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# The Discovery of Non-Basic Atrial Natriuretic Peptide Clearance Receptor Antagonists. Part 1

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**Abstract**—The cyclic peptide ANP 4-23 and the linear peptide analogue AP-811 have been shown to be selective ANP-CR antagonists. Via alanine scanning and truncation studies we sought to determine which residues in these molecules were important in their binding to the clearance receptor and the relationship between these two molecules. These studies show that several modifications to these compounds are possible which improve physical properties of these molecules while retaining high affinity for the ANP-CR. 

ANP is a member of a family of natriuretic peptide hormones, that includes atrial natriuretic peptide (ANP), brain naturetic peptide (BNP), and C-type naturetic peptide (CNP).[1,2] The natriuretic peptides demonstrate a number of actions on the cardiovascular system including: natriuresis, diuresis, and relaxation of vascular smooth muscle. ANP is a 28 amino acid cyclic peptide, which is produced in atrial myocytes in response to increases in heart rate and atrial stretch. There are two biologically and functionally distinct classes of ANP receptors.[3] The first is linked to guanylate cyclase and is thought to mediate the physiological effects of ANP via increases in intracellular cGMP levels. These guanylate cyclase-linked receptors are further divided into the ANP-A and ANP-B receptors according to their relative affinity for different natriuretic peptides. The second class of ANP receptors are not thought to mediate the cardiovascular effects of the hormone but rather serve to clear ANP from the extracellular circulation. This receptor is known as the atrial naturetic peptide clearance receptor (ANP-CR). The effects of ANP in vivo are short lived due to rapid clearance by the ANP-CR in the lung and via proteolytic cleavage by neutral endopeptidase (NEP) in the kidney.[4] Over 50% of exogenously administered ANP is cleared in a single pass through the pulmonary circulation, and this clearance is thought to be primarily mediated by the ANP-CR.[5,6] In a therapeutic application, infusion of ANP in patients with pulmonary hypertension (PH) produces a dose-dependent decrease in pulmonary arterial pressure, without adverse effects on systemic pressure or pulmonary gas exchange. This effect is in contrast to nonselective effects produced by other vasodilators, and suggests that ANP could be used to reduce PH.[7,8] Unfortunately, therapeutic treatment of PH by administration of ANP would be limited due to its short in vivo half-life. We reasoned that a more feasible approach to increasing pulmonary ANP levels would be through blockade of the ANP-CR, thus elevating endogenous levels of ANP in the pulmonary vascular bed. This strategy was perceived to have the additional advantage of selectively elevating ANP levels in the pulmonary vasculature, since once ANP enters the systemic circulation it is rapidly degraded by NEP.

A number of structure–activity studies have defined the key residues in ANP responsible for its binding to the ANP-A and ANP-B receptors.[9] Only a subset of these residues are necessary for potent binding to the ANP-CR. These are contained in the Phe-8 through Ile-15 sequence of the peptide. This has led to the identification of several highly potent and selective ligands for the ANP-CR (Fig. 1, Table 1). Among these are ANP 4-23, which is

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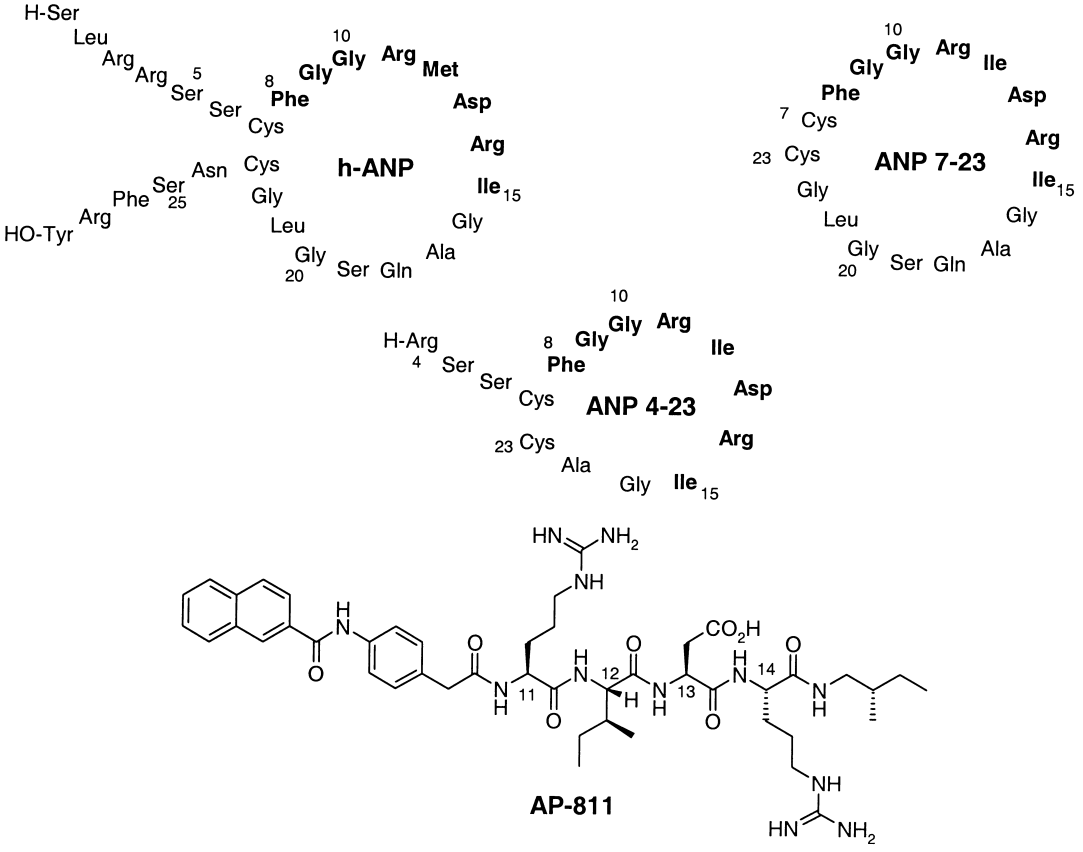


**Figure 1.** Structures of human ANP (h-ANP) and selective ligands for the ANP-CR. Affinities for these compounds are given in Table 1. Numbers in AP-811 structure refer to corresponding residues in ANP.

**Table 1.** Binding affinities[a] of known ligands at ANP-CR and ANP-A receptors

| Compound | ANP-CR $K_i$ (nM) | ANP-A $K_i$ (nM) | Selectivity ratio |
|---|---|---|---|
| **h-ANP** | 0.059±0.02 (9) | 0.65±0.12 (6) | 11 |
| **ANP 4-23** | 1.64 (2) | >10 μM (3) | 6097 |
| **AP-811** | 0.48±0.11 (9) | >10 μM (3) | >20,000 |

[a]Binding affinities refer to the displacement of [$^{125}$I] ANP from the ANP-A or ANP-CR receptor, and are mean ± SEM from (*n*) experiments in duplicate. For method of determination see ref 18.

a cyclic peptide related to ANP itself, and a modified linear peptide known as AP-811.[10,11] We hypothesize that the relationship between ANP and these two selective ANP-CR ligands is as follows: The Arg-Ile-Asp-Arg sequence in ANP 4-23 or AP-811 maps to the Arg-11 through Arg-14 sequence of ANP, the napthylamidophenylacetic acid portion of AP-811 serves as a mimic of Phe-8 to Gly-10 in ANP, and the 2-methylbutyl C-terminus of AP-811 is an Ile-15 side-chain mimic. While AP-811 is not orally bioavailable, we thought that this structure offered a promising starting point to develop an orally active, selective ANP-CR blocker.[12] The lack of oral bioavailability of AP-811 is not surprising considering that it has a molecular weight of 915 Da. Peptides of this size are generally poorly absorbed and/or rapidly metabolized. AP-811 contains several highly charged amino acids including two arginine residues, which likely also limits absorption. Recently, it has been shown that removal of similar basic residues improves oral bioavailability in thrombin inhibitors.[13,14] To address these issues, we initially investigated the structural requirements for the binding of AP-811 to the ANP-CR. An alanine scan of the peptide backbone was undertaken to assess the contributions of side-chain residues to binding affinity, and truncated N- and C-terminal analogues were examined to determine if meaningful reductions in molecular weight could be achieved by shortening the length of the molecule while retaining affinity for the ANP-CR. The results of these interesting studies are shown diagrammatically in Figure 2. Several important findings resulted from these investigations. The arginine side chains appear to play a minor role in binding, as either one can be removed with no loss of activity. This is in contrast to the known SAR of the binding of ANP to the ANP-A and ANP-B receptors, which demonstrated that both arginines are critical for significant receptor affinity.[9] However, when both arginine residues are replaced by alanine, a much larger, nonadditive decrease in binding affinity is obtained. Dramatic decreases in affinity occur upon replacing either the Ile or Asp residues with Ala, suggesting that these side chains are involved in important interactions with the receptor. The fact that the Arg-11 residue can be replaced by a nonbasic residue suggests that the Asp is not involved in conformational organization of the peptide through intramolecular salt bridge formation, and thus is probably involved in an interaction with the

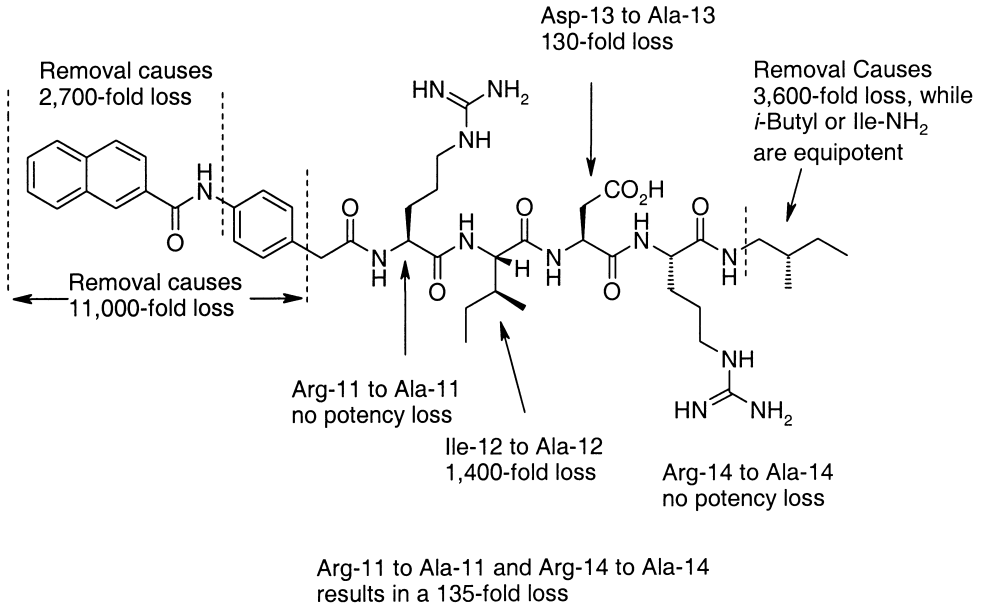


**Figure 2.** Changes in potency from modifications to AP-811. Numbers listed next to residues refer to the corresponding residue in ANP. Individual binding affinities are given in reference 17 and Table 2.

receptor. Less encouraging was the finding that both the N- and C-termini are required, presumably involved in important hydrophobic interactions with the receptor.

An NMR-derived structure of a truncated form of ANP, (ANP 7-23, Table 2) has been reported by the Fesik group.[15] We were interested in using this structural information to drive drug design efforts around AP-811, especially since internal efforts at determining an NMR structure of AP-811 had found it to be highly conformationally flexible in solution. However, to make use of this structural information we needed to know that ANP 7-23 had high affinity for the clearance receptor, and that it bound to the receptor using the same side chain interactions. This would increase our confidence that conformational information obtained from the ANP 7-23 NMR structure could be applied to the design of AP-811 analogues. Evaluation of ANP 7-23 found that the molecule was very potent at the ANP-CR, with a $K_i$ of 0.1 nM. We next carried out a limited alanine scan of residues in the Phe-8 to Ile-15 region of ANP 7-23 to see if similar effects to those found for AP-811 would be obtained.[16] The results of these modifications (Table 2) show a similar SAR exists between the two series of molecules. While the magnitude of the effect of the modifications differ between the two series, the rank ordering of importance of an individual residue to the binding affinity to the ANP-CR is identical between the series. Unfortunately, synthetic difficulties prevented the accessibility of the Arg-14 to Ala modification in ANP 7-23, but we presume the effect would be similar to that found for AP-811. These results suggest that these series share a similar binding mode, and that conformational information obtained in the NMR structure of ANP 7-23 would be of use in designing new acyclic analogues related to AP-811.

The data obtained from this study suggest that while it will be difficult to reduce the molecular weight of AP-811, significant reductions in the overall charge of the molecule are possible. These results are significant, since molecules of this size are typically absorbed from the intestine via the transcellular route, and only the neutral form migrates through the intestinal cell membrane to any significant extent. Future papers in this series will further illustrate the design concepts which ultimately led to compounds that elevate plasma ANP levels upon oral administration.

**Table 2.** Comparison of alanine scans between AP-811 and ANP 7-23

| | AP-811 | | ANP 7-23 | |
|---|---|---|---|---|
| Amino acid modification | $K_i$ (nM)[a] | Loss in activity | $K_i$ (nM)[a] | Loss in activity |
| None | 0.48±0.11 (9) | — | 0.10±0.04 (4) | — |
| Arg-11 to Ala | 0.3 (2) | No change | 0.49 (1) | 5-fold |
| Asp-13 to Ala | 62 (2) | 130-fold | 87 (2) | 870-fold |
| Ile-12 to Ala | 680±121 (3) | 1400-fold | 360 (2) | 3600-fold |
| Arg-14 to Ala | 0.4 (1) | No change | NA[b] | NA[b] |
| Arg-11 and Arg-14 to Ala | 65 (2) | 135 | NA[b] | NA[b] |

[a]Binding affinities refer to the displacement of [$^{125}$I] ANP from the ANP-A or ANP-CR receptor, and are mean data from (*n*) experiments in duplicate, (±SEM if $n>2$).
[b]Compound not available.

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12. Oral bioavailability studies were conducted in-house using male rats given a 30 mg/kg oral dose of AP-811. These studies failed to detect plasma levels of the parent compound using LC/MS detection. We thank Ms. Karin Kirkland for providing this information.
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16. ANP 7-23 and the alanine modified versions of this compound were custom synthesized by Cambridge Research Biochemicals, P.O. Box 461, Stockton-on-Tees, Cleveland, TS23 1XH UK. Structures of individual compounds are as follows:


ANP 7-23
Ac-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-$NH_2$

Ac-Cys-Phe-Gly-Gly-Arg-**Ala**-Asp-Arg-
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-$NH_2$

Ac-Cys-Phe-Gly-Gly-**Ala**-Ile-Asp-Arg-
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-$NH_2$

Ac-Cys-Phe-Gly-Gly-Arg-Ile-**Ala**-Arg-
Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-$NH_2$


17. Binding affinities of the truncated AP-811 compounds are as follows: phenylacetamido-Arg-Ile-Asp-Arg-amino-2(*S*)methylpentane $K_i$ 539 nM ($n=1$); acetamido-Arg-Ile-Asp-Arg-amino-2(*S*)methylpentane $K_i$ 2900 nM (n = 1) ; 4-[2-napthamido]phenylacetamido-Arg-Ile-Asp-Arg-$NH_2$ $K_i$ 2600 nM (n = 1). These compounds and the other AP-811 analogues shown in Table 2, were prepared in solution using standard Fmoc chemistry. Each peptide was purified by preparative HPLC (>95% purity) and was characterized by NMR, MS, and elemental analysis.
18. For receptor binding experiments and determination of antagonists $K_i$ values a method similar to published report (Koyama et al., ref 11) was employed. Briefly, membranes (48,000 g fraction) prepared from recombinant BV-Sf21 cells expressing the ANP-C receptor, or recombinant CHO cells expressing the human ANP-C or ANP-A receptors, were incubated in a Tris–HCl buffer (50 mM, pH 7.4, containing 0.1 M NaCl, 0.1 mM EDTA, 0.1% BSA and 10 μM Thiorphan), with a mixture of [$^{125}$I]ANP (Amersham, 0.02–0.05 nM) and tested compounds (range of 0.1 nM to 30 μM), on a 96-deepwell plate, for 1 h, at 23 °C and shaking. The bound receptor-ligand complex was separated from free ligand by washing with 50 mM/0.1 M NaCl Tris buffer, followed immediately by vacuum filtration on 96-well GF/C filter plates. The plates were air-dried overnight and following addition of 25 μL scintillation liquid they were sealed and counted in the Microbeta Trilux scintillation counter. Non-specific binding is determined by addition of cold ANP (1 μM). Computation of equilibrium binding constants ($K_D$ and $K_i$), receptor density ($B_{max}$), as well as statistical analysis were carried out using GraphPad 'PRISM' software. The difference between various binding affinities (-Log[$K_i$]) were tested with ANOVA and changes were considered significant when $P < 0.05$.