand binds to its receptor, stabilizing the active conformation, which then activates all signaling pathways. As the complexity of GPCR signaling became more apparent, a newer, more appropriate multi-state model was put forth. This model introduces the ability of different ligands can induce various conformational changes that are responsible for the activation of distinct downstream signaling pathways. This selective activation of effectors is referred to as biased signaling, or functional selectivity. In addition, there are ligands that bind topographically distinct sites from the orthorsteric binding site, allosteric modulators, and these can also stabilize different conformations of the receptor and therefore modulate positively or negatively the signaling pathways.

1.3.2 Biased signaling of GPCRs

As previously discussed, it is now well accepted that GPCRs can adopt multiple conformations that will then be responsible for the selective activation of different subsets of signaling pathways. Different orthosteric ligands were shown to have the capacity to bias receptor signaling (Fig. 1.5).

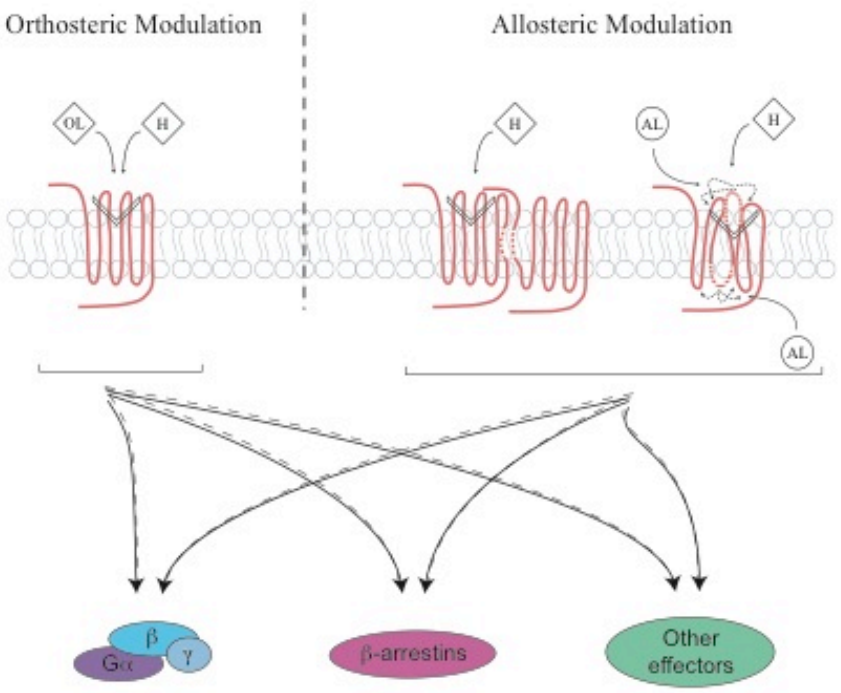
As we know, one category of signaling pathways that have attracted a lot of attention in recent years is β-arrestin-dependent signaling one. As such, β-arrestins, which were proteins thought to only desensitize GPCRs and target them for internalization, have been shown to also participate in receptor signaling. In the recent years, many have focused on orthosteric ligands that have the ability to bias signaling between G proteins and β-arrestin (Violin and Lefkowitz 2007; Rajagopal, Rajagopal et al. 2010; Luttrell 2013). A good example of such ligand is the angiotensin II analog SII [(Sar^1,Ile^5,Ile^8)-AngII], which does not lead to G protein-dependent signaling, while stimulating the activation of β-arrestins-dependent pathways (Holloway, Qian et al. 2002). This peptide promoted receptor internalization but showed a reduced MAPK activation and no G protein-dependent inositol phosphate production. Another example of such ligands was demonstrated for the β2-adrenergic receptor (β2AR). The well-characterized β-blocker Carvedilol, thought to be a classical antagonist, was shown to bias signaling downstream from this receptor by antagonizing the Gαs-mediated signaling, while still stimulating β-arrestin-dependent signaling (Wisler, DeWire et al. 2007). In addition, a study focusing on the dopamine D2 receptor (D2R) showed that three analogs of the second-generation antipsychotic aripiprazole (UNC9975, UNC0006, and UNC9994) are biased towards β-arrestin signaling (Allen, Yost et al. 2011). As it turns out, many of these biased ligands were associated with potential health benefits. For example, a study on the C-C chemokine receptor type 2 (CCR2)

highlighted the effects of GMME1, which selectively engages in G protein signaling, without β-arrestin recruitment (Rafei, Berchiche et al. 2009). This compound was shown to improve the state of autoimmune encephalomyelitis mice, and is thought to define a new class of medication for treating autoimmune diseases (Rafei, Campeau et al. 2009).

There also exists another type of signal bias, which can be seen between different G protein subtypes. An example of this was seen with parathyroid hormone-related peptide (PTHrP) derivatives, which bind the type 1 parathyroid hormone receptor (PTH1R). These ligands, PTHrP(2–36) and Bpa1-PTHrP(1–36), were shown to retain the ability to activate Gαs-mediated signaling, but were unable to activate Gαq-mediated signaling nor recruit β-arrestin to the receptor and subsequent desensitization (Bisello, Chorev et al. 2002). Another interesting study showed that atosiban, an oxytocin derivative acted as an agonist on the Gαi pathway was also acting as an antagonist on the Gαq pathway downstream from the oxytocin receptor (OTR), thus displaying biased signaling properties at the level of G protein subtypes. Of note, the OTR has the ability to couple to both G proteins, leading to opposite cellular responses. In fact, its coupling to Gαi inhibits cell proliferation, whereas its coupling to Gαq stimulates this downstream response (Reversi, Rimoldi et al. 2005).

Moreover, it is interesting to note that biased signaling is not only described between different G proteins and/or between G proteins and β-arrestins, but also between other signaling effectors. For example, AL-8810, which was previously described as an antagonist of the prostaglandin F2α receptor (FP)-mediated Gq activation, showed a bias towards the transactivation of the EGFR, leading to the activation of ERK1/2 (Goupil, Wisehart et al. 2012). Different signaling and functional outcomes can also be achieved when looking at β-arrestin–selective ligands such as SII and DVG [(Asp^{1},Val^{5},Gly^{8})-AngII], which are AngII analogs.

Though they both bind the AT1R and stimulate the recruitment of β-arrestin, DVG stimulates cellular proliferation but not migration, while SII produces the opposite effect on these cellular responses (Zimmerman, Beautrait et al. 2012). The examples provided in this section emphasize the diversity of signaling outcomes that result from the binding of various ligands to their cognate receptors. In addition, it is obvious that the old paradigm supporting the two-state model of GPCR activation may have been simplistic.



(Khoury et al, 2014)

Figure 1.5: Directing signaling by orthosteric and allosteric GPCR ligands, receptor complexes and domains.

Signaling occurs following the binding of endogenous hormones (H) to the orthosteric site on G protein-coupled receptor (GPCR), which involves different domains, mainly the transmembrane and extracellular ones. This leads to the activation of multiple signaling pathways that are balanced between the G proteins, β-arrestins, and/or other signaling effectors. Ligand-directed signaling (i.e.. biased signaling) can occur through the binding of either orthosteric (OL) or allosteric (AL) ligands to the receptor, which changes the balance of signaling between the effectors as compared to a ligand of reference. Formation of receptors complexes, such as dimers as well as reorientation of extra/intracellular domains of receptors can also lead to conformational rearrangements or be targeted for biased signaling.

1.3.3 Allosteric modulation of GPCRs

Another relatively new concept has provided us with an additional level of complexity and this is what we refer to as allosteric modulation. Allosterism was first described with the hemoglobin, where the binding of oxygen to a specific site increases the affinity of other oxygen molecules to the remaining unoccupied sites (Monod, Wyman et al. 1965). Over the years, this concept has proven to be widely spread for various types of proteins, and more recently for transmembrane receptors, such as GPCRs. Interestingly, numerous endogenous allosteric modulators have been identified, and shown to play crucial roles in keeping diverse biological functions mediated by this class of receptors. To date, the best characterized GPCR allosteric modulator is the G protein itself, which binds receptors and stabilizes their active conformation. Moreover, ions, such as Zn^{2+}, Na^{+}, and Ca^{2+} are other examples of endogenous molecules that have been shown to allosterically modulate GPCRs (May, Leach et al. 2007). In addition, other types of endogenous modulators, such as the small tripeptide PLG (Pro-Leu-Gly), also known as the melanocyte-stimulating hormone release inhibiting factor (MIF-1) has also been shown to act as an allosteric modulator on the D_2 and the D_4 dopamine receptors. PLG increases the affinity of dopamine for its receptors and the agonist-mediated inhibition of adenylyl cyclase (AC) (Mishra, Makman et al. 1999). Similarly to orthosteric ligands that have the capacity to bias GPCR signaling, allosteric ligands, which bind to topographically distinct sites from the endogenous ligands, can direct receptor signaling (Fig. 1.5). They can either exert positive, negative or neutral effects on receptor signaling. Specifically, allosteric ligands can influence the binding of the orthosteric hormone to its receptor, which can be independent from its impact on the signaling transduction promoted by the orthosteric ligand. Based on their effects on receptor signaling, these ligands have been divided into two main categories: positive allosteric

modulators (PAMs) and negative allosteric modulators (NAMs). Many advantages are known to be associated with the use of allosteric ligands in terms of fine tuning GPCR responses (Goupil, Laporte et al. 2012; Wootten, Christopoulos et al. 2013). First, because allosteric ligands bind sites on GPCRs that are more diverse in nature than the orthosteric ones, greater selectivity can be achieved with such ligands. Indeed, targeting specific receptors belonging to the same family subtype has been often challenging because of their highly conserved sequences and structures within their orthosteric binding sites. For instance, class C GPCRs, group II metabotropic glutamate receptors (mGluRs), including mGluR2 and mGluR3, were selectively targeted by a NAM, which only inhibited the glutamate-induced response of group II receptors without exerting any effect on groups I and III mGluRs (Hemstapat, Da Costa et al. 2007). Second, the effect of allosteric modulators on receptors is also saturable, since they are not competing with endogenous ligands. In other words, when all allosteric sites are occupied on receptors, no more effects are achieved. Third, most allosteric modulators are known to exert their function only in the presence of the endogenous ligand. Indeed, the allosteric ligand will modulate the receptors' conformation and signaling only when the endogenous hormone occupies its orthosteric site. However, some allosteric ligands have also been described to act as allosteric agonists and promote functional effects in the absence of orthosteric ligands and those are known as ago-allosteric modulators. Such ligands have also been referred to as "super-agonists", because they can act synergistically with the natural ligand. An example of ago-allosteric ligands is the phenylacetamide 1 and 2, which act directly on the free fatty acid receptor 2 (FFA2) (Lee, Schwandner et al. 2008).

Another property of allosteric ligands is also their ability to bias receptor signaling, a phenomenon that was described in the previous section. Such allosteric modulation has been

mainly studied in class A and C GPCRs. For example, a recent study characterized a new compound, PDC113.824, acting as an allosteric modulator on the class A GPCR, the prostaglandin F2α (FP) receptor. This compound was shown to act as a PAM on the agonist-mediated Gαq signaling pathway, while at the same time acting as a NAM on the Gα12 signaling cascade (Goupil, Tassy et al. 2010). Interestingly, this compound was able to significantly delay preterm labor in a mouse model. Another example of G protein-dependent biased signaling is revealed by an autoantibody, which targets a class C GPCR, the calcium sensing receptor (CaSR), which was identified in a patient with acquired hypocalciuric hypercalcemia (AHH). This particular antibody was shown to allosterically modulate the calcium-mediated potentiation of Gαq signaling, while inhibiting the Gαi response (Makita, Sato et al. 2007).

Interestingly, allosteric biased modulation of GPCRs is not only limited to G proteins vs. G proteins signaling but can also occur between G proteins and other signaling effectors, such as β-arrestins. For instance, the ATI-2341 compound acting on the C-X-C chemokine receptor type 4 (CXCR4) was recently shown to display differential ago-allosteric properties between G protein and β-arrestins signaling (Quoyer, Janz et al. 2013). ATI-2341 preferentially promoted the coupling of CXCR4 to Gαi- over Gα13-mediated signaling, but contrarily to the endogenous ligand, did not induce β-arrestin recruitment to the receptor. On the other hand, a study on the cannabinoid receptor 1 (CB1) showed that the ORG27569 compound acted as an NAM on the agonist-mediated Gαi signaling and a PAM on β-arrestin-dependent internalization of CB1; and acted as an ago-allosteric modulator on both receptor internalization and β-arrestin-dependent MAPK signaling (Ahn, Mahmoud et al. 2012; Ahn, Mahmoud et al. 2013). These examples highlight the diversity of GPCR allosteric modulators on both receptors and their ensuing

signaling (e.g. PAM and NAM effects). However, defining the mechanism by which these differentially direct downstream effectors and responses still remains empirical for most receptors.

1.3.4 Targeting GPCR domains

Because different domains of GPCRs (e.g. intracellular (ICL) and extracellular (ECL) loops and transmembrane domains (TM)) are known to participate in ligand and/or effector recognition and receptor dimerization, recent attention has also been drawn to understand the role of these domains in receptor conformation and signaling. It is well recognized that GPCRs can form oligomers within which conformational rearrangements of the receptors can impact signaling (Fig. 1.5) (Percherancier, Berchiche et al. 2005; Decaillot, Kazmi et al. 2011). An example is the heterodimerization of the class A GPCRs chemokine receptors CXCR4 and CXCR7 (Decaillot, Kazmi et al. 2011). This study showed that, when receptors are both in complex, agonist-mediated activation of Gαi is impaired, whereas β-arrestin is constitutively recruited to the dimer. This CXCR4/CXCR7 complex also led to a potentiation of different pathways downstream from β-arrestin, such as ERK1/2 and p38 activation, which ultimately resulted in an increased cell migration. The change from the G protein-dependent signaling normally induced by CXCR4 to a bias towards β-arrestin-dependent signaling is consequent of the conformational changes induced by its dimerization with CXCR7. In addition, a study used a peptide consisting of residues 276–296 of the TM6 of the β2-adrenergic receptor (β_2AR) to prevent homodimerization, and was able to show that the agonist-mediated cAMP production could be inhibited by this peptide. These results suggest that domains of GPCRs could be targeted to regulate receptors signaling in an allosteric fashion (Hebert, Moffett et al. 1996). In

addition, another study has also demonstrated that peptides derived from TM of CXCR4 and CCR5 could be used as specific receptor antagonists (Tarasova, Rice et al. 1999). Despite that transmembrane domains are involved in ligand binding and receptor activation, targeting these regions with peptides has proven to be difficult due to their hydrophobic nature.

On another hand, receptor intracellular loops (ICLs) are critical for GPCR signaling and can also be targeted to modulate receptor's responses (Table 1.2). For instance, studies using mimics of ICL3 from many different GPCRs, such as the adrenergic receptors α_{1B} and α_{2A} (α_{1B}AR and α_{2A}AR), as well as the muscarinic acetylcholine receptors M_1 and M_2 (M_1AChR and M_2AChR), have revealed the importance of this domain in G protein coupling/activation (Hawes, Luttrell et al. 1994). These peptides, which are derived from regions of receptors' loops were shown to disrupt both Gαq and Gαi coupling to their cognate receptors and to affect downstream signaling. Similar effects were also reported for the angiotensin II type 2 receptor (AT_2R) (Hayashida, Horiuchi et al. 1996). Moreover, because β-arrestins also bind ICLs, peptides derived from the ICL3 and ICL1 of receptors were also shown to block GPCR desensitization (Krupnick, Gurevich et al. 1994). For example, a synthetic peptide corresponding to the sequence of the full length of ICL3 of the luteinizing hormone/choriogonadotropin receptor (LH/CGR) was shown to reverse the agonist-mediated desensitization of adenylyl cyclase activity when incubated with membranes expressing LH/CGR by preventing the interaction between β-arrestin and the receptor (Mukherjee, Palczewski et al. 1999). Not only was targeting receptor's ICL shown to affect G protein and β-arrestin binding, but it was also demonstrated to bias GPCR signaling between these different pathways. Indeed, the ago-allosteric ATI-2341 compound, which belongs to the pepducin family and that is a short lipidated peptide of the ICL1 of the CXCR4, was shown to promote biased signaling between G proteins and β-arrestins

(Quoyer, Janz et al. 2013). Consistent with the idea that intracellular domains of GPCRs can be targeted to allosterically bias receptor signaling, a recent study on the β_2AR, using different antibodies directed against its intracellular domains, showed that the recruitment of β-arrestin to the receptor and the activation of G proteins were differentially affected (Staus, Wingler et al. 2014). Targeting GPCR's ICLs with peptide mimics has proven efficient for regulating receptor signaling. However, such approach suffers from the impediment of having to modify the peptides (e.g. lipidation etc.) for their cellular delivery. On the other hand, the modification of peptides with lipids would favor reaching higher plasma concentrations of peptide, which would presumably increase their activity as compared to a gene delivery approach.

The extracellular loops (ECLs) of GPCRs are also important in ligand binding and receptor activation (Table 1.2). Using site-directed mutagenesis, residues in the ECL2 (Phe(189), Trp(206), Phe(209), and Tyr(218)) of the V1α vasopressin receptor, which are highly conserved amongst GPCRs, were shown to be critical for receptor activation (Conner, Hawtin et al. 2007). Another study focusing on the parathyroid hormone 1 receptor (PTHR) used a similar approach and identified residues in the ECL3 (Trp-437 and Gln-440), which were important for PTH (1-34) binding (Lee, Luck et al. 1995). Mutagenesis studies of the adenosine A2B receptor have also implicated residues of the ECL1 in receptor activation (Peeters, van Westen et al. 2011). The ECL2 was also shown to be important for agonist binding and the activation of the M3 muscarinic acetylcholine receptor (M_3AChR) (Scarselli, Li et al. 2007), the C-C chemokine receptor type 5 (CCR5) (Samson, LaRosa et al. 1997), the dopamine D2 receptor (D2R) (Shi and Javitch 2004) and the complement factor 5α receptor (C5αR) (Klco, Wiegand et al. 2005). Other studies have also explored the contribution of ECL2 in stabilizing receptor conformations. For example, structural studies using NMR spectroscopy reveal that the ECL2 forms a cap that

stabilizes the inactive conformation of the receptor and changes its orientation upon receptor activation (Ahuja, Hornak et al. 2009).

The ECL domains of GPCR can also be targeted to allosterically modulate signaling. For instance, a recent study screened potential small molecule for their action on the relaxin/insulin-like family peptide receptor 1 (RXFP1), with the goal of finding agonists. They identified compound 8, which displayed agonistic effects on cellular responses such as cAMP production and cellular impedance. Interestingly, using different RXFP1 constructs and mutagenesis, they showed that the ECL3 of the receptor was required for this effect, suggesting that the compound interacts with an allosteric site within this extracellular loop (Xiao, Huang et al. 2013). Conversely, another study showed that the agonist effects of the allosteric modulators (phenylacetamide 1 and 2) on the FFA2 receptor were lost when its ECL2 was replaced with the one of the FFA3 subtype receptor (Smith, Ward et al. 2011). Moreover, antibodies against the ECL2 have been shown to modulate receptor activity. In the case of the somatostatin receptors (SSTR), antibodies directed against their ECL2 have agonistic-like properties, while having no effect on agonist binding to receptors. SSTR2, SSTR3 and SSTR5 selective antibodies were also shown to diminish cAMP production and decrease serotonin secretion leading to a suppressed proliferation of neuroendocrine tumor cells (Leu and Nandi 2010). Moreover, specific antibodies against the ECL2 of the CCR5 were shown to block HIV entry in cells (Blanpain, Vanderwinden et al. 2002). Similarly, a peptide mimic of the C-terminal portion of CCR5 was shown to act as a NAM for HIV-1 entry (Dogo-Isonagie, Lam et al. 2012). Not surprisingly, because ECLs can adopt different conformations in GPCR, either at basal state or upon receptor activation (Ahuja, Hornak et al. 2009; Wang, Wu et al. 2013), it can also be targeted to allosterically bias receptor signaling. For instance, a study on the vasopressin V2 receptor (V2R) using peptides

corresponding to a decapeptide of ECL1 (PPLLARAELA) or an octapeptide of ECL2's C-terminus (ALCRAVKY) showed physiological functional selectivity on different vasopressin-mediated responses in a non-competitive manner (Rihakova, Quiniou et al. 2009). Moreover, a peptide derived from a sequence overlapping the N-terminus of ECL2 and the TM4 of the prostaglandin F2α (FP) receptor, known as THG113 (ILGHRDYK), was able to block PGF2α-mediated contraction of the myometrium in a non-competitive manner (Peri, Quiniou et al. 2002). Supportive for the allosteric regulation of the FP receptor signaling, is the finding that a peptide-mimic of THG113, the PDC113.824 compound, was also able to induce functional selectivity on G protein signaling mediated by PGF2α (Goupil, Tassy et al. 2010). Also consistent with the idea that the ECL2 can be targeted to direct receptor signaling is the use of an allosteric modulator of the M2 and M4 muscarinic acetylcholine receptors, LY2033298 compound, that was shown to bias downstream signaling (Valant, Felder et al. 2012). Interestingly, a congener of the LY2033298, the LY2119620 compound was later shown, through co-crystallographic studies, to interact with residues of the ECL2, and to allosterically alter M2 receptor active conformation (Kruse, Ring et al. 2013). The GPCRs' ECLs represent promising targets for allostecically modulating receptors, as they are presumably more accessible than ICLs. In particular, ECL2 is an good target, because of its diversity amongst GPCRs and because it is involved in the binding of orthosteric ligands and in the signaling of many receptors. However, much more needs to be understood about how ECLs regulate ligand binding, receptor conformation and signaling, and how peptides derived from these regions affect these function.

Domains	Receptors	Functions	References
ICL	C-X-C chemokine receptor type 4	A pepducin derived from ICL1 acts as allosteric agonist	(Quoyer, Janz et al. 2013)
	β2 adrenergic receptor	Intrabodies targeting ICLs act as allosteric ligands	(Staus, Wingler et al. 2014)
	C-C chemokine receptor type 3	Different residues of ICLs are important for agonist-induced cellular responses (orthosteric)	(Auger, Pease et al. 2002)
	Dopamine D2 and D3 Receptors (D2R-D3R)	Certain residues in ICL2 are important for the agonist-induced translocation of arrestin3 (orthosteric)	(Lan, Teeter et al. 2009)
	α2-adrenergic receptor (α2AR)	Certain residues in ICL3 are important for agonist-induced signaling (orthosteric)	(Small, Forbes et al. 2000)
	Rhodopsin receptor	Mimics of the ICL3 and ICL1 allosterically blocked arrestin binding	(Krupnick, Gurevich et al. 1994)
	Luteinizing hormone/ choriogonadotropin receptor (LHCGR)	Mimic of the ICL3 allosterically blocked arrestin-dependent desensitization	(Mukherjee, Palczewski et al. 1999)
ECL	V1α vasopressin receptor (V1αR)	Residues of ECL2 are important for agonist binding and receptor activation (orthosteric)	(Conner, Hawtin et al. 2007)
	V2 vasopressin receptor (V2R)	ECL1-2 mimics act as bias, allosteric ligands	(Rihakova, Quiniou et al. 2009)
	M3 muscarinic acetylcholine receptor (m3AChR)	Residues of ECL2 are important for agonist-mediated signaling (orthosteric)	(Scarselli, Li et al. 2007)
	Somatostatin receptor (SSTR)	Anti-ECL2 antibodies act as selective allosteric agonists	(Leu and Nandi 2010)
	Prostaglandin F2α receptor (FP)	ECL2 mimic acts as an allosteric modulator	(Peri, Quiniou et al. 2002; Goupil, Tassy et al. 2010)
	C-C chemokine receptor type 5 (CCR5)	Anti-ECL2 antibodies allosterically block HIV entry	(Blanpain, Vanderwinden et al. 2002)

	C-C chemokine receptor type 5 (CCR5)	ECL2 mimic, acting as an allosteric modulator, blocks HIV entry	(Dogo-Isonagie, Lam et al. 2012; Thathiah, Horre et al. 2013)
	Parathyroid hormone 1 receptor (PTH1R)	Different residues of ECL3 are important for PTH (1-34) binding (orthosteric)	(Lee, Luck et al. 1995)
	Adenosine A2B receptor (A2BR)	Different residues of ECL1 are important for agonist-mediated receptor activation (orthosteric)	(Peeters, van Westen et al. 2011)

Table 1.2: Role of GPCRs intracellular (ICL) and extracellular (ECL) domains in receptors function

This list includes examples of studies that have outlined the roles of different GPCR domains. Some of these pinpoint different residues that, when substituted, will alter receptor function. Others describe antibodies that target different domains and affect signaling and finally, a few of these provide examples of domain mimics that affect signaling and subsequent cellular responses.

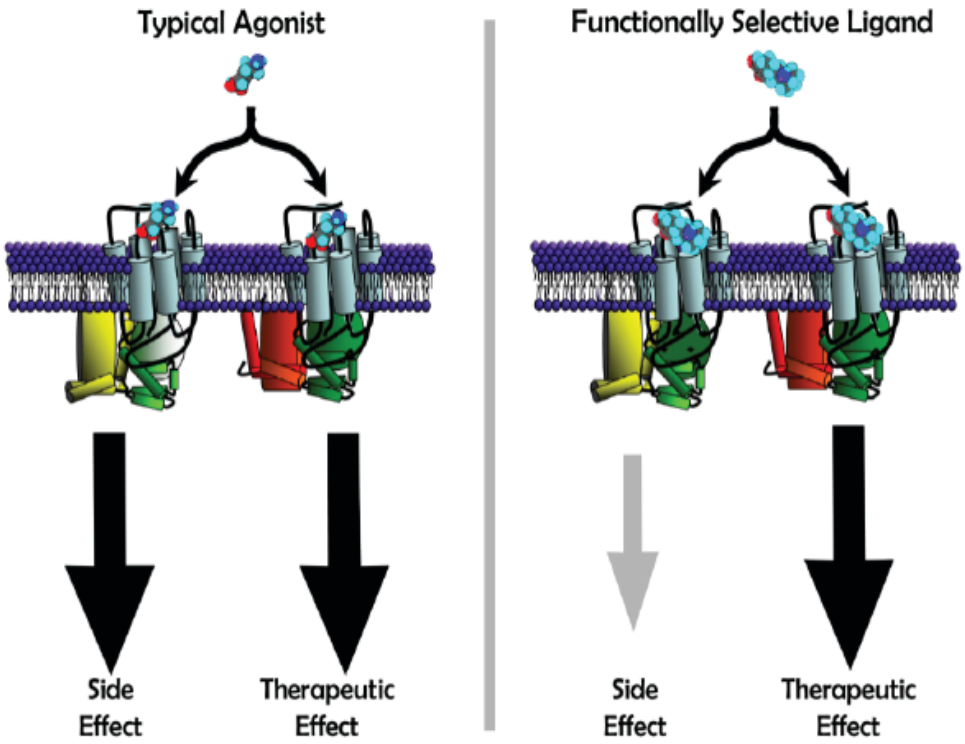
1.3.5 Therapeutic potentials of biased and allosteric ligands

Recently, great attention has been devoted to functional selectivity as a new paradigm applicable for the development of better therapeutic drugs with potentially fewer off-target and/or side effects (Fig. 1.6). Functionally selective ligands have the potential to discriminate between the signaling pathways responsible for the therapeutic effect and those that lead to unwanted effects.

An example is highlighted with the use of the biased agonist pilocarpine, which selectively acts on the M1 muscarinic acetylcholine receptor and shows positive therapeutic effects in different Alzheimer's disease models. Specifically, pilocarpine biased Gαq-mediated phospholipase C activation over the Gαs-mediated adenylyl cyclase stimulation, whereas the non-selective muscarinic agonist carbachol equally stimulated responses mediated by Gαs and Gαq (Fisher, Heldman et al. 1993; Gurwitz, Haring et al. 1994). Moreover, biasing β-arrestin-dependent signaling has also been shown to be potentially beneficial in heart diseases. For example, TRV120027 [Sar^1, Ile^5, D-Ala^8-OH]-AngII), a peptide antagonist of the AT1R-dependent Gαq pathway was recently shown to selectively induce β-arrestin signaling. TRV120027 increases cardiomyocyte contractility *in vitro,* and cardiac performance both in rats and dogs (Violin, DeWire et al. 2010; Boerrigter, Soergel et al. 2012), suggesting that this orthosteric biased ligand could be beneficial in acute heart failure treatment (Violin, DeWire et al. 2010). Another example is the biased ligand TRV130, which acts on the μ-opioid receptor and induces cAMP inhibition through a Gαi-dependent mechanism without inducing either β-arrestin recruitment or receptor internalization. This biased agonist, which has similar potency and efficacy on Gαi signaling as morphine, showed higher analgesic efficacy, lower respiratory

suppression and less gastrointestinal dysfunction when compared to morphine (DeWire, Yamashita et al. 2013).

Because allosteric ligands can bias GPCR signaling, such modulators also represent interesting opportunities for drug discovery (Table 1.3). Moreover, greater subtype selectivity amongst receptors can be achieved using allosteric ligands, which may also improve therapeutic benefits. For instance, the ADX10059 compound, a selective NAM for the mGluR5, was shown to improve symptoms in patients suffering from gastro-esophageal reflux disease (GERD) (Zerbib, Keywood et al. 2010; Zerbib, Bruley des Varannes et al. 2011). On the other hand, Reparixin (formerly Repertaxin), which acts as a NAM on both chemokine receptors, CXCR1 and CXCR2, shows promising therapeutic effects on the prevention of delayed graft dysfunction after kidney transplantation and early stages of breast cancer (Bertini, Allegretti et al. 2004; Ginestier, Liu et al. 2010). Research efforts focusing on targeting the ECL2 of GPCRs to allosterically modulate signaling, have also led to the development of new drugs. Maraviroc, a peptide that was originally developed from a mimic of the ECL2 of CCR5 (discussed in previous section), decreases the viral load in HIV-1 patients (Fatkenheuer, Pozniak et al. 2005). Because of the putative role of CCR5 in alloreactivity, Maraviroc is also in phase II clinical trial for acute graft-versus-host disease (GVHD). Moreover, the ECL2 mimic of the FP receptor, PDC31 (derived from the THG113 peptide), which was shown to inhibit preterm labor in different animal models, is now being evaluated in a clinical phase II trial for primary dysmenorrhea (Peri, Quiniou et al. 2002; Olson and Ammann 2007). Other examples of allosteric and biased drugs are currently under pre-clinical investigation or in clinical trials (Table 1.3).



(Mailman and Murthy, 2010)

Figure 1.6: Functional selectivity for better therapeutics

Biased ligands have the ability to selectively target the signaling pathways responsible for the therapeutic effect, while inhibiting the side effects. This type of signaling cannot be achieved using the typical agonists that activate all signaling pathways downstream from a receptor.

Receptors	Drugs	Indications	References
Calcium-sensing receptor (CaSR)	Cinacalcet (Marketed)	Hyperparathyroidism	(Goodman, Hladik et al. 2002)
C-C chemokine receptor type 5 (CCR5)	Maraviroc (Marketed)	AIDS/HIV	(Fatkenheuer, Pozniak et al. 2005)
C-X-C chemokine receptor type 1/2 (CXCR1/2)	Reparixin	Reperfusion injury in lung and kidney transplantation	(Bertini, Allegretti et al. 2004; Zarbock, Allegretti et al. 2008)
Prostaglandin F receptor (FP)	PDC31 (THG113.3)	Preterm labor* and primary dysmenorrheal†	(Olson and Ammann 2007)* (http://clinicaltrials.gov/show/NCT01250587)†
Metabotropic glutamate receptor 2 (mGluR2)	ADX71149	Schizophrenia	(Hashimoto, Malchow et al. 2013)
Metabotropic glutamate receptor 2/3 (mGluR2/3)	AZD8529	Schizophrenia	(http://clinicaltrialsfeeds.org/clinical-trials/show/NCT00985933)
Metabotropic glutamate receptor 5 (mGluR5)	AFQ056	Parkinson's disease levodopa-induced dyskinesia and fragile X syndrome	(Berg, Godau et al. 2011; Jacquemont, Curie et al. 2011)
	Dipraglurant (ADX48621)	Parkinson's disease levodopa-induced dyskinesia and dystonia	(Stocchi F. 2011) (http://www.addextherapeutics.com/rd/pipeline/dipra-ir/)
	ADX10059	Gastro-oesophageal reflux	(Zerbib, Keywood et al. 2010)
	RO4917523	Depression and Fragile X	(http://clinicaltrials.gov/ct2/show/study/NCT01517698)
	Fenobam	Fragile X	(Berry-Kravis, Hessl et al. 2009)
	STX107	Fragile X and autism	(http://clinicaltrials.gov/show/NCT01325740)

Table 1.3: Potential therapeutic usage of allosteric and biased GPCR signaling compounds/drugs

This table contains a list of allosteric ligands that, in some cases, display biased signaling properties. These particular compounds/drugs have either already been marketed for the treatment of a specific disease, or are currently under investigation in clinical trials.

1.4 Rationale and objectives of the study

Much effort has been dedicated over the past years in understanding biased signaling and allosteric modulation of G protein-coupled receptors (GPCRs). The types of ligands that display these types of signaling properties offer many theoretical advantages over the classical orthosteric ligands. Though there is great interest in developing such ligands, there is little known on how to go about designing these. One way to rationalize the design of new allosteric modulators is to look at regions that are known to be important for ligand binding and/or regulating the conformation of receptors. One such region that has been described as crucial for these functions is the second extracellular loop (ECL2) of GPCRs, which shows very little sequence homology between different GPCRs. Different residues of this region have been identified to be important for stabilizing the active conformation of the receptor while others were shown to be required for agonist binding and receptor activation (Conner, Hawtin et al. 2007; Scarselli, Li et al. 2007). This region has also been targeted by antibodies, which have a great impact on the signaling outcomes of those particular receptors (Blanpain, Vanderwinden et al. 2002; Leu and Nandi 2010). Finally, another approach that has proven to be very successful, involves designing mimics of the ECL2. For receptors such as the prostaglandin F2α (FP) receptor and the C-C chemokine receptor type 5 (CCR5), these mimics were shown to possess biased, allosteric properties and/or great therapeutic potential (Peri, Quiniou et al. 2002; Fatkenheuer, Pozniak et al. 2005; Goupil, Tassy et al. 2010; Dogo-Isonagie, Lam et al. 2012).

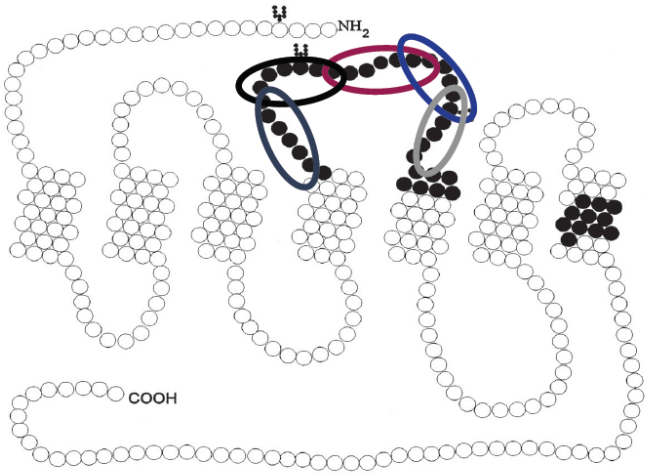
These studies suggest that the ECL2 of GPCRs both can bind ligands and contribute in maintaining a functional receptor conformation. Therefore, we can rationalize that the ECL2 is able to modulate GPCR signaling. My receptor of interest for my thesis work is the angiotensin

II type I receptor (AT1R), which is involved in regulating blood volume and vascular resistance, through the renin-angiotensin-aldosterone system (RAAS). Surprisingly, no allosteric modulators of this receptor have been designed up to date. The development of such ligands could offer alternatives to the commonly used treatments for cardiovascular diseases such as hypertension, acute heart failure and chronic heart failure. In certain cases, the first-line treatments are associated to side effects such as prolonged hypotension and reduced cardiac output (Smith, Ferguson et al. 2008). Based on the recent acquired knowledge on the importance of the ECL2, we hypothesized that this particular loop of the AT1R can be used as a putative allosteric modulator (Fig. 1.7). To test this, we synthesized five different octa- and nonapeptides that span the whole ECL2 of the AT1R. The main objectives of this project are the following:

1) Study the effect of these peptides on AngII-induced signaling and downstream cellular responses. Thus, we studied pathways such as ERK1/2 activation, IP1 production, calcium mobilization, which are know to be downstream of the Gq pathway. We also looked at more complex cellular responses like DNA synthesis (proliferation) and protein synthesis.

2) Study the structure/function relationship of these peptides, in order to understand how they are influencing AngII-dependent signaling. This part of our work will eventually allow us to identify the specific targets of the peptides and map the exact location of their binding sites. This is a more long-term goal, which will require the use of complex tools and assays.

A



Adapted from (Laporte, Boucard et al. 1999)

B

SC0021: I I H R N V Y F I
SC0022: E N T N I T V C A
SC0023: **F H Y E** S Q N S T
SC0024: T V C A **F H Y E**
SC0025: S Q N S T L P I

Figure 1.7: Five peptides derived from the second extracellular loop (ECL2) of the AT1R

A: Here is a representation of the angiotensin II type I receptor (AT1R) where the dark circles depict the ligand binding sites of AngII. The approximate positions of the five peptides that were derived from the ECL2 are circled. B: The exact sequences are provided, focusing on SC0023 and SC0024, the peptides of interest. In bold is the overlapping amino acid sequence of the aforementioned peptides.

CHAPTER 2:

Materials and methods

2.1 Materials

^{3}H-thymidine, ^{3}H-leucine and the ECL reagents were bought from Perkin Elmer (Waltham, MA). Angiotensin II was from Sigma-Aldrich (St Louis, MO). PGF2α was from Cayman Chemicals (Ann Arbor, MI). The SC00xx peptides were bought from Pepnome Inc. (Hong Kong, China). The Phospho-ERK1/2 (T202/Y204) mouse monoclonal antibody and total ERK1/2 rabbit polyclonal antibody were from Cell Signaling (Danvers, MA). Anti-mouse and anti-rabbit HRP-conjugated IgG were from Sigma-Aldrich (St. Louis, MO). Blotting paper, ECL membranes and films were bought from VWR. MEM, DMEM, Fetal bovine serum (FBS), L-glutamine and gentamicin were from were from Gibco, Life Technologies (Carlsbad, CA). G418 was bought from InvivoGen (San Diego, CA). Paraformaldehyde was from Thermo Scientific (Rockford, IL). Epidermal growth factor (EGF) was from Fitzgerald Industries International (Acton, MA). Bovine serum albumin (BSA) was from EMD Chemicals Inc (Gibbstown, NJ). DMSO was from Bioshop. Rapamycin, LY294002, PD98059, Go6983, SB203580 and AG1478 were from Calbiochem, EMD milipore (Billerica, MA). The IP-One HTRF® kit was bought from Cisbio (Bedford, MA).

2.2 Methods

2.2.1 Cell culture and transfections

All cells were grown at 37°C with 5% CO_2. The culture medium used for the HEK293 cells was MEM supplemented with 10% FBS, 5mM L-glutamine and 100 μg/ml gentamycin. The stable cell line expressing Flag-AT1R had 1μl of G418 (selection antibiotic) added for each mL of medium (MEM). Dr. Marc Servant provided us with the vascular smooth muscle cells (VSMCs) used for these assays (IRIC, Université de Montréal, Canada). The VSMCs were grown in

DMEM/low glucose supplemented with the same components as MEM. All transfections were done with a common method using calcium phosphate.

2.2.2 Western blots

Different cell types were used to perform these experiments. First, we used HEK 293 cells stably expressing the Flag-AT1R. We also used vascular smooth muscle cells (VSMCs) extracted from the rat. In the case of the VSMCs, these were seeded in 12-well plates (50 000 cells/well) and 24 hours later, they were serum-starved overnight. HEK 293 cells were also seeded in 12-well plates (100 000 cells/well) but only serum-starved for 30 minutes just before starting the pre-treatment. The cells were pre-treated for 30 minutes with the vehicle (medium) or with one of our peptides (SC00xx). Then, cells were treated for 5 minutes with increasing concentrations of AngII or PGF2α, the maximum dose being 1μM. Cells were solubilized in Laemmli buffer and the samples were ran on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Then, the membranes were blocked in PBS-Tween with 10% milk for 1 hour. Finally the membranes were probed with the appropriate antibodies in 1% milk (anti-pERK1/2), 1% BSA (anti-total ERK1/2). We used ImageJ in order to perform a semi-quantitative analysis by densitometry.

2.2.3 IP1 production

Two cell types were used for this experiment. First, HEK 293 cells stably transfected with the AT1R were used. These were seeded in 10 cm plates and 48 hours later, they were starved for 30 minutes. Second, VSMCs were also used but these cells were starved overnight, 24 hours later. Then, cells were resuspended in stimulation buffer and seeded in a 384-well plate (20 000 cells per well). These were pre-treated with vehicle (stimulation buffer), 10μM of SC0023 or SC0024 for 30 minutes and treated with increasing concentrations of AngII for 1 hour, allowing for the

accumulation of IP1. The IP-One HTRF® assay protocol was followed as the manufacturer's instruction. The time-resolved FRET signal was read on a Synergy 2 reader and these results were analyzed using a standard curve as a reference. The data was then plotted using the vehicle condition treated with 1μM of AngII as the maximum (100%).

2.2.4 [^{3}H]-thymidine incorporation

VSMCs were seeded in 24-well plates (15 000 cells/well). 24 hours later, the cells were serum-starved overnight. The next day, pre-treatment with any given compound (see figure legends) was done for 30 minutes followed by a treatment with AngII/EGF/PDGF for 24 hrs. For the last 4-6 hours of stimulation, 1 μCi of [^{3}H]-thymidine was added in every well. At the end of the 24 hours, cells were put on ice and washed with cold PBS1X. Then, 5% trichloroacetic acid (TCA) was added to the cells for 15 minutes. Finally, 0.2M NaOH was used to lyse the cells before scraping them and measuring the incorporated radioactivity by liquid scintillation spectrometry.

2.2.5 [^{3}H]-leucine incorporation

VSMCs were seeded in 24-well plates (15 000 cells/well). 24 hours later, the cells were serum-starved overnight. The next day, the cells were pre-treated with 10 μM of SC0024 for 30 minutes followed by a treatment with 1 μM of AngII for 24 hrs. For the last 4-6 hours of stimulation, 1 μCi of [^{3}H]-leucine was added in every well. At the end of the 24 hours, cells were put on ice and washed with cold PBS1X. Then, 5% trichloroacetic acid (TCA) was added to the cells for 15 minutes. Finally, 0.2M NaOH was used to lyse the cells before scraping them and measuring the incorporated radioactivity by liquid scintillation spectrometry.

2.2.6 Binding assay

HEK293 cells were seeded (800 000 cells/plate) in 10 cm plates. 24 hours later, one plate was transfected with 5 μg of Flag-AT1R, using calcium phosphate, while the second plate was not transfected (mock cells). 24 hours later, the cells were trypsinized and seeded in poly-L-ornithine-coated 24-well plates (100 000 cells/well). The next day, the medium was replaced by binding buffer with 0.2% of bovine serum albumin (BSA). The cells were then treated with either [^{125}I]-AngII (150 000cpm/well) or [^{125}I]-SC0023 (800 000cpm/well) in the presence or absence of cold ligands (see figure legends). Binding was done over-night at 4°C. Then, the cells were put on ice and washed 3 times with cold PBS1X, followed by 0.2M NaOH to lyse the cells. Each sample was then scraped and the radioactivity was analyzed at the γ-counter.

2.2.7 Calcium mobilization assay

HEK293 cells were seeded in 10cm plates (800 000 cells/plate). The next day, the cells were transfected with 5μg of Obelin and 5μg of AT1R. 24 hours later, the medium was changed. Another 24 hours later, the cells were pre-treated for three hours in tyrod buffer with 1% FBS, 1μM of coelenterazine and either vehicle (tyrod) or 10μM of SC0023/SC0024. After the three hours, the cells were counted and spun at 1000rpm in a centrifuge. The cells were then put back into a specific volume of their buffer (tyrod+coelenterazine+FBS+/-SC0023/24), in order to have 50 000 cells per 150μl. Increasing concentrations of AngII were plated into a 96-well plate, where cells were injected. The peak of luminescence was obtained using a Synergy 2 reader and the maximum values of each peak were retained for further analysis. All the data was normalized with the values we obtained in our controls. The controls were as follows: 10 μM of acetylcholine, 10 μM of carbachol and 1μM of the calcium ionophore A23187.

2.2.8 FRET, Raichu-RBD assay

For these experiments, we used HEK293 cells that were stably transfected with the AT1R. We plated 70 000 cells in 35 mm confocal dishes. 24h later, cells were transfected with 2.5ug of Raichu construct using a calcium phosphate method. The Raichu-RBD unimolecular biosensor is composed of a YFP moiety in the N-terminal domain (m2Venus), a central Rho-binding domain (RBD) of Rhotekin and a CFP moiety in the C-terminal domain. 24h later, the media was changed and another 24h later, the cells were serum-starved for 30 min and pre-treated with vehicle (medium) or 2 μM of SC0023. For the confocal microscope (Zeiss LSM-510), we used the 63X objective and proceeded to the FRET experiment. Using the program Zen2009, images were collected every 2 minutes for 20 minutes using live-cell microscopy at 37ºC. The settings that were used were: excitation at 405 nm for CFP and FRET, 514 nm for YFP. The energy transfer efficiency between the CFP (donor) and YFP (acceptor) was determined by calculating the ratio of the YFP over CFP fluorescence from three different regions of each cell, and corrected for background signal using the program Metamorph.

2.2.9 Ruffling assay

HEK293 cells that were stably transfected with Flag-AT1R were seeded in 6-well plates onto cover slips (50 000 cells/well). 48 hours later, the cells were serum-starved for 30 minutes, pre-treated with vehicle (medium) or 10 μM of SC0023 for 30 minutes and then treated with 1 μM of AngII for another 30 minutes. Then, they were fixed with paraformaldehyde (PFA) 4% for 5 minutes. Then, the cells were washed 4 times with PBS1X, before adding the blocking solution (PBS1X with 2% BSA) with 0.05% Triton-X-100: The cells were blocked and permeabilized for

15 minutes. Then, Phalloidin-Alexa488 was added at a 1/1000 dilution for 45 minutes. Finally, the cells were washed 3 times with PBS1X before mounting the cover slips on microscope slides (with mounting agent). The slides dried overnight and the next day, photos were taken of each condition using our confocal microscope. Cells were counted and categorized according to their respective phenotypes.

2.3 Statistical analysis

All graphs were plotted using GraphPad Prism, version 4.0. For figure 1,2,8,9, results were analyzed using a one-way ANOVA test. For figures 4,5,6,7,11, we used a two-one ANOVA test. All figures that were analyzed with ANOVA were followed by a bonferroni correction, in order to reduce the chance of false positives and were representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CHAPTER 3:

Results

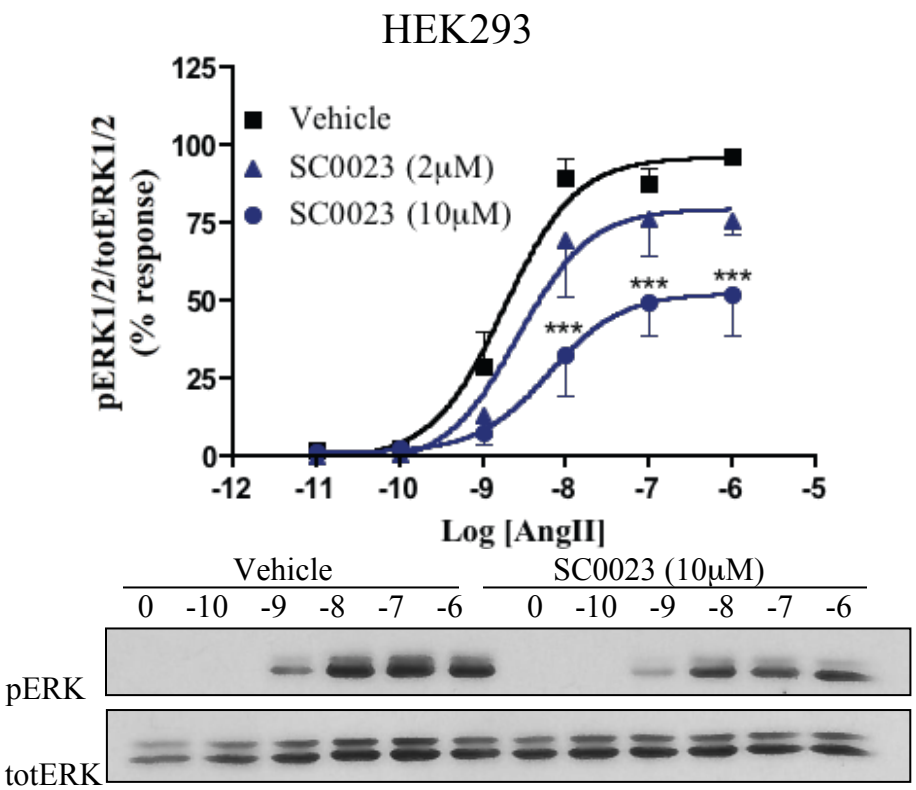
3.1 Effect of SC0023 on AngII-induced ERK1/2 activation

First, we wanted to see if there was any effect of the peptides that were derived from the ECL2 of the AT1R on a well-characterized AngII-induced pathway. In this case, we decided to study the ERK1/2 pathway, which is involved in responses such as cell proliferation (Zhao, Liu et al. 2005). We started with a heterologous system, HEK293 cells, that were stably transfected with the construct Flag-AT1R. We used western blotting as a biochemical assay to compare the ERK1/2 activation in the presence or absence of these peptides. We used increasing concentrations of AngII at a single time point- five minutes. Interestingly, the nonapeptide we refer to as SC0023 showed a dose-dependent inhibition of ERK1/2 activation in these cells (Fig. 3.1A). Since this inhibitory effect on MAPK activation, of about 50%, was seen only in presence of the AngII, the orthosteric ligand of AT1R, these results were the first that suggested that this peptide could be acting as a either an antagonist or a negative allosteric modulator (NAM) of this receptor. We were interested in knowing if this effect could be translated in a more physiological system, and proceeded in using a second cell type. As mentioned in a previous chapter, AngII exerts many of its effects in vascular smooth muscle cells (VSMCs), therefore making them a relevant model. Here, we used a primary cell line of rat VSMCs, which were extracted from the aorta of the animal. Since these cells endogenously express the AT1R, we were able to repeat the previous experiments and assess the effects of SC0023 on MAPK activation. SC0023 was able to decrease the AngII-induced activation of ERK1/2 by 50% in VSMCs (Fig. 3.1B). Though we cannot rule out the possibility that SC0023 is a partial antagonist of the AT1R, these results further highlighted the proposed NAM effect of this peptide on this response.

3.2 Effect of SC0023 on PGF2α-induced ERK1/2 activation

Next, in order to test the selectivity of SC0023 for the AT1R, we wanted to see if it could decrease the activation of ERK1/2 downstream from another receptor. The prostaglandin F2α (FP) receptor was a good candidate, given it is a GPCR, known to be expressed in VSMCs and is also an activator of this MAPK pathway. SC0023 did not display any significant modulatory effect on PGF2α-induced ERK1/2 activation (Fig. 3.2). These results suggest that SC0023 is acting selectively on the AT1R, which results in the inhibition of ERK1/2 activation.

A


HEK293
pERK1/2/totERK1/2 (% response)
Vehicle
SC0023 (2μM)
SC0023 (10μM)
Log [AngII]
Vehicle
SC0023 (10μM)
0 -10 -9 -8 -7 -6 0 -10 -9 -8 -7 -6
pERK
totERK

B

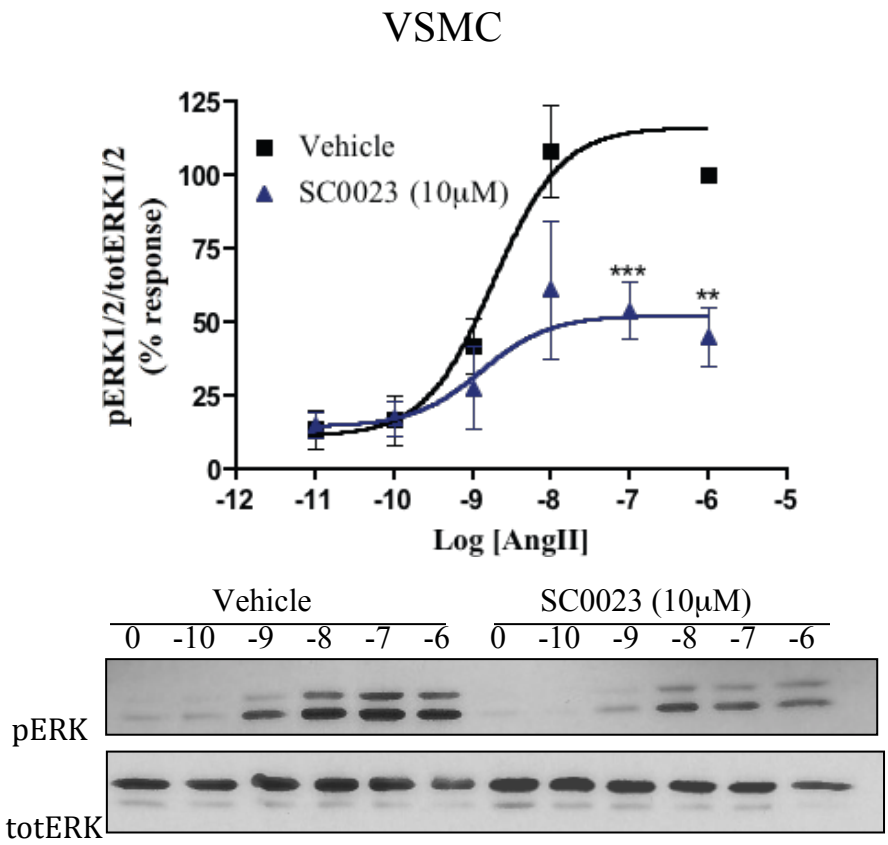

VSMC
pERK1/2/totERK1/2 (% response)
Vehicle
SC0023 (10μM)
Log [AngII]
Vehicle
SC0023 (10μM)
0 -10 -9 -8 -7 -6 0 -10 -9 -8 -7 -6
pERK
totERK

Figure 3.1: SC0023 decreases AngII-induced ERK1/2 activation in HEK293 cells and VSMC

A: HEK293 cells stably transfected with the AT1R were serum-starved for 30 minutes before being pre-treated for 30 minutes either with vehicle (medium) or with the indicated concentrations of SC0023. Then, the cells were treated for 5 minutes with increasing concentrations of AngII. B: VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes either with vehicle (medium) or with 10μM of SC0023. Then, the cells were treated for 5 minutes with increasing concentrations of AngII. A, B: A western blot was performed using anti-phospho- and anti-total ERK1/2 antibodies and the results were quantified using ImageJ by densitometry. These were then plotted using the vehicle condition treated with 1μM of AngII as the reference point (100%). Results were analyzed using a two-way ANOVA test followed by a bonferroni correction and are representative of at least three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

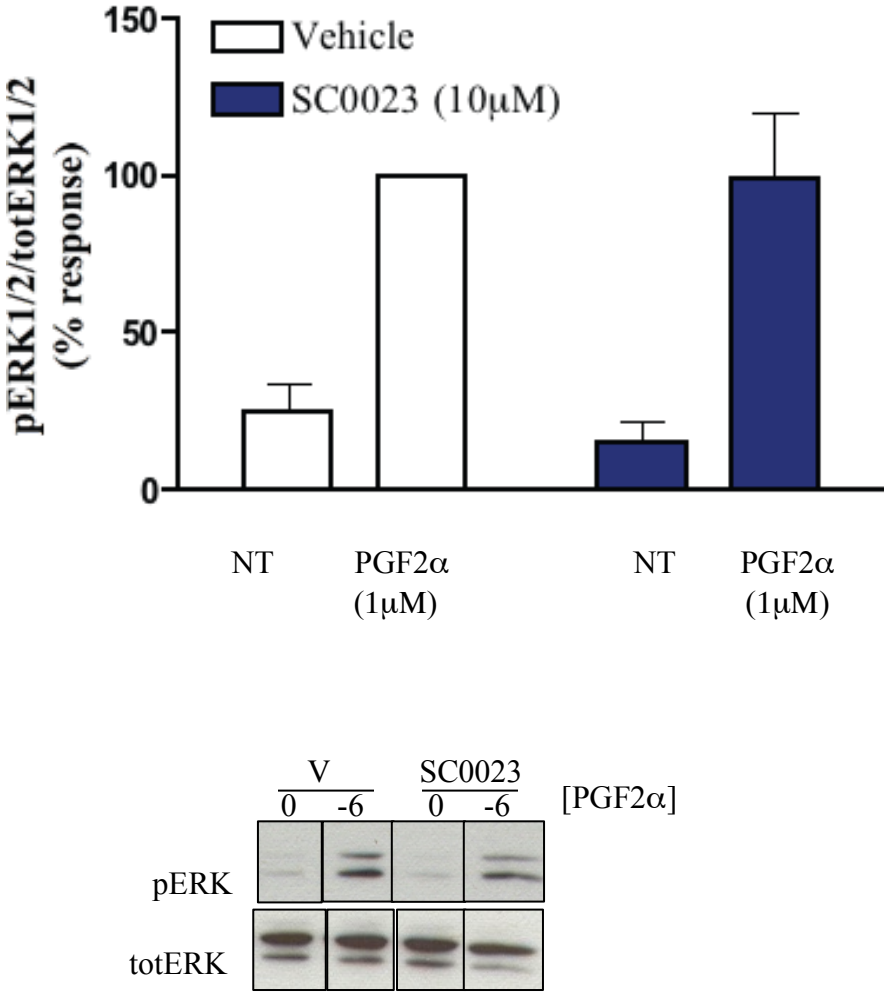


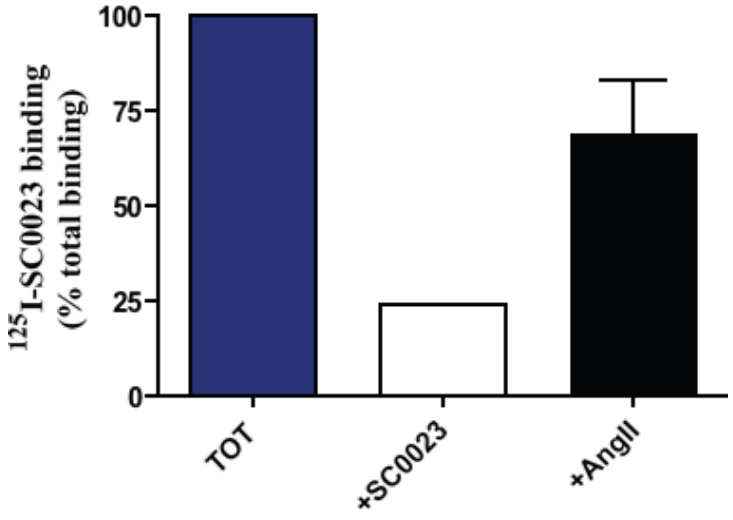
Figure 3.2: SC0023 has no effect on PGF2α-induced ERK1/2 activation in VSMCs

VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes either with vehicle (medium) or with 10μM of SC0023. Then, the cells were treated for 5 minutes with 1μM of PGF2α. The western blot was performed using anti-phospho- and anti-total ERK1/2 antibodies and the results were quantified using ImageJ by densitometry. These were then plotted using the vehicle condition treated with 1μM of PGF2α as the reference point (100%).

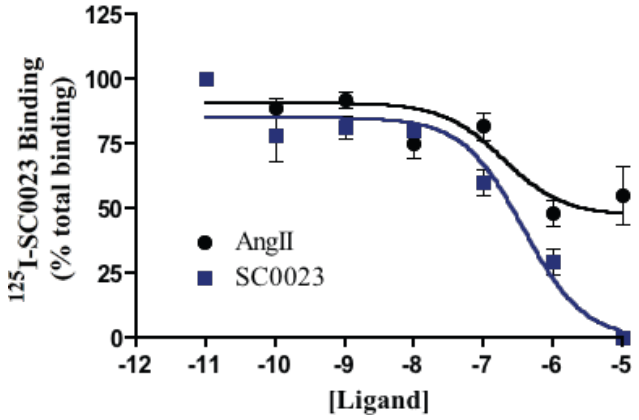
3.3 Binding of SC0023-I^{125} on HEK293 cells

We were next interested in validating that this effect was due to the allosteric regulation of SC0023 on AT1R. To answer this question, we decided to perform binding studies. The amino acid sequence of SC0023 contained a tyrosine, which allowed us to iodinate the peptide. The first set of experiments was performed on untransfected HEK293 (mock cells) and HEK293 cells transfected with 5 μg of AT1R in parallel. Cells were incubated with [^{125}I]-SC0023 alone or in the presence of 10 μM of SC0023 or 1 μM of AngII, over-night at 4°C. The results showed that cold SC0023 was able to displace radioactive SC0023, while AngII was only able to partially displace this binding (Fig. 3.3A). Minimal non-specific binding was seen in the mock condition (data not shown), suggesting that this peptide is binding specifically to the AT1R. Then, we proceeded in performing a dose displacement experiment where iodinated ligands (SC0023 or AngII) were incubated with increasing concentrations of the cold ligand (SC0023 or AngII). The EC50 for AngII and SC0023 were 4.7 nM and 376 nM, respectively (Fig. 3.3B, C). The slight displacement of [^{125}I]-SC0023 by AngII could be explained by two different mechanisms. Though this suggests that there may be negative cooperativity between the two ligands, where the binding of the allosteric ligand changes the conformation of the receptor in such a way that the affinity of the orthosteric ligand (AngII) for AT1R is reduced, we cannot exclude the possibility that SC0023 can partially compete with AngII binding. We were able to discard the possibility that the two ligands are competing for the same binding site because the curves show there is never a complete displacement of [^{125}I]-SC0023 by AngII and [^{125}I]-AngII by SC0023. These experiments suggest that SC0023 is binding to AT1R in an allosteric fashion, which in turn results in a negative cooperativity with AngII binding to the orthosteric binding site.

A


125I-SC0023 binding
(% total binding)
100
75
50
25
0
TOT
+SC0023
+AngII

B


125I-SC0023 Binding
(% total binding)
125
100
75
50
25
0
AngII
SC0023
-12
-11
-10
-9
-8
-7
-6
-5
[Ligand]

C

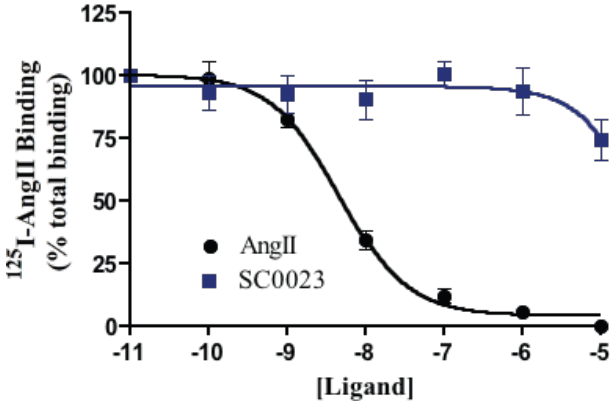

125I-AngII Binding
(% total binding)
125
100
75
50
25
0
AngII
SC0023
-11
-10
-9
-8
-7
-6
-5
[Ligand]

Figure 3.3: SC0023 binds AT1R in an allosteric fashion

A: Two conditions were used for this assay, HEK293 cells were either transfected with 5 μg of AT1R or used as a mock condition where no receptor was added. Both conditions were treated with ^{125}I-SC0023 and either 10 μM of SC0023 or 1 μM of AngII was added. The binding was done over-night at 4°C and performed as described in the *Materials and methods* section. The raw data from the mock condition (non-specific binding) was subtracted from the data collected from the cells that were transfected with the AT1R. Then, the data was plotted using the total binding with ^{125}I-SC0023 as a reference point (100%). B: HEK293 cells were transfected with 5 μg of AT1R. These were treated with ^{125}I-SC0023 and increasing concentrations of either SC0023 or AngII were added to the wells. C: HEK293 cells were transfected with 5 μg of AT1R. These were treated with ^{125}I-AngII and increasing concentrations of either SC0023 or AngII were added to the wells. B, C: Cells were allowed to reach a binding equilibrium over-night at 4°C. The next day, the results were collected and plotted using the maximum binding (not in presence of cold ligand) as a reference point (100%).

3.4 Effect of ECL2 peptides on AngII-induced VSMC proliferation

Based on our previous findings in terms of AT1R signaling, we wanted to next investigate if this effect on ERK1/2 phosphorylation could translate into physiological effects. Since ERK1/2 activation has been shown to be involved in cell proliferation and considering that VSMCs proliferate in response to AngII (Daemen, Lombardi et al. 1991; Zhao, Liu et al. 2005), we decided to use an assay that measures the amount of ^{3}H-thymidine incorporation, which is indicative of increased DNA synthesis and thus cell proliferation (Daemen, Lombardi et al. 1991). The VSMCs were pre-treated for 30 minutes with 10μM of the different peptides and then treated for 24 hours with AngII. The radioactive thymidine was only added in the last 4-6 hours, which allowed for a lower background signal. Surprisingly, though SC0023 was shown to strongly inhibit ERK1/2, this peptide did not show any significant effect on this cellular response (Fig. 3.4A). We tested other peptides in parallel to control for non-specific effects and to our surprise, the octapeptide SC0024, which has a four amino acid overlapping sequence with SC0023, potently inhibited proliferation (50-60% decrease), potentially acting as a NAM on this response (Fig. 3.4A). Amongst the other ECL2-derived peptides, while the effects of SC0021 and SC0022 were modest, pre-treatment with SC0025 resulted in a significant potentiation of this AngII-induced response.

We were also interested in knowing if SC0024 had any effect on the basal level of proliferation. To test this, we used VSMCs and treated them only with the allosteric modulators for a period of 24 hours. In this case, SC0024 showed no effect on the basal state of VSMCs proliferation, which indicates that this peptide requires the presence of AngII to exert its NAM effect (Fig. 3.4B). Because having no effect on their own is characteristic of most allosteric

modulators, this particular result further suggests that this peptide is indeed acting as a NAM of AT1R-mediated cell proliferation.

3.5 Effect of SC0024 on EGF- and PDGF-induced VSMC proliferation

To know whether this inhibitory effect is specific to the AT1R, we wanted to test the effect of SC0024 on the proliferation induced by other receptors. Therefore, we chose two receptor tyrosine kinases, which were shown to induce VSMC proliferation; platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) (Newby and George 1993; Zhan, Kim et al. 2003). In the case of PDGF, a concentration of 20ng/μl was used, which resulted in a response in the same range as with AngII. In terms of EGF, a concentration of 1ng/mL was used. Whether the cells were treated with PDGF or EGF, pre-treatment with SC0024 had no effect on the incorporation of ^{3}H-thymidine (Fig. 3.4C, D). These results show that SC0024 is a selective NAM of AngII-induced proliferation.

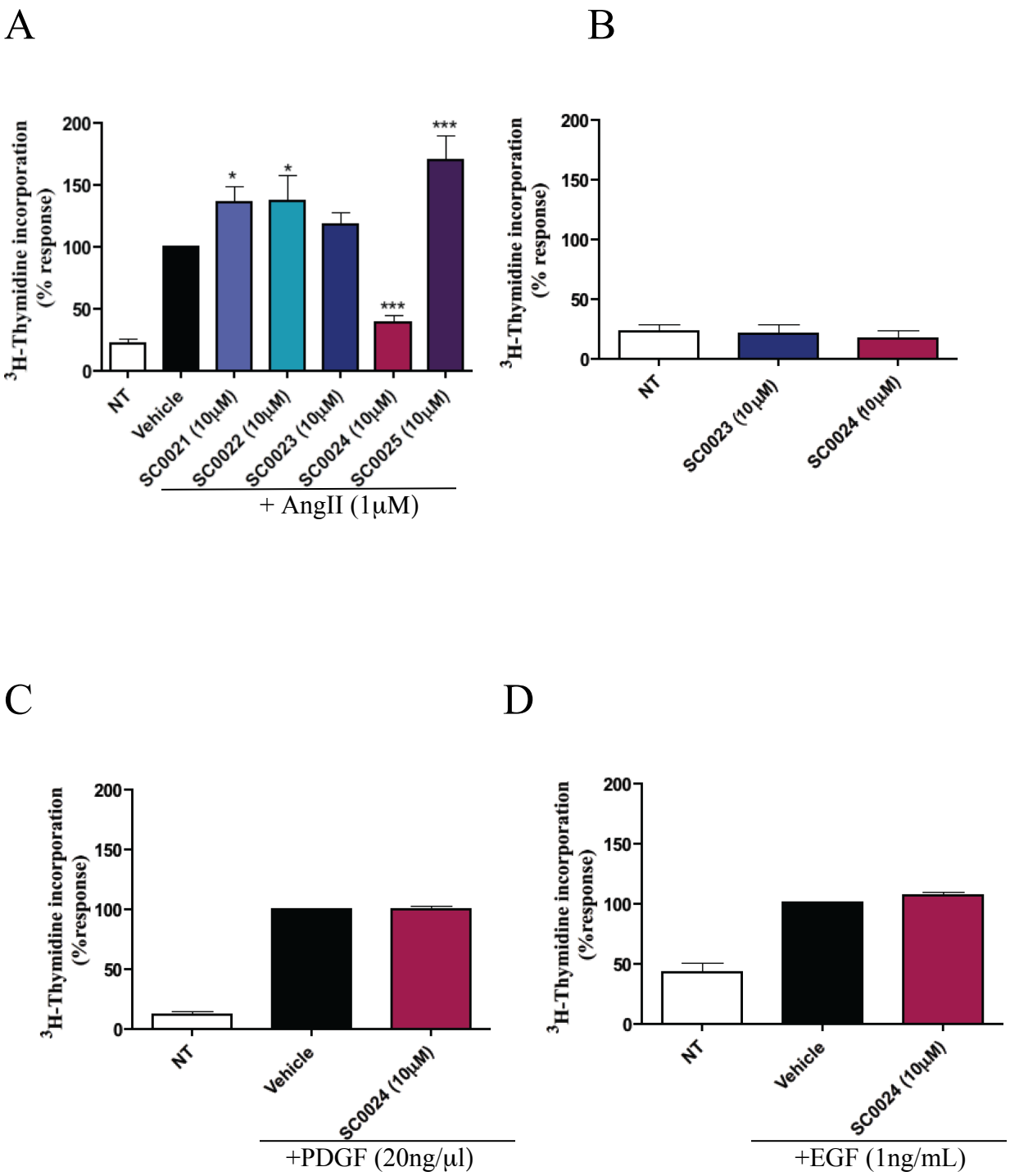

A
B
C
D
^{3}H-Thymidine incorporation (% response)
^{3}H-Thymidine incorporation (%response)
200
150
100
50
0
*
*
***
***
NT
Vehicle
SC0021 (10μM)
SC0022 (10μM)
SC0023 (10μM)
SC0024 (10μM)
SC0025 (10μM)
+ AngII (1μM)
SC0023 (10μM)
SC0024 (10μM)
+PDGF (20ng/μl)
+EGF (1ng/mL)

Figure 3.4: SC0024 inhibits VSMC proliferation induced by AngII, but not by PDGFR and EGFR

A: VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (medium) or 10μM of either SC0021/SC0022/SC0023/SC0024/SC0025. Then, they were treated with 1μM AngII for 24 hours. B: VSMCs were serum-starved overnight. The next day, the cells were treated with the vehicle (medium) or 10μM of either SC0023/SC0024**.** C: VSMCs were serum-starved overnight. The next day, the cells were treated for 30 minutes with the vehicle (medium) or 10μM of SC0024. Then they were treated with 20ng/μl of PDGF for 24 hours. D: VSMCs were serum-starved overnight. The next day, the cells were treated for 30 minutes with the vehicle (medium) or 10μM of SC0024. Then they were treated with 1ng/ml of EGF for 24 hours. A, B, C, D: For the last 4-6 hours of the treatment, ^{3}H-thymidine was added to the wells. The results were collected as described in *Materials and Methods* and these were then plotted using the vehicle condition treated with AngII, PDGF or EGF as the reference point (100%). Results were analyzed using a one-way ANOVA test followed by a bonferroni correction and are representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.6 Dose-response of SC0024 on AngII-induced VSMC proliferation

Next, we wanted to know the potency of SC0024 on the AngII-induced proliferation. In order to test this, increasing concentrations of the peptide were used in the presence of a single concentration of AngII. For this purpose, we used the same tritiated thymidine assay as previously described. SC0024 was used at concentrations of 0.5μM, 1μM, 2μM, 5μM, 10μM and 20μM, while treatment with AngII was kept at a single concentration of 1μM. The effect of this peptide started to be seen at 2μM and seemed to plateau at 20μM (Fig. 3.5). In conclusion, SC0024 inhibits proliferation downstream from AT1R with an apparent IC50 of approximately 3.5μM. These results showed that SC0024 is inhibiting AngII-induced proliferation in a dose dependent manner.

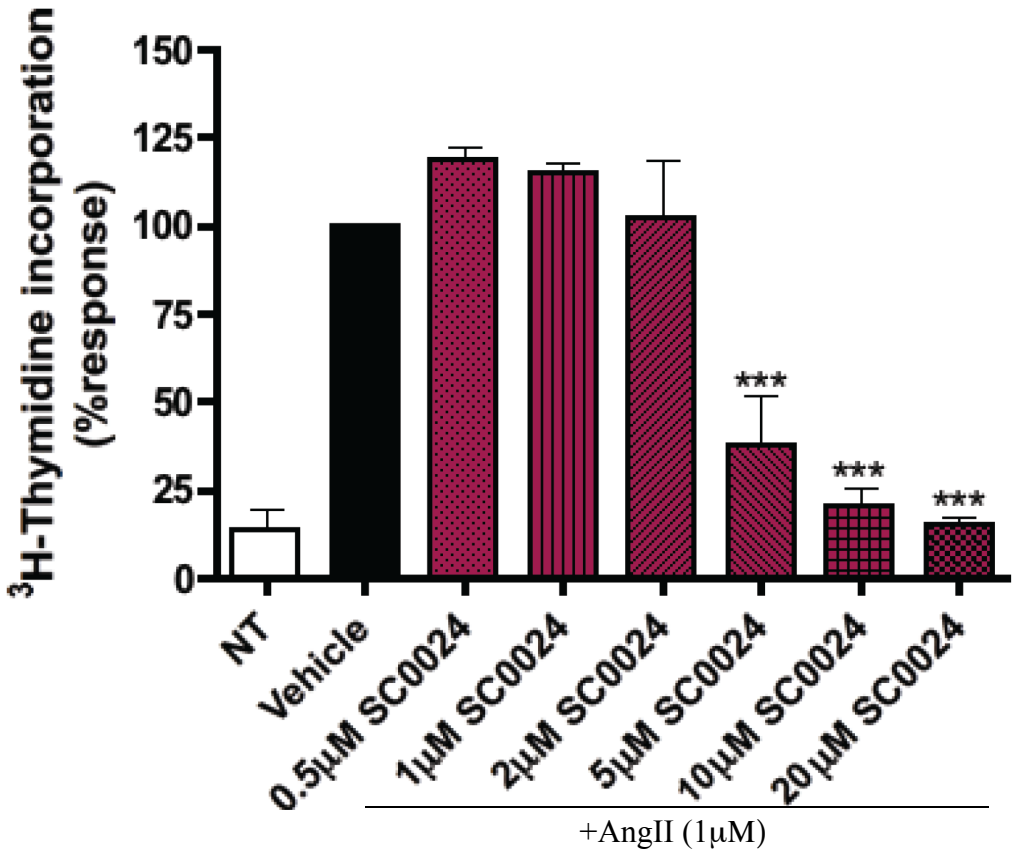


Figure 3.5: SC0024 inhibits AngII-induced proliferation in VSMCs with an IC50 of approximately 3.5μM

VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (medium) or different concentrations of SC0024 (0.5, 1, 2, 5, 10, 20μM). Then, they were treated with 1μM AngII for 24 hours. For the last 4-6 hours of the treatment, ^{3}H-thymidine was added to the wells. A liquid scintillation analyzer was used to collect the results and these were then plotted using the vehicle condition treated with AngII as the reference point (100%). Results were analyzed using a one-way ANOVA test followed by a bonferroni correction and are representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.7 Effect of different inhibitors on AngII-induced VSMC proliferation

Proliferation is a complex cellular response, which results from the integration of multiple pathways (Marrero, Schieffer et al. 1997; Dugourd, Gervais et al. 2003; Zhan, Kim et al. 2003; Zhao, Liu et al. 2005). As previously mentioned, we were interested in dissecting the main pathways that lead to VSMC proliferation, more particularly in response to AngII. To test this, we pre-treated VSMCs with different inhibitors and then treated them with AngII. Here, instead of using a 1μM AngII treatment, we used 100nM in order to maximize the effects seen in the presence of the inhibitors. We used the following inhibitors: 10μM of PD98059 (MEK inhibitor) (Hotokezaka, Sakai et al. 2002), 1μM of SB203580 (p38 inhibitor), 125nM of AG1478 (EGFR inhibitor) (Zhu, Liu et al. 2001), 2μM of Gö6983 (PKC inhibitor) (Kim, Kim et al. 2011), 10μM of LY294002 (PI3K inhibitor) (Vlahos, Matter et al. 1994) or 100nM of Rapamycin (mTOR inhibitor) (Raje, Kumar et al. 2004). The PKC inhibitor showed little effect on the AngII-induced proliferation, with a 25% decrease, while the p38 inhibitor showed a slight increase of this response. On the other hand, pre-treatment with the MEK inhibitor or the EGFR inhibitor resulted in a 60% inhibition. Finally, an inhibition of 80% was seen when cells were pre-treated with either the PI3K or mTOR inhibitors. We were therefore able to conclude that the MEK, PI3K and mTOR inhibitors were able to significantly decrease AngII-induced proliferation (Fig. 3.6). These results also suggest that the transactivation of EGFR plays a role AngII-induced VSMC proliferation. This was tested because it is well known that AngII can transactivate EGFR and that EGFR activation can lead to proliferation (Newby and George 1993). These results were able to pinpoint some of the pathways that would be interesting to investigate further, due to their implication in DNA synthesis and subsequence cellular proliferation. Future work would be required to really understand how SC0024 is in fact

inhibiting this response. Based on the few inhibitors that were tested, the PI3K-AKT-mTOR pathway would be of great interest.

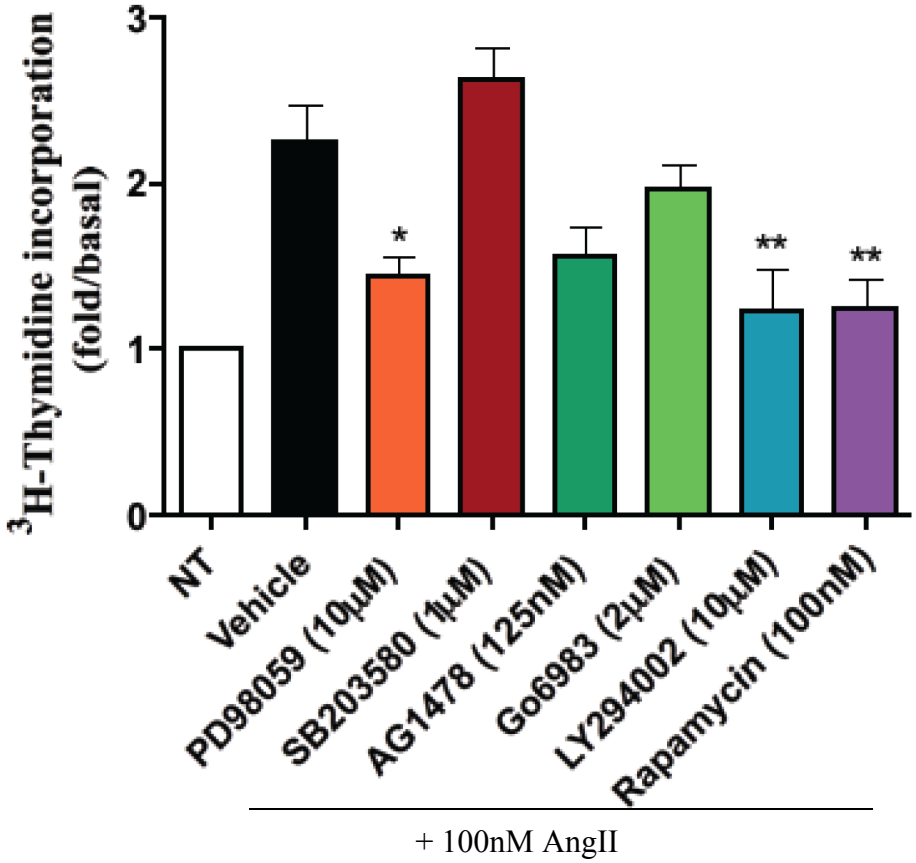


Figure 3.6: MEK, PI3K and mTOR inhibitors decrease AngII-induced proliferation in VSMCs. The EGFR inhibitor also tends to partially inhibit this response

VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (DMSO), 10μM of PD98059 (MEK inhibitor), 1μM of SB203580 (p38 inhibitor), 125nM of AG1478 (EGFR inhibitor), 2μM of Go6983 (PKC inhibitor), 10μM of LY294002 (PI3K inhibitor) or 100nM of Rapamycin (mTOR inhibitor). Then, they were treated with 100nM of AngII for 24 hours. For the last 4-6 hours of the treatment, ^{3}H-thymidine was added to the wells. A liquid scintillation analyzer was used to collect the results. The fold/basal increases were analyzed using a one-way ANOVA test followed by a bonferroni correction and are representative of three independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.8 Effect of SC0024 on AngII-induced protein synthesis in VSMCs

Asides from DNA synthesis, another downstream consequence of AngII treatment is the induction of protein synthesis (Berk, Vekshtein et al. 1989). Here, we wanted to test whether this response was affected by pre-treatment with SC0024. Using a similar method as for DNA synthesis, we measured the incorporation of ^{3}H-leucine to assess the extent of protein synthesis. VSMCs were pre-treated with vehicle (medium) or 10μM of SC0024 for 30 minutes before being treated with 1μM of AngII for 24 hours. Similarly, ^{3}H-leucine was only added for the last 4-6 hours of the treatment. As we expected, SC0024 not only acted as a NAM on DNA synthesis but also, to a lesser extent, on protein synthesis (Fig. 3.7). The inhibition that was seen in the presence of SC0024 for this set of experiments was around 30%.

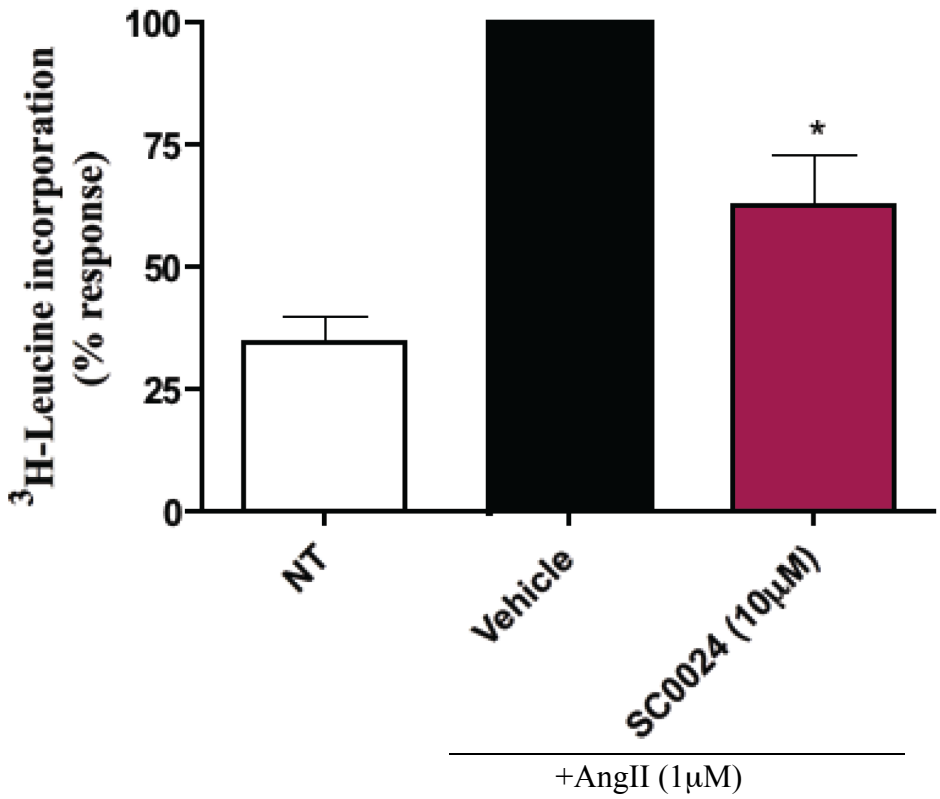


Figure 3.7: SC0024 decreases AngII-induced protein synthesis of VSMCs.

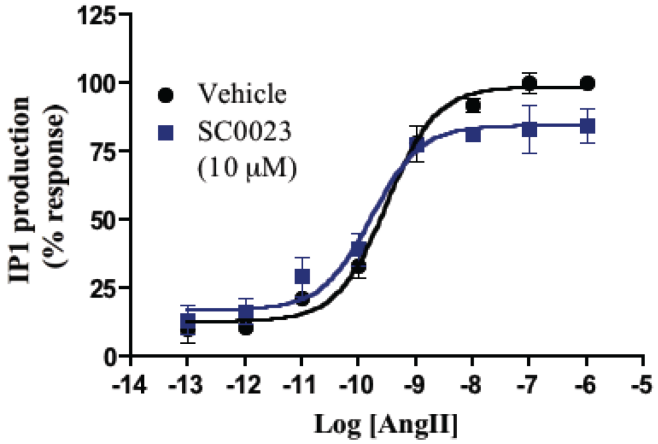
VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (medium) or 10μM of either SC0022/SC0023/SC0024. Then, they were treated with 1μM AngII for 24 hours. For the last 4-6 hours of the treatment, ^{3}H-leucine was added to the wells. A liquid scintillation analyzer was used to collect the results and these were then plotted using the vehicle condition treated with 1μM AngII as the reference point (100%).

3.9 Effect of SC0023 and SC0024 on AngII-induced IP1 production

Though these results on DNA and protein synthesis were very interesting, we were also interested in testing the effects of different peptides that were synthesized on pathways that are more upstream. Therefore, we proceeded to look at the second messenger inositol monophosphate (IP1), which is primarily thought to be downstream from Gαq (de Gasparo, Catt et al. 2000). Receptor activation is closely followed by the activation of phospholipase C (PLC), which will cleave phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG) (de Gasparo, Catt et al. 2000). Since the production and metabolism of IP3 is very rapid, it remains difficult to measure its activity, which is why most assays focus on its downstream metabolite IP1. For this purpose, we used a homogeneous time resolved fluorescence (HTRF) assay, in which lithium chloride (LiCl) is used to prevent the degradation of IP1 and allows for its accumulation. Here, we assessed the effects of the peptides on this response and to do so, we used both HEK293 cells stably expressing the AT1R and VSMCs. Increasing concentrations of AngII were used and the cells were treated for one hour, allowing for accumulation. On the first hand, pre-treatment with 10μM of SC0023 had very little effect in HEK293 cells, showing an inhibition of the efficacy of about 15% (Fig. 3.8A). The same experiment was done in VSMCs, where SC0023 strongly inhibited both the efficacy and the potency (50% inhibition and a one log rightward shift, respectively) of AngII-dependent IP1 production (Fig. 3.8B). These results suggest that SC0023 is acting as a NAM on this response. This discrepancy between the results in the two cell types is most likely due to the different nature of those cells, HEK293 cells being heterologous system in which the AT1R is often overexpressed. The peptide SC0024 was only tested in VSMCs, where it increased the potency of the response, acting as a positive allosteric modulator (PAM) (Fig. 3.8C).

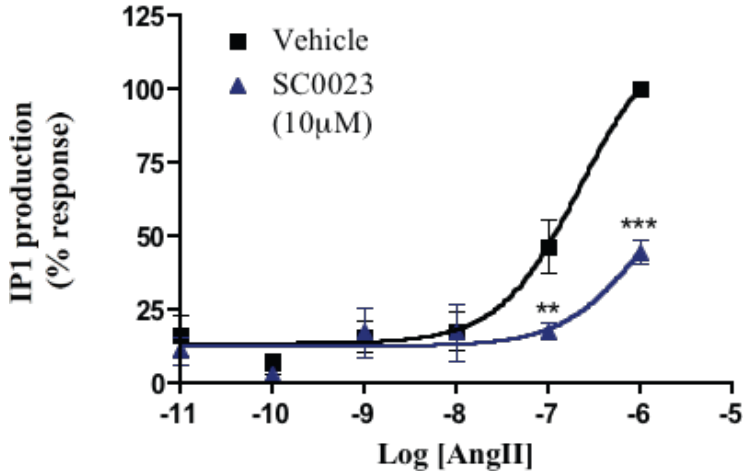
A

HEK293



B

VSMC



C

VSMC

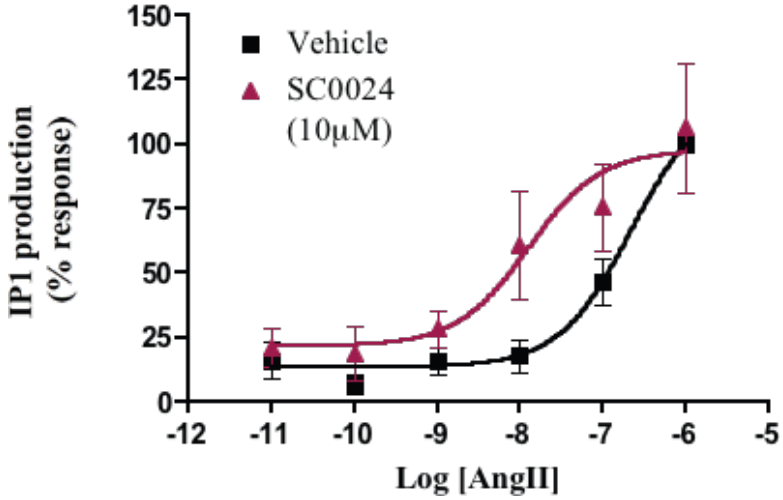


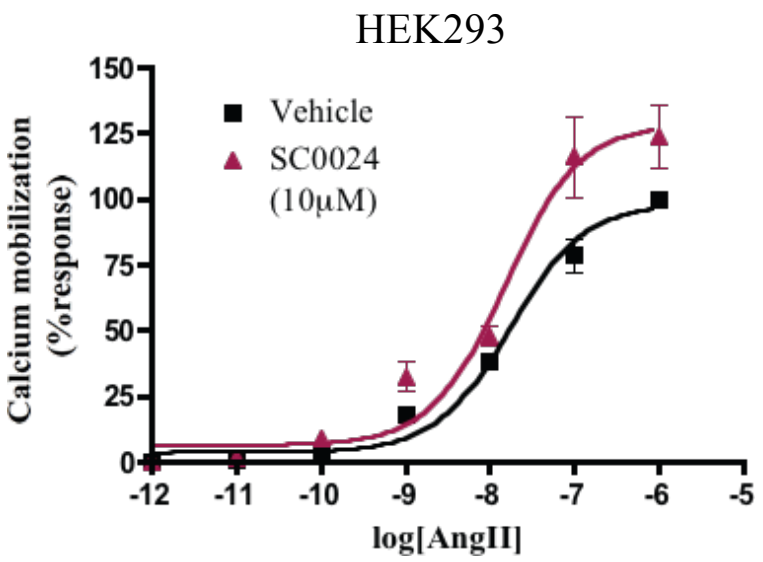
Figure 3.8: SC0023 decreases AngII-induced IP1 production, while SC0024 has a tendency to increase this response in VSMCs

A: AT1R cells (HEK293) were serum-starved for 30 minutes before being pre-treated with 10μM of SC0023 for another 30 minutes. B, C: VSMCs were serum-starved overnight, and the next day, the cells were pre-treated with 10μM of SC0023/SC0024 for 30 minutes. A, B, C: Then, the cells were treated with 1μM of AngII for 1 hour. The IP-One HTRF® assay protocol was followed as the manufacturer's instruction. After, the time-resolved FRET signal was read on a Synergy 2 reader. Finally, the results were plotted using the vehicle condition treated with 1μM AngII as the reference point (100%). The results were analyzed using a two-way ANOVA test followed by a bonferroni correction and are representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.10 Effect of SC0023 and SC0024 on calcium mobilization in HEK293 cells

The effects of inositol phosphate production on cellular responses are widespread. Indeed, when IP3 is produced, it can then bind to its receptor (IP3R) on the endoplasmic reticulum (ER)(Mikoshiba 2007). This will then activate the Ca^{2+} channels and release Ca^{2+} into the cytoplasm. Though this is merely one way by which calcium is released in response to AngII binding to AT1R, our previous results on IP1 production led us to wanting to study our peptides in the context of calcium mobilization. In this case, we decided to use a bioluminescence assay. In this case, the luciferase Obelin needs to be transfected, which is not done very effectively in primary cell lines such as VSMCs. Therefore, we used a heterologous system, HEK293 cells, since they can be readily transfected. Here, equal amounts of AT1R and Obelin was transfected in the cells, before being pre-treated for three hours with vehicle (tyrode buffer) or 10μM of SC0023/SC0024 and coelenterazine, the substrate for the luciferase. After the pre-treatment, cells were treated with increasing concentrations of AngII. In these experiments, SC0023 did not have any significant effect on calcium mobilization, which agrees with the results on IP1 production in HEK293 cells (Fig. 3.9B). The interesting result here was the non-significant but evident PAM effect of SC0024 on calcium release (Fig. 3.9A). The presence of this peptide resulted in an increase of approximately 25%. In short, in VSMCs, SC0024 acts as a NAM on proliferation and protein synthesis and as a PAM on IP1 production. In HEK293 cells, it acts as a PAM on calcium mobilization. Conversely, SC0023 acts as a NAM on ERK1/2 activation both in HEK293 and VSMCs. It also acts as a NAM on IP1 production in VSMCs.

A


HEK293
Vehicle
SC0024
(10μM)
Calcium mobilization
(%response)
150
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
log[AngII]

B

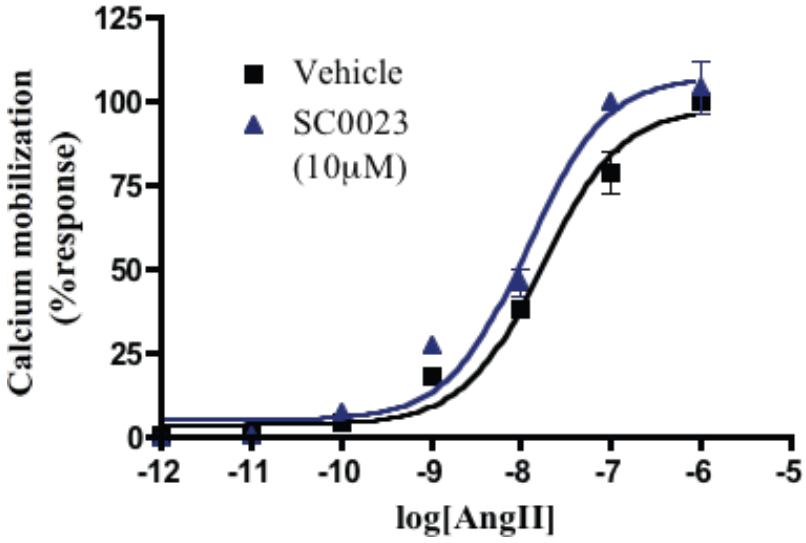

HEK293
Vehicle
SC0023
(10μM)
Calcium mobilization
(%response)
125
100
75
50
25
0
-12
-11
-10
-9
-8
-7
-6
-5
log[AngII]

Figure 3.9: SC0024 increases calcium mobilization in HEK293 cells

HEK293 cells were transfected with 5μg of Obelin and 5μg of AT1R in a 10cm plate. 48 hours later, the cells were pre-treated for three hours in tyrod buffer with 1% FBS, 1μM of coelenterazine and either vehicle (tyrod) or 10μM of SC0023/SC0024. Increasing concentrations of AngII were plated into a 96-well plate, where cells were injected. The peak of luminescence was obtained using a Synergy 2 reader and the maximum values of each peak were retained for further analysis. All the data was normalized with the values we obtained in our controls. The controls were as follows: 10 μM of achetylcholine, 10 μM of carbachol and 1μM of the calcium ionophore A23187. Finally, the results were plotted using the vehicle condition treated with 1μM AngII as the reference point (100%). The results were analyzed using a two-way ANOVA test followed by a bonferroni correction and are representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

3.11 Effect of alanine substitutions of SC0024 on AngII-induced VSMC proliferation

Another aspect that we were interested in studying is the relationship between the structure and the function of these peptides. We decided to focus on SC0024 given we had a robust assay that could be used as a tool. The first step towards understanding how this peptide is working was to synthesize a total of seven peptides, each having one alanine substitution at a position (Fig. 3.10A). The reasoning behind this is that alanines are simple amino acids that are not bulky and do not greatly alter the main-chain conformation of peptides (Cunningham and Wells 1989). Replacing one amino acid with an alanine can technically give its contribution to the function of SC0024 on AngII-dependent signaling. Once all these peptides were synthesized, we tested their functionality on the proliferative assay that was described earlier in this section. For the pre-treatment, a concentration of 10 μM was used for all peptides, while the treatment with AngII was kept at 1 μM. Many of the substitutions resulted in intermediate effects but interestingly, when positions 5-6-7 (F-H-Y) were substituted with an alanine, the inhibitory effect of SC0024 was lost (Fig. 3.10B). Another interesting result was that substituting the first residue, which is a threonine with an alanine actually made SC0024 even more efficacious at blocking AngII-induced proliferation of VSMCs. More importantly, we showed that positions 5-6-7 are important for their NAM effects this particular cellular response.

A

Nomenclature	Amino acid sequence
SC0024	T V C A F H Y E
SC0024-A1	**A** V C A F H Y E
SC0024-A2	T **A** C A F H Y E
SC0024-A3	T V **A** A F H Y E
SC0024-A5	T V C A **A** H Y E
SC0024-A6	T V C A F **A** Y E
SC0024-A7	T V C A F H **A** E
SC0024-A8	T V C A F H Y **A**

B

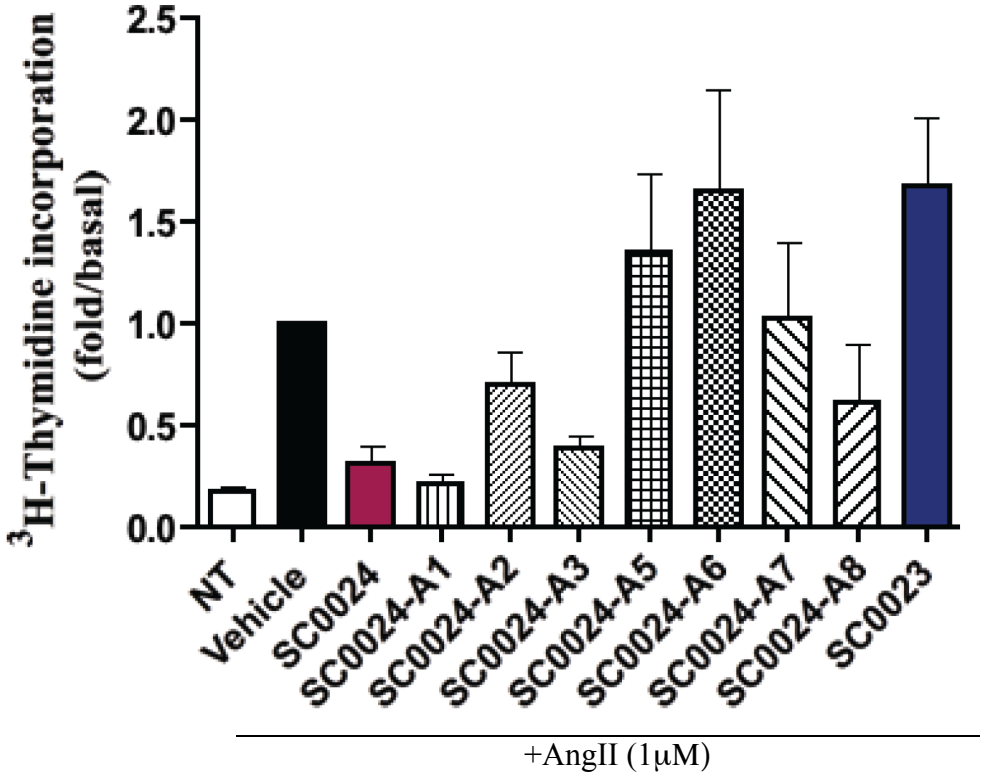


Figure 3.10: An alanine scan reveals that residues in positions 5-6-7 are important for the inhibitory effect of SC0024 on AngII-induced VSMC proliferation

A: Amino acid sequences of the peptides used in this experiment are listed here. B: VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (medium) or 10μM of SC0023, SC0024 or either one of the peptides containing alanine substitutions. Then, they were treated with 1μM AngII for 24 hours. For the last 4-6 hours of the treatment, ^{3}H-thymidine was added to the wells. The results were collected as described in *Materials and Methods* and these were then plotted using the vehicle condition treated with 1μM of AngII as the reference point (100%).

3.12 Effect of Bpa substitutions of SC0024 on AngII-induced VSMC proliferation

A long-term goal of this project is also to map the binding sites of these peptides. This will hopefully allow us to show their direct binding to the AT1R, and eventually show the exact location of the binding site on the receptor. To do so, we designed a peptide that would contain a benzoyl phenylalanine (Bpa) moiety that can be photo-activated when under a UV light (Boucard, Wilkes et al. 2000). The UV will photo-activate the molecule, which will then become reactive. The photo-activated benzophenone will then have the ability to make a covalent bond with a carbon-hydrogen bond (C-H) nearby, with over 50% efficiency (Chin, Martin et al. 2002). In this case, if the peptide binds to the AT1R, the Bpa moiety will be covalently bound to its receptor. This technique will permit us to immunoprecipitate (IP) the receptor and take the necessary steps in order to identify the binding site, most probably involving mass spectrometry. In our case, we decided to have two different peptides synthesized, one that has a Bpa replacing the amino acid in first position and one in fifth position (Fig. 3.11A). These were tested for functionality using the ^{3}H-thymidine incorporation assay. The SC0024 analogs were used at a concentration of 10 μM, and the cells were treated with 1 μM of AngII. While SC0024-Bpa1 lost its NAM effect on proliferation, a 50% inhibition was seen with SC0024-Bpa5, which was able to retain most of its inhibitory effect (Fig. 3.11B). These results confirm that we now have a very powerful tool that will allow us to identify the target of SC0024 and precisely map its binding site.

A

Nomenclature	Amino acid sequence
SC0024	T V C A F H Y E
SC0024-Bpa1	**Bpa** V C A F H Y E
SC0024-Bpa5	T V C A **Bpa** H Y E

B

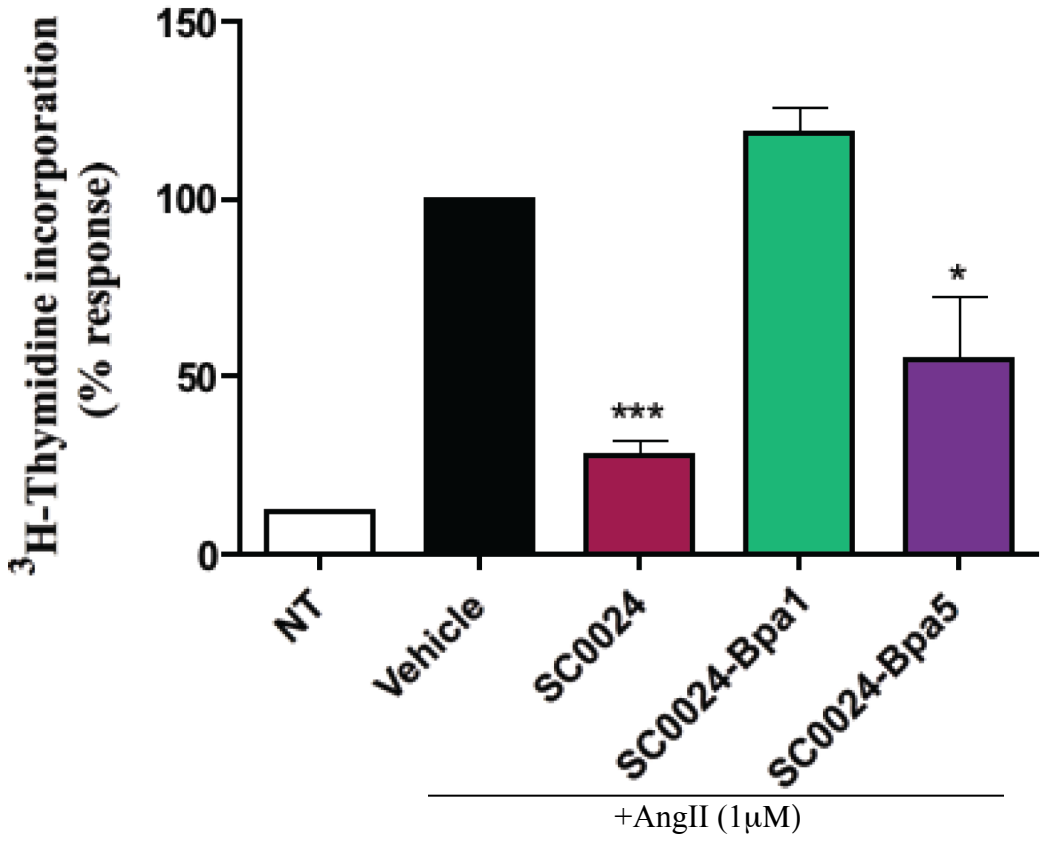


Figure 3.11: SC0024-Bpa5 decreases AngII-induced VSMC proliferation

A: Amino acid sequences of the peptides used in this experiment are listed here. B: VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (medium) or 10μM of SC0024-Bpa1/SC0024-Bpa5. Then, they were treated with 1μM AngII for 24 hours. For the last 4-6 hours of the treatment, ^{3}H-thymidine was added to the wells. A liquid scintillation analyzer was used to collect the results and these were then plotted using the vehicle condition treated with 1μM of AngII as the reference point (100%). Results were analyzed using a one-way ANOVA test followed by a bonferroni correction and are representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

CHAPTER 4:

Discussion

4.1 Summary

Here, we describe the characterization of putative biased, allosteric ligands, derived from the ECL2 of the AT1R. The AT1R is a GPCR responsible for the activation of multiple signaling pathways leading to various cellular/physiological responses and phenotypes, some of which were tested in the presence of our allosteric modulators (Fig. 13). For our first peptide of interest, SC0023, we demonstrated that it is acting as a NAM of AngII-induced ERK1/2 activation, both in HEK293 cells and in VSMCs, a primary cell line derived from the aorta of the rat. In VSMCs, we showed that SC0023 acted selectively on the AT1R, since it did not modulate PGF2α-mediated ERK1/2 activation. On another hand, this same peptide had no effect on AngII-mediated IP1 production and calcium mobilization in HEK293 cells but was shown to act as a NAM of the IP1 pathway in VSMCs, showing a discrepancy between different cell types. Also, though we hypothesized that the reduction in MAPK activation would translate to a downstream reduction response of DNA synthesis, this newly peptide was surprisingly shown to have no significant effect on AngII-induced VSMC proliferation. Finally, with the use of I^{125}-SC0023, we were able to show that this peptide is able to bind HEK293 cells that were transfected with the AT1R, while showing minimal non-specific binding in cells with no receptor. In terms of our second peptide of interest, SC0024, we discovered an almost entirely opposite signaling behavior, despite their overlapping amino acid sequences. First, SC0024 showed no effect on the AngII-mediated ERK1/2 pathway, but on the contrary, this peptide was shown to act as a potent NAM of VSMC DNA and protein synthesis. Finally, SC0024 acted as a PAM on AT1R-dependent IP1 production in VSMCs and on the calcium pathway in HEK293 cells. We can therefore conclude that our results on both of these peptides derived from the ECL2 of the

AT1R, suggest that they are acting as allosteric ligands displaying differential biased signaling properties.

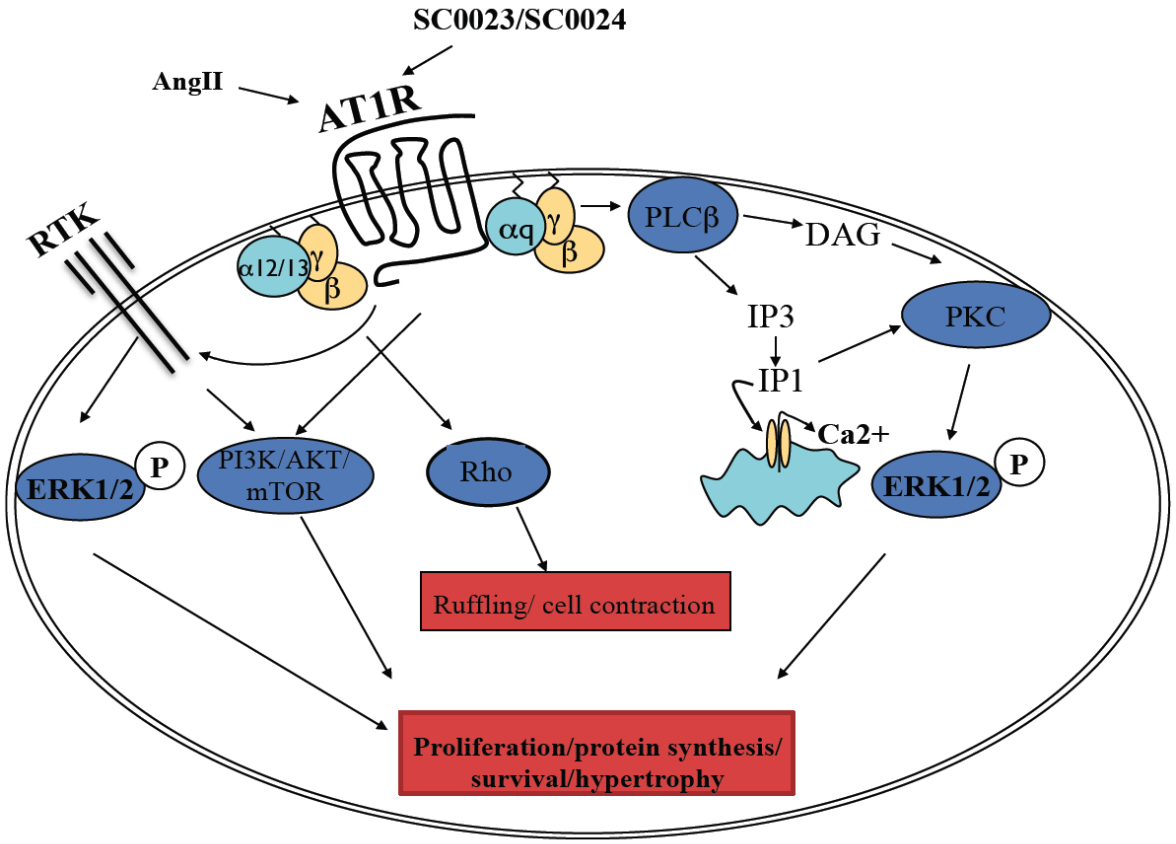


Figure 4.1: AT1R-dependent signaling and downstream cellular responses

The activation of the AT1R is responsible for the activation of multiple signaling pathways, some of which are shown here. The AT1R can also transactivate different RTKs, such as EGFR, which will in turn activate another subset of pathways. The ECL2-derived peptides were tested at different levels of these pathways, including on cellular responses such as proliferation.

An important question that can be raised from our results is the possibility for these peptides to act by a competitive mechanism. In terms of binding, when performing our experiments using either I^{125}-SC0023 or I^{125}-AngII, we observed a partial displacement of AngII by SC0023 and vice versa, but only at higher concentrations of either ligand. These results could suggest that this peptide is partially competing with AngII for its binding site instead of acting in an allosteric fashion. Though we cannot rule out this possibility, there exist other mechanisms for negative cooperativity, which could explain the partial displacement seen at these higher concentrations. Negative binding cooperativity occuring between two ligands is indeed a known characteristic of allosteric modulators (Conway and Koshland 1968; May and Christopoulos 2003). In other words, the binding of SC0023 could change the conformation of the receptor in such a way that the affinity of AngII for AT1R is reduced, without the need for the two ligands to compete for the same binding sites. However, for our second peptide of interest, SC0024, a preliminary result (n=1) shows that AngII does not displace the binding of its iodinated version in different cell types, suggesting that this peptide may be non-competitive, and showing little or no negative binding cooperativity for AngII binding on AT1R (data not shown). However, this result should be further validated. On the other hand, in terms of their effects on signaling, since both peptides were shown to exhibit differential effects on the various pathways studied, we can propose that SC0023 or SC0024 both act in a non-competitive manner.

Our results also show discrepancies between what is seen in HEK293 cells in comparison with what we observed in VSMCs. This is not surprising considering that HEK293 cells are used as a heterologous system in which we overexpress the AT1R, while VSMCs come from a primary cell line where the receptor is endogenously expressed. Major differences between cell-types have previously been reported, even between different clonal lines of HEK293 cell (Lin,

Boone et al. 2014). For example, one study reported that osteoblasts induce ERK1/2 activation both in a Gαq- and transactivation of EGFR-dependent manner, while in HEK293 cells very little evidence of transactivation is occurring for GPCR-mediated activation of MAPKs (Goupil, Wisehart et al. 2012). In our case, SC0023 was shown to potently decrease AngII-mediated IP1 production in VSMCs, while no significant effect was observed in HEK293 cells. However, different G proteins and pathways can lead to IP1 production, including Gαq/11, Gαi/o and Gα12/13 (Wu, Lee et al. 1992; Irie, Segi et al. 1994; Hains, Wing et al. 2006). Also, one clear difference between the two cell types is the potential for transactivation of RTKs by angiotensin, which can be seen in VSMCs (de Gasparo, Catt et al. 2000; Saito and Berk 2001). In fact, PDGF was shown to increase IP1 production and calcium mobilization in VSMCs (Roe, Hepler et al. 1989). In addition, EGF was also shown to activate PLCγ and lead to the production of inositol phosphates (Nishibe, Wahl et al. 1990). Interestingly, EGFR coupling to Gαi was shown to play a role in PLCγ activation and subsequent signaling events (Yang, Baffy et al. 1991). Consequently, SC0023 may be affecting the signaling in various ways in VSMCs to regulate IP1 production, which mechanisms seem not to be involved in HEK293 cells. Different means to test these pathways will be discussed in our future perspectives.

In addition, we showed that even though SC0023 had no significant effect on AngII-mediated IP1 production or calcium mobilization, it could partially inhibit AngII-induced ERK1/2 activation in HEK293 cells. This suggests that SC0023 is inhibiting ERK1/2 via a different pathway from the one leading to the production of inositol phosphates. Indeed, there are multiple studies that show G protein-independent/β-arrestin-dependent activation of ERK1/2 in HEK293 cells (Shenoy, Drake et al. 2006). Therefore, we can hypothesize that the effect of SC0023 on ERK1/2 could be mediated via a β-arrestin-dependent signaling pathway.

Moreover, the mechanism by which SC0024 potently inhibits VSMC proliferation remains to be elucidated. Consequently, this peptide should be tested on different pathways that are involved in DNA synthesis/proliferation. The involvement of ERK1/2 in the induction of AngII-dependent VSMC proliferation has been demonstrated in the past, though here we have characterized a NAM effect on proliferation, without any inhibition of MAPK (Zhao, Liu et al. 2005). However, it is also well recognized that proliferation results from multiple converging signaling pathways (Marrero, Schieffer et al. 1997; Dugourd, Gervais et al. 2003; Kim, Ahn et al. 2009; Zhao, Chen et al. 2010). As was shown in the results section, effectors such as AKT, PI3K and mTOR are good candidates for future experiments, since they were shown to be important for VSMC proliferation in response to AngII. Perhaps SC0024 modulates the activation of one of these effectors, which results in the net decreased of cell proliferation when treated with AngII. Another alternative would be that this peptide blocks the transactivation of one or several receptor tyrosine kinases (RTKs), such as EGFR or PDGFR. We showed that SC0024 did not affect EGFR- and PDGFR-induced VSMC proliferation but this does not rule out the possibility that the inhibitory effects of this peptide results from blocking their transactivation by AT1R. It is also possible that this suppression of VSMCs' proliferative state is not caused by a drastic effect on one pathway but rather by cumulative effects on multiple pathways that ultimately lead to inhibition of proliferation. In this case, it may be difficult to pinpoint one pathway and fully understand how this peptide is modulating AngII-mediated response.

In recent years, in order to develop new therapeutics, scientists have moved from the classical approach of reverse pharmacology to what is referred to as forward pharmacology.

Forward pharmacology is a target-based strategy, which in a situation like ours involves the search for compounds that would target specifically our receptor of interest. Indeed, this thesis work focused on the rational design of allosteric modulators based on the amino acid sequence of the ECL2 of GPCRs. We hypothesized that the ECL2 of GPCRs can be used as putative allosteric modulators, and chose the AT1R as a model. The choice of receptor was based on the knowledge that no allosteric modulator of the AT1R had ever been described as well as its inherent importance and contribution to the development of different pathologies, namely cardiovascular diseases. Our results on the effects of these ECL2 mimics indeed support the known literature on this particular subject. As others have already reported, this region of the receptor seems to be a good target for designing allosteric modulators, though the exact reason for this remains unknown (Peri, Quiniou et al. 2002; Leu and Nandi 2010; Dogo-Isonagie, Lam et al. 2012). Although it may not always be the case, the mechanism by which these ligands are able to influence the signaling of different GPCRs is likely to be by direct binding to the receptor in question. A way by which these mimics may bind to the receptor could be by directly acting on the ECL2. This would prevent the receptor from adopting the orthosteric-bound "fully" active conformation and would likely stabilize an intermediate conformation. Another alternative that could explain the mechanism of action of these mimics is that they are competing with the binding sites of the ECL2 itself. It is well accepted that the ECL2 changes its conformation/position when the receptor is activated, and is able to interact with the agonist, as already mentioned. In addition, upon activation, the ECL2 was shown to interact with different parts of the receptor, including different transmembrane (TM) domains (Wheatley, Wootten et al. 2012). In other words, one way to visualize this, would be that the peptides derived from the ECL2 bind to the regions of the receptor that usually make contact with the ECL2 itself. This

competition would induce conformational changes that could be responsible for the new signaling signature obtained in presence of these allosteric modulators. Here, challenges include trying to better understand the role played by GPCR domains such as the ECL2 in the conformation of receptors and how different allosteric ligands affect these domains to stabilize intermediate conformations resulting in different signaling signatures. In particular, because allosteric compounds may represent a promising new class of drugs, predicting their sites of binding on GPCRs and modes of action becomes increasingly important.

Moreover, the possibility of directing GPCR signaling with biased, allosteric ligands has opened new opportunities for developing more selective/effective drugs. Because, in principal, it would allow a better control of the signaling pathways involved in the underlying pathophysiology, side effects that may be engendered from the non-selective engagement of other GPCR subtypes and/or from unwanted downstream signaling responses could be greatly diminished. Although such ligands offer these advantages amongst others, much work is still required in order to fully benefit from their development and understand their mechanism. As we studied our peptides of interest, we realized that most GPCRs engage multiple downstream signaling pathways, which are in many cases cell, tissue and/or context specific. Accordingly, some of these ligands also seem to behave differently depending on which system is used to study them, as seen with SC0023 on IP1 production. These factors indeed add to the complexity of studying these ligands and emphasize how essential it is to better define the entire signaling signature of such ligands. These signatures should be defined for marketed drugs, orthosteric ligands as well as for putative biased and allosteric ligands to be studied. Particularly, some research should focus on these signaling behaviors in normal vs. pathological conditions, thus

highlighting both the similarities and differences. Establishing such signaling signatures becomes therefore essential in programs that aim at improving drug efficacy or at repurposing drugs.

4.2 Future work and preliminary data

I have attempted to establish a signaling signature for different peptides, more specifically SC0023 and SC0024 on different AngII-mediated effects, which had shown interesting but opposite effects. Given their differential effects on various pathways and their cell type specificity, this resulted in a relatively difficult task. Though the results discussed above were more focused on Gαq-dependent pathways and different downstream responses such as proliferation, there are many additional signaling pathways that would be interesting to study in the future. Such pathways that have not yet been investigated, but might play a role in AngII-dependent IP1 production, are the Gα12/13-mediated signaling pathways, which are known to lead to the activation of the Rho/ROCK (Siehler 2009). Although this relationship between Gα12/13 and Rho activation is well accepted, it has been shown that Gαq/11, in certain cases, can also activate Rho (Vogt, Grosse et al. 2003). However, with the goal of investigating the effect of SC0023 on AngII-mediated Gα12/13 pathways, we could use a Fluorescence Resonance Energy Transfer (FRET) assay. This assay makes use of a well-characterized unimolecular biosensor Raichu-RBD, which will allow us to study the activation of Rho (Fig. 4.2A) (Goupil, Tassy et al. 2010). Interestingly, preliminary results using HEK293 cells suggest that SC0023 may be inhibiting AngII-induced Rho activation (Fig. 4.2B-C).

In order to support the preliminary data suggesting there is a NAM effect of SC0023 on AngII-mediated Gα12/13-mediated pathways, I also decided to make use of another assay. Cell/membrane ruffling was chosen as a cellular response that is downstream from the activation

of Rho, and can be studied in HEK293 cells (Chhabra and Higgs 2007; Cotton, Boulay et al. 2007). Ruffling occurs when there is cytoskeleton reorganization within the cell, which is often a prelude for cell migration (Borm, Requardt et al. 2005). Though these results are still preliminary, SC0023 was also able to inhibit AngII-induced membrane ruffling (Fig. 4.3). These suggest that SC0023 is a NAM of AngII-mediated Rho activation and membrane ruffling in HEK293 cells, which are mostly Gα12/13-dependent responses. Given that differential effects of SC0023 on AngII-mediated IP1 production in HEK293 cells and VSMCs, these preliminary results suggest that the potent effect seen particularly in VSMCs is probably not mediated through a Gα12/13-dependent production of IP1. However, testing the activation of Rho in VSMCs would be important to support these preliminary data. In addition, the role of Gαi/o in the production of IP1 remains an interesting pathway to investigate, in both cell types. One way to test the involvement of the Gαi/o protein family is by using PTX (pertussis toxin), which can prevent this class of G protein from interacting with the receptor (Burns 1988). Moreover, in order to fully establish a signaling signature for this peptide, the direct effect of the peptides on the coupling of different G proteins should be tested. To measure G protein activation, the non hydrolysable GTP analog [35S]GTPγS could be used (Cooper, McMurchie et al. 2009). An immunosprecipitation of the alpha subunit of interest should allow studying the extent of coupling of each G protein in the presence or absence of SC0023 and SC0024. In addition, it would be interesting to know which cellular responses are affected by this peptide. For example, migration and contraction assays would be of great value, given the modulatory effects of SC0023 on Rho activation and cell ruffling in HEK293 cells.

In terms of our second aim, involving the structure/function studies, there are different assays that could lead to the identification of the targets and binding sites. Two approaches are

being undertaken to reach this goal. First, as mentioned in the results section, we are using the peptide with a benzoylphenylalanine (Bpa) modification, which can be used as a photoactivable probe. Having the analog SC0024-Bpa5 that retained its inhibitory effect on proliferation, we can use it as a tool for this part of our work. Unfortunately, this approach has proven to be difficult because of the obstacles that were faced when trying to characterize this particular compound. Once its functional effect was established, the next logical step was to perform binding studies to confirm its selective binding to AT1R. Though the iodination of the parent compound SC0024 was proven to be inefficacious, we did not encounter any problems with SC0024-Bpa5. Problems only arose during the analysis of the binding results. Preliminary data show that SC0024-Bpa5 is binding to VSMCs and HEK293 cells transfected with the AT1R but also to nonspecifically to HEK293 and COS-7 cells that were not transfected with the receptor (data not shown).

Though this was a minor setback, there are different ways to interpret these results. First, since we are not dealing with the parent compound, it is possible that the substitution of the amino acid in fifth position with a Bpa disrupted the structure of the peptide in such a way that its binding properties were modified. Another possibility is that SC0024 is in fact indirectly modulating AngII-dependent responses by binding to an entity that can then bind or be found in complex with the AT1R. This entity could in fact be present in all cell types used in the binding studies. Its effect would therefore be specific to AngII but would not be exerted through direct binding to AT1R. Also, it is possible that the iodinated form of SC0024-Bpa5 is "sticky" and that the selective binding to AT1R is buried under a greater amount of non-specific signal. In order to answer these questions, we are developing tools that will be used in the future.

In fact, another approach that is being developed, utilizes the rather new concept of "click chemistry". This approach has the advantage of using a biotin tag that will allow us to perform an immunoprecipitation (IP) of the ligand in complex with its target. This will also allow for the enrichment of the target and the use of mass spectrometry. For this, we had to synthesize another peptide that contained a propargylglycine (PAG). Again, we used the photoactivable SC0024-Bpa5, but added a PAG after the eighth position, which contains a glutamic acid. We started by testing the activity of this modified peptide on AngII-induced proliferation. Preliminary results show that this modified SC0024-Bpa5 has retained the capacity to inhibit proliferation (Fig. 4.4). Once it is confirmed that this modified peptide retained its effect, the next step will be to proceed to the crosslinking experiment. After the cells are put under a UV light in order to crosslink the peptide to the target, click chemistry will be used to biotinylate the ligand of interest. Then, we will perform an IP in order to concentrate ligand-bound complex. Provided that we finish with enough material, we will then be able to use mass spectrometry to ultimately identify the target.

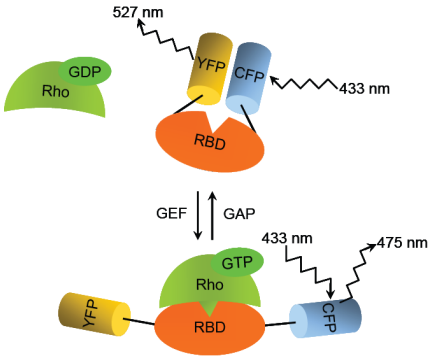
Finally, studying the structure-activity relationship of different peptides will be of great interest, as one of our future aims will be to increase the affinity and/or efficacy of the present peptides. This will be achieved by first synthesizing different analogs of the peptides and then testing their effects on selected functional assays (e.g. Proliferation assay). Having done an alanine-scan, we have already taken the first step towards understanding which amino acids are important for the activity of SC0024 on AngII-induced cell proliferation. To test the effect of lipophilicity and permeability of our peptide of interest, a second step would involve a lysine-scan and an aspartic-acid scan, which would either introduce a negative or a positive charge to our peptide. In addition, we will test the effect of steric hindrance by introducing a large amino acid such as tryptophan, in different positions of the peptide (Ortiz-Acevedo, Melendez et al.

2004). Also, though linear peptides can be used as therapeutic agents, most of these have the disadvantage of not being very stable since they can easily degraded by proteolytic enzymes. Accordingly, it would be valuable to design a cyclic peptide that would retain the activity of the parent compound but would also offer the advantage of having a better biological activity than linear peptides (Joo 2012).

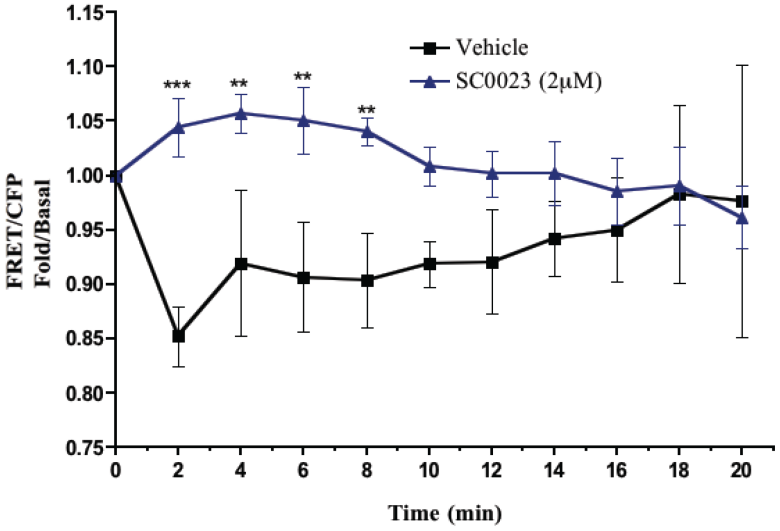
4.3 Conclusion

In conclusion, the characterization of the peptides derived from the ECL2 of the AT1R has resulted in different AngII-dependent effects. Defining the entire signaling signature for these different peptides would help us to understand their exact allosteric and biased effects, which could in turn lead us towards the rational design of such molecules. As we are aware that allosteric and biased ligands can be useful in terms of drug discovery, it would be important to thoroughly investigate the potential effects of such ligands. In addition, as mentioned earlier, defining the binding sites and confirming the targets of these peptides remains of great importance, and will be central to the future studies concerning this project.

A



B



C

High FRET (Rho inactivated)

Low FRET (Rho activated)

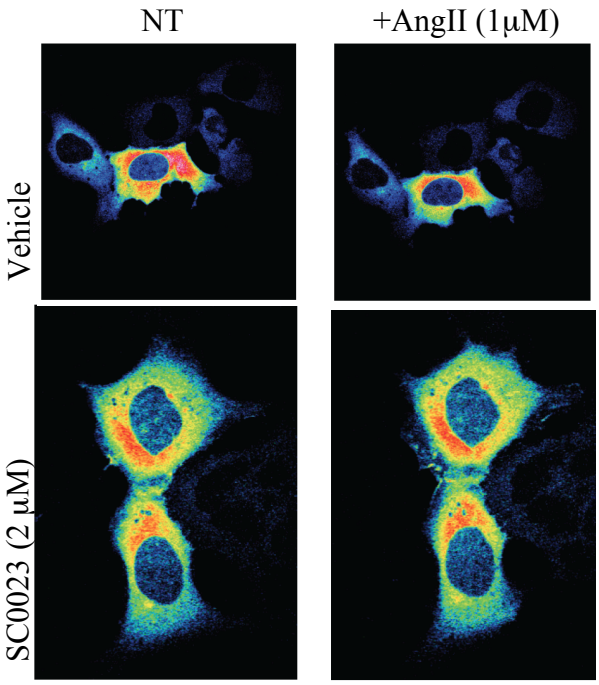
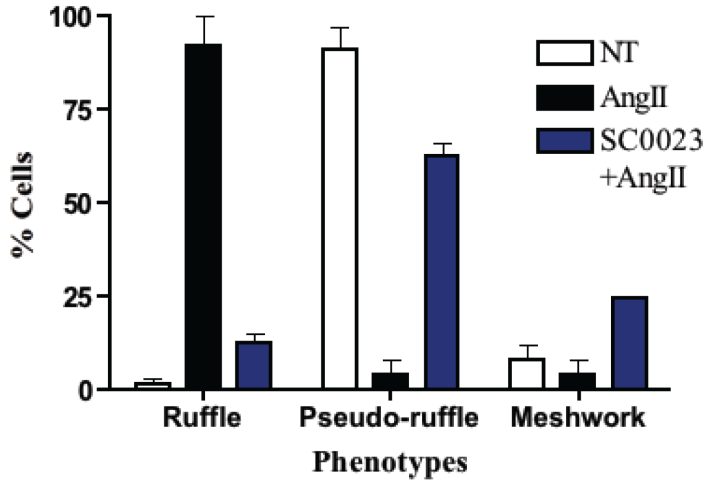


Figure 4.2: SC0023 inhibits the activation of Rho in HEK293 cells

A: A schematic of how the Raichu-RBD (rho binding domain) biosensor works. There is an initial FRET, which is lost upon Rho activation. B: Cells stably transfected with Flag-AT1R were plated in 35mm dishes. 24 hours later, the cells were transfected with 2.3μg of the biosensor Raichu-RBD. 48 hours later, the cells were serum-starved for 30 minutes followed by a pre-treatment with either vehicle (medium) or 2μM of SC0023. The FRET experiment was then performed on a confocal microscope using the program ZEN2009. The cells were stimulated with 1μM of AngII (time 0) and photos were taken before treatment and then every 2 minutes for 20 minutes. A FRET/CFP ratio was taken and then plotted as fold over basal. Results were analyzed using a 2-way ANOVA test followed by a bonferroni correction and are representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$. C: Images obtained by confocal microscopy showing the amount of FRET in each condition. A high FRET represents a state in which Rho is inactivated, while a low FRET shows there is activation of Rho.

A


100
75
50
25
0
% Cells
NT
AngII
SC0023
+AngII
Ruffle
Pseudo-ruffle
Meshwork
Phenotypes

B

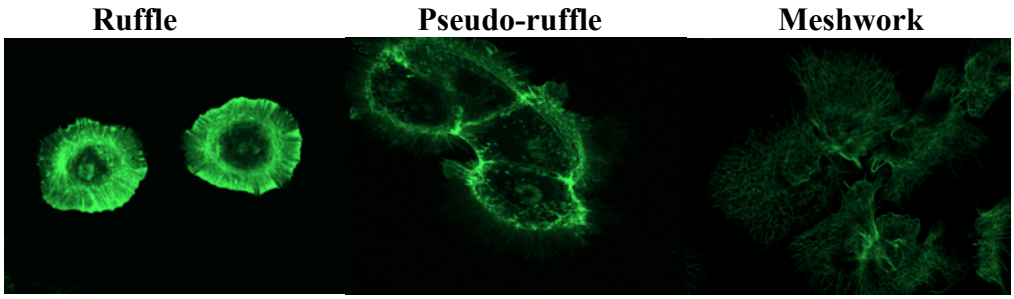

Ruffle
Pseudo-ruffle
Meshwork

Figure 4.3: SC0023 inhibits AngII-induced cell ruffling in HEK293 cells

HEK293 cells stably transfected with the AT1R were plated in 6-well plates onto microscopy slides. 48 hours later, the cells were serum-starved for 30 minutes, followed by a 30 minute pre-treatment with either vehicle (medium) or 10 μM of SC0023. Then, cells were treated with 1 μM of AngII for another 30 minutes. Then we followed our immunostaining protocol using Phalloidin-Alexa488 to stain actin, and mounted the slides on cover slips. Using the program ZEN2009 on our confocal microscope, multiple photos were taken for each condition. We then counted the cells displaying the different phenotypes and plotted them in percentages, in order to see the frequency of each phenotype. For each experiment, between 50 and 75 cells of each condition were used to compile the data.

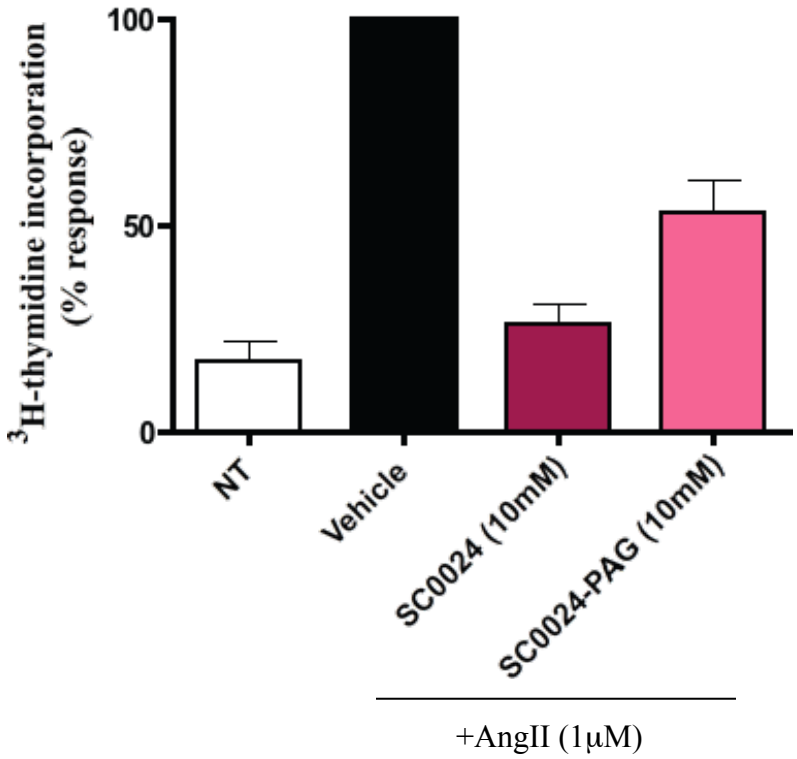


Figure 4.4: SC0024-Bpa5-PAG decreases AngII-induced VSMC proliferation

VSMCs were serum-starved overnight. The next day, the cells were pre-treated for 30 minutes with vehicle (medium) or 10μM of SC0024/SC0024-Bpa5-PAG. Then, they were treated with 1μM AngII for 24 hours. For the last 4-6 hours of the treatment, ^{3}H-thymidine was added to the wells. A liquid scintillation analyzer was used to collect the results and these were then plotted using the vehicle condition treated with 1μM of AngII as the reference point (100%)

References

Ahn, K. H., M. M. Mahmoud, et al. (2012). "Allosteric modulator ORG27569 induces CB1 cannabinoid receptor high affinity agonist binding state, receptor internalization, and Gi protein-independent ERK1/2 kinase activation." J Biol Chem **287**(15): 12070-12082.

Ahn, K. H., M. M. Mahmoud, et al. (2013). "Distinct roles of beta-arrestin 1 and beta-arrestin 2 in ORG27569-induced biased signaling and internalization of the cannabinoid receptor 1 (CB1)." J Biol Chem **288**(14): 9790-9800.

Ahuja, S., V. Hornak, et al. (2009). "Helix movement is coupled to displacement of the second extracellular loop in rhodopsin activation." Nat Struct Mol Biol **16**(2): 168-175.

Ali, M. S., B. Schieffer, et al. (1997). "Angiotensin II stimulates tyrosine phosphorylation and activation of insulin receptor substrate 1 and protein-tyrosine phosphatase 1D in vascular smooth muscle cells." J Biol Chem **272**(19): 12373-12379.

Allen, J. A., J. M. Yost, et al. (2011). "Discovery of beta-arrestin-biased dopamine D2 ligands for probing signal transduction pathways essential for antipsychotic efficacy." Proc Natl Acad Sci U S A **108**(45): 18488-18493.

Auger, G. A., J. E. Pease, et al. (2002). "Alanine scanning mutagenesis of CCR3 reveals that the three intracellular loops are essential for functional receptor expression." Eur J Immunol **32**(4): 1052-1058.

Baker, K. M. and J. F. Aceto (1990). "Angiotensin II stimulation of protein synthesis and cell growth in chick heart cells." Am J Physiol **259**(2 Pt 2): H610-618.

Berg, D., J. Godau, et al. (2011). "AFQ056 treatment of levodopa-induced dyskinesias: results of 2 randomized controlled trials." Mov Disord **26**(7): 1243-1250.

Berk, B. C., V. Vekshtein, et al. (1989). "Angiotensin II-stimulated protein synthesis in cultured vascular smooth muscle cells." Hypertension **13**(4): 305-314.

Berry-Kravis, E., D. Hessl, et al. (2009). "A pilot open label, single dose trial of fenobam in adults with fragile X syndrome." J Med Genet **46**(4): 266-271.

Bertini, R., M. Allegretti, et al. (2004). "Noncompetitive allosteric inhibitors of the inflammatory chemokine receptors CXCR1 and CXCR2: prevention of reperfusion injury." Proc Natl Acad Sci U S A **101**(32): 11791-11796.

Bisello, A., M. Chorev, et al. (2002). "Selective ligand-induced stabilization of active and desensitized parathyroid hormone type 1 receptor conformations." J Biol Chem **277**(41): 38524-38530.

Blanpain, C., J. M. Vanderwinden, et al. (2002). "Multiple active states and oligomerization of CCR5 revealed by functional properties of monoclonal antibodies." Mol Biol Cell **13**(2): 723-737.

Boerrigter, G., D. G. Soergel, et al. (2012). "TRV120027, a novel beta-arrestin biased ligand at the angiotensin II type I receptor, unloads the heart and maintains renal function when added to furosemide in experimental heart failure." Circ Heart Fail **5**(5): 627-634.

Borm, B., R. P. Requardt, et al. (2005). "Membrane ruffles in cell migration: indicators of inefficient lamellipodia adhesion and compartments of actin filament reorganization." Exp Cell Res **302**(1): 83-95.

Boucard, A. A., B. C. Wilkes, et al. (2000). "Photolabeling identifies position 172 of the human AT(1) receptor as a ligand contact point: receptor-bound angiotensin II adopts an extended structure." Biochemistry **39**(32): 9662-9670.
Burns, D. L. (1988). "Subunit structure and enzymic activity of pertussis toxin." Microbiol Sci **5**(9): 285-287.
Burson, J. M., G. Aguilera, et al. (1994). "Differential expression of angiotensin receptor 1A and 1B in mouse." Am J Physiol **267**(2 Pt 1): E260-267.
Chavkin, C., S. S. Schattauer, et al. (2014). "Arrestin-mediated activation of p38 MAPK: molecular mechanisms and behavioral consequences." Handb Exp Pharmacol **219**: 281-292.
Chhabra, E. S. and H. N. Higgs (2007). "The many faces of actin: matching assembly factors with cellular structures." Nat Cell Biol **9**(10): 1110-1121.
Chin, J. W., A. B. Martin, et al. (2002). "Addition of a photocrosslinking amino acid to the genetic code of Escherichiacoli." Proc Natl Acad Sci U S A **99**(17): 11020-11024.
Conner, M., S. R. Hawtin, et al. (2007). "Systematic analysis of the entire second extracellular loop of the V(1a) vasopressin receptor: key residues, conserved throughout a G-protein-coupled receptor family, identified." J Biol Chem **282**(24): 17405-17412.
Conway, A. and D. E. Koshland, Jr. (1968). "Negative cooperativity in enzyme action. The binding of diphosphopyridine nucleotide to glyceraldehyde 3-phosphate dehydrogenase." Biochemistry **7**(11): 4011-4023.
Cooper, T., E. J. McMurchie, et al. (2009). "[35S]GTPgammaS binding in G protein-coupled receptor assays." Methods Mol Biol **552**: 143-151.
Cotton, M., P. L. Boulay, et al. (2007). "Endogenous ARF6 interacts with Rac1 upon angiotensin II stimulation to regulate membrane ruffling and cell migration." Mol Biol Cell **18**(2): 501-511.
Cunningham, B. C. and J. A. Wells (1989). "High-resolution epitope mapping of hGH-receptor interactions by alanine-scanning mutagenesis." Science **244**(4908): 1081-1085.
Daemen, M. J., D. M. Lombardi, et al. (1991). "Angiotensin II induces smooth muscle cell proliferation in the normal and injured rat arterial wall." Circ Res **68**(2): 450-456.
de Gasparo, M., K. J. Catt, et al. (2000). "International union of pharmacology. XXIII. The angiotensin II receptors." Pharmacol Rev **52**(3): 415-472.
Decaillot, F. M., M. A. Kazmi, et al. (2011). "CXCR7/CXCR4 heterodimer constitutively recruits beta-arrestin to enhance cell migration." J Biol Chem **286**(37): 32188-32197.
DeWire, S. M., D. S. Yamashita, et al. (2013). "A G protein-biased ligand at the mu-opioid receptor is potently analgesic with reduced gastrointestinal and respiratory dysfunction compared with morphine." J Pharmacol Exp Ther **344**(3): 708-717.
Dogo-Isonagie, C., S. Lam, et al. (2012). "Peptides from second extracellular loop of C-C chemokine receptor type 5 (CCR5) inhibit diverse strains of HIV-1." J Biol Chem **287**(18): 15076-15086.
Dugourd, C., M. Gervais, et al. (2003). "Akt is a major downstream target of PI3-kinase involved in angiotensin II-induced proliferation." Hypertension **41**(4): 882-890.

Fatkenheuer, G., A. L. Pozniak, et al. (2005). "Efficacy of short-term monotherapy with maraviroc, a new CCR5 antagonist, in patients infected with HIV-1." Nat Med **11**(11): 1170-1172.
Fisher, A., E. Heldman, et al. (1993). "Selective signaling via unique M1 muscarinic agonists." Ann N Y Acad Sci **695**: 300-303.
Fredriksson, R., M. C. Lagerstrom, et al. (2003). "The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints." Mol Pharmacol **63**(6): 1256-1272.
Gilman, A. G. (1987). "G proteins: transducers of receptor-generated signals." Annu Rev Biochem **56**: 615-649.
Ginestier, C., S. Liu, et al. (2010). "CXCR1 blockade selectively targets human breast cancer stem cells in vitro and in xenografts." J Clin Invest **120**(2): 485-497.
Goodfriend, T. L., M. E. Elliott, et al. (1996). "Angiotensin receptors and their antagonists." N Engl J Med **334**(25): 1649-1654.
Goodman, O. B., Jr., J. G. Krupnick, et al. (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-450.
Goodman, W. G., G. A. Hladik, et al. (2002). "The Calcimimetic agent AMG 073 lowers plasma parathyroid hormone levels in hemodialysis patients with secondary hyperparathyroidism." J Am Soc Nephrol **13**(4): 1017-1024.
Goupil, E., S. A. Laporte, et al. (2012). "Functional selectivity in GPCR signaling: understanding the full spectrum of receptor conformations." Mini Rev Med Chem **12**(9): 817-830.
Goupil, E., D. Tassy, et al. (2010). "A novel biased allosteric compound inhibitor of parturition selectively impedes the prostaglandin F2alpha-mediated Rho/ROCK signaling pathway." J Biol Chem **285**(33): 25624-25636.
Goupil, E., V. Wisehart, et al. (2012). "Biasing the prostaglandin F2alpha receptor responses toward EGFR-dependent transactivation of MAPK." Mol Endocrinol **26**(7): 1189-1202.
Gurwitz, D., R. Haring, et al. (1994). "Discrete activation of transduction pathways associated with acetylcholine m1 receptor by several muscarinic ligands." Eur J Pharmacol **267**(1): 21-31.
Hains, M. D., M. R. Wing, et al. (2006). "Galpha12/13- and rho-dependent activation of phospholipase C-epsilon by lysophosphatidic acid and thrombin receptors." Mol Pharmacol **69**(6): 2068-2075.
Hashimoto, K., B. Malchow, et al. (2013). "Glutamate modulators as potential therapeutic drugs in schizophrenia and affective disorders." Eur Arch Psychiatry Clin Neurosci **263**(5): 367-377.
Hawes, B. E., L. M. Luttrell, et al. (1994). "Inhibition of G protein-coupled receptor signaling by expression of cytoplasmic domains of the receptor." J Biol Chem **269**(22): 15776-15785.
Hayashida, W., M. Horiuchi, et al. (1996). "Intracellular third loop domain of angiotensin II type-2 receptor. Role in mediating signal transduction and cellular function." J Biol Chem **271**(36): 21985-21992.
Hebert, T. E., S. Moffett, et al. (1996). "A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation." J Biol Chem **271**(27): 16384-16392.

Hemstapat, K., H. Da Costa, et al. (2007). "A novel family of potent negative allosteric modulators of group II metabotropic glutamate receptors." J Pharmacol Exp Ther **322**(1): 254-264.
Hepler, J. R. and A. G. Gilman (1992). "G proteins." Trends Biochem Sci **17**(10): 383-387.
Higuchi, S., H. Ohtsu, et al. (2007). "Angiotensin II signal transduction through the AT1 receptor: novel insights into mechanisms and pathophysiology." Clin Sci (Lond) **112**(8): 417-428.
Holloway, A. C., H. Qian, et al. (2002). "Side-chain substitutions within angiotensin II reveal different requirements for signaling, internalization, and phosphorylation of type 1A angiotensin receptors." Mol Pharmacol **61**(4): 768-777.
Hotokezaka, H., E. Sakai, et al. (2002). "U0126 and PD98059, specific inhibitors of MEK, accelerate differentiation of RAW264.7 cells into osteoclast-like cells." J Biol Chem **277**(49): 47366-47372.
Irie, A., E. Segi, et al. (1994). "Mouse prostaglandin E receptor EP3 subtype mediates calcium signals via Gi in cDNA-transfected Chinese hamster ovary cells." Biochem Biophys Res Commun **204**(1): 303-309.
Jacquemont, S., A. Curie, et al. (2011). "Epigenetic modification of the FMR1 gene in fragile X syndrome is associated with differential response to the mGluR5 antagonist AFQ056." Sci Transl Med **3**(64): 64ra61.
Joo, S. H. (2012). "Cyclic peptides as therapeutic agents and biochemical tools." Biomol Ther (Seoul) **20**(1): 19-26.
Khoury, E., S. Clement, et al. (2014). "Allosteric and biased g protein-coupled receptor signaling regulation: potentials for new therapeutics." Front Endocrinol (Lausanne) **5**: 68.
Kim, J., S. Ahn, et al. (2009). "Independent beta-arrestin2 and Gq/protein kinase Czeta pathways for ERK stimulated by angiotensin type 1A receptors in vascular smooth muscle cells converge on transactivation of the epidermal growth factor receptor." J Biol Chem **284**(18): 11953-11962.
Kim, S. Y., S. Kim, et al. (2011). "PKC inhibitors RO 31-8220 and Go 6983 enhance epinephrine-induced platelet aggregation in catecholamine hypo-responsive platelets by enhancing Akt phosphorylation." BMB Rep **44**(2): 140-145.
Klco, J. M., C. B. Wiegand, et al. (2005). "Essential role for the second extracellular loop in C5a receptor activation." Nat Struct Mol Biol **12**(4): 320-326.
Krupnick, J. G., V. V. Gurevich, et al. (1994). "Arrestin-rhodopsin interaction. Multi-site binding delineated by peptide inhibition." J Biol Chem **269**(5): 3226-3232.
Kruse, A. C., A. M. Ring, et al. (2013). "Activation and allosteric modulation of a muscarinic acetylcholine receptor." Nature **504**(7478): 101-106.
Lagerstrom, M. C. and H. B. Schioth (2008). "Structural diversity of G protein-coupled receptors and significance for drug discovery." Nat Rev Drug Discov **7**(4): 339-357.
Lan, H., M. M. Teeter, et al. (2009). "An intracellular loop 2 amino acid residue determines differential binding of arrestin to the dopamine D2 and D3 receptors." Mol Pharmacol **75**(1): 19-26.
Laporte, S. A., A. A. Boucard, et al. (1999). "Determination of peptide contact points in the human angiotensin II type I receptor (AT1) with photosensitive analogs of angiotensin II." Mol Endocrinol **13**(4): 578-586.

Laporte, S. A., R. H. Oakley, et al. (1999). "The beta2-adrenergic receptor/betaarrestin complex recruits the clathrin adaptor AP-2 during endocytosis." Proc Natl Acad Sci U S A **96**(7): 3712-3717.
Laragh, J. H., M. Angers, et al. (1960). "Hypotensive agents and pressor substances. The effect of epinephrine, norepinephrine, angiotensin II, and others on the secretory rate of aldosterone in man." JAMA **174**: 234-240.
Lee, C., M. D. Luck, et al. (1995). "Homolog-scanning mutagenesis of the parathyroid hormone (PTH) receptor reveals PTH-(1-34) binding determinants in the third extracellular loop." Mol Endocrinol **9**(10): 1269-1278.
Lee, T., R. Schwandner, et al. (2008). "Identification and functional characterization of allosteric agonists for the G protein-coupled receptor FFA2." Mol Pharmacol **74**(6): 1599-1609.
Leu, F. P. and M. Nandi (2010). "GPCR somatostatin receptor extracellular loop 2 is a key ectodomain for making subtype-selective antibodies with agonist-like activities in the pancreatic neuroendocrine tumor BON cell line." Pancreas **39**(8): 1155-1166.
Lin, S. Y. and T. L. Goodfriend (1970). "Angiotensin receptors." Am J Physiol **218**(5): 1319-1328.
Lin, Y. C., M. Boone, et al. (2014). "Genome dynamics of the human embryonic kidney 293 lineage in response to cell biology manipulations." Nat Commun **5**: 4767.
Luttrell, L. M. (2013). "Arrestin pathways as drug targets." Prog Mol Biol Transl Sci **118**: 469-497.
Makita, N., J. Sato, et al. (2007). "An acquired hypocalciuric hypercalcemia autoantibody induces allosteric transition among active human Ca-sensing receptor conformations." Proc Natl Acad Sci U S A **104**(13): 5443-5448.
Marrero, M. B., B. Schieffer, et al. (1997). "Role of Janus kinase/signal transducer and activator of transcription and mitogen-activated protein kinase cascades in angiotensin II- and platelet-derived growth factor-induced vascular smooth muscle cell proliferation." J Biol Chem **272**(39): 24684-24690.
May, L. T. and A. Christopoulos (2003). "Allosteric modulators of G-protein-coupled receptors." Curr Opin Pharmacol **3**(5): 551-556.
May, L. T., K. Leach, et al. (2007). "Allosteric modulation of G protein-coupled receptors." Annu Rev Pharmacol Toxicol **47**: 1-51.
Menard, J., T. T. Guyene, et al. (1991). "Renin release regulation during acute renin inhibition in normal volunteers." Hypertension **18**(3): 257-265.
Mikoshiba, K. (2007). "The IP3 receptor/Ca2+ channel and its cellular function." Biochem Soc Symp(74): 9-22.
Mishra, R. K., M. H. Makman, et al. (1999). "Modulation of agonist stimulated adenylyl cyclase and GTPase activity by L-pro-L-leu-glycinamide and its peptidomimetic analogue in rat striatal membranes." Neurosci Lett **269**(1): 21-24.
Mitchell, K. D., B. Braam, et al. (1992). "Hypertensinogenic mechanisms mediated by renal actions of renin-angiotensin system." Hypertension **19**(1 Suppl): I18-27.
Monod, J., J. Wyman, et al. (1965). "On the Nature of Allosteric Transitions: A Plausible Model." J Mol Biol **12**: 88-118.
Mukherjee, S., K. Palczewski, et al. (1999). "beta-arrestin-dependent desensitization of luteinizing hormone/choriogonadotropin receptor is prevented by a synthetic

peptide corresponding to the third intracellular loop of the receptor." J Biol Chem **274**(19): 12984-12989.
Newby, A. C. and S. J. George (1993). "Proposed roles for growth factors in mediating smooth muscle proliferation in vascular pathologies." Cardiovasc Res **27**(7): 1173-1183.
Nishibe, S., M. I. Wahl, et al. (1990). "Increase of the catalytic activity of phospholipase C-gamma 1 by tyrosine phosphorylation." Science **250**(4985): 1253-1256.
Ohtsu, H., H. Suzuki, et al. (2006). "Angiotensin II signal transduction through small GTP-binding proteins: mechanism and significance in vascular smooth muscle cells." Hypertension **48**(4): 534-540.
Oliverio, M. I., H. S. Kim, et al. (1998). "Reduced growth, abnormal kidney structure, and type 2 (AT2) angiotensin receptor-mediated blood pressure regulation in mice lacking both AT1A and AT1B receptors for angiotensin II." Proc Natl Acad Sci U S A **95**(26): 15496-15501.
Olson, D. M. and C. Ammann (2007). "Role of the prostaglandins in labour and prostaglandin receptor inhibitors in the prevention of preterm labour." Front Biosci **12**: 1329-1343.
Ortiz-Acevedo, A., M. Melendez, et al. (2004). "Tryptophan scanning mutagenesis of the gammaM4 transmembrane domain of the acetylcholine receptor from Torpedo californica." J Biol Chem **279**(40): 42250-42257.
Pals-Rylaarsdam, R., V. V. Gurevich, et al. (1997). "Internalization of the m2 muscarinic acetylcholine receptor. Arrestin-independent and -dependent pathways." J Biol Chem **272**(38): 23682-23689.
Peeters, M. C., G. J. van Westen, et al. (2011). "GPCR structure and activation: an essential role for the first extracellular loop in activating the adenosine A2B receptor." FASEB J **25**(2): 632-643.
Percherancier, Y., Y. A. Berchiche, et al. (2005). "Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers." J Biol Chem **280**(11): 9895-9903.
Peri, K. G., C. Quiniou, et al. (2002). "THG113: a novel selective FP antagonist that delays preterm labor." Semin Perinatol **26**(6): 389-397.
Pierce, K. L. and R. J. Lefkowitz (2001). "Classical and new roles of beta-arrestins in the regulation of G-protein-coupled receptors." Nat Rev Neurosci **2**(10): 727-733.
Quoyer, J., J. M. Janz, et al. (2013). "Pepducin targeting the C-X-C chemokine receptor type 4 acts as a biased agonist favoring activation of the inhibitory G protein." Proc Natl Acad Sci U S A **110**(52): E5088-5097.
Rafei, M., Y. A. Berchiche, et al. (2009). "An engineered GM-CSF-CCL2 fusokine is a potent inhibitor of CCR2-driven inflammation as demonstrated in a murine model of inflammatory arthritis." J Immunol **183**(3): 1759-1766.
Rafei, M., P. M. Campeau, et al. (2009). "Selective inhibition of CCR2 expressing lymphomyeloid cells in experimental autoimmune encephalomyelitis by a GM-CSF-MCP1 fusokine." J Immunol **182**(5): 2620-2627.
Rajagopal, S., K. Rajagopal, et al. (2010). "Teaching old receptors new tricks: biasing seven-transmembrane receptors." Nat Rev Drug Discov **9**(5): 373-386.
Raje, N., S. Kumar, et al. (2004). "Combination of the mTOR inhibitor rapamycin and CC-5013 has synergistic activity in multiple myeloma." Blood **104**(13): 4188-4193.

Reversi, A., V. Rimoldi, et al. (2005). "The oxytocin receptor antagonist atosiban inhibits cell growth via a "biased agonist" mechanism." J Biol Chem **280**(16): 16311-16318.
Ribas, C., P. Penela, et al. (2007). "The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling." Biochim Biophys Acta **1768**(4): 913-922.
Rihakova, L., C. Quiniou, et al. (2009). "VRQ397 (CRAVKY): a novel noncompetitive V2 receptor antagonist." Am J Physiol Regul Integr Comp Physiol **297**(4): R1009-1018.
Roe, M. W., J. R. Hepler, et al. (1989). "Platelet-derived growth factor and angiotensin II cause increases in cytosolic free calcium by different mechanisms in vascular smooth muscle cells." J Cell Physiol **139**(1): 100-108.
Saavedra, J. M. (1992). "Brain and pituitary angiotensin." Endocr Rev **13**(2): 329-380.
Saito, Y. and B. C. Berk (2001). "Transactivation: a novel signaling pathway from angiotensin II to tyrosine kinase receptors." J Mol Cell Cardiol **33**(1): 3-7.
Saito, Y. and B. C. Berk (2002). "Angiotensin II-mediated signal transduction pathways." Curr Hypertens Rep **4**(2): 167-171.
Samson, M., G. LaRosa, et al. (1997). "The second extracellular loop of CCR5 is the major determinant of ligand specificity." J Biol Chem **272**(40): 24934-24941.
Scarselli, M. and J. G. Donaldson (2009). "Constitutive internalization of G protein-coupled receptors and G proteins via clathrin-independent endocytosis." J Biol Chem **284**(6): 3577-3585.
Scarselli, M., B. Li, et al. (2007). "Multiple residues in the second extracellular loop are critical for M3 muscarinic acetylcholine receptor activation." J Biol Chem **282**(10): 7385-7396.
Seko, T., M. Ito, et al. (2003). "Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle." Circ Res **92**(4): 411-418.
Shenoy, S. K., M. T. Drake, et al. (2006). "beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor." J Biol Chem **281**(2): 1261-1273.
Shi, L. and J. A. Javitch (2004). "The second extracellular loop of the dopamine D2 receptor lines the binding-site crevice." Proc Natl Acad Sci U S A **101**(2): 440-445.
Siehler, S. (2009). "Regulation of RhoGEF proteins by G12/13-coupled receptors." Br J Pharmacol **158**(1): 41-49.
Small, K. M., S. L. Forbes, et al. (2000). "A four amino acid deletion polymorphism in the third intracellular loop of the human alpha 2C-adrenergic receptor confers impaired coupling to multiple effectors." J Biol Chem **275**(30): 23059-23064.
Smith, N. J., R. J. Ward, et al. (2011). "Extracellular loop 2 of the free fatty acid receptor 2 mediates allosterism of a phenylacetamide ago-allosteric modulator." Mol Pharmacol **80**(1): 163-173.
Smith, S. W., K. L. Ferguson, et al. (2008). "Prolonged severe hypotension following combined amlodipine and valsartan ingestion." Clin Toxicol (Phila) **46**(5): 470-474.
Staus, D. P., L. M. Wingler, et al. (2014). "Regulation of beta2-Adrenergic Receptor Function by Conformationally Selective Single-Domain Intrabodies." Mol Pharmacol **85**(3): 472-481.
Stocchi F., D. A., Hattori N., Hauser R.A., Lang A.E., Poewe W., Rascol O., Stacy M., Tolosa E., Trenkwalder C., Gao H., Nagel J., Gomez-Mancilla B., Merschhemke M., Ekin S. and

Abi-Saab W. (2011). "15th International Congress of Parkinson's Disease and Movement Disorders." Toronto, Canada.
Tarasova, N. I., W. G. Rice, et al. (1999). "Inhibition of G-protein-coupled receptor function by disruption of transmembrane domain interactions." J Biol Chem **274**(49): 34911-34915.
Thathiah, A., K. Horre, et al. (2013). "beta-arrestin 2 regulates Abeta generation and gamma-secretase activity in Alzheimer's disease." Nat Med **19**(1): 43-49.
Tomaschitz, A., S. Pilz, et al. (2010). "Aldosterone and arterial hypertension." Nat Rev Endocrinol **6**(2): 83-93.
Valant, C., C. C. Felder, et al. (2012). "Probe dependence in the allosteric modulation of a G protein-coupled receptor: implications for detection and validation of allosteric ligand effects." Mol Pharmacol **81**(1): 41-52.
van Koppen, C. J. and K. H. Jakobs (2004). "Arrestin-independent internalization of G protein-coupled receptors." Mol Pharmacol **66**(3): 365-367.
Violin, J. D., S. M. DeWire, et al. (2010). "Selectively engaging beta-arrestins at the angiotensin II type 1 receptor reduces blood pressure and increases cardiac performance." J Pharmacol Exp Ther **335**(3): 572-579.
Violin, J. D. and R. J. Lefkowitz (2007). "Beta-arrestin-biased ligands at seven-transmembrane receptors." Trends Pharmacol Sci **28**(8): 416-422.
Vlahos, C. J., W. F. Matter, et al. (1994). "A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002)." J Biol Chem **269**(7): 5241-5248.
Vogt, S., R. Grosse, et al. (2003). "Receptor-dependent RhoA activation in G12/G13-deficient cells: genetic evidence for an involvement of Gq/G11." J Biol Chem **278**(31): 28743-28749.
Wang, C., H. Wu, et al. (2013). "Structure of the human smoothened receptor bound to an antitumour agent." Nature **497**(7449): 338-343.
Weir, M. R. and V. J. Dzau (1999). "The renin-angiotensin-aldosterone system: a specific target for hypertension management." Am J Hypertens **12**(12 Pt 3): 205S-213S.
Wheatley, M., D. Wootten, et al. (2012). "Lifting the lid on GPCRs: the role of extracellular loops." Br J Pharmacol **165**(6): 1688-1703.
Wisler, J. W., S. M. DeWire, et al. (2007). "A unique mechanism of beta-blocker action: carvedilol stimulates beta-arrestin signaling." Proc Natl Acad Sci U S A **104**(42): 16657-16662.
Wootten, D., A. Christopoulos, et al. (2013). "Emerging paradigms in GPCR allostery: implications for drug discovery." Nat Rev Drug Discov **12**(8): 630-644.
Wu, D. Q., C. H. Lee, et al. (1992). "Activation of phospholipase C by the alpha subunits of the Gq and G11 proteins in transfected Cos-7 cells." J Biol Chem **267**(3): 1811-1817.
Xiao, J., Z. Huang, et al. (2013). "Identification and optimization of small-molecule agonists of the human relaxin hormone receptor RXFP1." Nat Commun **4**: 1953.
Yang, L. J., G. Baffy, et al. (1991). "Pertussis toxin-sensitive Gi protein involvement in epidermal growth factor-induced activation of phospholipase C-gamma in rat hepatocytes." J Biol Chem **266**(33): 22451-22458.
Zarbock, A., M. Allegretti, et al. (2008). "Therapeutic inhibition of CXCR2 by Reparixin attenuates acute lung injury in mice." Br J Pharmacol **155**(3): 357-364.

Zerbib, F., S. Bruley des Varannes, et al. (2011). "Randomised clinical trial: effects of monotherapy with ADX10059, a mGluR5 inhibitor, on symptoms and reflux events in patients with gastro-oesophageal reflux disease." Aliment Pharmacol Ther **33**(8): 911-921.

Zerbib, F., C. Keywood, et al. (2010). "Efficacy, tolerability and pharmacokinetics of a modified release formulation of ADX10059, a negative allosteric modulator of metabotropic glutamate receptor 5: an esophageal pH-impedance study in healthy subjects." Neurogastroenterol Motil **22**(8): 859-865, e231.

Zhan, Y., S. Kim, et al. (2003). "Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression." Arterioscler Thromb Vasc Biol **23**(5): 795-801.

Zhao, Y., X. Chen, et al. (2010). "Angiotensin II/angiotensin II type I receptor (AT1R) signaling promotes MCF-7 breast cancer cells survival via PI3-kinase/Akt pathway." J Cell Physiol **225**(1): 168-173.

Zhao, Y., J. Liu, et al. (2005). "Role of Ras/PKCzeta/MEK/ERK1/2 signaling pathway in angiotensin II-induced vascular smooth muscle cell proliferation." Regul Pept **128**(1): 43-50.

Zhu, X. F., Z. C. Liu, et al. (2001). "EGFR tyrosine kinase inhibitor AG1478 inhibits cell proliferation and arrests cell cycle in nasopharyngeal carcinoma cells." Cancer Lett **169**(1): 27-32.

Zimmerman, B., A. Beautrait, et al. (2012). "Differential beta-arrestin-dependent conformational signaling and cellular responses revealed by angiotensin analogs." Sci Signal **5**(221): ra33.