Receptors | Angiotensin Receptors

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In this article, we focus on one of the most intensely studied hormone-receptor systems in humans responsible for the regulation of blood pressure and water-electrolyte homeostasis. Since the last review chapter on Angiotensin Receptor (Inagami, 2004), there have been significant advances made in understanding the signal transduction mechanisms, pharmacology, and structural biology of these receptors as well description of a counter-regulatory arm of the renin-angiotensin system (RAS). There are four angiotensin (Ang) peptides, Ang II, Ang III, Ang IV, and Ang $(1-7)$, which meet the definition of an endogenous hormone that act through specific receptors. These cell surface Ang receptors, also called "interpreters of angiotensinergic signals", have been described in great detail in previous reviews (Forrester *et al.*, 2018; Karnik *et al.*, 2015). Angiotensin II, the classical blood pressure hormone binds and activates the type 1 (AT_1R) and type 2 (AT_2R) receptors. The Ang $(1-7)$ peptide is paired with multiple receptors, of which Mas receptor (MasR) and the Mas-related receptor (MRGD) are better studied. These four Ang receptors are seven-trans-membrane helical G protein-coupled receptors (GPCRs) that rely on heterotrimeric G proteins for mediating their cellular effects. These GPCRs are also capable of mediating β-arrestin biased signaling. The receptors for Ang IV (AT4Rs) are not GPCRs, instead they are type II single transmembrane Zn metalloproteases, IRAP (insulin regulated aminopeptidase), and its paralog AP-N (aminopeptidase-N). Ang III, Ang $(1-7)$ and Ang IV also function as surrogate ligands for AT_1R and AT_2R.

Careful studies have been performed by researchers for more than a century to understand several pathological states that result from RAS dysregulation in order to develop effective therapies. Indeed these efforts have resulted in two classes of highly successful and safe drugs, the angiotensin-converting enzyme inhibitors (ACEIs) and the AT1 receptor blockers (ARBs). Along with the importance of classical RAS, this article will highlight recent advancement in the field such as the role of counter-regulatory RAS, recent breakthroughs in defining the active state crystal structures of AT_1R and AT_2R; RAS components as targets of new diseases, the clinical potential of the approved drugs, and RAS targeting drugs under clinical trials.

Generation of Hormone Peptides of RAS and Consequences of Inhibition

All four angiotensin hormones are produced from the ten residues long amino-terminal segment (called Ang I) of angiotensi-nogen, a ≈ 65 kd serpin produced predominantly in the liver. Angiotensinogen gene knockout is lethal in mice, and it is the only source for the production of Ang I in the human proteome. The cascade of enzymes that are not localized to any one tissue but act on circulating and locally produced Ang I or its derivative fragments to generate the hormone peptides constitute RAS.
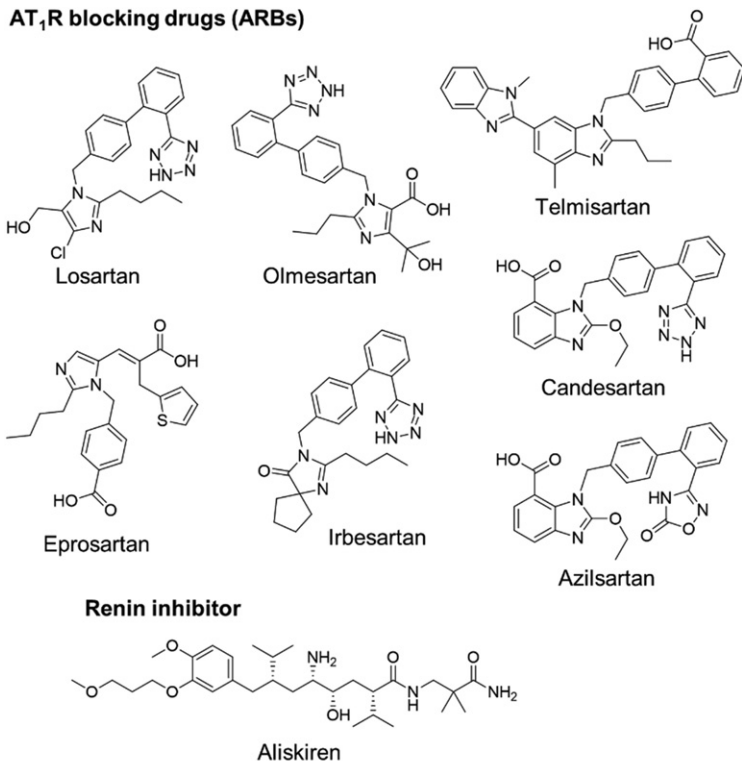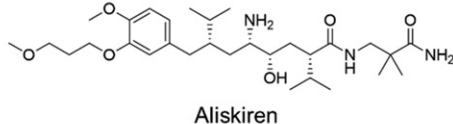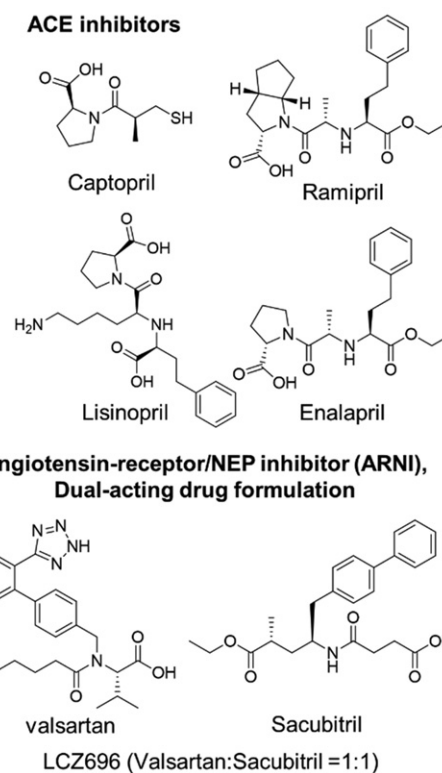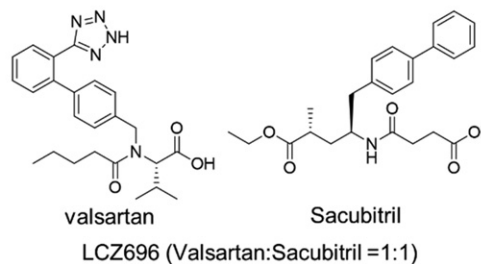
The initiating enzyme of the RAS cascade is renin, an aspartyl protease predominantly produced in the kidney that reacts exclusively with angiotensinogen, and cleaves only one peptide bond between residues 10 and 11 generating the decapeptide Ang I (Fig. 1). No hormonal activity is detected for Ang I, although its ubiquitous distribution may be attributed to Ang I-binding carrier proteins that show no receptor-like function to transmit any signal. The physiological significance of Ang I is that it is the only source for the hormone-generation role of RAS in body fluids and the intra- or inter-cellular apace. Many isoforms of renin exist that show tissue-specific variation in expression. Renin gene knockout in mice is not lethal.

An orally active non-peptide, a direct renin inhibitor, Aliskiren (Fig. 2) was FDA approved for the treatment of hypertension. It reduced plasma renin activity and hypertension, which are effects similar to those produced by ACEIs and ARBs. Aliskiren is well-tolerated with a relatively lower incidence of cough and angioedema (life-threatening airway swelling and obstruction) than those taking ACEIs. Gastrointestinal adverse effects upon taking Aliskiren is reported in some patients. The ALTITUDE trial studies (Novartis, 2014) reported increased adverse events (non-fatal stroke, renal complications, hyperkalemia, hypotension) with no apparent additional benefits in diabetic patients.

Release of renin by the kidney is under feedback control of circulating Ang II levels as well as many physiological factors, including ambient blood pressure, fluid volume, and electrolyte balance. Higher levels of prorenin than renin is released to plasma in disease states such as hypertension and diabetes. The question, how prorenin is activated outside the kidney led to the discovery of the prorenin receptor (PRR) (Batenburg *et al.*, 2004; Nguyen *et al.*, 2002). PRR is a ubiquitously expressed 350-amino acid protein, previously described as Na/H + ATPase, that binds prorenin and induces Ang I generation without proteolytic activation of prorenin. PRR expression levels are high in brain regions, which could be particularly relevant because the expression of classic RAS components is low. The PRR may contribute to angiotensin surges, and drugs to control renin activity by targeting PRR may be developed in the future.

The prohormone Ang I serves as the substrate for two prominent Zn protease angiotensin-converting enzymes, ACE and ACE2 abundantly expressed in the lungs but also in other tissues. Classical ACE is the same protein known as the kininase II in the kinin-kallikrein system. Thus, ACE converts Ang I to the vasoconstrictor octa-peptide hormone Ang II (Fig. 1), and it also degrades

Fig. 1 *The classical and counter-regulatory renin-angiotensin pathways with currently available and developmental drugs.* The classical RAS pathway is shown in dark red. Currently available antihypertensive drugs are shown in pink with italics. The counter-regulatory RAS pathway is shown in dark green. Compounds targeting this pathway are shown in violet, italics. Solid arrow indicates the synthesis of peptides, while the dotted arrow shows peptides targeting to their respective receptors. APA, aminopeptidase A; APN, aminopeptidase N; ACE2, angiotensin-converting enzyme 2; CPA, carboxypeptidase A; NEP, neutral endopeptidase; PEP, prolyl endopeptidase; THOP, thimet oligopeptidase; PCP, prolyl carboxyendopeptidase; PRCP, prolyl carboxypeptidase; AD, aspartate decarboxylase; AT_1R, Angiotensin II type 1 receptor; AT_2R, Angiotensin II type 2 receptor; Mas R, Mas receptor; MRGD, Mas-related G protein-coupled receptor member D.

vasodilator bradykinins. ACE activity increases blood pressure by producing Ang II, which induces constriction of blood vessels. This enzyme is the target of one of the most successful antihypertensive drugs, with a minimum of side effects and complications. Starting with captopril, a series of long-acting ACE inhibitors such as ramipril, enalapril, lisinopril (**Fig. 2**) have been highly successful drugs, primarily used for the treatment of high blood pressure, but have been effectively repurposed for treatment in heart failure, kidney damage in diabetes and fibrotic tissue damage.

ACE2 is a late addition to RAS, after the characterization of AT1 and AT2 receptors. It is a smaller molecule with high homology and functional similarity to ACE. However, it's enzymatic mechanism is different, being a carboxyl mono-peptidase it degrades Ang II to produce Ang $(1-7)$ (**Fig. 1**). Removal of the C-terminal residues from Ang I to form Ang $(1-9)$ combined then with the carboxyl di-peptidase activity of ACE also produces Ang $(1-7)$ and Ang $(1-5)$ (**Fig. 1**). Of these, Ang $(1-9)$ and Ang $(1-5)$ are inert degradation products. There are a few reports of the biological effects of these peptides that need further confirmation. Whereas Ang $(1-7)$ was first shown to activate phospholipase A2 to release arachidonic acid from phospholipids leading to the formation of prostaglandins through a GPCR, MasR, and later through the Mas related GPCR, MRGD. Both inhibitory and potentiating pharmacological agents targeting ACE2 have been disappointing in experimental systems without leading to clinically useful drugs as yet.

In recent literature actions of Ang II through AT_1R is described as classical RAS axis (**Fig. 1**) presumably because most of the known Ang II functions are mediated by the AT_1R. Further, AT_1R gene null mice do not survive, an outcome similar to knocking out the angiotensinogen gene. In contrast, knocking out genes for all other components of RAS is not lethal but demonstrate adaptive changes. Thus, the second axis of RAS, a counter regulator of the classical RAS axis (**Fig. 1**), is proposed consisting of AT_2R, MasR, and MRGD, their cognate ligands, and enzymes that produce these ligands. We will summarize recent advances concerning these two arms of RAS.

Fig. 2 *Chemical structures of the approved drugs targeting different components of the renin-angiotensin system.* AT$_1$R, Angiotensin II type 1 receptor; ACE, Angiotensin-converting enzyme; NEP, Neprilysin.

Classical RAS, Signaling Mechanisms and Topics of Current Interest in AT$_1$R Research

Classical RAS consisting of ACE/Ang II/AT$_1$R promotes almost all of the homeostatic regulatory functions of Ang II on cardiovascular, renal, and cerebral systems. The physiological levels of the hormone Ang II maintains normal blood pressure through regulation of peripheral resistance of vasculature, heart rate, cardiac output, neuronal control, and body fluid homeostasis through activation of the AT$_1$R. Whereas, overstimulation of AT$_1$R is associated with disease states such as hypertension, heart failure, cardiac hypertrophy, coronary artery disease, stroke, ischemic heart, diabetic nephropathy, arrhythmia and renal diseases (Khan, 2011; Lee et al., 2012; Vejakama et al., 2012; Vijayaraghavan and Deedwania, 2011).

Upon Ang II binding to AT$_1$R intracellular signals are mediated by heterotrimeric G-proteins (G$_{q/11}$, G$_{12/13}$, and G$_i$), which interact with the receptor, followed by the production of second messengers such as inositol trisphosphate, diacylglycerol, reactive oxygen species (ROS), and arachidonic acid. These molecules trigger the activation of downstream effectors like phospholipases C, A, and D. Furthermore, AT$_1$R activates various protein kinases intracellularly, including serine/threonine kinases such as mitogen-activated protein kinase (MAPK) family kinases, Protein kinase C (PKC), and Protein kinase B (PKB or Akt), receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (nRTKs). AT$_1$R also produces G protein-independent signals, such as β-arrestin signaling (Wei et al., 2003). Some novel and expanding Ang II signaling pathways investigated are Wnt/ β-catenin pathway, Notch pathway, NLRP3 (Nucleotide-binding domain and leucine-rich repeat-containing PYD-3) inflammasome and Hippo pathway which plays a variety of roles during developmental processes. Further, the key expanding areas include intracellular as well as extracellular organelle signal communications, signaling through posttranslational protein modification, cellular and tissue metabolic modulation, microRNAs, and long noncoding RNAs (Forrester et al., 2018).

Non-canonical AT$_1$R functions have been described that include ligand-independent activation of AT$_1$R through either mechanical stress or AT$_1$R -directed agoniztic autoantibodies or via receptor mutations (Liu et al., 2016; Mederos y Schnitzler et al., 2011; Storch et al., 2012; Unal et al., 2012; Wallukat and Schimke, 2014). These modes of activation may occur clinically, as in hypertension or preeclampsia (Wei et al., 2011; Zou et al., 2004). Candesartan and other inverse agonizts may show increased therapeutic effects in these disease conditions (Wei et al., 2011; Zou et al., 2004). AT$_1$R signaling properties could be altered in pathology when AT$_1$R forms both homodimers and heterodimers (AbdAlla et al., 2004, 2000; Ayoub et al., 2015; Bellot et al., 2015; de Lourdes Gonzalez-Hernandez et al., 2010; Goupil et al., 2015; Martinez-Pinilla et al., 2015; Rozenfeld et al., 2011; Siddiquee et al., 2013; Wnorowski and Jozwiak, 2014) in disease states like atherosclerosis, preeclampsia, and chronic kidney disease (AbdAlla et al., 2001, 2004; de Lourdes Gonzalez-Hernandez et al., 2010).

β-arrestin biased signaling in GPCRs is thought to be a relatively protective long-term effect of hormonal action. The discovery of β-arrestin biased AT$_1$R ligands preferentially activating the β-arrestin-mediated signaling pathway (Violin *et al.*, 2010; Wei *et al.*, 2003) has gained much attention due to therapeutic potential in treating cardiovascular diseases (Boerrigter *et al.*, 2011; Boerrigter *et al.*, 2012). The size of the side chain of eighth residue present in different Ang II analogs determines the relative strength of AT$_1$R signaling bias through G-protein and β-arrestin pathways (Domazet *et al.*, 2015; Rajagopal *et al.*, 2011; Zimmerman *et al.*, 2012). Ang II and other full agonizts with Phe8 efficiently activate the Gq pathway. Whereas, Ile8 substitution in S1I8 changes it to a partial agonist for Gq signaling. It has been reported that strong β-arrestin biased ligands (which functions through β-arrestin while deficient in Gq-mediated functions) have smaller side chains (e.g., Ala) or even deletion of eighth residue (Rajagopal *et al.*, 2011; Strachan *et al.*, 2014).

Crystal Structures of AT$_1$R

Crystallographic structures of both active and inactive conformational states are now available for AT$_1$R, which provide insights into the conformational dynamics of the receptor in agonist and antagonist bound states.

Two inactive state structures were solved first, with an experimentally used antagonist ZD7155 (PDB ID: 4YAY) and clinically used inverse agonist olmesartan (PDB ID: 4ZUD) (Zhang *et al.*, 2015a,b). For solving the structure, the human AT$_1$R was engineered for thermal stability by truncating at the N-terminal region (delete Met1, Thr7-Asp16) and C-terminal tail at the end of helix 8, and by inserting a thermos stabilized apocytochrome, b$_{562}$RIL (BRIL) at N-terminus. The final structure contains 289 out of 359 full-length residues of AT$_1$R. However, these modifications did not alter the pharmacological and functional properties of the receptor for antagonist binding and signaling (Zhang *et al.*, 2015b). High-accuracy computer model of hAT$_1$R that included deleted portions were generated (Singh *et al.*, 2018). The binding poses of experimentally used antagonists, and eight clinically used ARBs were found to be similar. Molecular dynamics simulation studies validated experimental Ki differences of ARBs as well as discriminatory mutagenesis data. Differences in spatiotemporal interactions of different ARBs was observed, which could account for efficacy differences reported for ARBs in various clinical trials. Nature and bonding energy contribution of some critical residues involved in binding all ARBs significantly differ. For example, Arg167^{ECL2} sidechain interacted with both imidazole and tetrazole groups in olmesartan but not in candesartan, losartan, and irbesartan. Instead, Lys199^{TM5} interacted with losartan and irbesartan. Valsartan interactions with Lys199^{TM5} is a weak water-mediated interaction. Both Arg167^{ECL2} and Lys199^{TM5} bond with two carboxylic groups of eprosartan, but mutating either of these residues do not affect eprosartan binding (Singh *et al.*, 2018). Initial structural papers also predicted that Ang II binding mode to differ from that of ARBs, but sharing critical contact residues (Tyr35$^{3.91}$, Trp84$^{2.60}$, Tyr87$^{2.63}$, Arg167^{ECL2}, Lys199$^{5.42}$, Ile288$^{7.39,}$ and Tyr292$^{7.43}$) which can account for the competitive antagonistic relationship of ARBs with Ang II (Singh *et al.*, 2018).

The active state structure of AT$_1$R bound to the Ang II analog [Sar^1, Ile^8]Ang II (S1I8) was elucidated at 2.9 Å resolution (PDB ID: 6DO1) (Wingler *et al.*, 2019a,b). The crystallization construct included all N-terminal residues and BRIL inserted into ICL3 as has been done for solving structures of many other GPCRs. In this configuration, the engineered receptor construct increased the affinity for Ang II ~2-fold and also displayed strong binding to an active-state conformation-specific AT$_1$R nanobody, Nb. AT110i1. This study demonstrated unequivocally the critical residues of AT$_1$R that bond with various sidechains in the peptide ligand and provided a structural interpretation for the movement of different segments of the receptor, which enable signaling. For instance, Pro7 and Ile8 of S1I8 (Phe8 in Ang II) interact with Trp84$^{2.60}$, Val108$^{3.32}$, Leu112$^{3.36}$, and Ile288$^{7.38}$, which are located at the bottom of the orthosteric binding pocket. Ile8 of the peptide also forms a hydrophobic contact with His256$^{6.51}$, a residue critical for AT$_1$R activation (Noda *et al.*, 1995). Unlike ARBs, the peptide ligand is involved in extracellular interactions with hydrophobic residues (Ile172^{ECL2}, Tyr92^{ECL1}, Val179^{ECL2}, and Ala181^{ECL2}) and charged residues (Asp281$^{7.32}$ and Asp263$^{6.58}$) confirming prior mutagenesis results (Feng *et al.*, 1995; Fillion *et al.*, 2013). The terminal carboxylate of the peptide interacts with Lys199$^{5.42}$. This interaction has been observed as a key contact in AT$_1$R activation by mutagenesis studies (Noda *et al.*, 1995; Fillion *et al.*, 2010). Due to inward shifts of TM5 and TM7, and changes in ELC2 conformation, the ligand-binding pocket is substantially constricted around the peptide in the active state receptor compared to the inactive structure. The peptide-binding site of the AT$_1$R showing S1I8-AngII binding residues of the receptor are shown in **Fig. 3**.

While comparing the active and inactive structures of AT$_1$R, the most notable conformational changes were observed on the intracellular side of the receptor, as observed in almost all activated GPCR structures (Manglik and Kruse, 2017). Outward displacement of TM6 by 11 Å, along with the rotation of TM5 away from the G protein/β-arrestin binding pocket and inward rotation TM7, was clearly observed. Additionally, a short α helix is formed by the reorganization of the ICL2, and a significant repositioning of helix 8 was also observed. Helix 8 in the inactive AT$_1$R structure (**Fig. 4**) follows an atypical conformation bent away from the membrane plane. On the other hand, the active structure (**Fig. 3**) displays the conventional position of Helix 8 parallel to the membrane. The structural findings validate several functional studies suggesting that the Helix 8 of AT$_1$R may be a critical motif for generating G protein independent signals.

The activation mechanism of AT$_1$R suggested by Wingler *et al.* suggests the movement of Trp253$^{6.48}$ and Tyr292$^{7.43}$ to avoid a steric clash with Phe8 sidechain of Ang II, resulting ultimately in the breakage of a hydrogen bond between Asn111$^{3.35}$-Asn295$^{7.46}$ present in the inactive structure (Wingler *et al.*, 2019a,b). A slightly different scheme for activation is suggested by MD simulation studies. This model suggests that Phe8 in Ang II is involved in a van der Waals "grasp" interaction with Ile288$^{7.39}$ in AT$_1$R. An induced mechanical strain pulls Tyr292$^{7.43}$ and breaks critical inter-helical H-bonds, first between Tyr292$^{7.43}$ and Val108$^{3.32}$ and
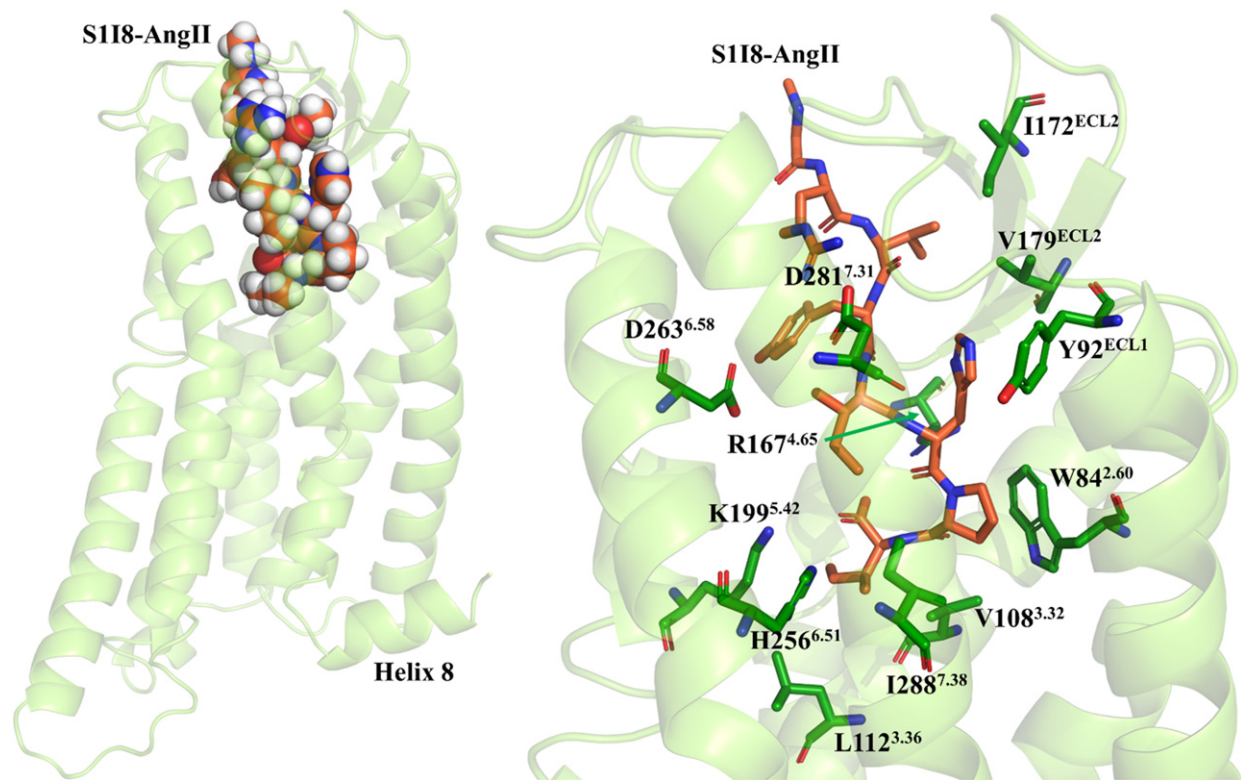
Fig. 3 *The peptide-binding site of the AT₁R showing S1I8-AngII binding residues of the receptor and position of helix 8 in an active state of AT₁R.* Sarcosine1, Isoleucine8-AngII is abbreviated as S1I8-AngII. The S1I8-AngII-binding residues of AT₁R are shown in green. S1I8-AngII is shown in orange.
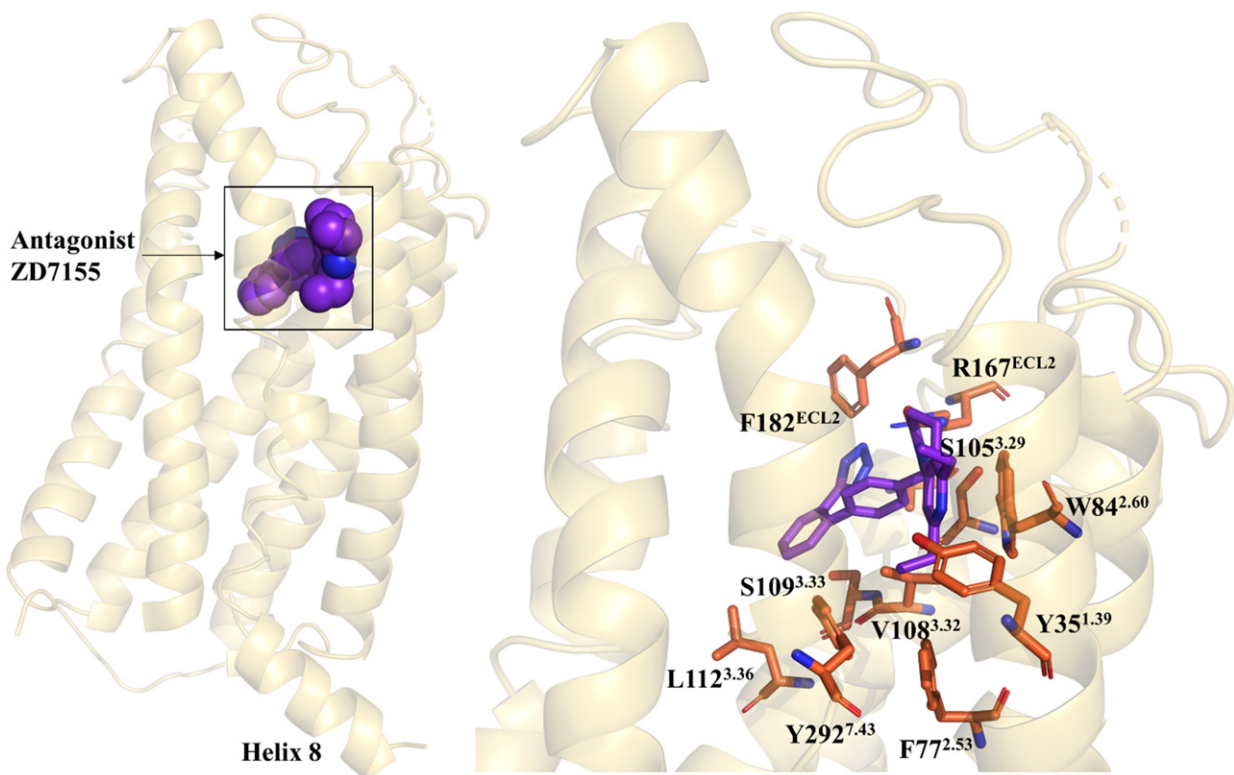
Fig. 4 *The ARB's binding site of the AT₁R showing ZD7155 (antagonist) binding residues of the receptor and position of helix 8 in an inactive state of AT₁R.* The antagonist ZD7155 binding residues of AT₁R are shown in orange. ZD7155 is shown in violet.

second between Asn111$^{3.35}$ and Asn295$^{7.46}$ (Singh et al., 2019). The consensus step in both schemes, breakage of hydrogen bond (s) between Asn111$^{3.35}$-Asn295$^{7.46}$ was shown to activate AT$_1$R ligand independently by mutagenesis studies (Noda et al., 1995; Singh et al., 2019; Wingler et al., 2019b).

Approved ARBs and Their Clinical Potential

Eight ARBs, azilsartan, candesartan, eprosartan, olmesartan, irbesartan, losartan, telmisartan, and valsartan are in clinical use as safe drugs primarily to control blood pressure. These drugs vary in their duration of action and efficacy when repurposed in the treatment of disorders of kidney, heart, lungs, fibrogenesis of liver, kidney, or heart and stroke prevention. Additional indications have been recommended. For example, telmisartan can improve glucose metabolism and lipid profile (Nedogoda et al., 2013). Losartan shows uricosuric activity (Nedogoda, 2011). Reduction in inflammatory processes in vessel walls was observed in patients with arterial hypertension and metabolic syndrome when treated with ARBs, thus decreasing their risk for cardiovascular disease and development of diabetes mellitus (Savoia and Schiffrin, 2007). Based on the experimental data, sartans are reported to have possible therapeutic effects in the treatment of autoimmune diseases such as rheumatoid arthritis (Silveira et al., 2013) and multiple sclerosis (Lanz et al., 2010).

AT$_1$ receptors are abundantly expressed on the endothelial cells of the blood-brain barrier, and these have been benefited by the neuroprotective actions of the ARBs (Saavedra et al., 2011). ACE inhibitors captopril and perindopril, and ARB's losartan, telmisartan, and candesartan have shown neuroprotective effects in animal Parkinson's disease models. These effects seem to be mediated by a decrease in the overproduction of reactive oxygen species (ROS) (Perez-Lloret et al., 2017). Preclinical and clinical data support potential antidepressant properties of ACEIs and ARBs. For example, captopril (Costall et al., 1990), losartan (Llano Lopez et al., 2012; Srinivasan et al., 2003), valsartan (Ping et al., 2014), irbesartan (Ayyub et al., 2017), telmisartan (Aswar et al., 2017), and Candesartan (Benicky et al., 2011; Saavedra et al., 2006) had positive effects on depression and reduced anxiety behavior, whereas other antihypertensive agents did not (Vian et al., 2017).

Dual-Acting Angiotensin-Receptor/NEP Inhibitor (ARNI)

Sacubitril/valsartan or LCZ696, called Entresto (developed by Novartis), is a novel dual-acting drug formulation comprising equimolar amounts of the ARB valsartan and the neprilysin inhibitor sacubitril (Sacubitril is a prodrug that is activated to sacubitrilat by de-ethylation via esterases). This drug combination increases neprilysin activity while inhibiting the harmful effects of the RAS, without affecting bradykinin and other neprilysin-derived vasoprotective factors. Sacubitril/ valsartan has demonstrated clinical efficacy in lowering blood pressure in patients with primary hypertension and patients with or without heart failure. The landmark clinical trial named PARADIGM-HF [Prospective comparison of ARNI (LCZ696) with ACEI (Enalapril) to Determine Impact on Global Mortality and morbidity in chronic Heart Failure] showed that LCZ696 was significantly more effective than enalapril in treating heart failure (McMurray et al., 2014; Mogensen et al., 2018). Sacubitril/ valsartan was approved by the FDA in 2015. This drug is now included in European (Ponikowski et al., 2016) and American (Yancy et al., 2016) clinical practice guidelines for the treatment of heart failure.

Counter-Regulatory RAS With its Importance and Limitations

Counter- regulatory or the non-canonical or the non-classical RAS primarily consists of Ang II/Ang III-AT$_2$R, and the ACE2-Ang-(1 − 7)-MasR axis. Generally, it is believed that the counter-regulatory RAS functions in a manner antagonistic to the deleterious effects of a dysregulated classical RAS, the Ang II-AT$_1$R axis. This includes actions of extensively studied ligand/receptor actions such as Ang (1 − 7)/MasR and Ang II or Ang III/AT$_2$R, as described earlier (Forrester et al., 2018). There are also reports suggesting the actions of Ang (1 − 9)/AT$_2$R on the cardiovascular system (Mendoza-Torres et al., 2018). Potential roles of ligands, ala-mandine, and Angiotensin A remain poorly characterized. Ang-(1 − 7) has also been suggested to function as a β-arrestin-biased agonist of AT$_1$R (Teixeira et al., 2017). Alamandine functions through MRGD and activates the AMP-activated protein kinase (AMPK)–nitric oxide (NO) pathway, which prevents Ang II-induced hypertrophy (Jesus et al., 2018). Angiotensin 1–5 has been shown to induce atrial natriuretic peptide (ANP) secretion through the Mas receptor and activating the PI3K–Akt–endothelial NO synthase pathway (Yu et al., 2016).

Different research groups have different opinions about whether Ang-(1 − 7) binds directly to the Mas receptor or not, and this has been reflected from the contradictory results published. Some showed that Ang-(1 − 7) can oppose the Ang II signaling as a result of heterodimerization between AT$_1$R and Mas receptor but not by direct binding to the Mas receptor (Gaidarov et al., 2018; Kostenis et al., 2005). However, others demonstrated the binding of radiolabeled or fluorescent Ang-(1 − 7) to the Mas receptor-expressing tissue (Santos et al., 2003). In summary, more research is required to confirm whether Ang-(1 − 7) acts as an endogenous agonist of the Mas receptor.
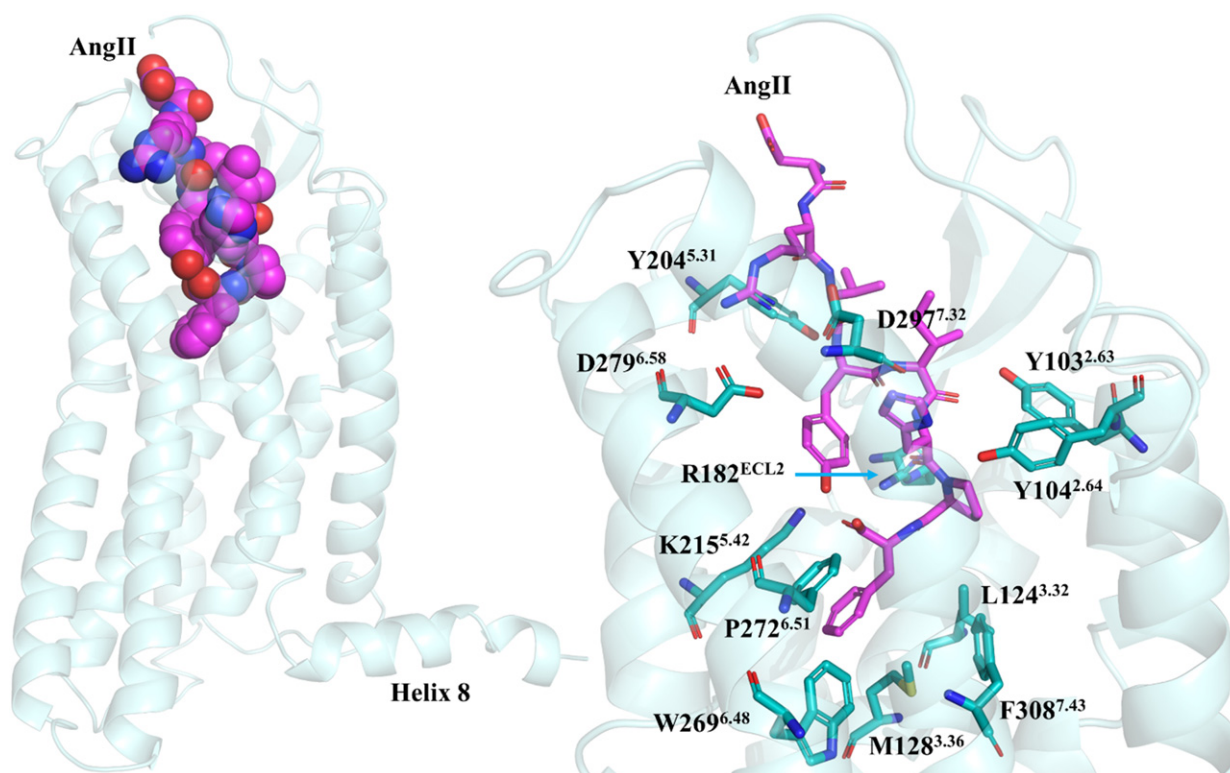
Fig. 5 *The peptide-binding site of the AT_2R showing Ang II binding residues of the receptor and position of helix 8.* The Ang II-binding residues of AT_2R are shown in teal. Ang II is shown in magenta.

Crystal Structures of AT_2R

AT_2R structures have been solved with either small-molecule ligands (quinazolinone-biphenyl tetrazole derivatives 1 and 2) named as compound 1 and compound 2 (Zhang *et al.*, 2017b). The compound 1 is AT_2R selective, while compound 2 is AT_1R/AT_2R dual ligand [PDB ID: 5UNF; 5UNG; 5UNH]. Following this study, the peptide ligand-bound AT_2R structures were solved with S1I8 (PDB ID: 5XJM) or the endogenous peptide, Ang II (PDB ID: 6JOD) (Asada *et al.*, 2018, 2020).

A nearly similar AT_2R construct was used to obtain both S1I8-Ang II-bound and Ang II bound crystal structures. The AT_2R-specific antibody, Fab4A03, was added during the purification of both the structures to increase thermal stability and facilitate crystallization. Both structures lack the putative C-terminal palmitoylation site (residue range: 35–346) and N-terminal glycosylation sites. The difference lies in the type and position of BRIL that was inserted in the third intracellular loop in S1I8 bound AT_2R and fused at N-terminus in Ang II-bound AT_2R. The S1I8-Ang II bound AT_2R sequence includes 35–346 of the 363 residues, while the Ang II bound AT_2R sequence includes 1–346 of 363 residues.

The structures demonstrated AT_2R to accommodate both peptides in a very similar binding mode. The Ang II sidechains interacted with AT_2R residues whose position and chemical characteristics are conserved, as seen in the AT_1R structure. Arg2 forms salt bridges with $Asp279^{6.58}$ and $Asp297^{7.32}$, which are conserved as $Asp263^{6.58}$ and $Asp281^{7.32}$ in AT_1R. The guanidinium group of $Arg182^{ECL2}$, which is conserved as $Arg167^{ECL2}$ in AT_1R bonds with carbonyl oxygens of His6 and Pro7 in Ang II. Phe8C-terminal carboxyl group forms a salt bridge with the side chain of $Lys215^{5.42}$, which is conserved as $Lys199^{5.42}$ in AT_1R. Phe8 of Ang II also interacts with $Leu124^{3.32}$, $Met128^{3.36}$, $Trp269^{6.48}$, $Phe272^{6.51}$, and $Phe308^{7.43}$. Ligand binding and mutagenesis experiments proved that $Tyr104^{2.64}$, $Met128^{3.36}$, $Lys215^{5.42}$, $Trp269^{6.48}$, $Phe272^{6.51}$, $Arg182^{ECL2}$, $Asp297^{7.32}$, and $Phe308^{7.43}$ play vital roles in Ang II binding. Although Fab4A03, AT_2R specific antibody used during crystallization, does not affect the conformation of the side chains critical for ligand binding in both Ang II-bound and S1I8-Ang II-bound AT_2R, ECL2 conformation on the surface is affected in both structures. The peptide-binding site of the AT_2R with Ang II binding residues of the receptor are shown in Fig. 5.

The residues at the bottom of the ligand-binding pocket and $Met128^{3.36}$ ($Leu112^{3.36}$ for AT_1R), seems to play a key role in AT_2R activation. Upon Ang II binding, $Met128^{3.36}$ moves toward $Phe308^{7.43}$ ($Phe308^{7.43}$ for AT_1R) to make room for Phe8 side chain of Ang II, leading to the rotation of TM7 possessing $Phe308^{7.43}$. These residues form hydrophobic core at the bottom of the ligand-binding cavity of AT_2R, as observed in AT_1R. The insertion of Ang II Phe8 residue into this hydrophobic core appears to trigger the activation of the receptor, where the conformational change is transferred through the region containing the internal lock, $Asn111^{3.35}$-$Asn295^{7.46}$ leads to the breakage of the hydrogen bond between these residues during AT_1R activation. In AT_2R, $Ser311^{7.46}$ is present in place of $Asn295^{7.46}$, and a hydrogen bond between $Asn127^{3.35}$ and $Ser311^{7.46}$ is not observed in the Ang

II-bound AT_2R structure. It is not clear whether these residues form hydrogen bond in the inactive conformation since AT_2R inactive structure is not available. However, AT_2R has been described as a constitutively active receptor (Miura and Karnik, 2000), therefore it may naturally lack the hydrogen bond between $Asn127^{3.35}$ and $Ser311^{7.46}$.

In support of this view, an active like conformation of AT_2R was captured (Asada et al., 2018, 2020; Zhang et al., 2017b). Structures showed that AT_2R lacks the key conformational locks seen in AT_1R: 1) TM5/TM6 phenylalanine cluster ($Phe208^{5.51}$/$Phe249^{6.44}$/$Phe250^{6.45}$) in AT_1R are replaced by aliphatic residues ($Leu224^{5.51}$/$Phe265^{6.44}$/$Ile266^{6.45}$) in AT_2R. In all the three crystal structures available for AT_2R (mentioned under structure of AT_2R section), these residues align with active AT_1R structure rather than inactive AT_1R. 2) The $Asn111^{3.35}$-$Asn295^{7.46}$ hydrogen bond in the AT_1R inactive state is not present in the AT_2R structure. Instead, AT_2R has $Ser311^{7.46}$ (in place of $Asn295^{7.46}$), and $Asn^{3.35}$ has moved away from the receptor core. 3) In AT_1R, T292F mutation increases the affinity towards Ang II while decreases towards antagonists. Whereas $Tyr292^{7.43}$ is substituted by $Phe308^{7.43}$ in the AT_2R, denoting a mutation-induced-stabilization of an active-like state. 4) Finally, AT_2R shows highly unusual behavior as it does not seem to signal through traditional G-protein- and β-arrestin-mediated signaling pathways, and this is potentially due to helix 8 overlap over the transducer-binding site of AT_2R (Zhang et al., 2017a).

The MasR and Mas-related GPCRs exhibit structural features of AT_2R that cause constitutive activation. In signaling experiments, high constitutive signaling by MasR is reported. Therefore intrinsic constitutive activation is likely a feature of these receptors. However, there has been no structural investigations focused on MasR and its activation by ligands such as Ang $(1-7)$ or related peptides.

## Targeting the Counter-regulatory Axis of RAS and Novel Therapeutic Approaches

AT_2R signaling and functions are better understood now and being translated to develop potential drugs targeting AT_2R. The developmental drugs are AT_2R agonizts with primary indications for use in fibrotic diseases and diabetic nephropathy. A non-peptide AT_2R agonist, Compound 21 (C21) (Fig. 6), has successfully completed phase I clinical testing and entered Phase II clinical study in patients with Pulmonary fibrosis in systemic sclerosis (SSc) and Idiopathic pulmonary fibrosis (IPF). In addition, a phase II study of C21 in patients with COVID-19 proposed. The rationale behind this study is to address an imbalance in the local RAS caused by ACE2 inactivation due to COVID-19 binding. C21 may suppress inflammatory mediators by acting directly on the AT_2R and bypass the way by which the virus disables the system (See Relevant Websites Section). Selective AT_2R agoniztic lanthipeptide MOR107 (MorphoSys, See Relevant Websites Section) previously known as LP-2 (by Lanthio Pharma See Relevant Websites Section) is currently being tested in a phase I clinical trial with a focus on oncology indications. The AT_2R antagonist EMA401 (Fig. 6) has completed a phase II clinical trial for the treatment of neuropathic pain (Rice et al., 2014). It is currently undergoing additional phase II studies (Novartis, 2020).

Central acting Aminopeptidase A (APA) inhibitors are in consideration for the treatment of neurogenic hypertension. Overproduction of Ang III in the brain exerts a tonic stimulatory control over blood pressure in experimental models of hypertension. Brain Ang III is generated by brain APA, and hence brain APA represents a promising target for the development of potent and selective central-acting antihypertensive agents. RB150/QGC001 $(4,4-dithio-\{bis[(3S)-3-aminobutyl sulfonic acid]\})$, a prodrug of selective and specific APA inhibitor EC33, later renamed as Firibastat (Fig. 6), was developed for clinical studies (Fournie-Zaluski et al., 2004). Orally administered RB150 crosses the hepatic, intestinal, and blood-brain barriers. The prodrug RB150 is cleaved by brain reductases on entry into the brain to generate two active molecules of EC33. This active molecule then inhibits the brain APA activity, block the formation of brain Ang III (Fournie-Zaluski et al., 2004), thus decrease BP and arginine-vasopressin release in deoxycorticosterone acetate salt rats and spontaneously hypertensive rats (Bodineau et al., 2008; Marc et al., 2012, 2018). A phase Ia clinical studies have shown that Firibastat is well tolerated in healthy human volunteers (Balavoine et al., 2014). A phase IIa trial was carried out in grade I and II hypertensive patients (Azizi et al., 2017), which suggests that firibastat treatment is very effective in decreasing daytime ambulatory systolic blood pressure (SBP) without causing hypotension. Later, a large phase IIb studies, NEW-HOPE (Novel Evaluation with QGC001 in Hypertensive Overweight Patients of Multiple Ethnic Origins), was carried out in overweight hypertensive patients and a significant BP-lowering efficacy and a safe tolerability profile were observed (NCT03198793) (Ferdinand et al., 2018) in those patients. If the proposed phase III trials to determine the efficacy succeed, firibastat could be the first of a new class of centrally acting antihypertensive agents. Based on all the clinical studies, firibastat may be especially effective in African Americans who are poor responders to blockers of the systemic RAS.

A recent study has shown that an oral formulation of hydroxypropyl-β-cyclodextrin/Ang-$(1-7)$ (Fig. 6) is effective in humans, as demonstrated by attenuating eccentric overload muscle damage (Becker et al., 2018). The effects of Ang-$(1-7)$ oral formulation in humans may open new possibilities for evaluating the actions Ang-$(1-7)$ and its therapeutic effects in patients.

RAS Components as Targets of New Diseases Including COVID-19

RAS is a complex, multifunctional system that possesses important roles beyond the cardiovascular system. Alterations in the expression of these components were shown to be involved in various diseases. For example, brain RAS involving the ACE2/Ang-$(1-7)$/Mas receptor axis and the Ang IV/insulin-regulated aminopeptidase pathways may play a role in Parkinson's and Alzheimer's diseases (Wright et al., 2013). ACE, Ang-$(1-7)$, AT2 receptors, and N-acetyl-Ser-Asp-Lys-Pro may have a role in
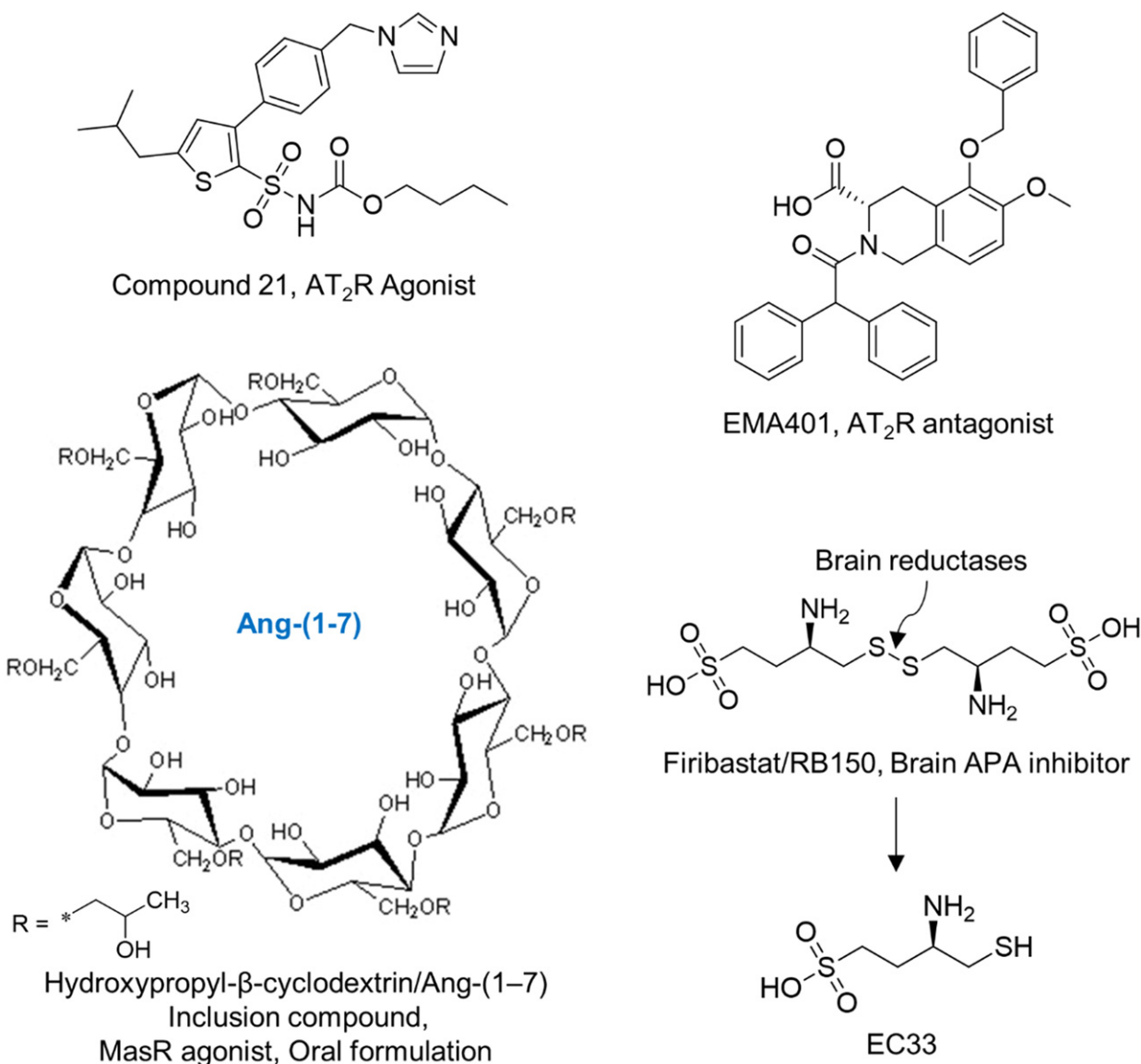
Fig. 6 *Chemical structures of the drugs which are under clinical trials targeting different components of the renin-angiotensin system.* AT₂R, Angiotensin II type 2 receptor; MasR, Mas Receptor; APA, Aminopeptidase A.

hematopoiesis (Rodgers and Dizerega, 2013). Aldosterone produced locally may have a pathogenic role (Aroor *et al.*, 2013; De Mello and Frohlich, 2014), and the ACE2/Ang-(1 − 7)/Mas receptor pathway may take part in reproduction, fetal programming, and cancer (Chappell *et al.*, 2014; Herr *et al.*, 2013). Activation of skeletal RAS plays a major role in bone diseases, such as arthritis, osteoporosis, and deterioration, as well as in fracture healing (Zhao *et al.*, 2019).

The recently emerged disease involving the RAS component is COVID-19 (Coronavirus Disease 2019) pandemic. The virus is named SARS-CoV-2 (severe acute respiratory syndrome coronavirus-2). The virus binds to the ACE2 receptor for entry into target cells. ACE2 prevents adverse effects of Ang II by degrading it to Ang-(1 − 7) (Donoghue *et al.*, 2000; Hamming *et al.*, 2007) and has been implicated in hypertension (Allred *et al.*, 2002; Crackower *et al.*, 2002), diabetes (Tikellis *et al.*, 2003), Ang-(1 − 7) regulation during pregnancy (Brosnihan *et al.*, 2003), heart failure and ventricular remodeling (Donoghue *et al.*, 2003; Zisman *et al.*, 2003). ACE2 predominantly expressed on epithelial cells of lungs, heart, kidney, and intestine (Donoghue *et al.*, 2000; Zhang *et al.*, 2020; Zhao *et al.*, 2020) is a functional receptor for the coronavirus (Li *et al.*, 2003). Interaction between the SARS-CoV-2 spike receptor-binding domain (RBD) and ACE2 has been shown in atomic-resolution by structural studies (Lan *et al.*, 2020; Wang *et al.*, 2020). These findings shed light on virus recognition, infection, and provide important structural information about the development of therapeutic treatment against this emerging virus.

Several comorbidities, including cardiovascular diseases, diabetes mellitus, and hypertension, are involved in COVID-19 patients with a severe course of progression and contributing to higher mortality risk (The Novel Coronavirus Pneumonia Emergency Response Epidemiology Team, china, 2020). Some research groups reported that both ACEI and ARBs could

substantially increase the mRNA expression of cardiac ACE2 (Ferrario *et al.*, 2005), which may increase virus susceptibility. However, due to lack of evidence about the potential negative impact of these medications on COVID-19 infection, European and American Societies of Cardiology expressed that ACEIs and ARBs are safe to continue and should be prescribed according to established guidelines (Sommerstein *et al.*, 2020). In conclusion, cardiovascular diseases and/or their therapy, by influencing ACE2 levels, may play a crucial role in infectiveness and outcome of COVID-19. Whether treatment or disease triggered upregulation of ACE2 affects the course of COVID-19 needs to be determined.

Acknowledgment

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