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Update on Angiotensin II Subtype 2 Receptor: Focus on Peptide and Non-peptide Agonists

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Running Title Page:

Update on Angiotensin II Subtype 2 Receptor Agonists

Number of text pages: 37

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Non-standard abbreviations

Full name	Abbreviations
Abdominal aortic aneurysm	AAA
Apolipoprotein E knockout	$ApoE^{-/-}$
Asymmetric dimethylarginine	ADMA
Atrial natriuretic peptide	ANP
Extracellular signal-regulated kinase	ERK
Human coronary artery endothelial cells	hCAECs
Human coronary artery vascular smooth muscle cells	hCAVSMCs
Human Embryonic Kidney-293	HEK-293
Idiopathic pulmonary fibrosis	IPF
Mitogen-activated protein kinase	MAPK
Mitogen-activated protein kinase phosphatase-I	MKP-I
N- nitro-L-arginine methyl ester	L-NAME
Nicotinamide adenine dinucleotide phosphate	NADPH
Protein tyrosine Phosphatase 1B	PT1B
Ras-related C3 botulinum toxin substrate 1	Rac1
Reverse Transcriptase-polymerase chain reaction	RT-PCR
Signal Transducers and activators of transcription	STAT
Spontaneously hypotensive rats	SHR
Src-homology-2-domain containing tyrosine phosphatase-1	SHP-1
Transforming growth factor β1	TGFβ1
Thromboxane A2 receptor	TxA2R
Tissue kallikrein-deficient receptor	$TK^{-/-}$
Vascular smooth muscle cell	VSMC

Recommended section assignment:

Drug Discovery and Translational Medicine

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Abstract

Angiotensin II (Ang II) is the most dominant effector component of the renin-angiotensin system (RAS) that generally acts through binding to two main classes of G protein-coupled receptors, namely Ang II subtype-1 receptor (AT1R) and angiotensin II subtype-2 receptor (AT2R). Despite some controversial reports, the activation of AT2R generally antagonizes the effects of Ang II binding on AT1R. Studying AT2R signaling, function, and its specific ligands in cell culture or animal studies have confirmed its beneficial effects throughout the body. These characteristics classify AT2R as part of the protective arm of the RAS that, along with functions of Ang (1-7) through Mas receptor signaling modulates the harmful effects of Ang II on AT1R in the activated classical arm of the RAS. Although Ang II is the primary ligand for AT2R, we have summarized other natural or synthetic peptide and nonpeptide agonists with critical evaluation of their structure, mechanism of action, and biological activity.

Significance Statement: AT2R is one of the main components of the RAS and has a significant prospective for mediating the beneficial action of the RAS through its protective arm on the body's homeostasis. Targeting AT2R offers substantial clinical application possibilities for modulating various pathological conditions. This review provided concise information regarding the AT2R peptide and non-peptide agonists and their potential clinical applications for various diseases.

Keywords: *Renin-angiotensin system, Angiotensin II, Angiotensin II subtype 2 receptor, AT2R ligands, Peptide*

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1 Introduction

The renin-angiotensin (Ang) system (RAS) is an intricate complex cascade of Ang peptides that elicits diverse biological functions. Its unique feature lies in balancing the two opposite arms composed of enzymes/peptides/receptors within the system to maintain normal physiological conditions. Initially, it had been identified as a circulating endocrine system responsible for blood pressure, fluid and electrolyte balance, and systemic vascular resistance (Stroth and Unger, 1999). Detailed biochemical and molecular studies indicated that the concentration of Ang peptides in the blood is too high to be attributed to the systemic generation of these peptides alone as they present with a rapid clearance in the systemic circulation. These findings led to the consensus that a parallel tissue RAS in addition to the circulating component exists. The widespread presence of Ang converting enzymes (ACE) and Ang peptides receptors in various tissues also supported the tissue RAS concept (Paul et al., 2006).

As depicted schematically in Fig. 1, angiotensinogen is a serum glycoprotein produced by the liver and serves as the precursor for all Ang peptides. The plasma renin cleaves angiotensinogen to form an inactive decapeptide, Ang I. Then, ACE cleaves Ang I into an octapeptide, Ang II, which binds to the Ang II subtype 1 receptor (AT1R). However, recent findings suggest that Ang I is also metabolized by ACE2 (an ACE homolog), carboxypeptidase, and neprilysin, to give other truncated peptides like Ang (1-9) and Ang (1-7) as well. Ang (1-7) binds to the Mas receptor (MasR) and evokes the opposite action to that of AT1R (Santos et al., 2000). The complexity of RAS does not end here as Ang II can itself bind to different subtypes of AT1R, i.e., Ang II subtype 2 Receptor (AT2R) (Chiu et al., 1989). Ang II can be further metabolized by aminopeptidase A to give Ang III or ACE2 to produce Ang (1-7). Furthermore, Ang III has also been cleaved by aminopeptidase N to generate Ang IV. It has been reported that Ang III and Ang IV can bind to both AT1R and AT2R, but they have more selectivity to AT2R (Stroth and Unger, 1999).

With several different peptides acting in the RAS, there is a delicate balance between the peptides working on the system's opposite functional arms. On the classical arm, the ACE/Ang II/AT1R forms a

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dominant axis where Ang II evokes vasoconstriction, aldosterone secretion, increases sympathetic tone, and promotes cellular growth and proliferation. Its overactivation has been implicated in cardiovascular disorders, cancer, metabolic disorders, inflammation, and neurological disorders (Deshayes and Nahmias, 2005; Benigni et al., 2010; Gebre et al., 2018). On the protective arm, ACE/Ang II/AT2R acts as a protector by eliciting functional antagonism to AT1R. As the AT2R is scarcer in its expression than AT1R, the effects observed are subtle. The AT2R cannot be neglected as its stimulation results in protective effects such as vasodilation, antiproliferation, natriuresis, and anti-fibrosis (De Gasparo and Siragy, 1999). Another axis, ACE2/Ang (1-7)/MasR, also functions as a protective arm acting opposite to that of AT1R signaling. As the paper's focus lies in the ACE/Ang II/AT2R axis, here we discuss more the scope and implications of AT2R (Unger et al., 1996).

The rapid burst in the information about the complex and interconnected RAS, especially during the last 30 years, has brought the existence of AT2R into the limelight. Although belonging to the same receptor type, AT2R is widely diverse in terms of structure, basal confirmation, tissue distribution leading to different and opposite physiological activity to that of AT1R. There has been a well-developed characterization of AT1R due to more discoveries and exploration in the scientific domain. The difficulty in getting a proper cell line, lack of a suitable animal model, the discrepancy in the initial structural model, and tissue distribution have contributed to the maintenance of overall enigmatic characteristics of this receptor. For any receptor's proper characterization, it is of utmost importance to identify and develop agonists and antagonists. In this aspect, AT2R falls behind its counterpart AT1R; however, lately, efforts have been made to optimize its functions based on endogenous agonists. Most of the agonists reported are the peptides modified from Ang II, and only one small molecule named Compound 21 (C21) has been successfully established as an AT2R agonist. The lack of suitable pharmacokinetic characterization and pharmaceutical parameters have caused its little to no penetration into successful clinical applications but carries immense potential to do so (Steckelings et al., 2011).

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Well-developed literature reviews serve a significant role in adding existing knowledge available in any field and act as a tool for more scientific discoveries. Here we have found that there is limited information in the literature available regarding the AT2R specifications, function, agonist, and antagonist (Juillerat-Jeanneret, 2020). To facilitate research for further studies, we have provided an updated review of the structural and biological properties of AT2R. Along with this, we aim to provide a common platform for all agonists used to date with the critical analysis.

1.1 AT2R protein structure

The AT2R is a member of the superfamily G protein-coupled receptor. It has seven transmembrane (TM) helical domains with an extracellular N-terminal and intracellular C- terminal on either side of the membrane (De Gasparo et al., 2000). The receptor contains 5 N-glycosylation sites, 5 Ser/Thr phosphorylation sites, and 14 Cys residues. AT2R shows β hairpin conformations in the extracellular loop2 (ECL2), forming two disulfide bridges linking N-terminus with ECL3 (Cys^{35}-Cys^{290}) and helix III with ECL2 (Cys^{117}-Cys^{195}). The cDNA reading frame encodes a 363-amino acid protein with an approximate molecular weight of 41 KDa, although the molecular weight varies according to cell types due to differences in glycosylation (Kambayashi et al., 1993; Nakajima et al., 1993).

The AT2R is encoded by the gene residing on the X-chromosome and has three exons with the third exon's entire coding region (Lazard et al., 1994). Several cis-regulatory regions control the promoter activity of the AT2R gene (Agtr2), and its expression is downregulated by cyclic adenosine monophosphate (cAMP) with the inhibition of both the gene transcription and messenger ribonucleic acid (mRNA) stability (Murasawa et al., 1996). The confinement of AT2R in the X-chromosome's long arm may contribute to the variation of AT1R: AT2R level according to the gender, with this ratio being higher in males than females (Silva-Antonialli et al., 2004).

1.2 The divergent state of AT2R

Although AT1R and AT2R belong to the same receptor type, they share just 34% of homology, explaining their polar apart biological activity. The highest homology level occurs in a TM domain, and

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only a few commonalities are observed in the N-terminal and the loops between both receptors (De Gasparo et al., 2000). The third intracellular loop (ICL) and C-terminal have the lowest homology. The third ICL is regarded as the main site for G-protein coupling site accounted for its atypical signal transduction (Hayashida et al., 1996).

As both of the Ang II receptors have a similar affinity to endogenous ligand Ang II (3-4 nM), it gave rise to the theory that receptors share homogeneity in the Ang II binding site (Table 1). The earlier receptor mutational studies suggest that some essential commonalities between the two receptors exist, but there were divergent mechanisms within each subtype to complement the shared basic binding processes (Heerding et al., 1998).

Apart from divergent amino acid residues between Ang receptors, they also differ in their basal state's conformational structure. Generally, agonist activates G-protein-coupled receptors (GPCRs), but atypical AT2R remains in the constitutively active state without agonist receptor activation. The crystallography studies done in the human AT2R bound to the high-affinity antagonist revealed that it displays active-like conformation. The helix VIII's unusual conformation in AT2R regulates AT2R non-canonical activity by sterically blocking G protein/β arrestin and switching to coupling with G protein/β arrestin upon post-translational modifications and its environment (Zhang et al., 2017).

A series of AT1R/AT2R receptor chimeras was designed to characterize structural determinants responsible for cell signaling. This study revealed that unlike hydrophobic residue in GPCRs, the intracellular loop1 (ICL1) of AT2R has polar and charged residue, namely Gln^{72} and Lys^{73}. This polar residue destabilizes the interaction between helix VIII and ICL1 but promotes interaction of this helix with TM6 leading to the atypical conformation of the AT2R helix VIII domain. Other than this, the divergent C-terminal also aided in the atypical AT2R active conformations (Connolly et al., 2019). Additionally, the internal lock (Asn^{111}-Asn^{295}) of AT1R, which has to be dismantled for active conformation, was also observed in AT2R (Asn^{127}-Ser^{311}), but there were no hydrogen bonds between

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internal locks. This difference in the internal lock hinted that AT2R remains in a different basal state compared to typical AT1R (Asada et al., 2020).

The ligand-independent apoptosis was observed in the AT2R transfected cultured fibroblasts, epithelial cells, and vascular smooth muscle cells (Miura and Karnik, 2000). The GPCRs undergoing homo or hetero oligomerization is a common effect to induce cell signaling. The presence of Cys residue in the extracellular loops in the AT2R leaves an open site for both inter and intramolecular disulfide bonds. In another study, homooligomerization of AT2R in the serum-free pheochromocytoma (PC12W) cell line was proved by immunoblotting, and apoptotic cell signaling was observed without the input of any agonist for AT2R (Miura et al., 2005). The overexpression of AT2R by gene transfection in the lung adenocarcinoma (Pickel et al., 2010), rat insulinoma (INS-1) (Liu et al., 2015), human bladder cancer cell lines (Bac) (Pei et al., 2017) promotes apoptosis. Ang II or AT2R antagonists did not modulate this apoptotic effect, implicating that it was due to the constitutive activation of AT2R. The overexpression of AT2R also impaired insulin secretion in INS-1 and inhibited angiogenesis in the Bac.

Consistent with the constitutively active conformation theory, AT2R does not undergo desensitization and internalization, unlike its AT1R counterpart. The immunofluorescence microscopy studies in the human embryonic kidney cell line detailed that AT2R is not internalized into endosomes but localized in the plasma membrane upon agonist stimulation (Hein et al. 1997).

In the rat mesenteric artery, the administration of Ang II or CGP42112A, an AT2R agonist, evoked concentration-dependent vasorelaxation in the presence of an AT1R blockade. There was attenuation of vasoconstriction effects evoked by the Ang II via AT1R, but AT2R mediated relaxation was seen consistently in the same low dose of agonist, suggesting lack of AT2R internalization during the short term or long term AT1R blockade (Widdop et al., 2002).

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2 Distribution and expression of AT2R

2.1 Tissue distribution

The early studies conducted on the tissue distribution of AT2R were based on autoradiography, ligand binding, and in situ hybridization. They revealed that AT2Rs are predominantly found in the fetal tissues whose expression decreases rapidly after birth. In adults, their expressions were limited to the tissues of the brain, heart, adrenal glands, vascular endothelium, kidney, myometrium, and ovary (Steckelings et al., 2005; Singh and Karnik, 2016). This led to the opinion that AT2R is important in the growth, development, and differentiation process. In contrast, AT2R knockout mice ($AT2R^{-/-}$) had normal embryogenesis without any birth defects failing the commonly held notion (Hein et al., 1995). There was no clear justification provided between the accepted notion and its failed outcome, thus bringing this whole theory into controversy.

Later, western blot analysis conducted on the brain stem, kidney, and liver from male fetuses (3 days before birth), male neonates (3 days after birth), male and female adults (8 weeks old), and male aged (28 months old) rats showed increased AT2R expression with age contrary to the decline theory. They found that the tissues of the brain stem, liver and kidney fetuses, and neonates exhibited a significantly lower AT2R protein expression and higher AT1R expression compared with adult rats (Yu et al., 2010). Similar kinds of results were obtained when major mice organs like the heart, lung, liver, kidney, brain, and spinal cord were analyzed for protein expression (Gao et al., 2012). However, skin tissues showed different results as AT2R was upregulated, and AT1R was downregulated from fetal life to adulthood (Yu et al., 2014). This expression profile was supported by immunofluorescence studies, but no significant differences were found in AT1R and AT2R mRNA levels among fetal, neonatal, and adult mice. This study also highlighted the inverse relationship between AT1R and AT2R expression pattern suggesting the role of AT1R in fetal development and AT2R in adulthood (Gao et al., 2012).

Although no solid consensus has been reached regarding the discrepancy, it should be noted that western blotting measures total AT2R, including cytoplasmic (immature receptor) and plasma membrane receptor

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(mature receptor). Further investigation is needed using more specific antibodies against AT2R to explain the diametrical apart theory regarding tissue distribution of AT1R and AT2R expression. However, autoradiography and ligand binding studies only determine plasma membrane receptors (Yu et al., 2010). Secondly, western blot analysis has not been done extensively in all subspecific tissues like ligand binding, in situ hybridization, or autoradiography. More studies to explain the diametrical apart theory regarding tissue distribution of AT1R and AT2R expression are needed.

2.2 AT2R expression in pathological condition

Another unique feature of the AT2R is that its expression is upregulated in pathological conditions compared to normal physiological settings. The study done by (Ruiz-Ortega et al., 2003) showed that renal expression of AT2R increased when Ang II was systemically infused in the different experimental models of renal injury due to inflammation, apoptosis, and proteinuria. Similar results were obtained when AT2R expression was analyzed by real-time reverse transcriptase-polymerase chain reaction (RT-PCR), western blotting, and immunofluorescence labeling in cerebral ischemic models of rats. An increase in the density of AT2R was found centered around the peri-infarct zone when focal cerebral ischemia was induced, but the expression of AT1R remained unaltered (Li et al., 2005). A single-cell RT-PCR performed in the adult rat cardiomyocytes one day before and after myocardial infarction (MI) also showed the augmented AT2R expression after MI (Busche et al., 2000). Not only was this observed in various rat models of tissue and vascular injury, sciatic and optic nerve transection models also showed a marked surge in the AT2R expression suggesting its role in the healing process and maintaining normalcy (Steckelings et al., 2005; Namsolleck et al., 2014).

2.3 Factors affecting AT2R expression

The expression of AT2R is tightly regulated by intracellular and extracellular growth factors, including the growth stage of the cell and cell type. In the fibroblast cell line R3T3, known to express AT2R alone, the density of AT2R was low in the actively growing state but substantially increased in the confluent state. When serum and growth factors like bovine fibroblast factor, insulin-like growth factor-I, and

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transforming growth factor-β were added to quiescent R3T3, and another exclusively AT2R expressing cell line, PC12W, the AT2R expression was found to be inhibited. The decreased AT2R expression was attributed to their negative influence on the gene transcription of AT2R. The time frame in the incubation of nerve growth factor (NGF) also affected the AT2R mRNA expression in primary neuronal cell culture. Extracellular factors like Ang II or AT2R agonist CGP42112A stimulated AT2R in a time and concentration-dependent manner in the R3T3 cell line. Apart from this, the cell type on which growth factors are acted upon also influenced AT2R expression. For example, treatment of insulin in cultured neurons downregulates the AT2R, and in vascular smooth muscle cells (VSMC) upregulates the AT2R expression (Gallinat et al., 2000). Likewise, growth factors (phorbol ester, lysophosphatidic acid, and basic fibroblast growth factor) markedly suppressed mouse AT2R mRNA expression in the R3T3 but not in the VSMC (De Gasparo et al., 2000).

2.4 Controversial nature of AT2R in different disease states

Despite much evidence on AT2R beneficial effects, it is not too far from the controversy. There is some evidence contradicting the traditionally held belief that AT2R imposes its beneficial effects by antagonizing the Ang II through AT1R. The higher expression of AT2R in rat cardiomyocytes induced by adenovirus resulted in constitutive hypertrophy (D'Amore et al., 2005). It has been reported that ventricular myocyte-specific overexpression of AT2R promotes the development of dilated cardiomyopathy and heart failure in transgenic mice. It was more pronounced in mice with the highest copy number of 34, which resulted in heart failure and death (Yan et al., 2003).

Similarly, in another study, it has been shown that overexpression of AT2R in the heart of transgenic mice worsened cardiomyocyte hypertrophy, cardiac fibrosis, and upregulation of transforming growth factor β1 (TGFβ1) mediator for fibrotic and inflammatory responses. The cardioprotective effect of AT2R was prominent in the mice having low to medium copy numbers (i.e., 2-4). The mice with a high copy number (i.e., 9) showed deleterious effect post-myocardial infarction mediated through upregulated TGFβ1 and reactive oxygen species in the heart. The mechanism by which increased AT2R expression

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interacts with inflammatory mediators is still unknown (Xu et al., 2014). The possible explanations for observed discrepancies could be related to the relative AT2R to AT1R density ratio and signal pathway followed. There was a pattern followed which stated that the density of AT2R should be around 35-40% of AT1R to show beneficial effects (Masaki et al., 1998; Kurisu et al., 2003) and higher than that or equal to AT1R would start showing detrimental effect (Yan et al., 2003; D'Amore et al., 2005; Xu et al., 2014). Besides, the cellular signaling pathway involved in receptor activation also plays a significant role in determining aftermath physiological effects (Chow and Allen, 2016). The contribution of AT2R received more criticism when its stimulation tends to activate the same signaling pathway causing cardiomyocyte hypertrophy and cardiac fibrosis in parallel rather than in the opposite direction of AT1R activation. It has been reported that the AT2R gene-targeted knockout mice do not demonstrate hypertrophy and pressure overload, and cardiac contractile functions remained normal (Senbonmatsu et al., 2000; Ichihara et al., 2001). This effect was explained based on reduced protein synthesis and kinases activity, which was observed in AT2R knockout mice (Senbonmatsu et al., 2000). Matsushita et al. documented the AT2R mediated vascular osteogenesis through extracellular signal-regulated kinase (ERK), which was abolished by PD123319. These opposite effects have been attributed to the secondary signaling mechanism. Besides, the binding of PD123319 is not exclusive to AT2R, and it binds to alamandine receptor, MAS-related G-protein-coupled receptor D, as well (Matsushita et al., 2015). Zulli et al. reported that inhibition of AT2R results in opposing vasoactive effects in the diseased human radial artery, but the AT2R-mediated vasodilation remains dominant. Although the reason behind the dichotomized result of AT2R activation has largely remained unexplained, in-depth research of the signaling mechanism is warranted to demystify these observed unconventional effects (Zulli et al., 2014).

There is some evidence showing that AT2R activation has a similar effect as its counterpart. These observations diminish the feasibility of AT2R as a viable therapeutic target. However, the lack of a clear explanation of AT2R knockout animal models' different behavior and why no follow-up studies confirm the same finding raises doubt. The activation of both angiotensin receptors in the same direction may pose

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a risk, but synergistic beneficial effects could also be achieved during the AT1R blockade. In summary, before making any definite conclusion about the AT2R importance as a drug target, it is very crucial to clarify the impact of any variables involved in AT2R mediated pharmacological actions.

3 Signaling pathway for the AT2R

After the establishment of AT2R as a GPCR, similarities and divergent characteristics between Ang receptors were drawn. It was found that AT1R acts as a typical GPCR and couples to a wide variety of G protein in the same cell type, whereas AT2R is atypical and limited to Gi (Hansen et al., 2000) and some unknown G proteins (Buisson et al., 1995; Bedecs et al., 1997). This receptor is known to follow three different molecular events: 1) activation of protein phosphatases and protein dephosphorylation, 2) regulation of the nitric oxide (NO)–3,5cyclic guanosine monophosphate system (cGMP), and 3) stimulation of phospholipase A2 (PLA2) and release of arachidonic acid (AA) (Nouet and Nahmias, 2000). We briefly discusedd the AT2R signaling pathway, which has been reviewed in detail elsewhere (Sadashiva S. Karnik et al.).

3.1 Activation of protein phosphatases and protein dephosphorylation

The AT2R stimulation has been widely accepted to cause downregulation of growth factor-induced intracellular cascades, leading to kinase activation and protein dephosphorylation. Here the AT2R mediates the activation of 3 different Tyr, Ser, or Thr phosphatases, and they are Src homology -2 domain-containing Tyr phosphatase-1 (SHP-1), protein phosphatase 2A (PP2A), and mitogen-activated protein (MAP) kinase phosphatase (MKP)-I. All three phosphatases are responsible for AT2R mediated inactivation of the ERK cascade in different cell types. The inactivation of the ERK causes obstruction in the signaling transmission receptor on the surface of the cell to the DNA in the nucleus of the cell, thus inhibiting cell growth, proliferation and aiding in apoptosis (Fig. 2).

SHP-1 is a widely expressed inhibitory protein Tyr phosphatase (PTP) in the cells, acting as a checkpoint for unwanted cell growth. In the study done in the N1E-115 neuroblastoma cell line, AT2R mediated the activation of SHP-1 and interrupted the growth factor-induced ERK pathway. The activation of SHP-1

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mediated by AT2R is pertussis toxin insensitive independent of G_i signal (Bedecs et al., 1997). Rather, SHP-1 coupling may actually involve an atypical G-protein scaffolding mechanism, $G_{\beta\gamma}$-independent constitutive association of the receptor with G_s and SHP-1 (Feng et al., 2002). Similarly, PP2A is another important and versatile enzyme acting as the regulator of the cell cycle. The activation of PP2A by AT2R in cultured neurons from newborn rats has been reported to show the inhibition of ERK MAP kinase (MAPK) activities important for apoptosis (Shenoy et al., 1999).

Another highly conserved phosphatase, MKP-1, is also involved in down-regulating ERK cascades. The AT2R stimulation in the cardiac myocytes resulted in the activation of vanadate sensitive dual-specificity (Tyr and Thr) phosphatase leading to inactivation of the MAPK/ERK (Fischer et al., 1998). In the PC12W cells, the treatment of Ang II via AT2R caused stimulation of MKP-1 and impairment in the MAPK/ERK activation. The interference in this intracellular level led to the inactivation of cell survival factor B-cell lymphoma-2 (Bcl-2) in those cells, thus resulting in apoptosis (Horiuchi et al., 1997).

The AT2R mediated SHP-1 activation is also known to cause dephosphorylation of signal transducer and activator of transcription (STAT) pathway responsible for mediating cell differentiation, cell migration, and cell growth. In the study done in AT2R complementary deoxyribonucleic acid (cDNA) transfected VSMCs, inactivation of STAT was observed via the inhibition of serine phosphorylation, thereby resulting in the decrease of proto-oncogene c-fos transcription (Horiuchi et al., 1999). This is how AT2R recruits diverse intracellular pathways in various cell lines to show the same effect, i.e., protein dephosphorylation. Some of the pathways are reported in a similar cell line, hinting that more than one mechanism is involved and acts as an efficient cell lock system at different levels at various time points (Nouet and Nahmias, 2000; Nahmias and Boden, 2004). Furthermore, the level of AT2R protein expression also determines the apoptosis of cultured fibroblasts, epithelial cells, and VSMCs. Ligand independent apoptosis was also observed that involved a signaling pathway that included activation of p38 MAPK and caspase 3 (Miura and Karnik, 2000; Miura and Karnik, 2002).

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The AT2R activation has been found to be a negative regulator of scaffolding protein caveolin-1 (CAV1), inducing melanoma and breast cancer migration and invasion. This ability of AT2R is linked to activation of the protein tyrosine phosphatase 1B (PTP1B), dephosphorylation of CAV1, and inhibition of the CAV1/ Ras-related protein 5A (Rab5)/Ras-related C3 botulinum toxin substrate 1 (Rac1)signaling axis. Here, the AT2R stimulation was also shown to block CAV1-enhanced melanoma metastasis in a preclinical animal model (Martínez-Meza et al., 2019).

3.2 Regulation of the NO–cGMP pathway

Being an endogenously produced autacoid, NO controls a variety of biological processes, including vasodilation, neurotransmission, cell growth, apoptosis, and inflammation. Most of the biological effects of NO are thought to be mediated via stimulation of cGMP. Here, AT2R stimulation increases protein and gene expression of endothelial nitric oxide synthase (eNOS), facilitating NO production (Carey et al., 2000). This increased NO generation and subsequent rise in cGMP was observed in various cell lines like cultured bovine endothelial cells, dog coronary arteries, and isolated perfused rat renal arteries (Steckelings et al., 2005). The activation of cGMP promotes vasodilation through the inhibition of cytosolic-free calcium levels by several mechanisms. These mechanisms include: i) inhibition of inositol 1,4,5-trisphosphate (IP3)-mediated calcium release from intracellular stores, ii) removal and sequestration of intracellular calcium through calcium pump mechanisms, and iii) both direct and indirect inhibition of the extracellular calcium influx through voltage-gated calcium channels (Tsai and Kass, 2009).

The enhanced AT2R mediated NO/cGMP pathway promoted neuronal differentiation and outgrowth in NG10815-15, neuroblastoma, and glial cell lines (Côté et al., 1998). Later AT2R mediated NO/cGMP pathway and AT2R mediated MAPK (p42/p44 MAPK) were also found going hand in hand for the neuron differentiation in the same cell line model, suggesting more than one way for AT2R mediated action (Gendron et al., 2002) and different cell lines like PC12W (Zhao et al., 2003). On the other hand, AT2R encourages gastrointestinal sodium and water absorption by a pathway that induces stimulation of

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the sympathetic nervous system and NO/cGMP cascade. This way, AT2R acts as a functional antagonist for AT1R mediated inhibition of sodium and water absorption (Jin et al., 1998).

The RAS and kinin pathway have long been considered as partners for cardiovascular homeostasis. AT2R and kinins play an important role in cardioprotection and vasodilation, opposing the biological effects of AT1R. Siragy et al. attributed the increase of renal bradykinin (BK) to non-AT1R and hinted at the possible role of AT2R (Siragy et al., 1996). It was confirmed that the protective vasodilator response is mediated by the renal production of BK and NO through AT2R (Siragy and Carey, 1999) and these are reviewed briefly by the same group (Carey et al., 2000). Similarly, in the stroke-prone spontaneously hypertensive rats (SHR), continuous infusion of Ang II causes an increase in cGMP level (Fig. 3). This response was abolished by N-nitro-L-arginine methyl ester (L-NAME), an inhibitor of NO-synthase (NOS), AT2R blocking as well as using BK2 receptor (B2R) antagonist, supporting the role of BK/NO/cGMP (Gohlke et al., 1998).

The mechanism by which AT2R controls the release of BK and modulates the production of downstream vasoactive factors has been addressed in several experimental settings. One of the earliest pieces of evidence was given by Tsutsumi et al., where they overexpressed vascular smooth muscle-specific (VSM-specific) AT2R gene in mice (Tsutsumi et al., 1999). Here, AT2R mediated the acidosis by the inhibition of Na^+/H^+ exchanger activity, thus promoting kininogenase activity in aortic VSM cells and releasing BK. The increased level of BK enhanced endothelial BK2 receptor (B2)–mediated vasodilation through activation of the NO/cGMP system, resulting in an AT2R-mediated depressor effect.

Bergaya et al. explored the vascular kallikrein-kinin system, responsible for vascular kinins production, including BK. They recorded and compared the increase in perfused arteries' flow rate in wild-type animals ($TK^{+/+}$) and tissue kallikrein-deficient mice ($TK^{-/-}$). They found out that the AT2R antagonist PD123319 significantly reduced flow-induced dilation in $TK^{+/+}$ mice but had no significant effect in $TK^{-/-}$ mice. Furthermore, B2R antagonist, HOE-140, significantly reduced the response to flow in the wild-type animals ($AT2R^{+/+}$), but not in $AT2R^{-/-}$ mice, stating that functional AT2R and B2R are codependent

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on each other to elicit flow-dependent dilation (Bergaya et al., 2004). It has been confirmed that the cardioprotective effect of AT1R blockade is mediated via B2R and AT2R (Messadi-Laribi et al., 2007).

Dimerization between Ang and BK receptors has added a new dimension to understanding the nature of RAS and kinins pathway interaction. AT1R and B2R have been shown to undergo heterodimerization and demonstrate an inverse physiological relationship (Su, 2014). Similarly, Abadir et al. showed that there is a direct molecular interaction between AT2R and B2R. The authors illustrated that AT2R and B2R in the membrane of the PC12W cells are in close molecular proximity that gives rise to receptors dimerization resulting in enhanced NO and cGMP production (Abadir et al., 2006). This dimerization emphasizes that AT2R and B2R work in concert to amplify the expected biological effect in a mutually dependent way. Although this concept seems very novel with little literature support, this gap of information has given rise to the question about whether this phenomenon is particular to PC12W cells or it can be observed in various cell lines. Therefore, more investigation is needed to validate this concept in detail.

Furthermore, Zhu et al. attempted to shed light on the relationship between AT2R and kinins with their signaling mechanism using mouse coronary artery endothelial cells concerning kallikrein activation. It has been reported that AT2R-stimulation increases in prolyl carboxypeptidase (PRCP; a plasma prekallikrein activator) activation mediated by the tyrosine phosphatase SHP-1, which in turn stimulates the PRCP-dependent prekallikrein-kallikrein pathway(Fig. 3). Subsequently, PRCP cleaves the complex of high molecular weight kininogen and plasma prekallikrein to kallikrein generating BK. These events trigger the activation of serial cascades of NO/cGMP and subsequently cause vasodilation (Zhu et al., 2012). AT2R is also found to inhibit proximal tubular Na^+/K^+-ATPase, an active tubular sodium transporter, via NO/cGMP pathway in proximal tubules isolated from Sprague-Dawley rat, thus unraveling the mechanism by which the AT2R mediates dilation and natriuresis (Hakam and Hussain, 2006) (Fig. 3).

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3.3 Stimulation of PLA2 and release of AA

PLA2-AA is one of the common mechanisms responsible for diverse cellular functions. Here PLA2 is an enzyme that hydrolyzes phospholipids into AA. This AA acts as a precursor for different metabolites mediated by cyclooxygenase, lipoxygenase, or cytochrome P450 monooxygenase into a wide range of biologically active compounds (Balsinde et al., 2002). In the epithelial cells of the proximal renal tubule and cardiac myocytes, Ang II activated PLA2 via AT2R and induced sustained release of AA (Fig. 4). The elevated AA influences ion transport, thus playing an important role in the natriuresis and intracellular pH (Lokuta et al., 1994; Jacobs and Douglas, 1996). Similarly, Ang II binding to its neuronal AT2R in the brain also showed involvement modulation of membrane ionic currents and firing rate through the AA release and its metabolism by 12-lipoxygenase through serine/threonine phosphatase PP2As (Zhu et al., 2000).

The sustained release of the AA by the AT2R pathway also gives rise to the various epoxy derivatives of AA, dependent on cytochrome P450. These metabolites serve as an upstream mediator of MAPK in the renal cells (Dulin et al., 1998). Another study reported it was reported that Ang II activates the Tyr kinase- Shc-Grb2-Sos complex pathway, which is a growth factor modulating the signaling pathway. Through this unique pathway, there is an activation of p21 ras protein, an important cell signaling protein, due to the exchange of guanosine diphosphate (GDP) to guanosine triphosphate (GTP) in the renal cell. These studies provided a basis for linkage between AT2R and receptor Tyr kinase through lipid secondary messenger (Jiao et al., 1998).

4 AT2R ligands

Figure 5 presents the chemical structure of AT2R ligands as several natural peptides and some synthetic non-peptide small molecules.

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4.1 Angiotensin peptides

4.1.1 Ang II

Ang II, a main effector peptide in the RAS, is considered the primary endogenous agonist for AT2R. These eight amino acid peptides (Asp^1-Arg^2-Val^3-Tyr^4-Ile^5-His^6-Pro^7-Phe^8) have a similar binding site for both receptors but with different pharmacophores for each receptor subtypes (Miura and Karnik, 1999). The modifications of all Ang II side chains affected binding to the AT2R at nearly similar extents, whereas binding to the AT1R is significantly affected by modifications at side-chain positions of amino acids 2, 4, 6, and 7. They further revealed that the AT1R is in a constrained conformation and is activated only when bound to Ang II. In contrast, the AT2R is in a relaxed position, and no single interaction is critical for binding**.**

Rosenstrom et al. performed glycine scans where each amino acid in Ang II is replaced with Gly, and subsequent peptides analogs are tested for receptor binding activity. This study divulged that Arg^2 and positive charge in the N-terminal side chain were important for Ang II binding to the AT2R (Rosenström et al., 2004b).

The VSMCs showed reduced proliferation and inhibited MAPK activity when transfected AT2R cells were stimulated by Ang II (Nakajima et al., 1995). When the PC12W and R3T3 cells (that exclusively expressing AT2R) were treated with Ang II, it resulted in apoptosis via dephosphorylation of MAP kinase (Yamada et al., 1996). Otherwise, potent vasoconstrictor Ang II also shows a vasodilatory effect in the mesenteric microvessels involving the large-conductance, calcium- and voltage-activated potassium channel (Dimitropoulou et al., 2001). In contrast, there is evidence where Ang II vasodilatory effect via AT2R alone is not sufficient to exert hypotension effect without blockage of AT1R in the background (Gohlke et al., 1998). The vasodilation in SHR was observed due to the stimulation of the BK/NO/cGMP pathway. This has led to support of the hypothesis that the antagonistic effect of AT1R by Ang receptor blockers (ARBs) is a more successful therapy for hypertension as it makes Ang II in the tissue and plasma available to bind with AT2R.

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Indeed, several studies in human coronary microarteries (Batenburg et al., 2004) and resistance vessels of diabetic hypertensive patients (Savoia et al., 2007) have also reported a functional vasodilatory role for the AT2R. Oliverio et al. supported the same theory that vaso-regulation of AT2R comes into play only after the suppression of AT1R. They reported no alteration in blood pressure during the infusion of Ang II in mice lacking AT1R genes where AT2R modulated the blood pressure (Oliverio et al., 1998). When AT1R is blocked by an ARB (such as candesartan), the expression of AT2R is seen to be upregulated regionally in the *in vivo* rat model of reperfused myocardial infarction, having a beneficial effect on the infarct size and LV (left ventricle) dysfunction (Jugdutt and Menon, 2004). Some studies suggest that after administration of the ARBs, the circulating and tissue level of Ang II markedly increases, leading to an overstimulation of AT2R, indicating that the effect of ARBs could also be mediated by their action on AT2R (Parlakpinar et al., 2011). This was also tested in post-MI heart failure experimental models where cardioprotective effects were seen in the presence of AT1R blockade alone (Oishi et al., 2006). The AT2R agonist treatment alone has presented the promising result that its activation stills remain relevant (Chang et al., 2011). Nevertheless, whether AT2R therapy can act alone or it can be used as combination therapy with AT1R blockade remains debatable.

A similar kind of observation was made in terms of Ang II action on neuronal differentiation. When cultured Schwann cells expressing both AT1R and AT2R, treated with Ang II, the expression of the neurite-promoting protease nexin-1 decreased. The blockade of the AT1R and stimulation of the AT2R led to several-fold increases of nexin-1 favoring nerve regeneration (Bleuel et al., 1995). However, another study reported that Ang II via AT2R alone promoted the axonal elongation of postnatal rat retinal explants and dorsal root ganglia neurons *in vitro* and axonal regeneration of retinal ganglion cells after optic nerve crush *in vivo* (Lucius et al., 1998). The controversy regarding whether Ang II alone or background restriction in the AT1R is needed for the AT2R mediated activity pushed the need for more selective AT2R agonists forward, giving rise to modified Ang II and non-peptide agonists (Table 2).

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4.1.2 Modified Ang II peptide

When Ang II binds to the angiotensin receptors, the side chains of amino acids Val^3 and Ile^5 of Ang II are involved in productive hydrophobic interaction with one another in the receptor cavity. This produces turn conformations centered at Tyr^4 that generally serve as a recognition trigger in the peptide receptor interaction. Small variations of the turn geometry in the 3-5 region of Ang II result in a drastic loss in the AT1R binding affinity while having an only minor impact on the AT2R binding affinity (Schmidt et al., 1997). Ang II was optimized to make several derivatives of Ang II by mimicking γ-turn scaffold for AT2R selective activity. Most of the papers reported *in vitro* radioligand binding assay on rat liver membrane for AT1R and pig uterine membrane for AT2R, which are listed in Table 3. The binding affinity of Ang II, 4 amino-Phe^6-Ang II are taken as standard and compared with the modified peptide analogs.

One of the earliest reported Ang II analogs having a high binding affinity to AT2R is synthesized by the cyclization of Val^3 and Ile^5. The incorporation of thioacetalization in Ang II resulted in the compound (Table 2 and supplementary fig. 1, compound 1) having better AT2R selectivity compared to native Ang II (Lindman et al., 2003). Similarly, other Ang II analogs were synthesized by including tyrosine-functionalized 5,5-bicyclic thiazabicycloalkane dipeptide mimetics in the place of Tyr^4-Ile^5 residues. The α,α disubstituted chimeric amino acid derivative and on-resin bicyclization to a cysteine residue were assimilated while the peptide synthesis of Ang II gave rise to the compounds (Table 2 and supplementary fig. 1, compound 2 and 3) having equipotent affinity to AT2R with Ang II in nanomolar range but with far better AT2R selectivity (Johannesson et al., 2004). However, because of the fact that biological activity of these synthesized analogs is not evaluated, their agonistic activity cannot be confirmed. Here, we have reported modified Ang II compounds having a higher binding affinity to AT2R produced from the mentioned synthetic procedure in Table 3.

Apart from the γ-turn mimetic scaffold, it is discovered that the position of the guanidine group of the Arg^2 residue in space, in relation to the Tyr^4 side chain and the N-terminal end, was also critical for AT2R

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binding affinity (Table 2 and supplementary fig. 1, compound 4) (Rosenström et al., 2004b). The synthesized Ang II analog encompassing a benzodiazepine-based γ-turn-like scaffold having a suitable Arg^2 position in the sequence displayed high AT2R selectivity and exhibited AT2R affinity in the low nanomolar range. The same group (Rosenström et al., 2005) later improved benzodiazepine γ-turn mimetic by positioning at 9^{th} of benzodiazepine rather than 7^{th} position for serving as a handle for the attachment of N-terminal residue (Table 2 and supplementary fig. 1, compound 5). Additionally, this brought a 10-fold increase in the affinity to AT2R, which further supported the fact that a more favorable position for a geometrical turn will give rise to highly selective AT2R selective ligands. Furthermore, these pseudo peptides are able to show typical AT2R agonist activity by stimulating outgrowth of neurites and activating p42/p44 MAPK, anti-proliferative activity in the PC12Wcells (Rosenström et al., 2004a; Rosenström et al., 2005).

Several Ang II analogs having γ-turn mimetics scaffold are developed by the incorporation of 1,3,5-trisubstituted aromatic scaffolds in place of Val^3-Tyr^4-Ile^5, Val^3-Tyr^4, or Tyr^4-Ile^5. The compound (Table 2 &3, compound 6) having aromatic scaffolds replacingTyr^4-Ile^5 was reported to have maximum binding affinity in the nanomolar range amongst all the pseudo peptides due to the presence of Val^3 improving receptor interaction. This pseudo peptide showed equivalent agnostic activity to that of Ang II by stimulating neurite outgrowth at the equal concentration in AT2R expressing NG1085-15 cell line but no agonistic effect in an AT1R functional assay (Georgsson et al., 2005).

Another study by Jedhe et al. explored the inclusion of peptide-based hydrogen bonding-directing reverse-turn scaffold into an Ang II sequence. The foldamer-based Pro-Amb scaffold (Proline and 3-amino-2-methoxy-benzoic-acid dipeptide, Table 2 and supplementary figure 1, compound 7) is incorporated by replacing Val^3-Tyr^4 in Ang II structure. Nuclear magnetic resonance and circular dichroism studies confirmed γ turn-like conformation in Pro-Amb analogs in aqueous solution pointed out to robust bifurcated hydrogen bonding. Although derived compounds showed AT2R agonistic activity,

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i.e., neurite outgrowth in the NG1085-15 cell line, no information was disclosed regarding quantification of receptor binding affinity (Jedhe et al., 2016).

The Ang II binding to AT2R, other than being tolerant to the alteration in the amino acid structure, is also insensitive to the truncation of the Ang II sequence, unlike the AT1R counterpart as reported previously (De Gasparo et al., 1991). The acetylated pentapeptides Ac-Tyr-Val-His-Pro-Phe and Ac-Tyr-Val-His-Pro-Ile were reported to have high selectivity and Ki values in the nanomolar range, but no information was divulged regarding agonistic and/or antagonistic properties. Taking these as lead compounds, several different bicyclic aromatic scaffolds are introduced in the Tyr^4-Val^5 region that gave rise to 13 different pseudo peptides. Among them, the compound having 1,3,5-trisubstituted aromatic scaffolds with Ile in C-terminal (Table 2&3, compound 8) is found to have an equivalent affinity as Ang II to AT2R, whereas more than 10000-fold less as Ang II to AT1R. This compound also exerted agnostic effects at the AT2R based on its ability to induce neurite outgrowth. This study highlighted the lipophilic aliphatic C- terminal more favors AT2R interaction than aromatic side chain (Georgsson et al., 2006).

Another interesting approach in the field of peptidomimetics is the introduction of the β-amino acid in place of natural amino acids. This means an additional methylene group in the peptide backbone will be incorporated without a change in the side chain. A modification as simple as this, however, can affect the binding and stability of the peptides like Ang II, as reported by Jones et al. In this study, they have tested binding affinity and agonistic activity of each Ang II analogs by substituting individual β-amino acid in the sequence of the native ligand Ang II. From the competition-binding assay performed in the human embryonic kidney (HEK) cells transfected with either AT1R and AT2R, the β-Tyr^4-Ang II and β-Ile^5-Ang II are found to bring 1000-fold AT2R selectivity compared to Ang II. Besides that, β-Ile^5-Ang II is also found to have a 10-fold increase in half-life compared to a native peptide (28 minutes) in the plasma stability test with more active metabolites. These all gave good explanations on why only this compound is able to evoke both vasorelaxations in mouse aortic rings as well as *in vivo* depressor activity in SHR at a low level of AT1R blockade (Jones et al., 2011).

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4.1.3 Ang III, an Ang II metabolite:

The omnipresent aminopeptidase hydrolyses N-terminal Asp^{1} of Ang II to give heptapeptide, Ang III (Arg-Val-Tyr-Ile-His-Pro-Phe) (Fig. 1 and Table 2). This endogenous peptide has reduced potency towards AT1R that has been explained by the importance of interaction between Asp^{1} residues of Ang II with the His^{183} of the AT1R for the pre-activation process during binding. On the contrary, removal of the side chain Asp^{1} increased Ang III's affinity to AT2R significantly, thus explaining the non-essentiality of Asp^{1} of Ang II to AT2R binding. The radioligand binding studies done on the human myometrium abundantly expressing AT2R showed that relative affinities to AT2R were found to be Ang III > Ang II > Ang I > PD123319 > Ang (1-7) > Ang IV > Losartan (Bouley et al., 1998).

Interestingly, when the Gly scan was performed on the Ang II and Ang III, it was found that a positive charge on the N-terminal of Ang III is not required for high AT2R affinity but seems to be more important in Ang II. The *in vitro* radioligand binding studies on pig uterine membrane showed that replacement of Arg^{1} residue by Gly only reduced affinity to AT2R by two folds (Ang III K_i=2.2 nM, Gly^{1}-Ang III K_i=5.4 nM). However, a nine-fold decrease in the affinity was observed after the replacement of Arg^{2} residue by Gly (Ang II K_i=0.6 nM, Gly^{2}-Ang II K_i=55 nM). This suggested that Ang II and Ang III may bind differently to the AT2R (Rosenström et al., 2004a). However, there is a dearth of researches on the molecular mechanism behind the binding of Ang III to AT2R, leaving a knowledge gap in this area.

The radioligand binding performed in the rat or human uterine membrane of human adrenal tissues for AT2R binding studies generally gives variable affinity values. Therefore, to reduce confounders responsible for possible mismatch in binding and functional assay, a study done was done in the stably transfected with either AT1R or AT2R in the HEK-293 cells. The assessment of relative AT2R/AT1R selectivity of major endogenous Ang peptides from the radioligand binding studies in HEK cell revealed the affinity order as CGP112A > C21 ≥ PD123319 >>> Ang (1-7) ~Ang III >>> Candesartan. This adds

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further weight to the notion that the shorter endogenous peptides are preferred ligands for AT2R (Bosnyak et al., 2011).

The appreciating AT2R/AT1R selectivity of Ang III over its parent peptide, when extrapolated for beneficial biological activity, supported the theory that Ang III was the preferred endogenous ligand for AT2R. A functional assay for Ang II and its metabolites is performed in the rat coronary vascular bed with or without AT1R or AT2R blockade. Ang III and Ang IV all showed vasoconstriction mediated by the AT1R though at a lower potency than Ang II, and vasorelaxation effect was also seen subsequently. But at the AT2R blockade, a powerful potentiated vasoconstriction effect was observed after treatment with Ang III even on nM concentrations (van Esch et al., 2008).

Natriuresis is considered one of the prominent AT2R mediated biological activities. When uninephrectomized rats were administered Ang II and Ang III via renal interstitial at equimolar level, Ang III evoked a strong natriuresis effect on AT1R blockade (Padia et al., 2006). Furthermore, when the metabolism of Ang III by aminopeptidase N was blocked by the administration of inhibitor compound PC-18, an augmented natriuretic response was observed. This effect was observed in uninephrectomized rats when Ang III was administered at an equimolar level with AT1R blockade (Padia et al., 2007). The natriuretic response was reversed in both of the studies when AT2R antagonist PD123319 was given, supporting the fact that Ang III is the preferred ligand for AT2R mediated renal natriuresis.

Other than renal natriuresis, Ang III is also reported to favor cardiac natriuresis by stimulating Atrial Natriuretic Peptide (ANP) via AT2R. The stimulating effect of Ang III (1 M) on stretch-induced ANP secretion was blocked by the pretreatment with AT2R antagonist but not by AT1R or MasR antagonist. The Ang III-stimulated ANP secretion is decreased by pretreatment with an inhibitor of phosphoinositide 3-kinase (PI3K), Protein kinase B (PKB), NOS, soluble guanylyl cyclase, or protein kinase G (PKG), suggesting that Ang III mediates cardiac natriuresis through AT2R/PI3K/PKB/NO/PKG pathway (Park et al., 2013b). Along with improved coronary flow and ANP stimulation, Ang III also participated in the

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cardioprotective effect against ischemia/reperfusion injury from the activation of antioxidant enzymes and inhibition of apoptotic enzymes via the AT2R (Park et al., 2013a).

4.1.4 Modified Ang III

Ang II, being an endogenous agonist for AT2R, has a lot of scope for developing highly AT2R selective ligands by the alteration in its amino acids. Overseeing the success of β-substitution in Ang II in designing AT2R selective ligands, Del Borgo et al. have followed a similar trend by individual β-amino acid substitution in its original sequence of Ang III. The competition binding assays are performed in the HEK cells transfected with either AT1R or AT2R treated by all β-substituted Ang III analogs. Amongst all, β-Pro^{7}-Ang III was found to be > 20000-fold more selective for AT2R than AT1R compared to Ang III that has 10-fold selectivity for AT2R. This compound also caused both *in vitro* relaxation and *in vivo* depressor effects in conscious SHR in the presence of a low amount of AT1R blockade. β-Pro^{7}-Ang III was at least 10-fold more potent at AT2R and approximately 10-fold more AT2R-selective than the best functionally active AT2R agonists derived from the previous β-substituted scan using Ang II as the template (Del Borgo et al., 2015). β-Pro^{7}-Ang III also induced encouraging renal vasodilatory and natriuretic effects, a classic AT2R agonist activity, in both male and female normotensive rats. This study highlighted specific roles for the AT2R in the regulation of renal function according to gender since renal vasodilator response was much greater in female rats than males (Krause et al., 2020).

4.1.5 Ang (1-7)

Ang (1-7) is one of the major peptides in the protective axis of RAS, which mediates its action via MasR (Table 2 and fig. 5). Although Ang (1-7) shows 500-fold less affinity than Ang II, it has 40 times more AT2R selectivity than Ang II, suggesting Ang (1-7) can serve as an endogenous ligand for AT2R (Bosnyak et al., 2011). There is not much evidence of how Ang (1-7) binds to AT2R, but there is a possibility that the binding pattern may be similar to Ang II to AT2R, but the discrepancy in C-terminal may explain the loss of affinity to the receptor.

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Ang (1-7) evoked vasodepressor effect in SHR and Wistar rats in the presence of low dose AT1R blockade. The AT2R mediated response of Ang (1-7) was attenuated by AT2R antagonists as well as B2R antagonist, NOS inhibitor, and L-NAME inhibitor, suggesting AT2R-mediated action involving BK-NO cascade (Walters et al., 2005). Ang (1-7) was reported to have a biphasic effect on the proximal tubule Na^+-ATPase activity in which stimulation of sodium reabsorption was mediated by AT1R on the lower concentration (1 pM), and inhibition of sodium reabsorption was mediated by AT2R at higher concentration (10 nM). This shows a delicate balance of Ang (1-7) between Ang receptor levels for the proper functioning of electrolytic response. The AT2R mediated action of Ang (1-7) involved Gi/o protein/cGMP/PKG pathway (Lara et al., 2006). Ang (1-7) also induced AA release for prostaglandin production mediated through AT2R (Muthalif et al., 1998).

As the tissue-protective and regenerative actions of MasR and AT2R are similar and belong to the protection arm of the RAS, there has been discussion that there is a liaison between receptors in the form of dimerization of receptors or nonspecific ligands interaction (Villela et al., 2015). There is still a need for future research to elaborate the exact mechanism for dimerization of receptors, whereas the non-specificity of the Ang (1-7) can be seen in the Apolipoprotein E knockout mice ($ApoE^{-/-}$) for vascular and atheroprotective effects. AT2R and MasR antagonists block these effects suggesting that the Ang (1-7) actions were mediated by both receptors (Tesanovic et al., 2010). A similar kind of observation is made when Ang (1-7) evoked autophagy of the brain was dampened by both AT2R and MasR antagonists in SHR (Jiang et al., 2013). The comparison between synthetic MasR agonist (having no affinity to AT1R and AT2R) and Ang (1-7) would have given a clear picture regarding this possible mechanism.

4.1.6 Ang IV

The metabolic breakdown of Ang III by aminopeptidase B or N gives rise to the hexapeptide fragment Ang IV (Val–Tyr–Ile–His–Pro–Phe) (Table 2 and fig. 5). This short peptide Ang IV is reported to have a role in memory processing, improved cognitive behavior, and cardioprotection, primarily known to be mediated through novel Ang II type 4 receptor (AT4R) (Vanderheyden, 2009). Besides having a high

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binding affinity to AT4R, Ang IV also binds to other Ang receptors like AT1R and AT2R but with less binding affinity. Bosnyak et al. showed in their study that the shorter the endogenous peptide is, the better selectivity towards AT2R will be. Ang IV is found to be 200 times more selective for AT2R than AT1R and has five times more affinity to AT2R but low AT1R affinity compared to Ang (1-7) (Bosnyak et al., 2011).

The chronic infusion of Ang IV (1.44 mg/kg/day) to six-week-old ApoE $^{-/-}$ mice improved endothelial dysfunction due to the increased NO bioavailability. This effect was attenuated when AT4R antagonist divalinal-Ang IV or AT2R antagonist PD123319 was administered to the mice. In addition to this, there was a slight improvement in the endothelial dysfunction when AT1R blocker candesartan was coadministered to the group infused with Ang IV when compared to Ang IV infused group alone (Vinh et al., 2008b). Later the same group carried out another study to know the effect of chronic infusion of Ang IV (0.72 mg/kg/day) in 32-week-old ApoE$^{-/-}$ mice at advanced atheroma stage. They were able to show the vasoprotective effect at the lower dose, which they attributed to the increased AT4R expression at the advanced atheroma stage, corroborated by the AT4R density study (Vinh et al., 2008a). Although Ang IV has a high affinity for AT4R, there is an interplay between AT2R and AT4R for the beneficial biological outcome of this endogenous peptide. Consistent with the cross-talk between AT2R and AT4R, Ang IV also showed cerebroprotective action in the rat model of embolic stroke. Internal carotid infusions of increasing doses of Ang IV (0.01, 0.1, and 1 nmol/0.1 mL in saline) dose-dependently decreased mortality, neurological deficit, and cerebral infarct size in rats. The cerebroprotective action of Ang IV was completely abolished during AT4R antagonist administration and partially blocked during AT2R antagonist treatment (Faure et al., 2006).

The heterogeneous receptor stimulation for Ang IV is not limited to non-AT1Rs. There has been evidence that Ang IV can stimulate AT1R as well. Ang IV showed bidirectional response on chronic infusion to the Ang II-induced abdominal aortic aneurysm (AAA) in 12-week-old ApoE$^{-/-}$ mice. At a medium dose (1.44 mg/kg/day), Ang IV provided protective effect by inhibiting inflammatory response and matrix

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metalloproteinase activity via stimulation of AT4R and AT2R, but at the higher dose (2.88 mg/kg/day), Ang IV abolished these beneficial effects by a switching stimulation to AT1R (Kong et al., 2015). The receptor stimulation here plays an important role in the outcome of Ang IV that is dependent on various factors like dose and pathological conditions reinstating the fact that two opposite arms of Ang receptors are acting against each other. In-depth research in the future clarifying the complicated binding and signaling mechanisms of the receptor of RAS regarding Ang IV will definitely help to unearth more potential therapeutic benefits of this shorter Ang peptide.

4.2 Synthetic AT2R peptide agonist

4.2.1 CGP42112A

CGP42112A (Table 2 and fig. 5) was initially developed as an Ang II antagonist (N-α-nicotinoyl-Tyr-Lys-(N-benzyloxycarbonyl-Arg)-His-Pro-Ile and one of the first synthetic agonists that helped to define the heterogeneity of Ang receptors (Whitebread et al., 1989). As the interaction of C-terminal Ang II with the inner half of the TM3 domain of AT2R remains crucial for AT2R binding affinity, CGP42112A was also developed by retaining the carboxy-terminal of Ang II with the end aromatic chain replaced by aliphatic Ile, thus favoring even more affinity and selectivity. However, the way in which amino-terminal deletion of residue impacts Ang II binding to AT2R, CGP42112As are independent for these kinds of alterations suggesting a different kind of ligand domain interaction at the N-terminal (Yee et al., 1998). The retrospective analysis in the chemical evolution of AT2R peptide agonists by molecular binding modes and affinity estimations with the free energy perturbation method has shown that CG42112A presents a guanidine group on a branched-like structure mimicking the side-chain of Arg^2 in Ang II, which explains its strong affinity to AT2R (Vasile et al., 2020).

CGP42112A behaves as a full agonist both *in vitro* and *in vivo*, and AT1R binding actually occurs at relatively high concentrations (i.e., $>1\times10^{-5}$ M). Additionally, CGP42112A still remains the highest-affinity AT2R agonist to date ($K_i = 2\times10^{-10}$ M) and shows approximately ten-fold greater AT2R selectivity to that of AT1R than the best synthetic small molecule C21. Although CGP42112A has high selectivity, it

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has low potency and also acts as an agonist for AT1R in high concentrations; this made CGP42112A an unfavorable compound to be definitive of result with respect to its affinity and function toward AT2R (Hallberg et al., 2017).

4.2.2 Novokinin

Novokinin is a synthetic peptide derived from chymotrypsin digest, ovokinin (2-7) (Arg-Ala-Asp-His-Pro-Phe), of egg albumin (Table 2 and fig, 5). The ovokinin (2-7) is found to elicit vasorelaxation mediated by AT2R with a binding affinity of 210 μM. It evoked hypotensive effect in SHR at the dose of 10 mg/kg after oral administration (p.o.). Later this was modified to the [Pro^2, Phe^3] -ovokinin (2-7), which evoked anti-hypertensive effects at a dose of 0.3 mg/kg corresponding to more than 30-fold times less dose compared to ovokinin (2-7) after p.o. (Matoba et al., 2001).

In the quest of designing more potent bioactive peptides, the alanine scan is performed, which aided in determining suitable amino acid residues for each individual position. Like, Arg^1 residue at N-terminal is crucial for anti-hypertensive effects. Similarly, Pro at 2^{nd} and 5^{th} position conferred resistance to gastrointestinal degradation by protecting it from the action of enzymes like aminopeptidase and carboxypeptidase without affecting vasorelaxant activity. The aromatic Phe^3 is replaced with aliphatic Leu to escape degradation by chymotrypsin-type protease. They also established that aromatic amino acid residue with high hydrophobicity is required at the carboxyl-terminal, and Trp gives them the best results in terms of anti-hypertensive activity. This replacement of 4 amino acids out of the original 6 [ovokinin (2-7)] gives them a more potent peptide called novokinin. This novokinin peptide (Arg-Pro-Leu-Lys-Pro-Trp) (Table 2) has superior binding affinity at 7×10^{-6} M, and its bioactivity is seen at a lesser dose compared to its parent peptide (Yamada et al., 2002; Yoshikawa et al., 2013).

Novokinin induced relaxation in the mesenteric artery pre-constructed by phenylephrine at the concentration of 10^{-5} M. They also showed encouraging anti-hypertensive effect in the SHR at a dose of 0.03 mg/kg (saline) intravenous (i.v.)) and 0.1 mg/kg (emulsified in 30% egg yolk) after p.o. but no effect in normal hypotensive Wister Kyoto rat. This hypotensive effect was blocked by AT2R antagonist,

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cyclooxygenase (COX) inhibitor, and prostaglandin I_2 (IP) receptor antagonist but also insignificantly by NOS inhibitor. This proved the observed biological action was majorly mediated through IP receptor downstream of the AT2R, but the contribution of NO cannot be ruled out completely. Similarly, it did not induce hypotension in normal and AT2R deficient mice (Yamada et al., 2008). The study done by Mutlu et al. had tried to shed light on the role of the NO pathway for novokinin. Here in this study, novokinin was administered intraperitoneally (i.p.) at a dose of 0.1mg/kg for two weeks to the salt-fed and L-NAME-induced hypertensive rats. Novokinin showed a decline in the enzymes responsible for end-organ damage like asymmetric dimethylarginine (ADMA), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, and Rho kinase level induced in hypertension; however, these changes did not reach statistical significance (Mutlu et al., 2015).

Besides the vasorelaxant and anti-hypertensive effects, novokinin was also found to affect the central nervous system. In the study done by Ohinata et al., novokinin presented with anorexigenic effects at 100 mg/kg/mice (p.o.) and 30 nmol/mouse (intracerebroventricular (i.c.v.)) administration (Ohinata et al., 2009). This effect was mediated by the prostaglandin E receptor 4 (EP_4) system via AT2R as it was reversed by AT2R antagonist, COX inhibitor, and EP_4 antagonist. Similarly, another central nervous effect of novokinin was reported by (Yamada et al., 2009), where it exerted an anti-opioid effect. Here, centrally administrated novokinin (30 nmol/mouse) inhibits the antinociceptive effect of morphine (μ agonist) in mice, as evaluated by the tail-pinch test. This effect was mediated by the EP_3 system via AT2R as this was reversed by AT2R antagonist, COX inhibitor, and EP_3 antagonist.

In addition to this, novokinin also exerted gastroprotective effects via the AT2R-Prostaglandin (PG)s pathway (Zhang et al., 2016). Here, it inhibited basal gastric acid secretion and protected gastric mucosa from alcohol-induced injury in a dose-related manner in rats after i.c.v. administration at the dose of 50 and 100 nmol/rat, respectively. This effect was occluded by AT2R antagonist and COX inhibitor.

The further exploration of the multitude of therapeutic effects of novokinin had revealed that it had a beneficial role in asthma as well (Patel et al., 2019). Novokinin was found to lower lung inflammation

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and airway reactivity at the dose of 0.3 mg/kg, i.p. in allergen sensitized mice mediated via AT2R. The downstream mechanism of novokinin in inflammatory diseases like asthma is yet unknown, highlighting the fact that there is much more information needed to be known regarding its beneficial effects.

In contrary to reports on beneficial effects of novokinin, Pechlivanova et al. has reported that the novokinin infusion in the cerebral ventricles exacerbated diabetes-induced hyperalgesia and provoked anxiety-like behavior but improved spatial memory in streptozotocin-induced type I diabetic rats. In this study, novokinin has been administered continuously at a dose of 0.6 µg/rat/day via i.c.v. infusion for 28 days by osmotic minipumps. The initial data suggested that prolonged activation of AT2R brings plastic and functional changes, thus participating in the pathogenesis of diabetes mellitus-induced complications in the function of the nervous system (Pechlivanova et al., 2018).

4.2.3 NP-6A4:

Although there is no information regarding the chemical structure in the public domain, a patented agonist, NP-6A4, is established as one of the new members of the AT2R agonist family. It has been developed by Novopyxis Inc. (Boston, MA, United States), which has garnered FDA's Orphan Drug designation for the treatment of pediatric cardiomyopathy (Sharma et al., 2020), and its efficacy was tested in A549 human lung cell line acting same as when lungs are infected with SARS-CoV2 (Celebi et al., 2020).

Mahmood et al. had performed one of the primary investigations in this novel peptide, where they analyzed its cardioprotective effect on the basis of the cell survival of mouse cardiomyocyte HL-1 cells and primary cultures of human coronary artery VSMC (hCAVSMCs)s subjected to serum starvation. These serum-starved cells represented the nutrient-deficient stress condition associated with ischemic heart disease, thus contributing to the significant loss of cardiovascular cells via cell death. It had been reported that NP-6A4 increased cell viability and Myeloid Cell Leukemia 1 expression, an important protein essential for cell survival and viability of cardiomyocytes and VSMCs. This effect was blocked by the application of an AT2R antagonist (Mahmood and Pulakat, 2015).

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Further investigation on this peptide signaling mechanism in human coronary artery endothelial cells (hCAECs) and hCAVSMCs revealed that at the concentration of 1 μM, this peptide decreased phosphorylation of Jun-N-terminal kinase and suppressed reactive oxygen species, thus showing an anticytotoxic effect. Additionally, NP-6A4 (5 μM, 12 h) also increased expression of eNOS and generation of NO in hCAECs, and pretreatment with PD123319 (20 μM) suppressed this effect partially (65%). NP-6A4 increased Agtr2 mRNA and AT2R protein expression (1 μM, 12 h) in all human cells tested and activated a positive feedback loop for the AT2R signaling mechanism (Toedebusch et al., 2018).

Recently reported by Sharma et al. had explored the anti-inflammatory and vasoprotective effects of AT2R in the animal model of Ang II-induced AAA. Male ApoE$^{-/-}$mice were pre-treated subcutaneously with NP-6A4 (2.5 mg/kg/day) or vehicle for 14 days prior to Ang II administration, and treatments were continued for 28 days. NP-6A4 was shown to reduce aortic stiffness of the abdominal aorta and improve aortic distensibility significantly. However, it did not affect the maximal intraluminal aortic diameter or AAA incidences. These data suggested that the effects of AT2R agonist on vascular pathologies are selective, affecting the aortic stiffness and proteolytic activity without affecting the size of AAA (Sharma et al., 2020).

4.3 Non-Peptide AT2R agonist

Although a lot of efforts were made to synthesize AT2R selective agonist based on the compound L-162.782 (fig. 5), which has a similar affinity to both AT1R and AT2R, it was not until 2004 that a selective non-peptide agonist named C21 was developed. C21 was designed based on the mimicking of the three C-terminal amino acids His-Pro-Phe of Ang II essential for binding to Lys^{215} of AT2R (Georgsson et al., 2007). A compound containing a biaryl scaffold responsible for Ang receptor bindings which are connected to three functional groups was designed having: i) a lipophilic side chain, ii) a sulfonyl carbamate group, and iii) a methylene group attached to a bicyclic nitrogen heterocycle (fig. 6).

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Structural activity relationship studies show that methyl imidazole or triazole structure is essential for specific binding to AT2R with high affinity. With regard to the lipophilic side chain, it has previously been reported that small structural alterations of this group also seemed to reduce the affinity of the ligands to the AT2R; therefore, the isobutyl group was considered as most suitable. Similarly, the alkoxy part of the sulfonyl carbamate group was crucial to improving AT2R selectivity. The ligand binding and pharmacokinetic studies of C21 showed that it has K_i = 0.4 nM to AT2R, K_i =>10 µM to AT1R, and bioavailability is 20-30% after (p.o.) with the half-life of 4 hours in the rat (Wan et al., 2004; Hallberg et al., 2018).

Vasile et al. reported a detailed structural activity relationship of C21 by the binding modes and affinity estimations with the free energy perturbation method. It has been stated that C21 is anchored by electrostatic interactions of the central sulphonyl carbamate with charged side-chains of TM2 and TM5. The isobutyl group would be located in a deeper hydrophobic cavity within the TM region, which specifically facilitates the active receptor conformation (Vasile et al., 2020).

Being the most potent of the series of selective non-peptide AT2R agonists that have been synthesized and reported so far, C21 has served as a research tool and entered in several clinical trials in recent years. As deduced from a large number of *in vivo* studies, C21 demonstrates a variety of protective actions in a large number of diverse experimental disease models and has been reviewed extensively (Paulis et al., 2015; Hallberg et al., 2018). C21 has been established as a reliable compound for neuro and renal protective effects. Recently, it presented with the promising result as an anti-inflammatory and antifibrotic agent in the lungs, which granted it approval to be tested in clinical trials in the inflammatory disease setting such as COVID-19.

Contrary to the long-accepted theory that C21 action is only limited to activation of AT2R, it also has a low affinity to thromboxane A2 receptor (TxA2R). The vasorelaxant activity of C21 was tested in pigs pericardial and mice mesenteric arteries, which were pre-contracted with U46619 (a TxA2 agonist). The

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C21 induced vasorelaxation in U46619 contracted vessels did not block by the administration of AT2R antagonists. Furthermore, C21 inhibits U46619 induced platelet aggregation (Steckelings et al., 2015). Findings by Fredgart et al. suggest C21 as a high-affinity ligand for AT2R and low-affinity blocker for TxA2R, which inhibits TxA2-mediated vasoconstriction and platelet aggregation (Fredgart et al., 2015). These data collectively suggest that C21 is also a ligand for the TxA2R.

The optimal requirements for any compound to be considered as a solid AT2R agonist are expressing adequate receptor binding affinity, selectivity, and AT2R mediated biological action. The assessment of AT2R agonists has been tested by a binding assay using HEK-293 cell lines stably transfected with AT1R and AT2R. This assay is more appropriate and preferred over radioligand binding assays performed in rat liver and pig uterine membrane as it reduces possible confounders like tissue heterogeneity and differential Ang receptors expression. One of the preliminary steps for testing AT2R mediated biological effects is the application of antagonist PD123319, in which the antagonist effectively blocks AT2R agonistic activity. For this purpose, several *in vitro* and *in vivo* studies were utilized peptide and non-peptide agonists of AT2R using different cell lines such as NG1085-5 (Gendron et al., 2002; Georgsson et al., 2007; Jedhe et al., 2016), PC12W (Zhao et al., 2003; Rosenström et al., 2004a; Rosenström et al., 2005) and R3T3 (Yamada et al., 1996) or SHR (Widdop et al., 2002; McCarthy et al., 2009; Del Borgo et al., 2015) and $ApoE^{-/-}$ (Vinh et al., 2008b; Yamada et al., 2008; Sharma et al., 2020) animal models. There are also other studies that utilized transfected and transgenic mice for testing the biological activity of AT2R agonists.

5 Clinical Significance of AT2R

The cell lines originated from the human have long been an essential tool in identifying the pathophysiological and functional role of AT2R (Ishiguro et al., 2015; Ryan et al., 2016; Wang et al., 2020). The human data supporting the role of AT2R in vascular and cardiovascular pathophysiology is not in full supply, but the anti-inflammatory and antioxidative aspects of AT2R have been explored in the clinical realm. Owing to the fact that only one agonist, C21, has reached the level of extensive research

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for its suitable pharmacokinetics parameters, C21 has been successfully completed Phase I clinical trials (Steckelings et al., 2017). This compound has been patented by Viocore Pharma, a pharmaceutical company focused on rare diseases. This compound is currently in phase II clinical trials for testing its safety and efficacy in a rare pulmonary disease like idiopathic pulmonary fibrosis (IPF) (AB, 2020). Furthermore, the observed remarkable expression of AT2R in alveolar type 2 progenitor cells in the adult human lung suggested a beneficial role in COVID-19 infection (Tornling et al., 2021). The preliminary investigation in COVID-19 infected patients has shown encouraging evidence that C21 restores respiratory function in a double-blind, randomized, placebo-controlled Phase II trial of COVID-19 (AB, 2020). Collectively, the results of research on C21 have definitely supported the feasibility of the future establishment of AT2R as a promising pharmacological drug target.

6 Conclusion

The AT2R has promising pharmacological implications as it opposes AT1R mediated action. The AT2R stimulation has shown anti-proliferative, apoptotic, cell differentiation, vasodilatory, and neuronal modulation activity. The upregulated AT2R in the disease state, if properly channelized, can give us beneficial therapeutic applications. In this review, we discussed the natural or synthetic peptide and non-peptide small molecule agonists and covered their structure, mechanism of action, and biological activity. The Ang II acts as an endogenous ligand for AT2R; however, there is an urgent need for an agonist that offers better selectivity with a high affinity to the AT1R. The structural modification of Ang II peptide offers better metabolic stability; however, it is not suitable enough for the development of a lead compound for the treatment of various diseases. Until now, C21 is considered to be the most selective AT2R agonist with maximum binding affinity. This compound has reached clinical trials for IPF treatment and other indications such as COVID-19. Here in this article, we aimed at collecting information regarding AT2R and its agonists with recent updates that will help the researchers towards their future research.

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Conflict of interest

No author has an actual or perceived conflict of interest with the contents of this article.

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Figure legends

Fig 1. Schematic diagram of the RAS

Fig 2. The schematic diagram for signaling mechanism protein phosphatases and protein dephosphorylation Src homology -2 domain containing Tyr phosphatase-1 (SHP-1), protein phosphatase 2A (PP2A), mitogen-activated protein (MAP) kinase phosphatase (MKP)-I, signal transducers and activators of transcription (STAT), β-cell lymphoma-2 (Bcl-2), caveolin-1 (CAV1) protein tyrosine phosphatase 1B (PTP1B) Ras-related protein 5A (Rab5), Ras-related C3 botulinum toxin substrate 1 (Rac1).

Fig 3. Schematic diagram AT2R mediated NO and cGMP pathway. Bradykinin (BK), nitric oxide (NO), cyclic Guanosine monophosphate (cGMP), prolylcarboxypeptidase (PRPC), high molecular weight kininogen plasma kallikrein (HMWK.PK) (Karnik et al., 2015).

Fig 4. Schematic diagram for PLA2/AA pathway, protein phosphatase 2A (PP2A), mitogen-activated protein (MAPK), p21 ras protein (Karnik et al., 2015).

Fig 5. Chemical structures of some AT2R ligands.

Fig 6. Scaffold for AT2R agonist. Adopted from (Hallberg et al., 2017) with permission.

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Tables

Table 1. Comparison of amino acid residues in the AT2R and AT1R involved in Ang II binding. Adopted from (Asada et al., 2020)

Amino acids of Ang II	Residue in AT2R	Equivalent residue in AT1R	The region in the receptor	Interaction
Arg^{2}	Asp^{279}	Asp^{263}	TM6	Form salt bridge for ligand binding
	Asp^{297}	Asp^{281}	TM7	Stabilize the ligand-binding pocket
Ile^{5}	Tyr^{103}	-	TM2	Carbonyl oxygen of Ile form hydrogen bonds with Tyr
His^{6} Pro^{7}	Arg^{182}	Arg^{167}	ECL2	Carbonyl oxygen of His and Pro form hydrogen bonds with the guanidinium group of Arg
C-terminal of Phe^{8}	Lys^{215}	Lys^{199}	TM5	Form salt bridge with Lys
Phe^{8}	Leu^{124}	-	TM3	Form the hydrophobic core for ligand binding pocket
	Met^{128}	-	TM3	
	Trp^{269}	Trp^{253}	TM6	
	Phe^{272}	-	TM6	
	Phe^{308}	Phe^{308}	TM7	

Table 2. Modified angiotensin analogs with their binding affinity to both AT1R and AT2R

Compound [a]				
	Ki (nM) ± SEM		**AT2R Selectivity (AT1R/AT2R)**	**Reference(s)**
	AT1R[b]	**AT2R**[c]		

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Ang II	0.31 ± 0.08	0.63 ± 0.16	0.5	(Lindman et al., 2003)
	1.1 ± 0.4	1.2 ± 0.4	0.9	(Johannesson et al., 2004)
4-Amino Phe6-	3296 ± 154	1.97 ± 0.16	1670	(Lindman et al., 2003)
Ang II	> 1000	1.2 ± 0.4	> 800	(Johannesson et al., 2004)
Losartan	25 ± 5	-		(Lindman et al., 2003)
	1.9 ± 0.6	-		(Johannesson et al., 2004)
Compound 1	>10000	35	> 286	(Lindman et al., 2003)
Compound 2	>10000	1.0 ± 0.1	> 10000	(Johannesson et al., 2004)
Compound 3	>10000	3.7 ± 0.3	> 1000	(Johannesson et al., 2004)
Compound 4	>10000	3.0 ± 1.1	> 1000	(Rosenström et al., 2004a)
Compound 5	>10000	0.3 ± 0.01	> 10000	(Rosenström et al., 2005)
Compound 6	>10000	1.85 ± 0.1	> 5000	(Georgsson et al., 2005)
Compound 7	>10000	0.5 ± 0.03	> 20000	(Jedhe et al., 2016)
Compound 8	>10000	0.7 ± 0.01	>14000	(Georgsson et al., 2006)
Novokinin[d]	6.85×10^{-4}	7.34×10^{-6}	93	(Yamada et al., 2008)

	IC_{50} value (M)[e]			
	AT1R	**AT2R**		
Ang III	2.11×10^{-8}	6.48×10^{-10}	33	(Bosnyak et al., 2011)
β-Pro^7-Ang III[f]	1×10^{-5}	4.68×10^{-10}	21377	(Del Borgo et al., 2015)
Ang (1-7)	1×10^{-5}	2.46×10^{-7}	41	(Bosnyak et al., 2011)
Ang IV	1×10^{-5}	4.86×10^{-8}	206	(Bosnyak et al., 2011)
CGP42112A	1×10^{-5}	2.33×10^{-10}	42683	(Bosnyak et al., 2011)
NP-6A4	-	-	-	-
C21	1×10^{-5}*	2.29×10^{-9}	4367	(Bosnyak et al., 2011)

[a] Chemical structures are listed in Fig. 5 and S1-Fig.1, [b]rat liver membrane, [c] pig uterus membrane, [d] binding affinity done on HEK (human embryonic kidney)-293 cells, [e] binding affinity done on HEK (human embryonic kidney)-293 cells, [f] β-Pro7-Ang III is one of the best representations of modified Ang III.

Supplementary fig. Structure of Ang II and its modified compounds

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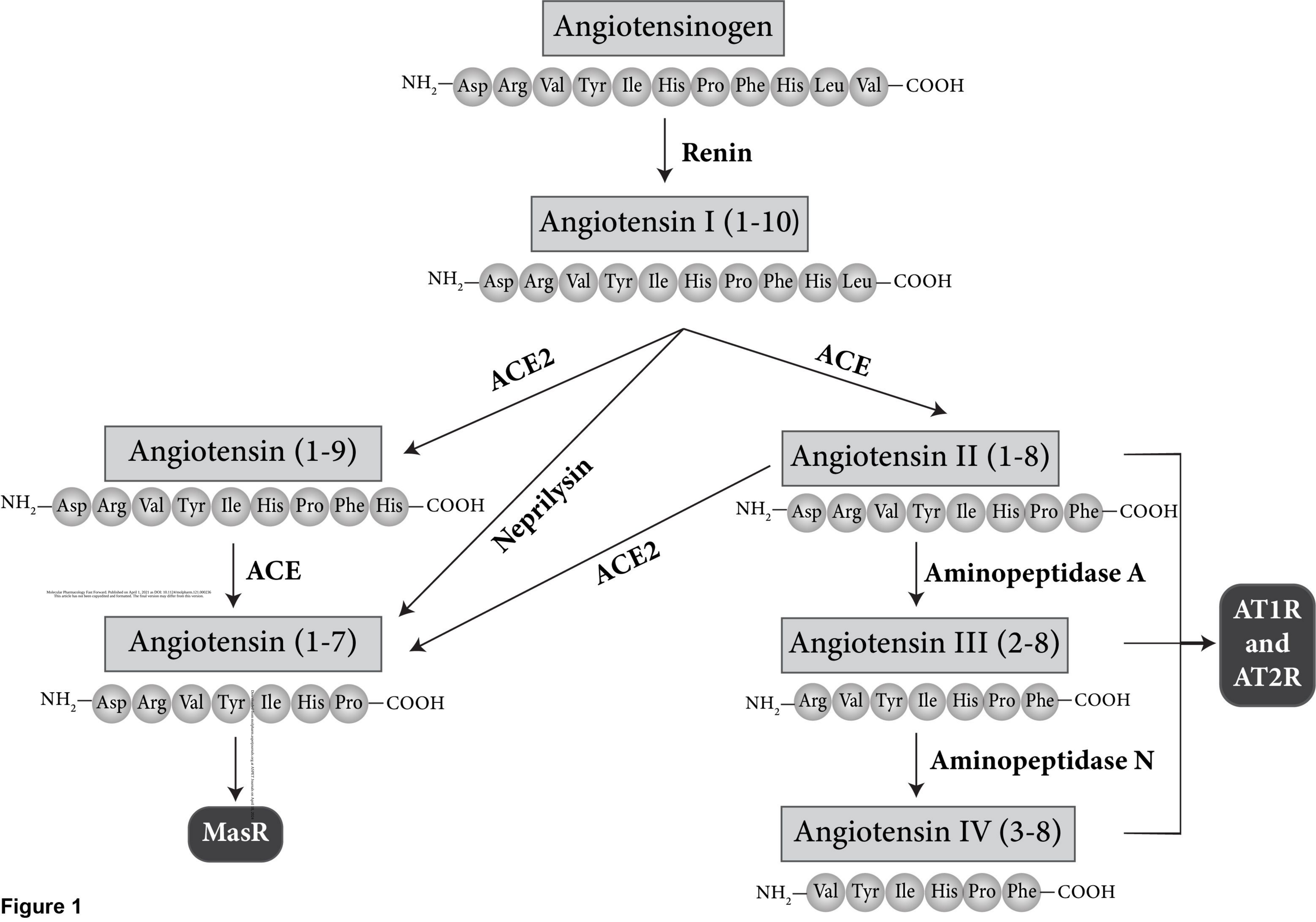

Figure 1

AT2R agonist

AT2R

Ga_S

$Ga_{i/o}$

SHP-1

MKP-1

PTPase

PP2A

PTP1B

p38 MAPK and Caspase

STAT-1

BCL-2

MAPK

Cav1

cFOS

ERK1/2

Rab-5/Rac-1

Biological activity

Apoptotic effect
Antigrowth effect
Anti-proliferative
Cellular differentiation
Anti-metastatic

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Figure 2

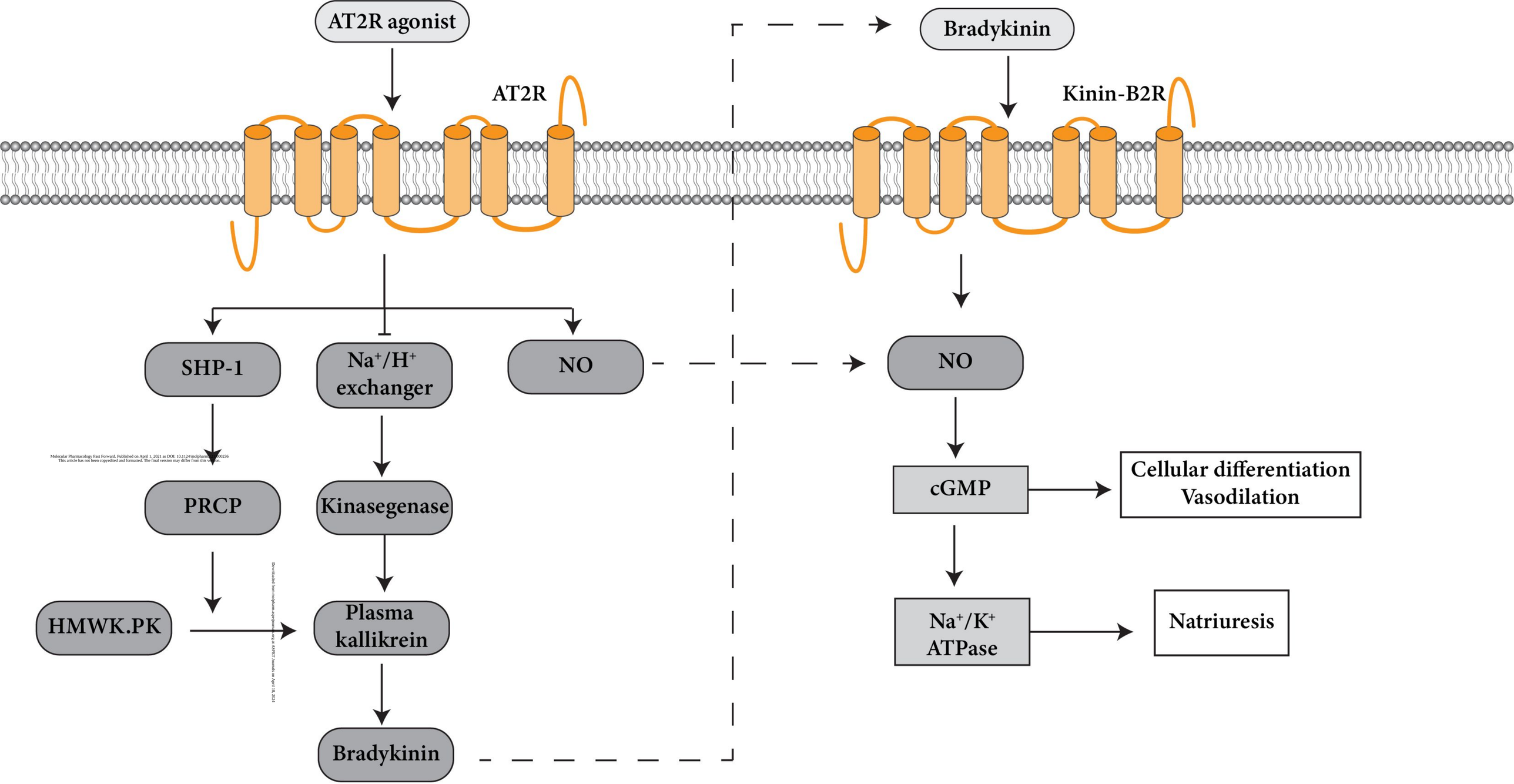

Figure 3

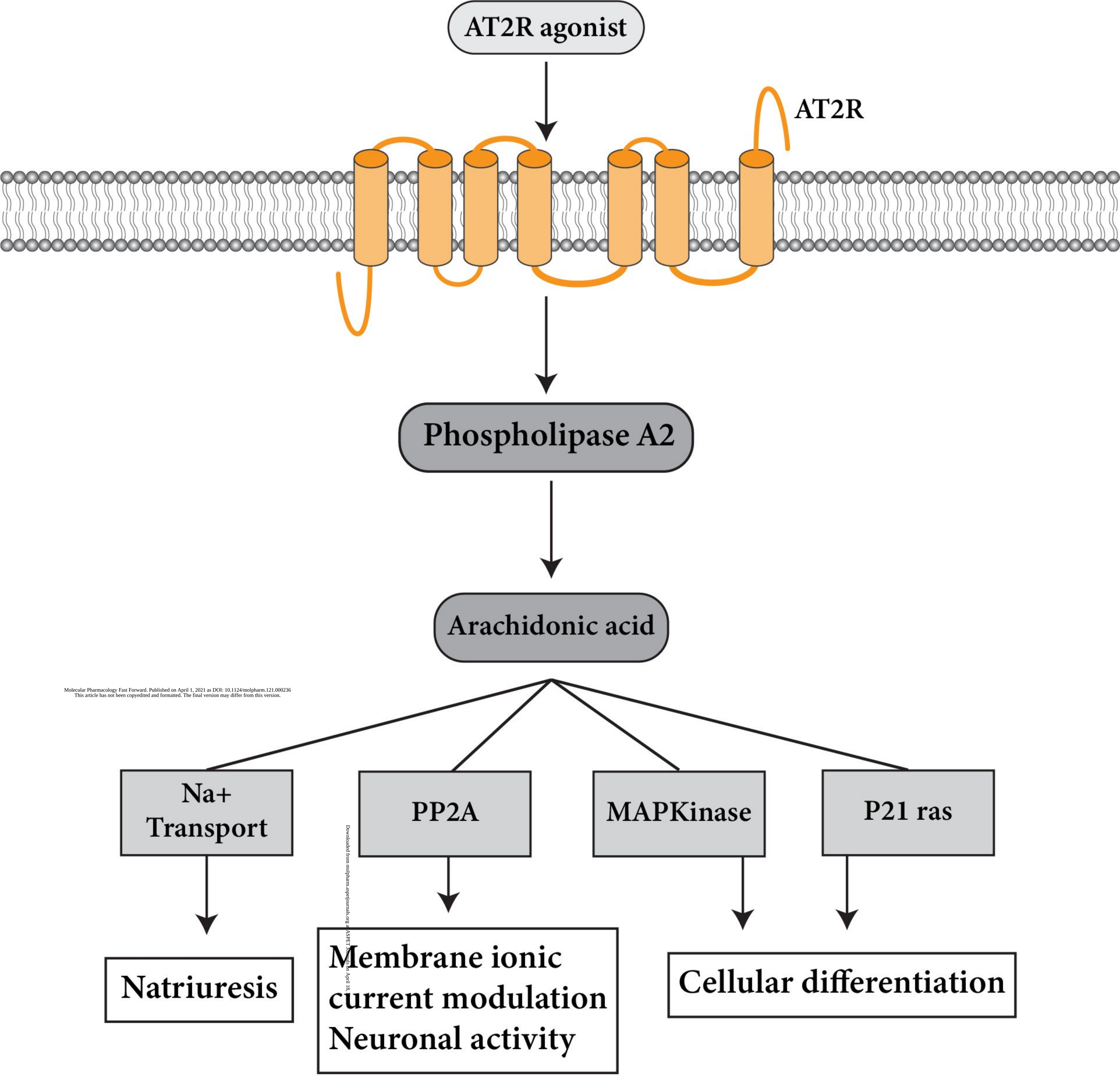

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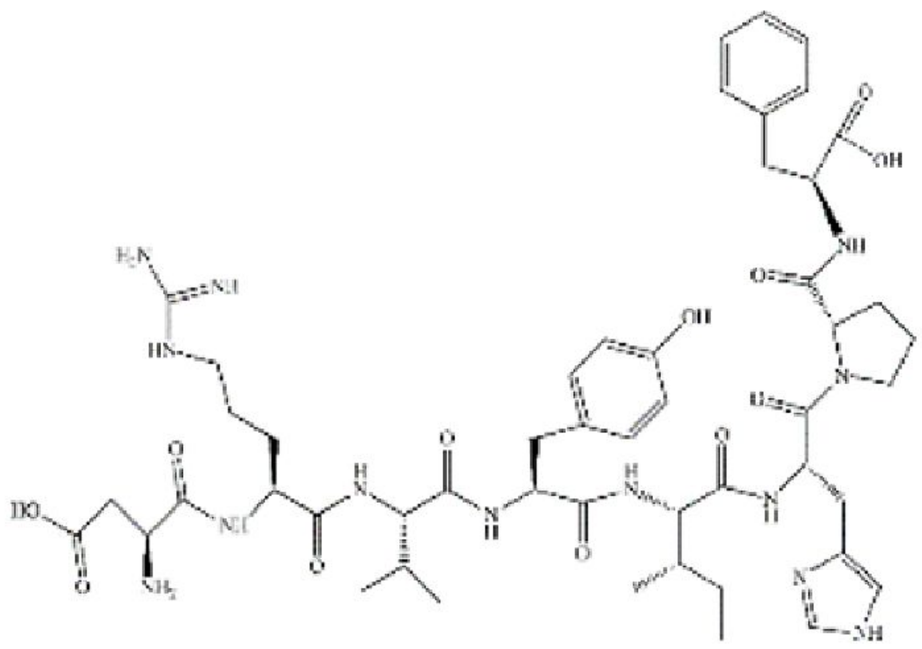
Figure 4



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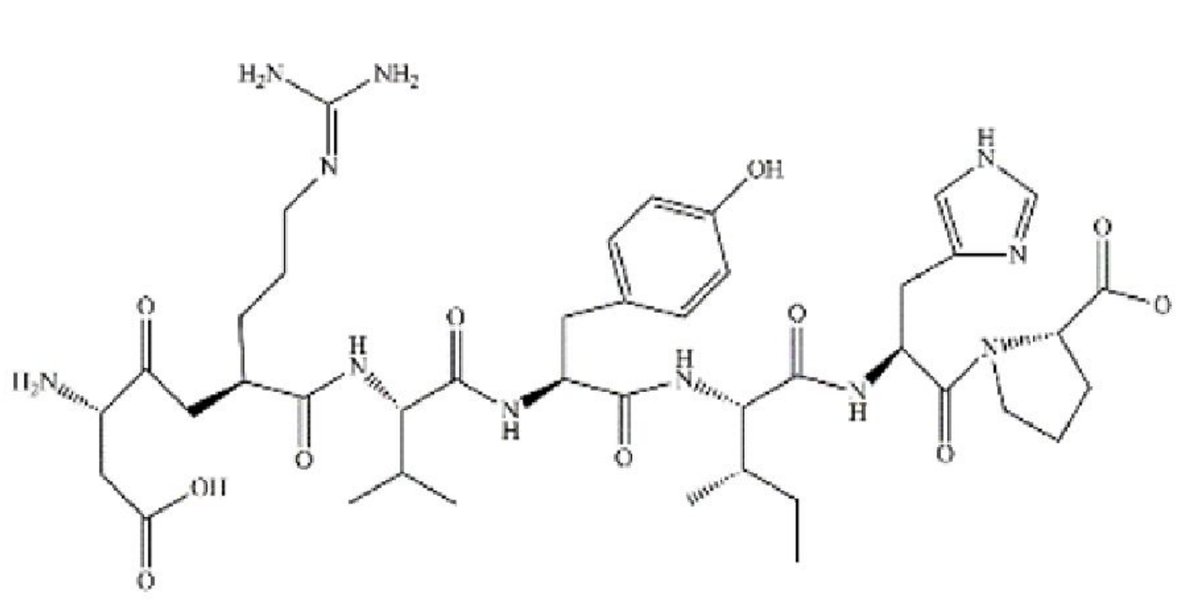
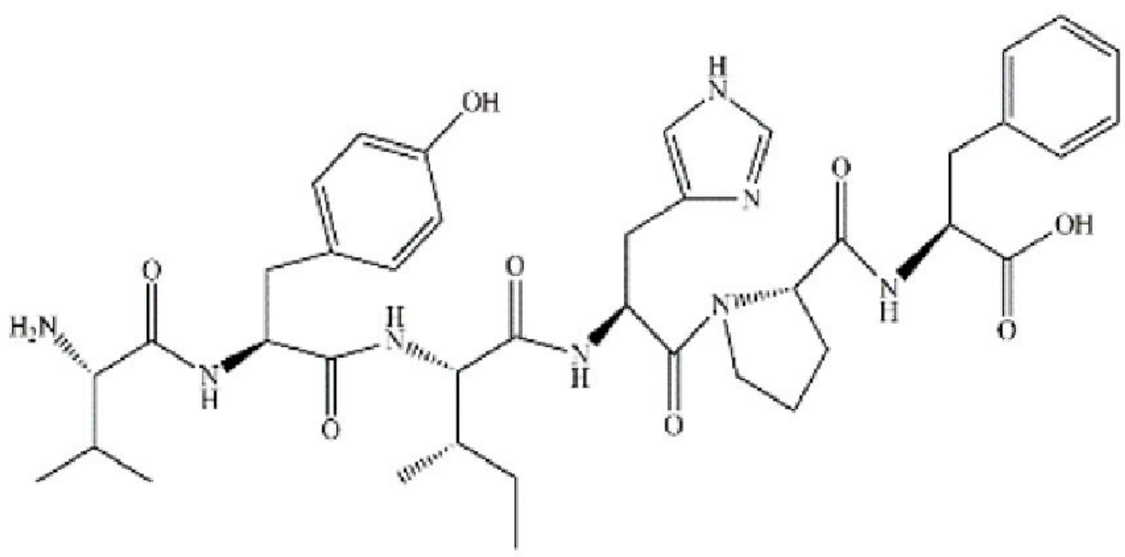


Ang (1-7)



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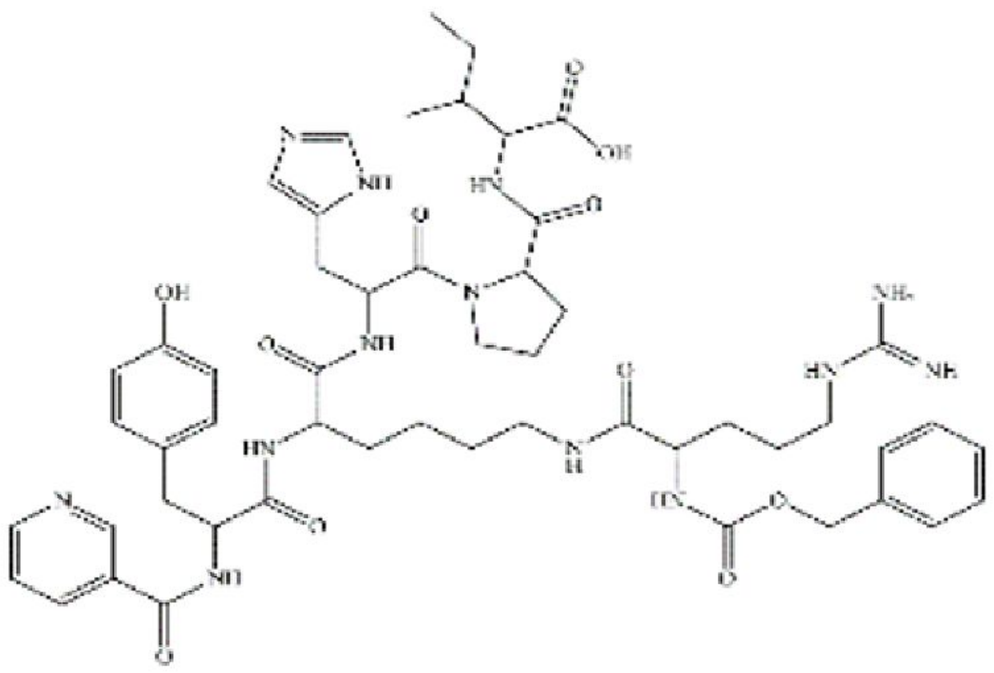

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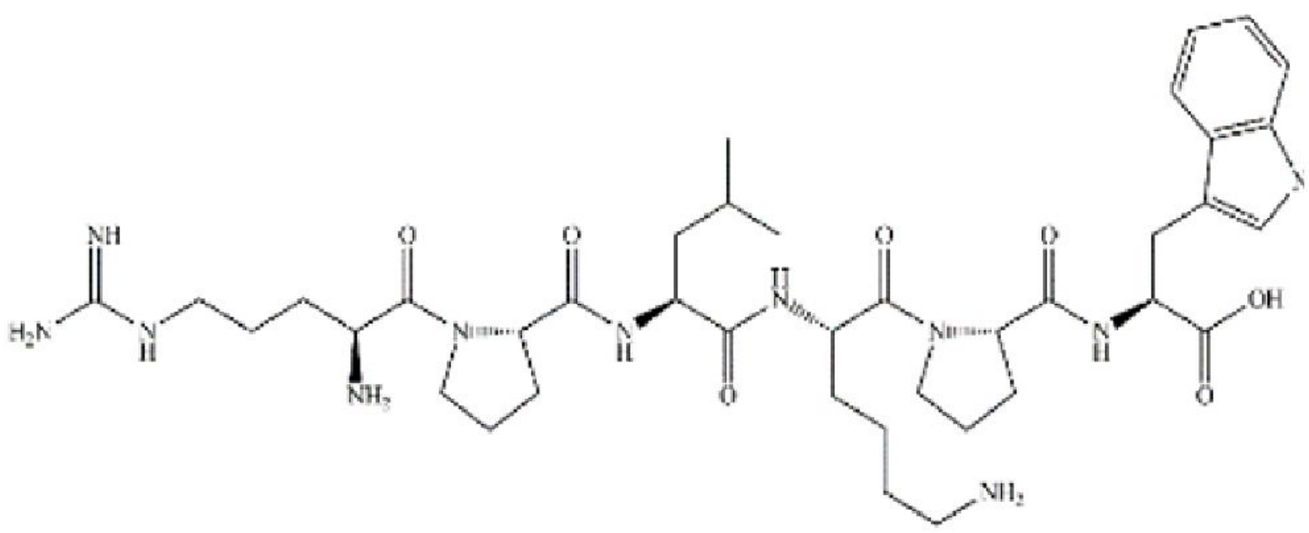
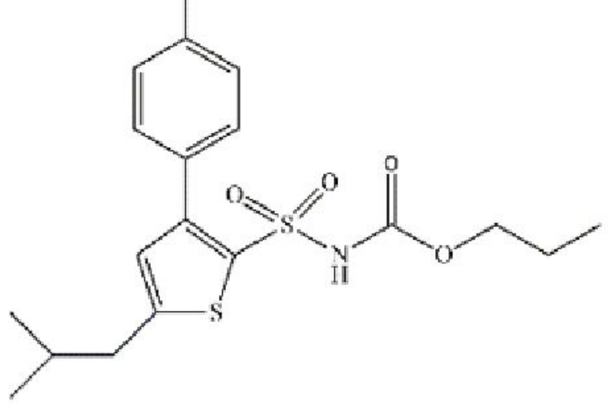


Novokinin



C21

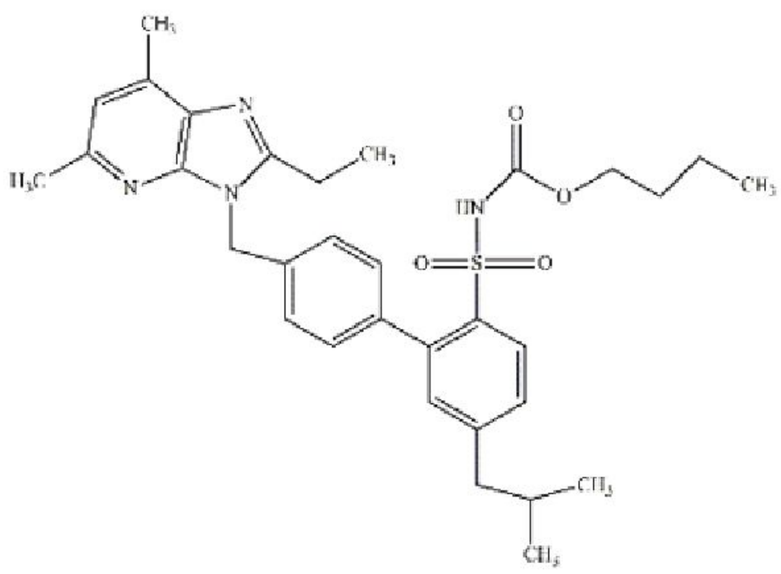


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Figure 5

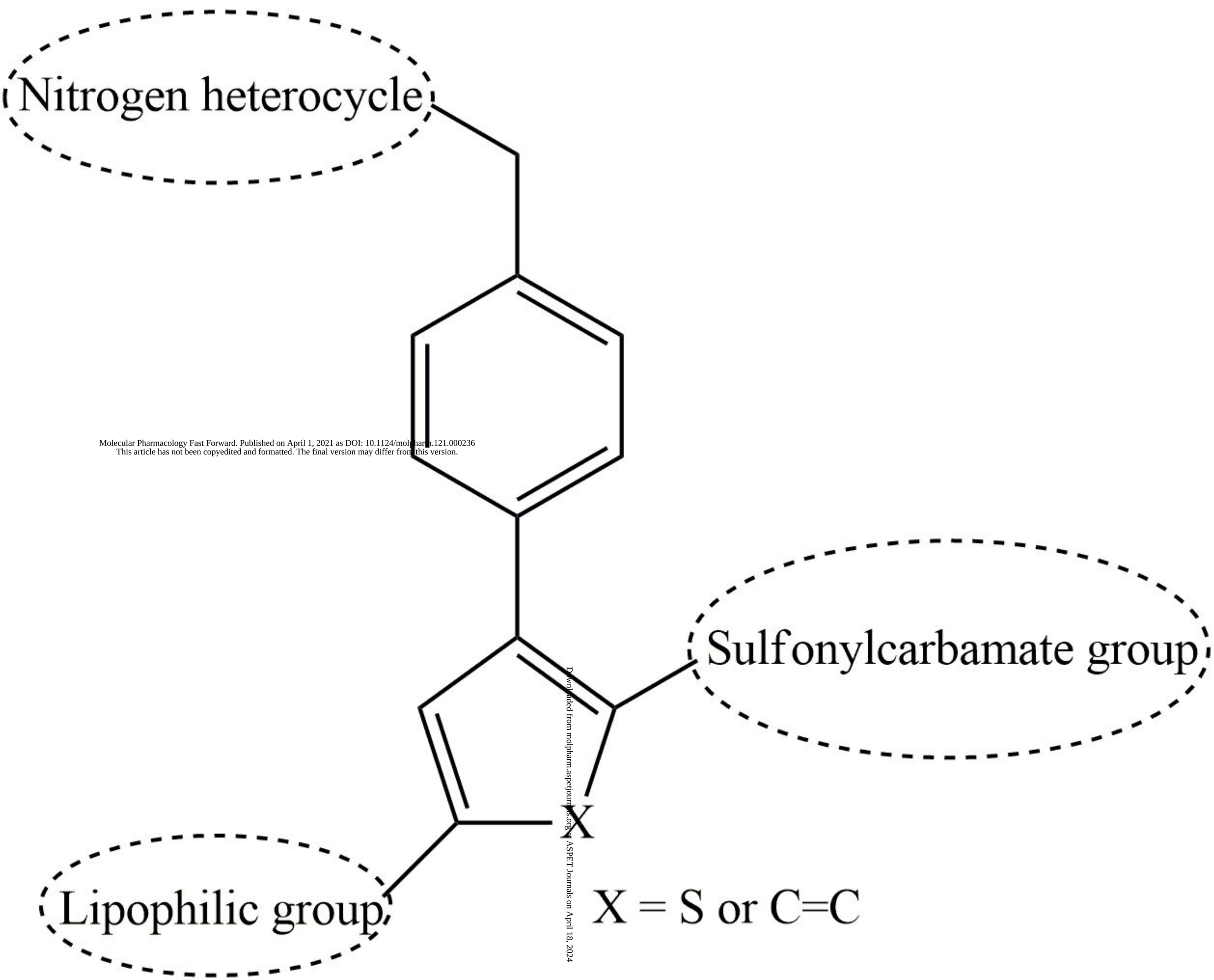

Figure 6