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Single Peptide Backbone Surrogate Mutations to Regulate Angiotensin GPCR Subtype Selectivity

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Through backbone amide bond substitutions with 1,4-disubstituted 1,2,3-triazole rings in strategic positions, the proteolytic stability in human plasma is enhanced and simultaneously

the AT₂R/AT₁R G-protein-coupled receptor (GPCR) subtype selectivity is tuned while retaining the neurotrophic effects of peptide ligands derived from [Y]⁶-Angiotensin II.

Through backbone amide bond substitutions with 1,4-disubstituted 1,2,3-triazole rings in strategic positions, the angiotensin GPCR subtype selectivity can be regulated

Peptidomimetics

click chemistry

competition-binding experiments

G-protein-coupled receptors (GPCRs)

neurotrophic effect

peptidomimetics

Mutating the side-chains of amino acids in a peptide ligand, with unnatural amino acids, aiming to mitigate its short half-life is an established approach. However, it is hypothesized that mutating specific backbone peptide bonds with bioisosters can be exploited not only to enhance the proteolytic stability of parent peptides, but also to tune its receptor subtype selectivity. Towards this end, four [Y]⁶-Angiotensin II analogs are synthesized where amide bonds have been replaced by 1,4-disubstituted 1,2,3-triazole isosteres in four different backbone locations. All the analogs possessed enhanced stability in human plasma in comparison with the parent peptide, whereas only two of them achieved enhanced AT₂R/AT₁R subtype selectivity. This diversification has been studied through 2D NMR spectroscopy and unveiled a putative more structured microenvironment for the two selective ligands accompanied with increased number of NOE cross-peaks. The most potent analog, compound **2**, has been explored regarding its neurotrophic potential and resulted in an enhanced neurite growth with respect to the established agent C21.

G-protein-coupled receptors (GPCRs) are the targets of approximately 35 % of approved drugs^[1] and are major signal transducers in higher eukaryotes. GPCRs exist as subtypes and although they share sequence and structure homology, they can present opposing activities. This is the case of the renin-angiotensin system (RAS), a pathway involved in intravascular volume regulation, where two receptors exist: the AT₁R

(Angiotensin Receptor subtype 1) and the AT₂R (Angiotensin Receptor subtype 2). The AT₁R is responsible for cell proliferation, angiogenesis, oxidative stress, and cardiovascular dysfunctions, whereas the AT₂R has been reported to counteract several of the effects mediated by the AT₁R, conferring cardio-protection,^[2] antiproliferation, antiinflammation, and neuroregeneration.^[3] Recently, we identified that AT₂R can provide therapeutic opportunities in melanomas,^[4] human pancreatic ductal adenocarcinoma,^[5] and breast cancer.^[6] In addition, we showed that AGTR₁ CpG island methylation in serum could serve as a novel biomarker of metastatic melanoma.^[4] These discoveries were feasible owing to the establishment of our selective compound, [Y]⁶-Angiotensin II, for the AT₂R.^[6] With an increasing body of evidence indicating that selective targeting of AT₂R can provide a better treatment for many different diseases,^[7] there is an imminent need to further develop angiotensin receptor subtype-selective ligands.

Traditionally, synthetic peptides have served as invaluable tools to probe the selectivity of peptidergic GPCR, like AT₁R and AT₂R. Conventionally, side-chains of a lead peptide sequence are mutated. However, to further exploit peptides as drugs, the main obstacle that has to be surpassed is their inherent low stability in vivo, mostly owing to their fast proteolytic degradation. In this frame, various methods have been invoked to mitigate their instability through the installation of amide-bond backbone surrogates such as triazole,^{[8,} 9] four-membered oxetane ring,^[10] thioamide or ester bonds,^[11] and methylated amide bonds.^[12] However, despite being considered crucial for peptide stabilization, peptide backbone modification is not considered yet as a tool to tailor the fundamental pharmacological properties of bioactive peptides such as receptor subtype selectivity.

Previously, we discovered a very potent and selective peptide analog of angiotensin II (AII), [Y]⁶-Angiotensin II designated as [Y]⁶AII (His⁶ is substituted by Tyr⁶), which presented a 18 000-fold AT₂R/AT₁R GPCR subtype selectivity and low nanomolar affinity for the AT₂R.^[6] This is of high importance as the AT₂R is responsible for tumor progression in some cancers, tissue repair, and neuron regeneration and is thus an important pharmaceutical drug target. However, sensitivity to proteases and consequently its low metabolic stability is a limitation for its further clinical development. To overcome this

problem, we conceived the design of four novel analogs that bear 1,2,3-triazoles in strategic locations within the sequence of [Y]⁶AII, as shown in Figure 1A. Along the sequence of each analog of [Y]⁶AII, one conventional amide bond was mutated with a 1,4-disubstituted 1,2,3-triazole (the synthetic procedure is depicted in Scheme S1 and the characterization details are presented in Figures S1-S24 and also in Table S1 in the Supporting Information). We selected the specific backbone positions because a modification of Phe or Pro might impede the AT₂R selectivity^[13] and the stabilization of the Asp-Arg bond with a triazole was not prioritized, as the cleavage of this bond leads to the active peptide Ang(2-8).^[14] The triazole heterocycle mimics the geometric, steric, and electronic features of an amide bond and likewise participates in hydrogen bonding and dipole-dipole interactions.^[15]

As a consequence, a crucial factor that should be evaluated first is the human plasma stability and whether the backbone location of the bioisostere affects the half-life of the peptidomimetics. For this, we set up LC-MS protocols to monitor their stability in human plasma (Figure 1B). After 24 h of plasma incubation, the four analogs were fully stable with a degradation percentage of about 10 %, whereas only 64 % of the initial concentration of [Y]⁶AII remained intact from plasma proteases activity. It became evident that the replacement of amide bonds by the 1,2,3-triazole bioisosteres in different backbone positions profoundly enhanced the stability of [Y]⁶AII in human plasma.

Afterward, the binding affinity and selectivity of the synthesized analogs of [Y]⁶AII were evaluated, as they should retain similar or enhanced values with respect to the parent peptide. [Y]⁶AII possesses enhanced binding affinity towards AT₂R with respect to the native hormone,^[6] and to this end, we utilized human embryonic kidney cells (HEK-293), stably transfected with either the AT₁R or the AT₂R, and determined the relative selectivity of the new analogs. Competition for ¹²⁵I-[Sar¹, Ile⁸]AII (Figure S25 in the Supporting Information) binding in the HEK-293 cells transfected with the AT₁R was observed only for AII and none of the compounds **1--4** illustrated any competitions (Figure 2A). On the other hand, in HEK-293 cells transfected with AT₂R, competitive binding for ¹²⁵I-[Sar¹, Ile⁸]AII was observed with all compounds (**1--4**); however, compounds **2** and **4** had superior affinity in comparison to compounds **1** and **3** (Figure 2B). To determine the relative AT₂R selectivities of the

synthetic ligands, the ratio of IC₅₀ values at AT₁R/AT₂R was evaluated (Table S2 in the Supporting Information). The rank order of affinity at AT₂R was compound **2** > compound **4** > compound **3** > compound **1**.

Two of the novel [Y]⁶AII analogs presented enhanced AT₂R/AT₁R subtype selectivity with respect to AII. Specifically, compound **2** and compound **4** were >3611- and 1336-fold selective, respectively, for AT₂R over AT₁R. In contrast, the other two compounds presented minimal AT₂R selectivity (5- and 64-fold for compound **1** and compound **3**, respectively), although their affinities for AT₂R were reduced compared with AII itself. The IC₅₀ values for AT₂R were calculated as 2 μM, 2.8 nM, 155 nM, and 7.5 nM for compounds **1**, **2**, **3**, and **4**, respectively (Table S2 in the Supporting Information). However, it is worth noting that as these compounds failed to displace AT₁R binding appreciably, their relative AT₂R selectivities may be conservative estimates.

Based on the monitored diversification of the AT₂R/AT₁R selectivity into two groups of compounds, we set out to explore the structural microenvironment of the four analogs in solution with high-resolution 2D NMR spectroscopy. The full resonance assignment was achieved for all analogs (Tables S3--S6 in the Supporting Information). By overlapping the NH/HA region of the 2D ¹H-¹H TOCSY NMR of all analogs (Figure 3A), it is evident that the signals of compounds **2** and **4** are significantly more spread than those of compounds **1** and **3** (peaks of compound **2** in the ranges of 7.38--8.75 and 3.84--5.19 ppm and of compound **4** in the ranges of 7.21--8.87 and 4.01--5.07 ppm; peaks of compounds **1** in the ranges 7.54--8.61 and 3.99--4.81 ppm and of compound **3** in the ranges 7.25--8.57 and 3.98--4.72 ppm). Similarly, the NH-NH NOE signals, according to ¹H-¹H 2D NOESY experiments (Figure S26 in the Supporting Information), of compounds **2** and **4** between their residues are more spread and reach higher ppm values, with respect to compounds **1** and **3**, suggesting the formation of more compact structures in solution. Interestingly, the number of cross-peaks between the NH protons of amino acids and the neighboring triazole ring proton (TriH) increases going from compounds **1** and **3** (with one and two cross-peaks, respectively) to compounds **4** and **2** (with three and four cross-peaks, respectively), as shown in Figure 3B.

This trend is in accordance with the stratification of the compounds according to the AT₁R and AT₂R affinity experiments, and specifically on their relative AT₂R selectivities. Compounds **2** and **4**, which present enhanced relative selectivity for AT₂R, present similar NMR spectroscopic data, with respect to compounds **1** and **3** bearing lower AT₂R selectivity. The enhanced number of cross-peaks between NH and TriH, recorded for compounds **2** and **4**, could potentially indicate the formation of a more compact structural microenvironment driven by the triazole ring, with respect to compounds **1** and **3**. The observed compound stratification by NMR spectroscopy is proportionate to the recorded AT₂R/AT₁R subtype selectivity values. This could imply that the favorable stereo-electronic environment offered by the specific localization of the triazole rings^[16] in compounds **2** and **4** could potentially be responsible for the recorded more favorable affinity for AT₂R.

Finally, based on the neuroprotective features associated by AT₂R, we set to investigate the neurotrophic action of our lead molecule, compound **2**, and compare it with the established non-peptide agonist compound 21 (C21), which has been reported to increase AT₂R-mediated neurite growth by Namsolleck et al.,^[17] (synthesis is briefly described in Figure S27 in the Supporting Information). Mouse embryonic cortical neurons exposed for 48 h to the proteolytically stable compound **2** showed increased neurite growth, as shown in Figure 4. Our results indicate that compound **2** enhanced neurite growth in primary neuronal cultures by 50±10 % (increase in the area covered by neurites), an effect that was slightly enhanced compared with the established AT₂R selective non-peptide agonist C21 (40±6 %). Therefore, our lead compound **2** may be considered as a novel therapeutic approach for the treatment of spinal cord injury. A relevant activity could be expected for the compounds **1**, **3**, and **4**, though owing to their reduced AT₂R selectivity they have not been evaluated in this assay.

In summary, we synthesized four peptidomimetic analogs of [Y]⁶AII in which we mutated four amide bonds with 1,4-disubstituted 1,2,3-triazoles. These mutations led to an enhanced proteolytic profile with respect to the parent compound [Y]⁶AII. Furthermore, two of the four backbone mutations (compounds **2** and **4**) were demonstrated to retain their AT₂R/AT₁R selectivity, whereas the backbone mutations in compounds **1** and **3** resulted in

reduced receptor binding and AT₂R subtype selectivity. This differential binding and selectivity profile was also projected in the recorded 2D NMR spectra. Specifically, a more structured microenvironment was recorded (chemical shifts are dispersed with higher spectral width, and with a higher number of NOE cross-peaks) for compounds **2** and **4**, than for the other two compounds. Evaluation of the neurotrophic effect of compound **2** indicated that backbone modification between Tyr⁴ and Ile⁵ led to an enhanced neurite growth with respect to the established agent C21. In conclusion, triazole bioisoster could pave the way for its exploitation beyond its traditional use to enhancing the stability of proteolytically unstable therapeutics. Installing the triazole ring in specific peptide backbone locations could regulate the backbone structure microenvironment of the peptide and modulate its affinity for GPCRs. Thus, the peptide backbone could be exploited in a heterodox way and specifically as a way to tailor the selectivity in GPCR subtypes and at the same time enhance the proteolytic stability of the parent peptides.

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Conflict of interest

I.E. Valverde and T.L. Mindt are inventors of patent WO2012/156511 and N.M. Grob and T.L. Mindt of patent WO 2019/057445 A1.

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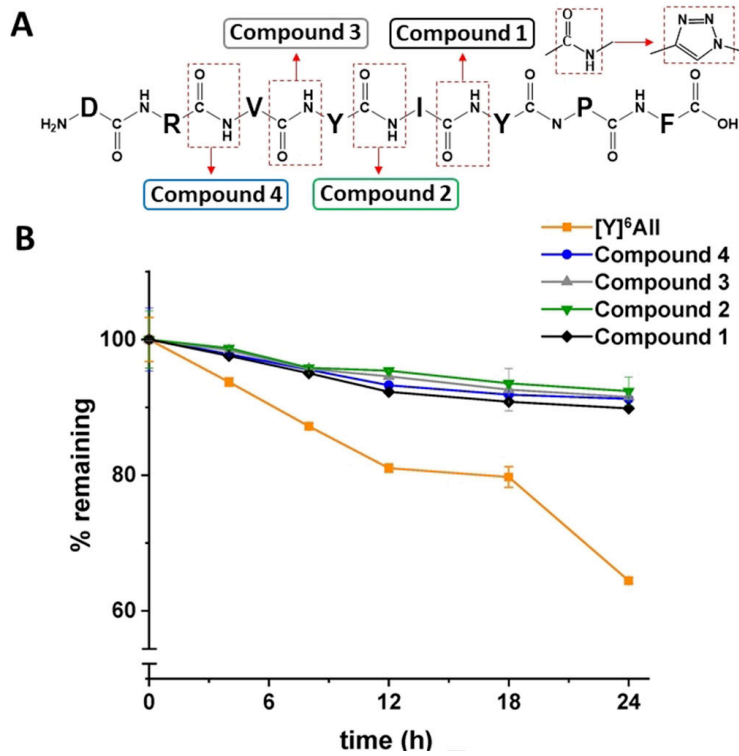


Figure 1 (A) Structures of the four analogs of $[Y]^6AII$ in which specific amide bonds have been replaced with triazole isosteres. (B) Human plasma stability of $[Y]^6AII$ and its analogs bearing 1,2,3-triazole isosteres in various backbone locations (standard deviation is indicated with error bars and the experiment was conducted in triplicate).

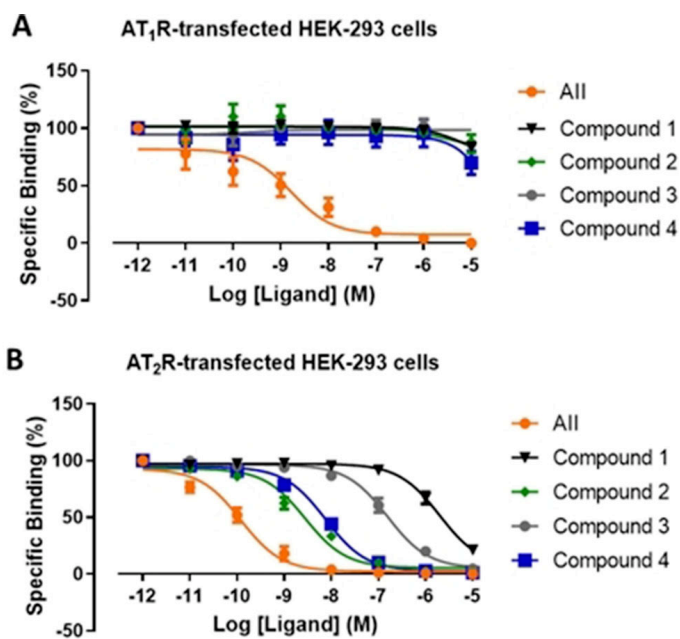


Figure 2 Competition-binding experiments for AII and the four peptidomimetic analogs against ^{125}I -[Sar¹, Ile⁸]AII binding performed in HEK-293 cells stably expressing: (A) AT₁R or (B) AT₂R. Results are the competition binding from three separate experiments (each in triplicate).

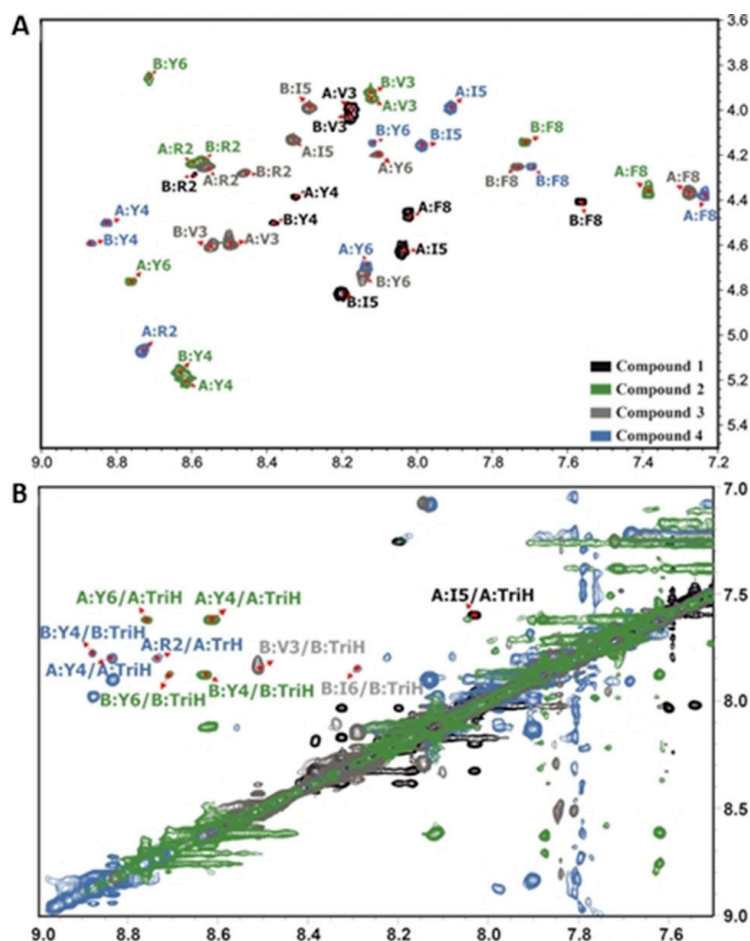


Figure 3 (A) Overlapping of the NH/HA region of the 2D ^1H - ^1H TOCSY spectra of the four analogs. (B) Overlapping of the NH/NH region of the 2D ^1H - ^1H NOESY spectra of the four analogs. Cross peaks in compound 1 are colored in black, in compound 2 are colored in green, in compound 3 are colored in gray, and in compound 4 are colored in blue. Label 'A' refers to the *cis* conformers and 'B' to the *trans* conformers. 'TriH' stands for the proton of the triazole ring.

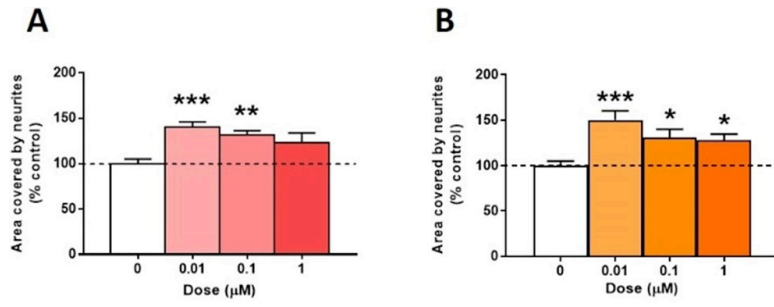


Figure 4 Neurotrophic effect of the non-peptide compound C21 (A), compared with compound **2** (B), in mouse cortical neuronal cells, at different concentrations. The graphs show the area covered by neurites per field of view, as means \pm SEM. * P <0.05, ** P <0.01, *** P <0.001 for treatment vs. control group. Experiments were conducted in triplicate.