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Graphical abstract

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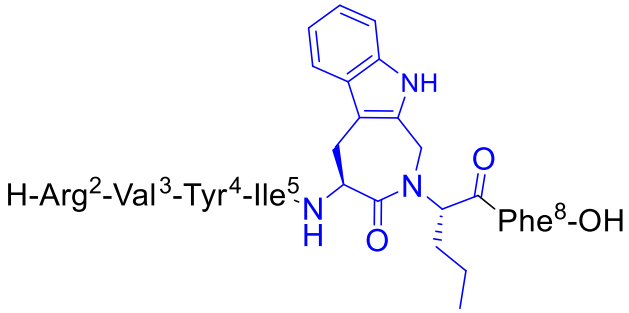

[des-Asp^1,Aia^6-Nva^7]-Ang II (**SBL-AII-10**)

IC_{50}(AT1R) >10000 nM; IC_{50}(AT2R) 24.44 nM

$t_{1/2}$ (human plasma) > 12h

Using Conformational constraints at position 6 of Angiotensin II to generate compounds with enhanced AT2R selectivity and proteolytic stability

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Abstract: The Renin-Angiotensin System (RAS) plays a crucial role in numerous pathological conditions. Two of the critical RAS players, the angiotensin receptors AT1R and AT2R, possess differential functional profile, although they share high sequence similarity. Although the main focus has been placed on AT1R, several epidemiological studies have evidenced that activation of AT2R could operate as a multimodal therapeutic target for different diseases. Thus, the development of selective AT2R ligands could have a high clinical potential for different therapeutic directions. Furthermore, they could serve as a powerful tool to interrogate the molecular mechanisms that are mediated by AT2R. Based on our recently established high affinity and AT2R selective compound [Y]^6-AII we developed several analogs through modifying aminoacids located at positions 6 and 7 with various conformationally constrained analogues to enhance both the selectivity and stability. We report the development of high-affinity AT2R binders, which displayed high selectivity for AT2R versus AT1R. Furthermore, all analogues presented enhanced stability in human plasma with respect to the parent hormone Angiotensin II as also [Y]^6-AII.

Key Words: Angiotensin II, Constrained peptides, Receptor affinity, Receptor selectivity, Proteolytic stability

The biological importance of G protein-coupled receptors (GPCRs) is well established, as they regulate several physiological and pathological processes. Thus, GPCRs constitute a constant and significant pharmacological target for drug development. Angiotensin II (Ang II) is an octapeptide hormone that plays an essential role in the renin-angiotensin system (RAS). Angiotensin II (Ang II; H-Asp^{1}-Arg^{2}-Val^{3}-Tyr^{4}-Ile^{5}-His^{6}-Pro^{7}-Phe^{8}-OH) binds to the Angiotensin type 1 (AT1R) and type 2 (AT2R) receptors with similar affinities [1]. In some cases activation of the AT2R opposes the effects mediated by AT1R [2]. The latter include vasoconstriction leading to increased blood pressure, cell growth, cardiac, renal, vascular hypertrophy, and fibrosis [3]. These indications are the molecular basis for the successful clinical application of AT1R antagonists. In contrast, Ang II binding to the AT2R leads to vasodilation, and in some cases cell apoptosis, and antiproliferation. Moreover, AT2R activation was shown to be involved in tumor suppression, tissue repair, and nociception[4-7]. Recently, we demonstrated that RAS has both oncogenic and tumor suppressor functions in melanoma. The inhibition of AT2R could provide therapeutic opportunities in melanoma treatments and may also serve as a novel biomarker of metastatic melanoma [8]. Therefore, the balance between the different functional responses of AT1R and AT2R seems to play an essential role in the pathophysiology of several diseases [9]. Both receptors are recognized with similar affinity by the native hormone Ang II, [10, 11] and hence, potent, stable and selective AT2R ligands, either serving as agonists or antagonists, are not only important research tools for the elucidation of the physiological role of this receptor subtype, but have also therapeutic potential [12, 13].

Previously, we discovered a potent and selective peptide analogue of Angiotensin II, named [Y]^{6}–A II (whereby histidine at position 6 of Angiotensin II has been substituted by tyrosine) presented a 18000 fold AT2/AT1 subtype selectivity and a very low nanomolar affinity for AT2R [14]. This compound also proved to be potent *in vivo* against human pancreatic ductal adenocarcinoma [15]. These results align with the profile of AT2R, being involved in some cases as tumor suppression, tissue repair, and neuron regeneration. From this study it became evident that substituting the amino acid at position 6 in the native hormone angiotensin II by other aromatic groups could enable a higher selectivity profile for AT2R over AT1R. Furthermore, the

substitution of the α-amino acids in Ang II by their β^3-*homo*-amino acid congeners was shown to reduce AT1R binding without diminishing AT2R binding and led to the identification of β^3-*h*Tyr^4-Ang II and β^3-*h*Ile^5-Ang II exhibiting ≈1000-fold AT2R selectivity, of which the β^3-*h*Ile^5 analogue, but not the β^3-*h*Tyr^4 analogue, had *in vivo* vasodepressor activity [16]. Since Ang III, the *C*-terminal heptapeptide of Ang II or [des-Asp^1]Ang II, is less potent at the AT1R, a similar β^3-*homo*-amino acid substitution experiment was performed, leading to β^3-*h*Pro^7-Ang III as a highly selective (>20000-fold) AT2R agonist with vasodepressor activity in spontaneously hypertensive rats and renal vasodilatory and natriuretic effects in normotensive rats [17, 18].

Along these lines, conformational constraints were considered for further establishment of structure-activity relationship studies. The introduction of conformational constraints into bioactive peptides has attracted considerable attention in the past decades. A multitude of studies has demonstrated that these modifications can, for instance, enhance the receptor selectivity and the metabolic stability of a peptide [19-21]. During the evaluation of Ang IV (H-Val^1-Tyr^2-Ile^3-His^4-Pro^5-Phe^6-OH) analogues, it was noticed that the replacement of the His^4-Pro^5 sequence by distinct conformationally constrained fragments led to metabolically stable, potent, and selective analogues [22]. Gratifyingly, most of these Ang IV analogues (**Figure 1**) had lost all affinity for the AT1R. Further optimization resulted in the highly potent, selective, and metabolically stable Ang IV analogue **IVDE77**: H-β^2-*h*Val-Tyr-Ile-Aia-Nva-Phe-OH **(Figure 1),** which does not activate the AT1R.[23] Based on these observations and especially on our recently established [Y]^6-AII potent compound where we consolidated the importance of the position 6 in generating highly selective analogues for AT2R with aromatic aminoacids, motivated us to explore the effect of replacing the His^6 residue in Ang II by various conformationally constrained analogues of tryptophan and tyrosine (e.g., Aia and HO-Tic, **Figure 1**) against AT2R. The synthesized analogues were validated in terms of binding potential to the AT1R and AT2R through radioligand competition-binding experiments in HEK-293 cells, stably transfected with either the AT1R or the AT2R. In addition, the stability of the peptide analogues was evaluated in human plasma.

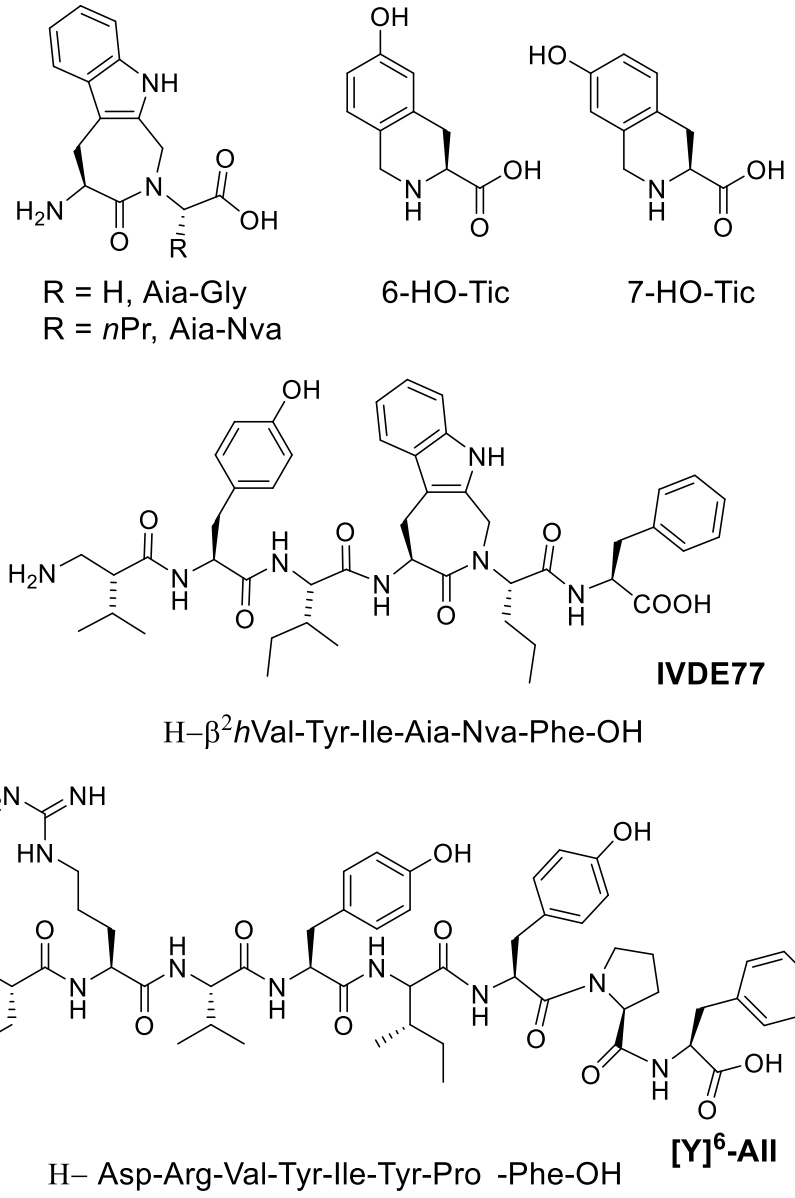
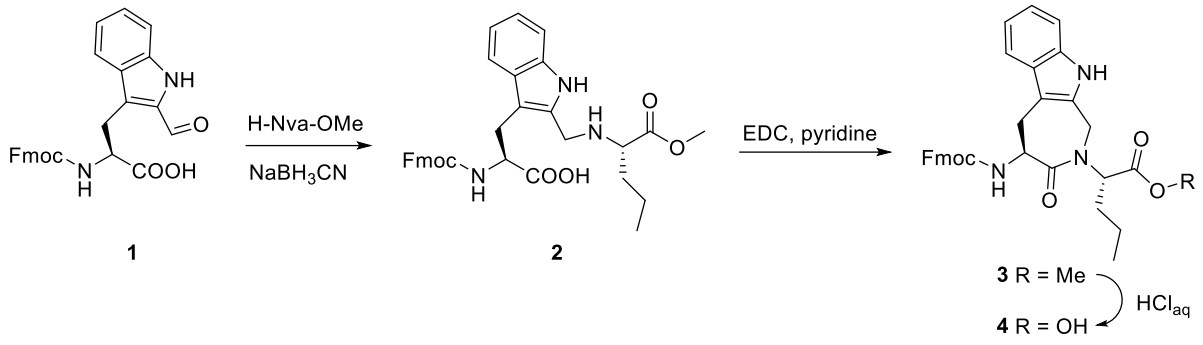


Figure 1. Structures of the conformationally constrained analogues used to replace the His-(Pro) sequence in Ang II, and structure of the highly potent and selective Ang IV analogue for AT1R **IVDE77** and the highly potent and selective AT2R analogue [Y]^{6}-AII.

The Ang II and [des-Asp^{1}]-Ang II **7-16** sequences were prepared by incorporation of the constrained building blocks using manual Fmoc/*t*Bu solid-phase peptide synthesis on L-Phe-loaded Wang resin using DIC/HOBt activation (see experimental section). While Fmoc-L-(7-HO)-Tic-OH was obtained from commercial sources, Fmoc-L-(6-HO)-Tic-OH was prepared by a Pictet-

Spengler condensation of formaldehyde with *m*-Tyr, according to literature procedures, [24,25] and followed by Fmoc-protection via reaction with Fmoc-OSu in the presence of Na_2CO_3 [26]. The dipeptidomimetic Fmoc-L-Aia-Gly-OH was made as described previously, [27] while dipeptidomimetic Fmoc-L-Aia-L-Nva-OH was prepared from Fmoc-2-formyl-L-Trp-OH **1** and H-L-Nva-OMe (Scheme 1).

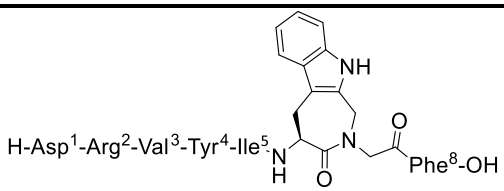
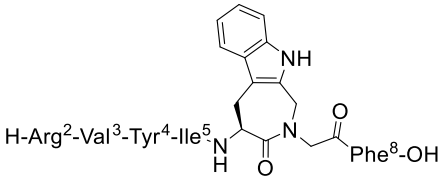
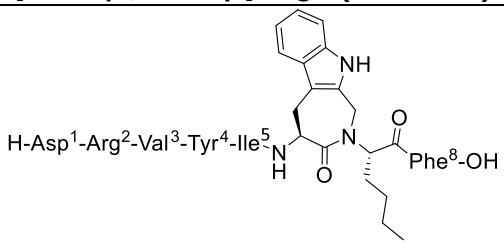
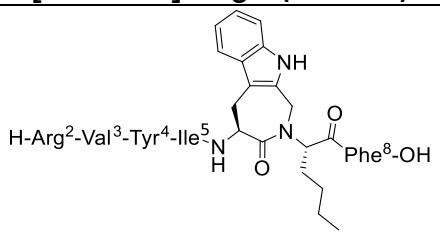
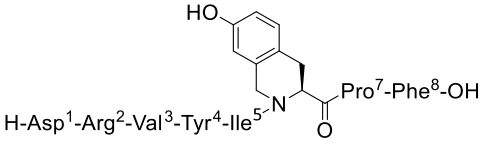
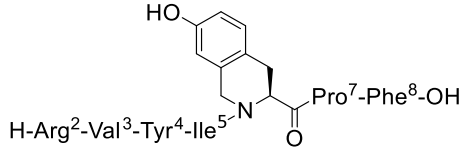
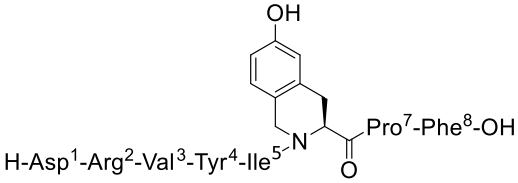


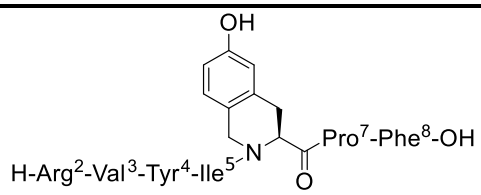
Scheme 1. Synthesis of Fmoc-Aia-Nva-OH **4**.

Fmoc-2-formyl-L-Trp-OH **1** was synthesized by SeO_2-induced ring-opening oxidation of (*S*)-Fmoc-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (Fmoc-Tcc) in 52% yield, as described earlier. [27,28] Next, reductive amination of aldehyde **1** with commercially available HCl.H-L-Nva-OMe ester in the presence of $NaCNBH_3$ yielded secondary amine **2**, which was subsequently lactamized to the constrained dipeptidomimetic Fmoc-L-Aia-L-Nva-OMe **3**. Finally, methyl ester **3** was quantitatively converted into its corresponding carboxylic acid **4** using acid-catalyzed hydrolysis [29]. The peptides were purified by preparative reversed-phase HPLC to assess the purity of >95%. Their sequence is shown in **Table 1.**

Table 1. Structure of the Ang II analogues, their affinities for the AT1R and AT2R, and calculated AT2R/AT1R selectivities.

Compound number	Compound structure and name	AT1R-IC_{50} (nM ± SEM)	AT2R-IC_{50} (nM ± SEM)	AT2R-fold selectivity
5	H-Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-His[6]-Pro[7]-Phe[8]-OH **Ang II**	10.2 ± 1.5	0.617 ± 1.280	16.5
6	H-Asp[1]-Arg[2]-Val[3]-Tyr[4]-Ile[5]-**Tyr[6]**-Pro[7]-Phe[8]-OH **[Y][6]-AII**	72550 ± 1.7	4.0 ± 1.4	18000

Compound number	Compound structure and name	AT1R-IC_{50} (nM ± SEM)	AT2R-IC_{50} (nM ± SEM)	AT2R-fold selectivity
7	 **[Aia[6]-Gly[7]]-Ang II (SBL-AII-7)**	>10000	45.74 ± 1.37	>219
8	 **[des-Asp[1],Aia[6]-Gly[7]]-Ang II (SBL-AII-8)**	>10000	106.6 ± 1.93	>94
9	 **[Aia[6]-Nva[7]]-Ang II (SBL-AII-9)**	>10000	70.29 ± 1.30	>142
10	 **[des-Asp[1],Aia[6]-Nva[7]]-Ang II(SBL-AII-10)**	>10000	24.44 ± 1.70	>409
11	 **[7-HO-Tic[6]]-Ang II (SBL-AII-11)**	>10000	89.22 ± 1.29	>112
12	 **[des-Asp[1],7-HO-Tic[6]]-Ang II (SBL-AII-12)**	133.9 ± 2.80	118.8 ±1.90	1.1
13	 **[6-HO-Tic[6]]-Ang II (SBL-AII-13)**	174.3 ± 1.83	59.28 ± 2.10	2.9

Compound number	Compound structure and name	AT1R-IC_{50} (nM ± SEM)	AT2R-IC_{50} (nM ± SEM)	AT2R-fold selectivity
14	 **[des-Asp^1,6-HO-Tic^6]-Ang II (SBL-AII-14)**	2010 ± 2.41	315.4 ± 1.5	6.4

IC_{50} values for AT1R and AT2R were determined by the displacement of [^{125}I]-[Sar^1,Ile^8]-Ang II from HEK-293 cells stably expressing the AT1R or AT2R. Results are the competition binding data obtained from three separate experiments (each in triplicate). Data for compound **6** are taken from Ref 14.

The IC_{50} values of the synthesized analogues to both AT1R and AT2R were determined in HEK-293 cells, stably transfected with either AT1R or the AT2R, as previously described [14]. [^{125}I]-[Sar^1,Ile^8]-Ang II was used as the radioligand in the competition assays at concentrations ranging from 1 pM to 10 µM. The results are shown in **Figure 2** and **Table 1.** None of the analogues **6–11** were able to displace the radioligand appreciably in the HEK-293 cells transfected with AT1R up to a concentration of 10 µM. The different hydroxy-Tic-containing Ang II analogues **12–14** showed some affinity for the AT1R, with [des-Asp^1,7-OH-Tic^6]-Ang II **12** having an IC_{50} value of 133.9 nM. In contrast, most of the analogues showed nanomolar range affinities for the AT2R. The rank order of affinity at AT2R was [Y]^6-AII **6** > [des-Asp^1, Aia^6-Nva^7]-Ang II **10** > [Aia^6-Gly^7]-Ang II **7** > [Aia^6-Nva^7]-Ang II **9** > [7-HO-Tic^6]-Ang II **11** > [des-Asp^1, Aia^6-Gly^7]-Ang II **8** >> [des-Asp^1, Asp^1, 7-HO-Tic^6]-Ang II **12**, [6-HO-Tic^6]-Ang II **13** and [des-Asp^1,6-HO-Tic^6]-Ang II **14 (Table 1).**

Since the target was to identify compounds with high affinity and selectivity for the AT2R, the AT2R selectivity (AT2R-fold selectivity) was calculated as the ratio of IC_{50} values at AT1R to that at AT2R (**Table 1**). As reported, [Y]^6-AII **6**, the highly selective AT2R ligand, exhibited a marked 18000-fold AT2R *versus* AT1R selectivity. Five of the Ang II analogues (**7**, **8**, **9**, **10**, and **11**) presented enhanced AT2R/AT1R subtype selectivities, ranging from 94 up to 409, although their affinities for AT2R were reduced when compared to Ang II **5** itself. Three analogues (**12**, **13** and **14**) presented insignificant AT2R/AT1R subtype selectivity.

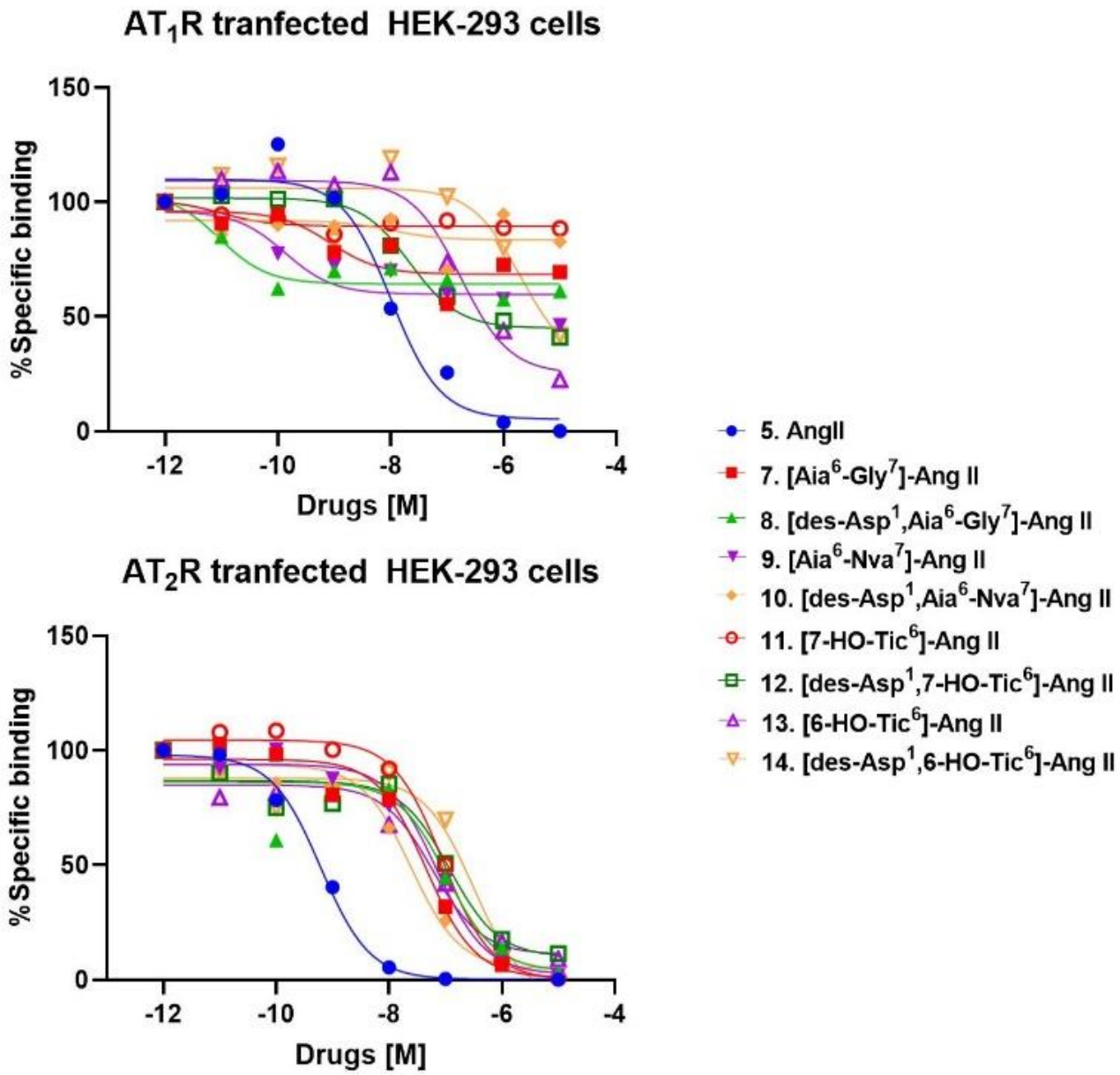


Figure 2. Radioligand competition-binding experiments performed in HEK-293 cells stably expressing (a) AT1R or (b) AT2R where increasing concentrations of modified peptide analogues **7-14** were tested against [^{125}I]-[Sar^{1},Ile^{8}]Ang II binding. Each point represents the mean for three separate experiments, each performed in triplicate. IC_{50} values are listed in Table 1.

Incubation experiments in human plasma were conducted to assess the impact of the constrained amino acids on the proteolytic stability of each Ang II analogue. An LC-MS method was developed to monitor their concentration at different time intervals, after incubation in human plasma. Separation of the analogues was achieved by reversed-phase chromatography, using a C18 column and a water/acetonitrile gradient method. For the quantification of the analogues, a triple quadruple mass spectrometry system was used in positive electrospray ionization mode, using multiple reaction monitoring (MRM). The most abundant transitions were determined and selected, as shown in **Table 3**. The 24h plasma stability profile of [Y]^{6}-AII **6** and the analogues **7**-**14** in human plasma is presented in **Figure 3**. After an incubation period of 24h, all the Ang II analogues' concentration remained above 50% of the initial concentration. The highest degradation rate was shown by [Y]^{6}-AII **6** retaining slightly more than half of its initial concentration after 24h. [Aia^{6}-Gly^{7}]-Ang II **7** and its [des-Asp^{1}] analogue **8** displayed a slower degradation rate approximately 70% of their initial concentration remaining after 24h (**Figure 3A**). In contrast, [Aia^{6}-Nva^{7}]-Ang II **9** and [des-Asp^{1}, Aia^{6}-Nva^{7}] **10** demonstrated no improvement on stability comparing to [Y]^{6}-Ang II **6**, all having a similar degradation rate (**Figure 3B**). In contrast, [7-HO-Tic^{6}]-Ang II **11** was found to have the highest stability of all analogues, retaining more than 90% of its initial concentration after 24h. The [des-Asp^{1}] analogue **12** is degraded somewhat faster than **11**, retaining more than 70% of its initial concentration after 24h (**Figure 3C**). Finally, [6-OH-Tic^{6}]-Ang II **13** and [des-Asp^{1}, 6-OH-Tic^{6}] **14** demonstrated similar stability compared to [Y]^{6}-AII **6** (**Figure 3D**). In general, all pairs of Ang II and [des-Asp^{1}]-Ang II analogues demonstrated a similar degradation rate, except for [des-Asp^{1},7-HO-Tic^{6}]-Ang II **12**, which had a higher degradation rate than [7-HO-Tic^{6}]-Ang II **11** (**Figure 3C**).

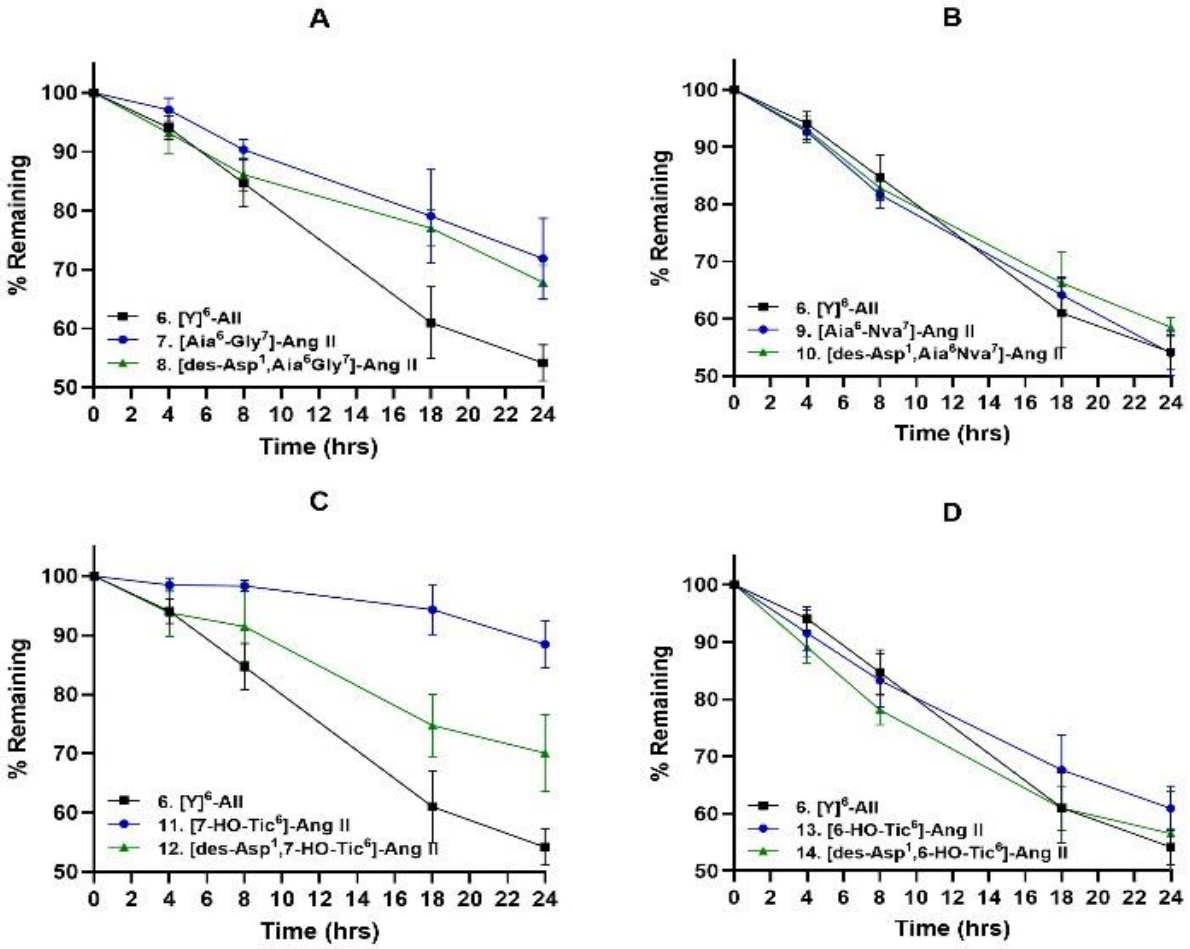


Figure 3. Plasma stability profile of all analogues compared to [Y^6]-AII (error bars representing the deviation).

The results reported here show that substitutions in the His^6-Pro^7 region of the amino acid sequence of Ang II differentially modified the peptidic ligands' affinity for AT1R and AT2R. As expected, native Ang II **5** displaced the radioligand in both AT1R and AT2R-transfected HEK-293 cells. The introduction of the constrained amino acids in the Ang II or [des-Asp^1]-Ang II sequence resulted in Ang II analogues **7-14,** which displayed little or no affinity for the AT1R (**Table 1**). In contrast, several analogues displayed IC_{50} values for the AT2R in the nM range. Interestingly, when altering the His-Pro dipeptide to the conformationally constrained indole analogue Aia-Gly

(see Figure 1, analogues **7** and **8**), the AT2R affinity was reduced compared to that of **5** but it showed excellent receptor subtype selectivity due to the very low AT1R affinity. The addition of the Nva side chain at position 7, to provide analogue **9**, resulted in a further reduction in AT2R affinity for the full Ang II sequence, but in a four-fold increase in AT2R affinity for the [des-Asp^1]-analogue **10**. As a result, **10** displayed the highest selectivity of this series of analogues. This indicates that the conformational constraint imposed by the Aia residue is not tolerated by the AT1R, but is favored by the AT2R [29]. In combination with its high metabolic stability, the high selectivity makes [des-Asp^1,Aia^6-Nva^7]Ang II **10** an interesting compound for further exploration aimed to unraveling the role of AT2R. Remarkably, for all other constrained analogues, the octapeptides are more potent at the AT2R than the corresponding des-Asp analogues. This differentiation could potentially be explained by discrete conformational features adopted by the specific peptides due to the larger hydrophobic structure of Aia^6-Nva^7 with respect to the other evaluated compounds bearing chemotypes of reduced comparable size. The [Aia-Nva] analogue IVDE77 (**Figure 1**) was also the most potent and selective AngIV analogue in a comparable series of compounds [23]. The substitution of the His^6 by a 6- or 7-HO-Tic residue, which are conformationally constrained Tyr analogues, did not, except for **11**, resulted in compounds with high affinity and selectivity for AT2R. This observation might indicate that this type of constraint is not leading to a conformation that can be recognized by the AT2R. These substituted Tic residues orient the aromatic ring away from the *C*-terminus of the Ang II peptide (cfr. Tic residues favor χ1 gauche (-) or gauche (+) conformations [19, 20]), consequently leading to a less compact conformation which was previously reported to give way to fewer favorable interactions with the AT2R [14]. In contrast, some of these analogues, such as **12** and **13**, show appreciable affinity for the AT1R, whereas the other constraints abolished this affinity. This may indicate that the AT1R tolerates this orientation of the side chain more than the AT2R. The [7-HO-Tic^6] -Ang II **11** analogue showed the highest stability in human plasma, with more than 90% of the compound remaining intact after 24 hours of incubation. Since the 7-HO-Tic substitution also generates AT2R selectivity, it is a valuable replacement for the His^6 residue, provided that the affinity of the Ang II analogue can be improved.

Our present studies further demonstrate and are in accordance to our former findings [14] that the amino acid at position 6 is crucial for AT2R selectivity. The replacement of the His^6-Pro^7 dipeptide by the constrained Aia-Nva, resulted in the Ang III analogue **10**, bearing nM affinity for the AT2R and high metabolic stability in human plasma. It further demonstrates that local conformational constraints of side chains are a valuable tool to induce receptor selectivity and metabolic stability. [20, 21]

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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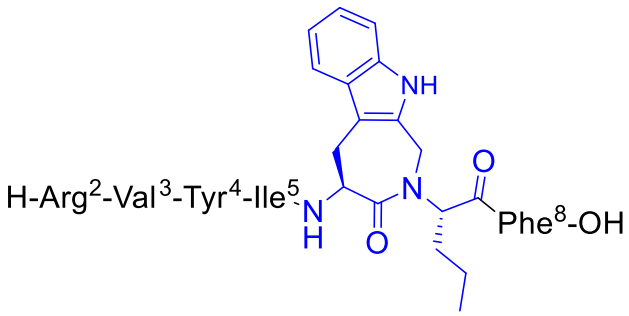
Graphical abstract



[des-Asp^{1},Aia^{6}-Nva^{7}]-Ang II (**SBL-AII-10**)

IC_{50}(AT1R) >10000 nM; IC_{50}(AT1R) 24.44 nM

$t_{1/2}$ (human plasma) > 12h