Angiotensin II vs Its Type I Antagonists: Conformational Requirements for Receptor Binding Assessed from NMR Spectroscopic and Receptor Docking Experiments

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The conformations of three angiotensin II (AII) peptide antagonists ([Sar1]-AII(1−7)-NH$_2$, [Sar1,Val5,Ala8]-AII and the AII antipeptide, [Glu1,Gly2,Val5,Val8]-AII) were assessed in a lipid medium. A common backbone turn was identified through modeling and spectroscopic studies. The His6 residue acted as a pivoting point beyond which each peptide adopted two distinct conformations. One principle conformer resembled that previously determined for AII while the other was designated as an AII antagonist like conformer. A computational overlay between the nonpeptide antagonist, Losartan, and both the AII and the AII like conformation of [Sar1,Val5,Ala8]-AII revealed common pharmacophoric points with RMS deviations between 1 and 1.5 Å. Both the AII conformer and the AII antagonist like conformer of [Sar1,Val5,Ala8]-AII were docked into a model of the AT$_1$ receptor. Receptor residue Phe289 and Asp281 provided good contact points for both peptides. Some differences were also noted. The terminal carboxyl of AII contacted Lys199 of the receptor while that of [Sar1,Val5,Ala8]-AII bridged Arg23 at the top of helix 1. The Asp1 side chain of AII interacted with His183 of the receptor.

Introduction

The octapeptide hormone angiotensin II (AII) is the primary active pressor agent produced by the renin−angiotensin cascade. It plays a central role in the regulation of blood pressure and electrolyte homeostasis.[1,2] The actions of AII are mediated through two distinct cell surface receptors referred to as AT$_1$ and AT$_2$. Both receptors have been cloned in recent years.[3−6] Hydropathy analyses of the two membrane proteins suggest that they are members of the larger superfamily of G-protein-coupled receptors. In adults, most of the known cardiovascular effects of AII occur via the AT$_1$ receptor.[7]

The importance of AII in hypertension and cardiovascular diseases has rendered the pharmacological blockade of AII formation and action of major therapeutic value. Two distinct paths of drug development aimed at restraining the action of AII have thus been established in recent years. Researchers first searched for inhibitors of the angiotensin-converting enzyme (ACE), which is responsible for the cleavage of the AII precursor peptide to its active form.[1] Early on, this path led to the discovery and commercialization of drugs such as Captopril[8] and Enalapril.[9] A second strategy has focused on designing antagonists of AII that specifically block the binding of the hormone to its AT$_1$ receptor and/or prevent receptor activation.

Several peptide antagonists of AII have been successfully prepared.[10−13] AII peptide antagonists are however unsuitable drug candidates due to their poor oral bioavailabilities and short plasma lifetimes. Their three-dimensional conformations nevertheless constitute a useful starting point for small molecule ligand design.[14,15] The successful nonpeptide antihypertensive drug, Losartan, is one example of an AII antagonist rationally designed from the peptide hormone's C-terminal conformation.[15]

Structure−activity studies centered on AII have demonstrated the importance of residues Tyr4, His6, and Phe8 for biological activity.[16,17] For example, agonist vs antagonist behavior is highly dependent on the nature of the amino acid in position 8.[12,13,18,19] Type I AII antagonists characterized by slow receptor resensitization rates are produced by replacing the C-terminal phenylalanine in AII with an aliphatic amino acid (Ile, Ala, Thr), whereas type II AII antagonists are obtained by modifying the AII Tyr4 hydroxyl group.[16,20,21] Compounds in this second class behave as reversible and competitive antagonists. Substituting the aspartic acid in position 1 with a Sar or a succinamoyl group furthermore improves the stability of AII analogues toward hydrolysis by amino peptidase and thus increases their duration of action.[10,11] The biological differentiation established via the nature of the amino acid at position 8 has led to a hypothesis suggesting that residues 1−7 define receptor specificity whereas residue 8 invokes receptor agonism. A potent antagonist, [Sar1]-AII(1−7)-NH$_2$, was rationally designed based on this argument.[22]

Knowledge of the conformational features that distinguish agonist from antagonist behavior among the reported AII analogues should facilitate the rational design of nonpeptide AII antagonists. Numerous con-

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AII	H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH
[Sar¹]-AII-NH₂	H-Sar-Arg-Val-Tyr-Ile-His-Pro-NH₂
[Sar¹,Val⁵,Ala⁸]-AII	H-Sar-Arg-Val-Tyr-Val-His-Pro-Ala-OH
AII antipeptide	H-Glu-Gly-Val-Tyr-Val-His-Pro-Val-OH

Figure 1. Chemical structures of AII and AII type I antagonists used in this study.
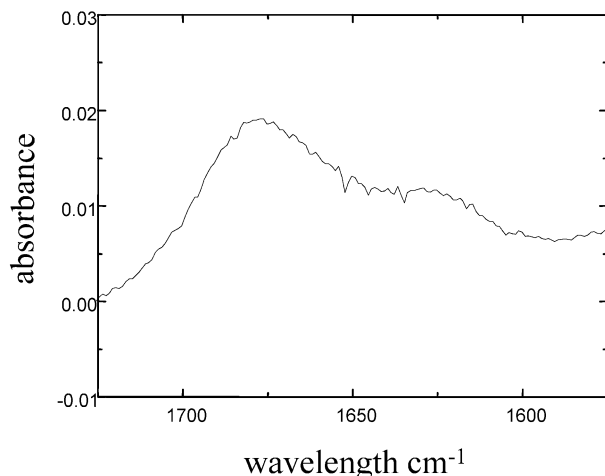

Figure 2. Amide I region of the FTIR spectrum of AII antipeptide recorded at 30 °C.

formational studies have led to some general conclusions as to how AII analogues fold in solution and as to what conformational features are likely important for receptor binding and activation. Spectroscopic studies of AII and [Sar1]-AII have shown that the agonists adopt a folded hairpin like conformation characterized by a trans His6−Pro7 peptide bond.[23−26] Additional common features include a clustering of the three aromatic rings and a charge relay system involving the Tyr4 OH, His6 imidazole, and C-terminal carboxyl.[23,26,27] One reported investigation examined conformational differences between AII and type I AII antagonists in dimethyl sulfoxide (DMSO). Whereas AII adopted the expected Tyr4, Ile5, His6 bend, there was no evidence of a folded conformation for any of the antagonists studied.[28]

Reported here are the results of conformational studies carried out on three AII peptide antagonists using NMR and Fourier transform infrared (FTIR) spectroscopic techniques in conjunction with molecular modeling. Their chemical structures are shown in Figure 1. The goal was to identify conformational similarities among the different types of AII antagonists that unilaterally distinguish them from the folded conformation associated with AII in a membrane mimetic environment. The first selected antagonist, [Sar1,Val5,Ala8]-AII, belongs to the type I class of AII antagonists. The second peptide, [Sar1]-AII(1−7)-NH₂, while lacking the C-terminal Phe8, nevertheless contains a modification at the critical eighth residue in AII. In this respect, the peptide resembles the type I AII antagonists. A third studied antagonist known as the AII antipeptide was originally encoded from the (−)mRNA sequence complementing the AII (+)mRNA.[29] This octapeptide is structurally related to AII. Four of its amino acids (Val3, Tyr4, His6, and Pro7) are identical, whereas another two represent conservative replacements (Asp1 to Glu1
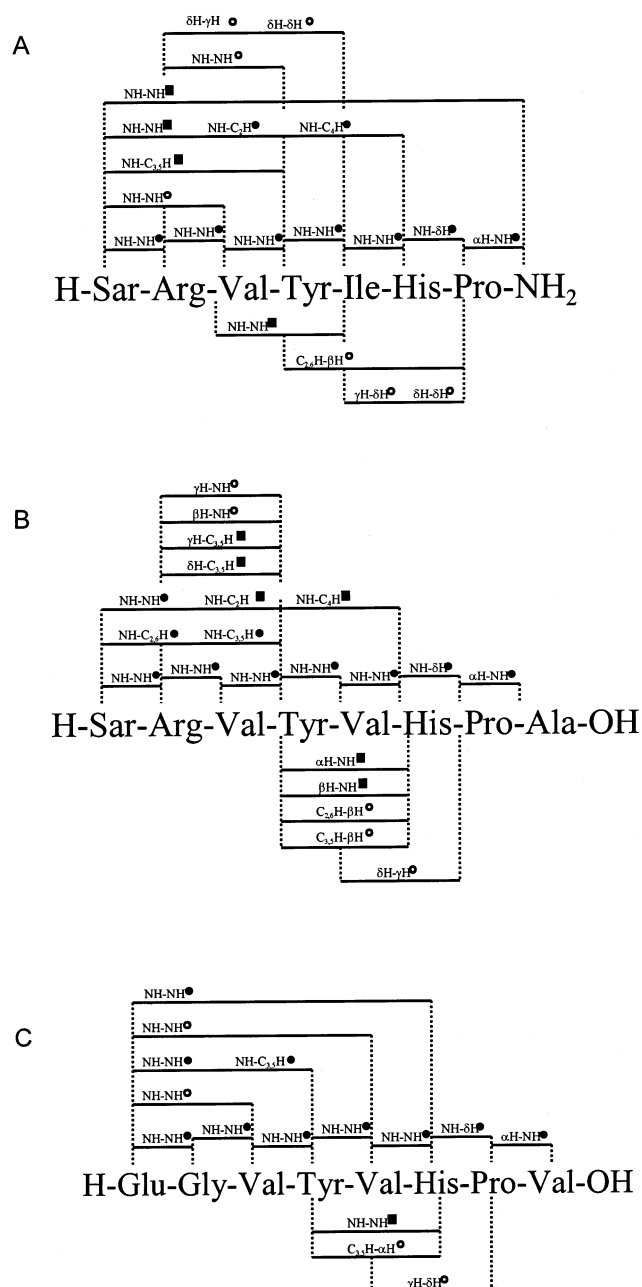

Figure 3. Summary of the medium range NOEs observed for (A) [Sar1]-AII(1−7)-NH₂, (B) [Sar1,Val5,Ala8]-AII, and (C) AII antipeptide at 30 °C. The symbols employed reflect the NOE intensities according to ●, strong; ○, medium; and ■, weak.

and Ile5 to Val5). The antipeptide possesses a valine at position 8, which suggests it belongs to the type I antagonist class of compounds.

Results

FTIR Spectroscopy. The amide I region in the FTIR spectrum obtained for the AII antipeptide is shown in Figure 2. An intense absorption band is observed at 1676 cm⁻¹, distinct from the amide I band at 1655 cm⁻¹. A band located between 1676 and 1683 cm⁻¹ was also observed for the other peptides studied. According to assignments in model compounds, such a signal either can be attributed to the presence of a β-turn structure or may be due to the absorption of the arginine side chain, which absorbs in the region 1673 ± 3 cm⁻¹.[30,31] Because the antipeptide does not include an Arg in its
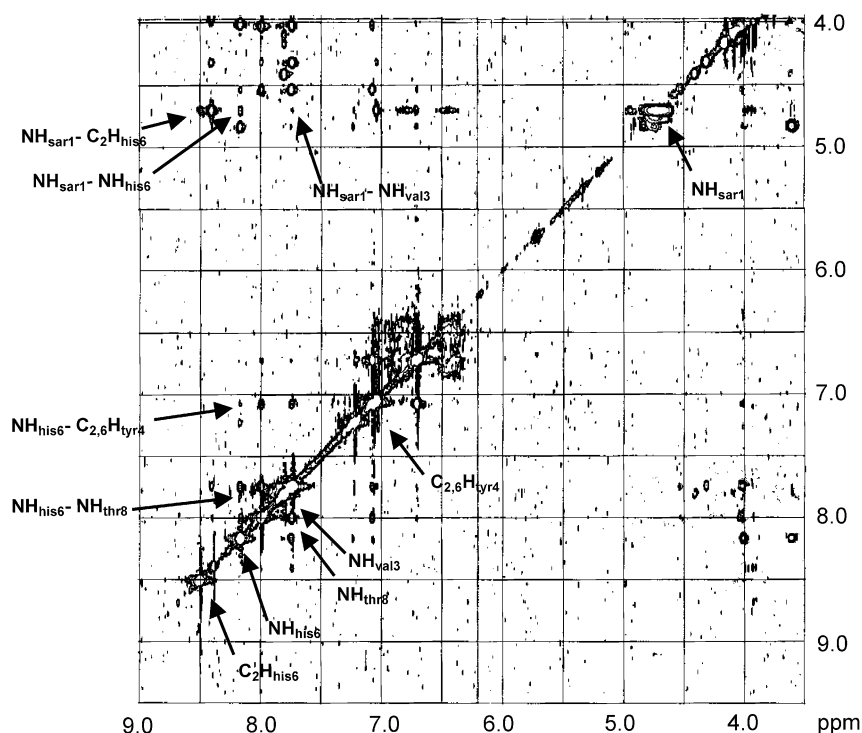
Figure 4. Portion of the NOESY spectrum acquired for [Sar1,Val5,Ala8]-AII at 30 °C with a mixing time of 100 ms.

composition (see Figure 1), its absorption band at 1676 cm^{-1} can only be attributed to the presence of a β-turn. According to published tables, the band at 1676 cm^{-1} corresponds to a type II β-turn.[30]

NMR Results. Conformational order for the three peptides in the micelle solution was evident from their respective nuclear Overhauser enhancement spectroscopy (NOESY) spectra. Numerous structurally diagnostic nuclear Overhauser effects (NOEs) were observed in all cases. Shown in Figure 3 is a summary of the NOEs cited for each peptide. Two common patterns of NOEs were observed amongst the three AII antagonists studied. The first pattern includes strong sequential backbone NH—NH NOEs, while the second and perhaps more informative pattern includes several medium to long range NOEs involving amino acids in positions 1 and 6. The latter is an indication of common and defined conformational order within the peptides. A portion of the NOESY spectrum for [Sar1,Val5,Ala8]-AII with conformationally relevant NOEs annotated is displayed in Figure 4. The information obtained from the NOE cross-peaks was converted to distance constraints used for molecular modeling.

The change in chemical shift of the amide protons was studied as a function of temperature. One- and two-dimensional ^{1}H spectra were acquired for each peptide at 5° intervals starting at 30 °C and ending with 50 °C. The amide temperature coefficients ($\Delta\delta/\Delta T \times 10^3$ ppm/K) at each temperature interval were calculated and then averaged. Figure 5 reports a summary of the temperature coefficient data for each of the three peptides studied. The temperature coefficient for the amino acid in position 1 (Sar or Glu) was not reported since fast exchange of its amide proton with the solvent rendered the NH signal invisible. As shown in Figure 5, the average amide proton chemical shift displacement for each amino acid is similar among the three ana-
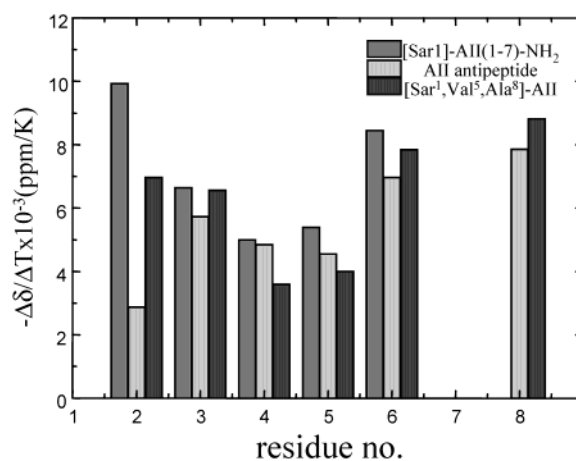

Figure 5. Amide proton temperature coefficients ($\Delta\delta/\Delta T \times 10^3$ ppm/K) as a function of amino acid position for the AII antagonists studied.

logues studied. An exception occurs for the amino acid in position 2. This position is occupied by an Arg in the case of [Sar1]-AII(1−7)-NH$_2$ and [Sar1,Val5,Ala8]-AII, whereas it is a Gly in the AII antipeptide (see Figure 1). For the shorter sequence [Sar1]-AII(1−7)-NH$_2$, the NMR amide proton signal associated with Arg2 decreases rapidly as the temperature increases and then disappears at 40 °C due to fast solvent exchange. It is therefore well-exposed to the aqueous milieu. Lower amide temperature coefficient values (<6) were consistently measured for amino acids in positions 4 and 5 as well as Gly2 in the case of the antipeptide. This result indicates that the amide protons associated with residues 4 and 5 are more shielded from the aqueous environment than others in the peptide sequence. The presence of a hairpin turn structure in the peptide backbone likely accounts for the observed amide proton shielding.

Table 1. Selected φ, ψ Angles for Conformers of AII Analogues

	AII like conformer		AII antagonist like conformer	
peptide	φ, ψ Ile5	φ, ψ His6	φ, ψ Ile5	φ, ψ His6
AII	−105, +138	+87, +140		
[Sar1]-AII(1−7)NH$_2$	+60 to +81, −51 to −72	−134 to −166, +78 to +120	−86 to −143, −50 to −75	−135 to −165, +68 to +130
[Sar1,Val5, Ala8]-AII	−72 to −100, −50 to −65	+59 to +80, +53 to +62	−72 to −91, −50 to −62	−150 to −162, +53 to +62
antipeptide			−61 to −92, −43 to −69	−124 to −170, +61 to +79

Molecular Modeling Results. The lowest energy conformations for each peptide studied contained a reverse turn structure between residues 2 and 5. Therefore, the backbone atoms of these four residues were used to superimpose the various conformations of each peptide onto one another. The first residue (Sar1 or Glu1 in the case of the antipeptide) was able to adopt multiple configurations and was not well-defined in this study. The reverse turn in each case is the result of a large number of conformationally diagnostic NOEs observed in the appropriate region. In addition, there were strong NH−NH (i, $i + 1$) NOEs involving residues 2−5 and consequently, these NH protons point inward and are in close proximity to each other. The C-terminal segments of the peptides were more flexible and less well-defined. In the case of [Sar1,Val5,Ala8]-AII, the C terminus diverged into two sets of conformations extending in different directions following the His6 residue. This is a result of two different observed φ angles for the His6 residue. One set of conformations contains φ angles around +60 to +80° and the other set of conformations contains φ angles in the −150 to −162° range. Two defined sets of conformations were also found for [Sar1]-AII(1−7)-NH$_2$, although in this case the C terminus diverged following the Ile5 residue. This was again a result of two sets of allowed φ angles at the Ile5 residue. Clusters of lowest energy conformations superimposed in the turn region from residues 2−5 are shown in Figure 6 for each of the three peptides studied. For comparison, the previously determined conformation of AII in a lipid micelle environment[23] is overlayed in boldface format on each peptide superposition. In all cases, the C terminus adopts distinct orientations to either the left or the right-hand side of the hairpin turn when viewed from the side with the N terminus projecting into the page. Both [Sar1]-AII(1−7)-NH$_2$ and [Sar1,Val5,Ala8]-AII extend their C-terminal residues in well-defined conformations oriented to either the left or the right-hand side of the backbone turn, whereas that of the antipeptide lies solely to the left. Unlike in the case of the antagonists, the C terminus of AII projects uniquely to the right-hand side of the backbone turn. Herein lies the main conformational difference between the peptide antagonists and the natural agonist sequence. Listed in Table 1 are φ and ψ angles accessible to Ile5 and His6 for peptides in this study as well as for the principal conformation of AII as determined from a previous study.[23] Two distinct sets of angles describing the conformational ensembles are included in Table 1. The first set of angles describes conformers resembling AII (AII like conformers) while conformers differing from AII at the C terminus are modeled by the second set of angles. Structures in the latter category are referred to as the AII antagonist-like conformers.

Losartan was superimposed on the predominant conformation of AII derived from previous NMR stud-
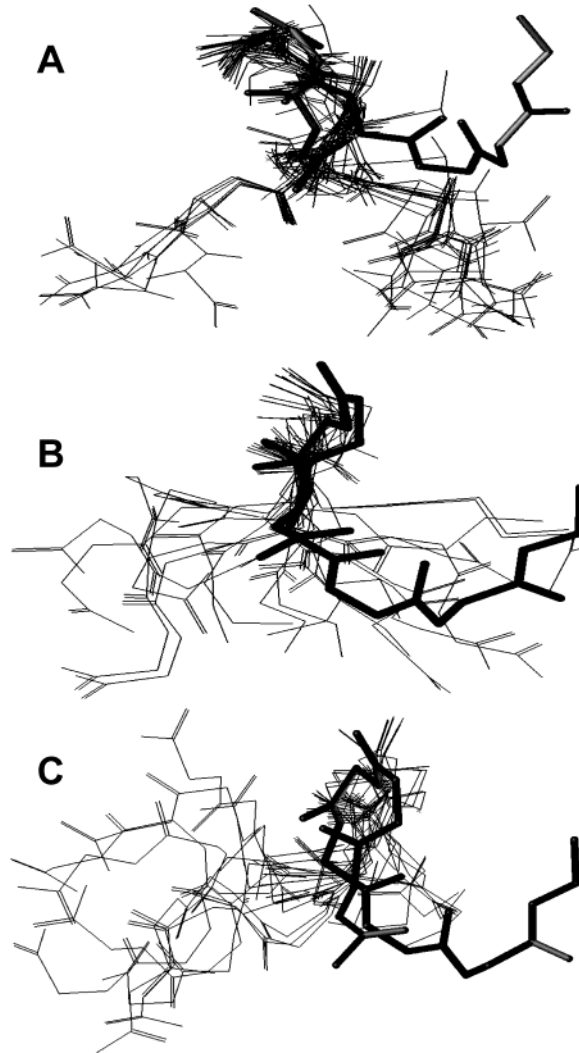

Figure 6. Side view representation of the superposition of final backbone structures for (A) [Sar1,Val5,Ala8]-AII, (B) [Sar1]-AII(1−7)-NH$_2$, and (C) the AII antipeptide. The bold type structure superimposed on each ensemble is that of AII determined previously.[23]

ies[23] as well as the subset of AII like conformers calculated for [Sar1,Val5,Ala8]-AII. Good fits were obtained in both cases with RMS deviations generally around 1.0−1.5 Å. The overlay between AII and Losartan is shown in Figure 7. When viewing the comparisons, certain specific points of match become apparent. Namely, Losartan's tetrazole fits well with the peptide's C-terminal carboxylate group, the hydroxymethylimidazole component of Losartan is superimposed on the peptides' His6 imidazole group, and Losartan's *n*-butyl chain matches the peptides' Ile5 (or Val5) carbon chain (Figure 7). This result suggests that the AII like conformer available to the peptide antagonists in the present study could share a common receptor binding site with both AII and Losartan.
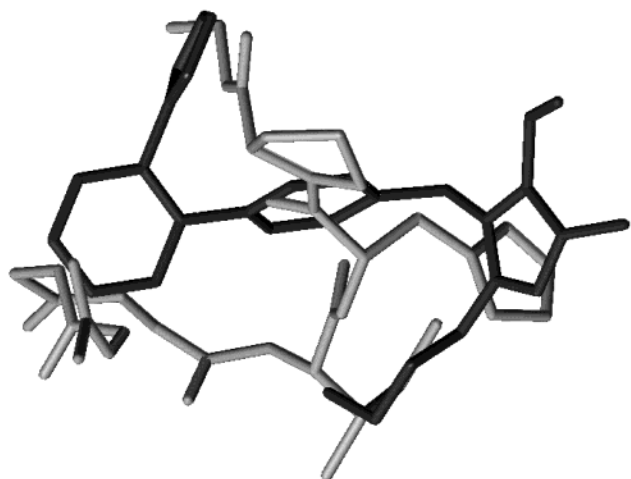
Figure 7. Conformational overlay of Losartan (black) and AII (grey) showing the best pharmacophoric fit.

Shown in Figure 8 is the proposed AII antagonist-like conformation of [Sar1,Val5,Ala8]-AII docked into a model of the AT$_1$ receptor determined previously and refined in the present study using the recently published crystal structure coordinates of rhodopsin.[32,33] A complex between AII and its AT$_1$ receptor is also shown in Figure 8 for comparison. As can be seen in the model, the principle pharmacophoric components of the peptides interact well with certain receptor residue side chains. The C-terminal carboxyl of the antagonist

projects in the direction of Asp281 of the receptor and may interact with Arg23 at the top of helix 1. Conversely, the terminal carboxyl of AII interacted with Lys199 of the receptor. In Figure 8, a ligand—receptor Arg2—Asp281 interaction was consistently observed. Furthermore, both peptides docked into the AT$_1$ receptor with their His6 side chain interacting with Phe289 of the receptor. Another observed point of contact was between the Asp1 side chain of AII and His183 of the receptor (Figure 8).

Discussion

The conformations of structurally distinct AII peptide antagonists were examined in a lipid environment by NMR and FTIR spectroscopy. A backbone turn located between residues 2 and 5 was a common feature found in all three peptides. This hairpin turn is not unique to the AII antagonist conformations reported here but rather represents a motif, which has surfaced from many conformational studies of AII and its agonist analogues using a variety of computational and spectroscopic techniques.[23–26,34,35] Several groups have thus postulated the turn conformation may be important for AII agonist activity. For example, the conformation of AII when examined in a neutral DPC phospholipid environment exhibited a well-defined hairpin turn along the peptide backbone.[23] An aromatic cluster involving the side chains of Tyr4, His6, and Phe8 was a second noteworthy feature in the deduced conformation for AII.
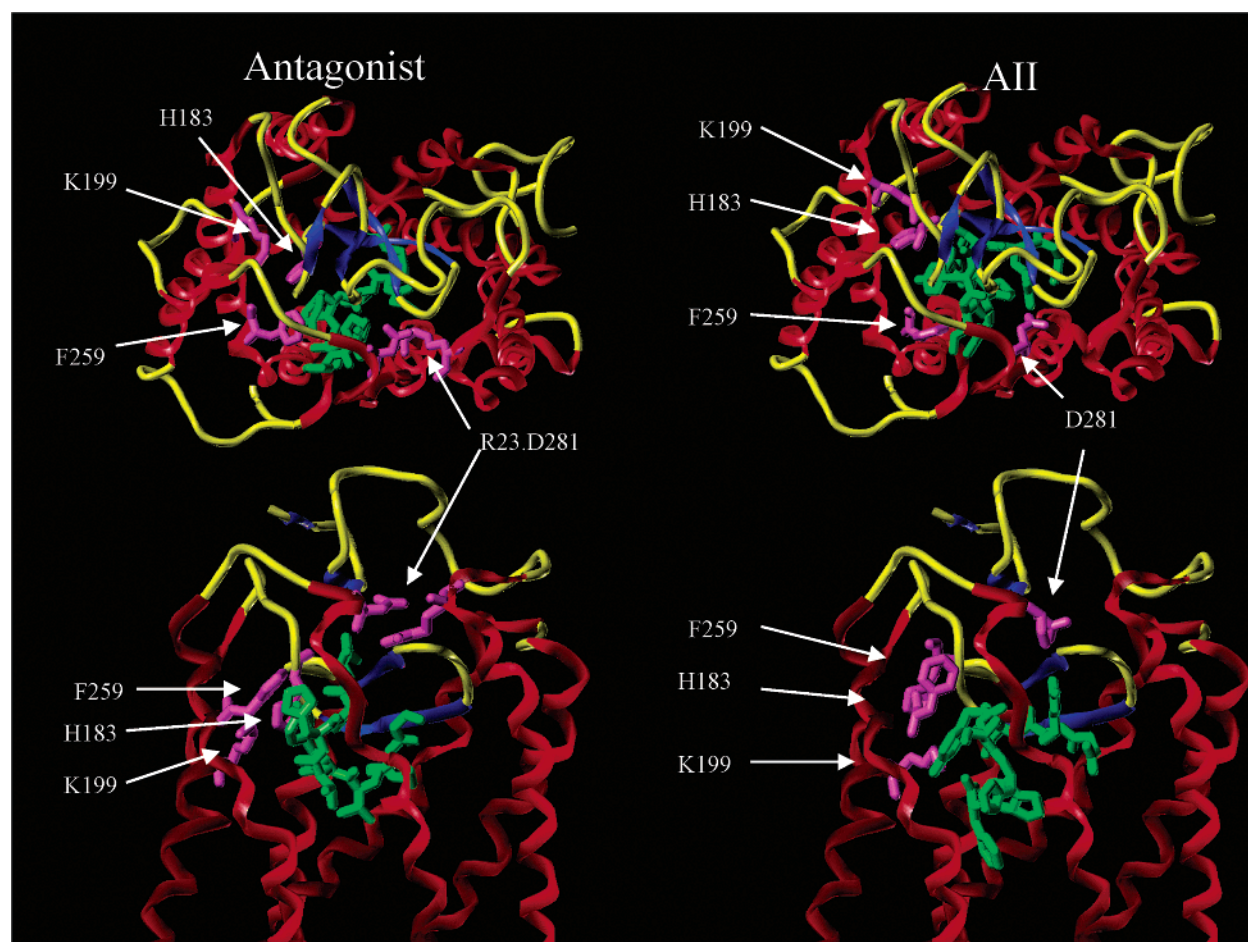

Figure 8. Proposed antagonist conformation of [Sar1,Val5,Ala8]-AII (left) and AII (right) docked into an AT$_1$ receptor model. Top and bottom models correspond to top and side views of the receptor, respectively.

This model of AII superimposes very well with the antagonist conformations reported here along the main chain turn between residues 2 and 5 (Figure 6), thus suggesting that this turn feature may be a common element for receptor binding but not necessarily for receptor activation. Additional support for this argument stems from a separate investigation involving synthesis and testing of secondary structure mimetics aimed at stabilizing a turn centered on the region 3−5 in AII. Introduction of the inverse γ-turn resulted in two diastereoemeric peptidomimetics analogues of AII with receptor affinity almost equal to that of the parent hormone.[36]

Results from structure−activity relationship studies of AII analogues suggest that distinct components of the peptides are responsible for AT_1 receptor binding and activation. Residues Arg2, Tyr4, His6, and Phe8 as well as the terminal carboxyl were identified as key pharmacophoric elements based on these studies.[16−19,37,38] Four of these five key elements, excluding the Phe8 side chain, are considered important for binding, whereas the C-terminal phenylalanine is necessary for activation. These conclusions are based in part on studies carried out on truncated AII analogues. For example, [Sar1]-AII is a potent AII agonist whereas [Sar1]-AII(1−7)-NH2 is an AII antagonist.[22,26,39] However, [Sar1]-AII(1−7)-NH2 maintains some intrinsic agonist activity suggesting that at least one residue in addition to Phe8 in the sequence is required for receptor activation and furthermore this antagonist is able to adopt AII's bioactive conformation.[22] In fact, this partial agonist behavior is a general property observed among the AII type I antagonists.[7,37] The truncated analogue AII-(4−8) is a full agonist but exhibits weaker receptor affinity as compared to AII, indicating that Arg2 only plays a role in binding.[40] An important discovery was the effect of replacing the Phe8 in [Sar1]-AII with a nonaromatic hydrophobic residue.[13,18] In all cases, an AII antagonist resulted. An aromatic group at the C terminus is thus of critical importance for AT_1 receptor activation. Likewise, Tyr4 is considered necessary for receptor activation since its methylation produced a fully competitive reversible antagonist.[16]

The model reported here can explain the above observations from a conformational point of view. The main difference between the AII agonist and the antagonist conformations in a membrane mimetic environment lie in the conformational space accessible to the peptides C terminus. The last three residues of AII can veer only to one side of the common backbone conformation, whereas the C terminus of the peptide antagonists containing either an aliphatic residue or no residue at position 8 are able to access the opposite side of the molecule as well. When the Phe8 is present, intramolecular interactions possibly promote a conformation that correctly positions the critical residues for receptor activation. The presence of an aromatic cluster involving Tyr4, His6, and Phe8 may thus restrict the peptide's C terminus to adopting a one-sided conformation in the case of the agonist AII. The importance of this aromatic ring cluster for receptor activation has also been demonstrated through synthesis and testing of a novel cyclic AII peptide analogue designed to promote a clustering of the three aromatic rings Tyr4, His6, and

Phe8. The constrained peptide exhibited AII agonist activity when tested in the rat uterus assay and in anesthetized rabbits.[41]

The antagonists however lack the additional force provided by a third aromatic ring required for keeping the C terminus from accessing other regions of conformational space. For example, when the Phe8 residue is removed as in the case of [Sar1]-AII(1−7)-NH2, some subset of the conformational ensemble still maintains the AII conformation thus explaining its intrinsic agonist activity. However, a second predominant conformation also exists, which could allow the peptide to bind without activating the receptor. The conformational preferences at the C terminus differentiating AII from its peptide antagonists correlate well with the notion of biological activity centered primarily on the C terminal residues of AII.

Site-directed mutagenesis experiments have confirmed that Asp281 of the AT_1 receptor interacts directly with Arg2 of angiotensin II.[42] This interaction was satisfied when the peptide conformer reported here for the type I antagonist [Sar1,Val5,Ala8]-AII and previously for AII[23,32] were docked into an AT_1 receptor model. Mutagenesis studies have also suggested that His183 of the AT_1 receptor is involved in binding Asp1 of AII.[42] This interaction is satisfied for AII when docked into the current receptor model. A second interaction between His6 of AII and Phe259 of the AT_1 receptor, as proposed on the basis of mutational studies,[43] is observed in the receptor−ligand complex models reported here for the type I antagonist and for AII.[32] The receptor model and conformational data described in the present manuscript in addition to previously reported data from receptor mutagenesis studies therefore collectively present a good argument supporting a common mode of binding for AII and the type I antagonists within the peptide N-terminal region up to and including His6.

With respect to the C terminus of the peptide ligands, other models derived from receptor mutations suggest that Lys199 of the receptor binds to the C-terminal carboxyl group of AII.[44] The same interaction was noted when AII was docked to the AT_1 receptor model. Lys199 is furthermore believed to provide a common binding site for AII and the tetrazole derivative component of nonpeptide antagonists originally designed from the His6−Phe8 portion of an NMR spectroscopic-derived AII model.[14,45] In support of this hypothesis, Losartan exhibited good overlay with the previously determined conformation of AII in a membrane mimetic environment[23,32] as well as with the AII like type I antagonist conformation of [Sar1,Val5,Ala8]-AII reported here. Absence of a Tyr4 mimetic in Losartan may thus be responsible for its antagonist rather than agonist activity despite its ability to mimic the conformation of the agonist C terminus.[46] The AII antipeptide and [Sar1,Val5,Ala8]-AII antagonists both contain a C-terminal carboxyl and are thus good candidates for interaction with Lys199 of the receptor. However, studies in which K199 was mutated to an alanine examined only the agonist response to AII and its analogues.[44] The agonist potency and/or receptor affinity of the type I antagonist toward a K199A mutant of the AT_1 receptor has not yet been tested. It is therefore reasonable to hypothesize that the C-terminal

carboxyl moiety in the antagonist conformation (i.e., non-AII like conformation) examined in the present study binds differently to the AT_1 receptor when compared to AII. Indeed, when the non-AII like conformer of [Sar1,Val5,Ala8]-AII was docked into the receptor model, residue 8 interacted with Arg23 at the top of the first helix of the receptor. This interaction may thus partially stabilize the predominant bound conformations of the antagonists. Further mutagenesis studies are required to fully test the role of Arg23. However, the fact that the terminal carboxyl in the proposed non-AII like antagonist conformation readily finds a suitable counterion when docked into the receptor model supports a primary differentiation of agonist vs antagonist activity based on orientation of the two C-terminal residues. The third studied peptide, [Sar1]-AII(1−7)-NH₂, lacks an amino acid at the eighth position altogether. Thus, a missing Phe8−K199 interaction is likely the principal contributing factor to its lack of agonist activity.

An alternative explanation for the differences in biological activity may stem from unique binding sites on the receptor for AII vs its peptide antagonists. However, this is unlikely for the reasons described above. Furthermore, [Sar1]-AII(1−7)-NH₂ behaves as a truly competitive and fully reversible AII antagonist both in vivo and in vitro,[47] suggesting that it shares a common binding space with AII.

Conclusion

In conclusion, it was determined that the AII type I peptide antagonists adopt a well-defined turn structure resembling that previously found for AII under similar solution conditions.[23] This hairpin structure therefore appears to be a common receptor recognition feature for both AII and its type I antagonists. According to the models derived in the present study, AII agonist receptor activity is distinguished from antagonist behavior via the orientation of the two C-terminal residues with respect to the common hairpin motif. This hypothesis is supported through observed compatibility between modeled receptor−peptide interactions involving established NMR solution conformations and published mutagenesis data. The results serve as a novel starting point for the development of nonpeptide antagonists from the C-terminal conformation of the AII type I antagonists.

Experimental Section

Materials. Angiotensin II (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-OH, AII) and AII antipeptide (H-Glu-Gly-Val-Tyr-Val-His-Pro-Val-OH) were purchased from Sigma (St. Louis, MO). [Sar1]-AII(1−7)-NH₂ and [Sar1,Val5,Ala8]-AII were purchased from Bachem (Torrance, CA). Sodium dodecyl-d_{25} sulfate (SDS) and deuterium oxide (+ 0.15% w/w TPS) were obtained from CDN Isotopes (Pointe-Claire, QC). All chemicals were used as received without further purification.

Sample Preparation. Samples for NMR studies were prepared by dissolving 1.1−1.3 mg of peptide and 28 mg of deuterated SDS-d_{25} in 500 μL of 50 mM sodium phosphate buffer (90% H₂O/5%D₂O). A 25 μL aliquot of D₂O (+0.15% w/w 3,3,3-trimethylsilylpropionate) was added as an internal reference to each sample. The pH of the resulting solutions was adjusted to 6.2−6.3 by adding optimal amounts of NaOD or DCl (from CIL Andover, MA). FTIR samples were more concentrated consisting of 2.1−7.5 mg of peptide plus 30 mg of SDS in 175 μL of 50 mM sodium phosphate buffer.

FTIR Measurements. A FTS-25 Bio-Rad (Cambridge, MA) spectrometer equipped with a water-cooled globar source and a medium-band mercury−cadmium−telluride detector was employed for the FTIR studies. For each sample, 100 scans were acquired with a 2 cm^{-1} resolution. Raw data were Fourier-transformed using a triangular apodization function. Spectra were corrected for the medium contribution by subtracting the spectrum of the solvent (phosphate buffer plus SDS). The sample was placed between two windows, made of CaF₂, and separated with a Teflon spacer of 5 μm. The cell was then mounted in a brass holder and placed in the FTIR sample room. The temperature was computer-controlled at 30 °C using a thermopump.

NMR Measurements. NMR measurements were carried out on a Bruker DMX-600 spectrometer, operating at 30 °C. ¹H chemical shifts were evaluated using the sequential assignment method. Two-dimensional TOCSY and NOESY spectra were acquired in the States-TPPI phase sensitive mode with 2K data points in F2. A total of 512 $t1$ increments were collected for each data set followed by zero-filling along F1. Final spectral dimensions were 6000 Hz (2K data points) and 600 Hz (1K data points) along F2 and F1, respectively. Mixing times of 100 and 200 ms were employed for NOESY experiments. TOCSY spectra were acquired with a mixing time of 50 ms. Gradient water suppression was achieved using the WATERGATE technique. Chemical shifts were referenced internally to 3,3,3-trimethylsilylpropionate.

Structure Calculations. All calculations were performed using the software package SYBYL (Tripos associates, St. Louis, MO) on a Silicon Graphics Indigo2 Extreme workstation. Procedures used were essentially as described in a previous publication[23] with some minor modifications. The Tripos force field was used for the energy calculations, and a dielectric constant of 10 was employed. Partial charges were calculated by the method of Gasteiger and Marsili.[48] The observed NOE cross-peaks were assigned as strong, medium, or weak based on their relative intensities and then converted to upper-bound distance constraints of 2.5, 3.5, and 5.0 Å, respectively. The lower-bound distance constraints were set at the sum of the van der Waals distances for the corresponding atoms. Pseudoatoms were employed for all methylene groups, and a 1.0 Å correction factor was added to these distance constraints.

Using the standard fragment library, a random structure for each peptide was generated. These structures were subjected to an unrestrained molecular dynamics simulation at 600 K for 1000 ps. Backbone dihedral angles were monitored along the dynamics trajectory. Ramachandran plots for individual amino acid residues displayed multiple transitions between the allowed regions, indicating a reasonable sampling of conformational space. Conformations were sampled every 4 ps during the simulation, resulting in 250 randomized structures. Internuclear distances derived from cross-peak intensities in NOESY spectra acquired for each peptide were incorporated as distance constraints for all subsequent calculations. NOE-derived distances involving degenerate side chain methyl groups of valine and isoleucine were not used. For each peptide, a total of between 40 and 50 intramolecular distance constraints were applied. Each of the 250 random structures for each peptide was minimized. To enforce the distance ranges, a square-well distance restraining force constant of 10 kcal/mol was employed. From the ensemble of minimized structures, the best 50 were selected for further analysis based on their energies and compatibility with the NMR data.

The 50 selected structures for each peptide were subjected to five cycles of restrained simulated annealing. The annealing protocol consisted of an initial equilibration step during which the temperature was gradually increased to 600 K over a 2 ps period, followed by 5 ps at 600 K. This was followed by gradual cooling to 300 K over a 3 ps period. The resulting structures were minimized using 3000 steps of conjugate gradient minimization. The annealing cycle was then repeated an additional four times for a total of five cycles. The structures for each peptide were then ranked according to energy.

Structures within 10 kcal/mol of the lowest energy structure were retained for further analysis. This resulted in approximately 10 structures for each peptide. The final group of structures for each peptide displayed no NOE-derived distance violations greater than 0.4 Å. All dihedral angles were measured, and the structures were superimposed with each other based on the backbone atoms of the first five residues.

Losartan was built using the standard fragment library in SYBYL and minimized. The torsional angles were set to the proposed bioactive conformation reported by Mavromoustakos et al.[41] Losartan was then superimposed with the principle conformations of [Sar1,Val5,Ala8]-AII and AII[23] using the following functional groups: (i) Losartans's hydroxymethyl-imidazole with the peptides' His6 imidazole group, (ii) Losartan's *n*-butyl chain with the peptides' Ile5 (or Val5) carbon chain, (iii) Losartan's tetrazole with the peptides' C-terminal carboxylate group, and (iv) Losartan's spacer phenyl ring with the peptides' pyrrolidine group of Pro7.

A molecular model of AII bound to the AT$_1$ receptor has previously been reported (B. C. Wilkes et al., 1999).[32] This model of the AT$_1$ receptor has since been refined to conform to the more accurate amino acid placement found in the recently published crystal structure of rhodopsin.[33] A preliminary model of one of the antagonists ([Sar1, Val5, Ala8]-AII) bound to the AT$_1$ receptor has been prepared using a previously described procedure.[32] This complex was minimized, and a short (20 ps) molecular dynamics simulation was performed.

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