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Design and synthesis of metabolically stable atrial natriuretic factor analogs

Amino- and carboxy-terminal stabilization

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Two analogs of rat atrial natriuretic factor, $rANF_{7-28}$-NH_2 and [Mpr^7,Ala^{20},D-Arg^{27}]$rANF_{7-27}$-NH_2, were prepared by the solid-phase method. These peptides had 2-fold and 7-fold less affinity, respectively, than $rANF_{1-28}$ in binding to membranes prepared from cultured aortic smooth muscle cells, and both peptides were 5-fold less potent than $rANF_{1-28}$ in relaxing serotonin-contracted rabbit aortic rings. $rANF_{7-28}$-NH_2 was rapidly degraded by rat kidney homogenates but [Mpr^7,Ala^{20},D-Arg^{27}]$rANF_{7-27}$-NH_2 had enhanced stability against rat kidney homogenate degradation. However, this in vitro stability did not translate into an extended duration of action in vivo.

Atrial natriuretic factor; Proteolytic degradation

1. INTRODUCTION

The discovery of a family of potent vasodilatory, natriuretic, and diuretic peptides (collectively referred to as atrial natriuretic factors) has led to considerable research activity (for reviews see references [1–5]). As with other key regulatory peptide hormones, the intrinsic biological activity of atrial natriuretic factor (ANF) is short lived. In the case of ANF, this presumably allows a precise and proper balance of the contribution of ANF to the maintenance of extracellular fluid volume. We wished to exploit the desirable biological activities of ANF and prepare analogs that showed a prolongation of biological action. Central to this approach is the ability to modify the parent structure profile against enzymatic degradation while maintaining biological activity.

The rational design of metabolically stable analogs requires an understanding of proteolytic inactivation in vivo. However, the in vivo enzymatic degradation of peptide hormones is a complex process. Kidney homogenate preparations are often used as in vitro models of peptide degradation [6]. We determined the products of rat (r)ANF_{7-28}-NH_2 degradation by a soluble rat kidney homogenate preparation [7]. The initial degradation products are the C-terminal hydrolyzed compounds $rANF_{7-27}$, $rANF_{7-26}$, and $rANF_{7-25}$. We proposed that the initial proteolysis of rANF $rANF_{7-28}$-NH_2 (by rat kidney homogenate) involved a trypsin-like cleavage of the Arg^{27}-Tyr^{28} amide linkage.

This report describes the synthesis and biological profile of $rANF_{7-28}$-NH_2, and [Mpr^7,Ala^{20},D-Arg^{27}]$rANF_{7-27}$-NH_2 (which was designed to resist proteolysis).

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2. MATERIALS AND METHODS

2.1. *Peptides*

Peptides were assembled by the stepwise solid-phase method [8]. Protected peptides were prepared beginning with 0.5 mmol resin by standard synthetic techniques using an automated peptide synthesizer. N^{α}-*tert*-Butyloxycarbonyl (Boc) protected amino acids and *p*-methyl-benzhydrylamine resin (1.1 mequiv./g) were purchased from Peptides International (Louisville, KY). The cycle used for incorporation of each Boc amino acid is shown in scheme 1. After completion of the stepwise assembly of the required sequence the N-terminal Boc group was removed using 60% TFA, 1.5% anisole in CH_2Cl_2 and the resin dried in vacuo. Peptides were deprotected and removed from the resin with anhydrous liquid HF (10 ml/g resin) containing 10% anisole for 45 min at 0°C. After removal of the HF in vacuo, the peptides were extracted with 30% acetic acid, rinsed with ethyl ether, and lyophilized. Purification of peptides utilized gel filtration on Sephadex G-25F, SP-Sephadex cation-exchange chromatography, and preparative HPLC. Homogeneity was assessed by analytical reversed-phase HPLC (two solvent systems), amino acid analysis, and FAB-MS.

2.1.1. (S-pMeBz1)3-mercaptopropionic acid

To a cooled solution (0°C) of 3-mercaptopropionic acid (5 g, 47 mmol) in THF (40 ml) under N_2, NaH (3.7 g, 61.1% oil dispersion, 94 mmol, portionwise over 5 min), and *p*-methylbenzyl bromide (8.7 g, 47 mmol, dropwise over 20 min) were added. The suspension was stirred overnight at room temperature, DMF (60 ml) was added, stirring continued for 0.5 h, and then 10% $NaHCO_3$ (50 ml) was added. The solvents were removed under reduced pressure, the residue suspended in H_2O (500 ml), and washed with EtOAc (3 × 100 ml). The aqueous phase was acidified to pH 3 with 5 N HCl, extracted with EtOAc (3 × 100 ml), the combined EtOAc washings were extracted with saturated NaCl (3 × 50 ml), dried (Na_2SO_4), and the solvent removed under reduced pressure to give 8.6 g of a gray oil. A portion of the oil (7.1 g) was purified by preparative liquid chromatography (Waters Prep 500) using EtOAc/hexanes (3:7). The desired fractions were pooled, the solvent removed under reduced pressure, and the residue recrystallized from warm EtOAc/hexanes (20 ml:100 ml) to give the desired product (5.97 g, 60%, m.p. 74–76°C [9]).

2.1.2. H-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-NH_2 (rANF$_{7-28}$-NH_2).

Synthesis was initiated by charging the reaction vessel with *p*-methylbenzhydrylamine resin (0.45 g, 0.50 mmol). The protected peptide resin corresponding to the title compound was obtained after stepwise coupling of the appropriate N^{α}-Boc-amino acids. Trifunctional amino acids were protected as follows: N^{α}-Boc-Cys(pMeBzl)-OH; N^{α}-Boc-Ser(Bzl)-OH; N^{α}-Boc-Cys(pMeBzl)-OH; N^{α}-Boc-Arg(Tos)-OH; N^{α}-Boc-Asp-(Chx)-OH. A portion of the protected peptide resin (1.2 g) was treated with liquid HF/anisole for 45 min at 0°C. After evaporation of HF, the product was extracted with 100 ml 30% AcOH, washed with Et_2O (3 × 25 ml), and extracted with an additional 30% AcOH (3 × 20 ml). The AcOH extracts were combined and freeze dried. The peptide powder was dissolved in 1.8 l H_2O and the pH adjusted to 8.3 with aqueous ammonia. A solution of 0.01 M $K_3Fe(CN)_6$ (27 ml) was added over 10 min and stirring continued for 30 min. The pH was adjusted to 5.0 with AcOH and excess ferro- and ferricyanide removed by the addition of 10 ml BioRad AG3-X4A(Cl^- form) anion-exchange resin. After stirring for 30 min the resin was removed by filtration and the solution concentrated in vacuo (30°C) to 10 ml. The cyclized peptide was desalted on a Sephadex G-25F column (2.5 × 85 cm) using 30% AcOH. Appropriate fractions were pooled and freeze dried (225 mg). The peptide powder was dissolved in 10% AcOH/20 mM NaOAc and applied to an SP-Sephadex cation-exchange column (1 × 45 cm). The peptide was eluted with a linear salt (NaCl) gradient from 0 to 1.5 M in 300 ml of the same buffer. The major peak was pooled, lyophilized, desalted by gel filtration on a Sephadex G-25F column (2.5 × 85 cm), and appropriate fractions pooled and lyophilized to give 99 mg material. This material was purified to homogeneity by preparative gradient HPLC (30% B→40% B over 25 min, flow rate 9 ml/min, Vydac 218TP1022 [2.2 × 25 cm], A = 0.1% TFA, B = 0.1% TFA, 60% MeCN). The lyophilizate from 6 injections (about 15 mg each) yielded 24 μmol of the title compound (overall yield 9.6% based on initial resin substitution). Amino acid analysis: Asp 1.77 (2), Glu 1.01 (1), Ser 1.84 (2), Gly 5.08 (5), Ala 1.05 (1), Arg 2.89 (3), Tyr 0.90 (1), Ile 1.80 (2), Leu 0.99 (1), Phe 1.89 (2). FAB mass spectrum: $[M+H]^+$ calc. 2373; found 2373.

	Time (*min*)
60% TFA, 1.5% anisole in CH_2Cl_2	2
60% TFA, 1.5% anisole in CH_2Cl_2	17
4 × CH_2Cl_2	0.5
2 × 25% DIEA in DMF	1
2 × DMF	1
1st coupling[a] in DMF	22
2 × DMF	1
25% DIEA in DMF	1
3 × DMF	1
2nd coupling[a] in CH_2Cl_2	22
2 × DMF	1
25% DIEA in DMF	1
3 × DMF	1
Capping (25% acetic anhydride in CH_2Cl_2)	10
4 × CH_2Cl_2	1

[a] 2-fold excess of preformed symmetrical anhydride (Arg, Asn, and Gln 4-fold excess of preformed 1-hydroxybenzotriazole esters)

Scheme 1.

2.1.3. Mpr-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Ala-Leu-Gly-Cys-Asn-Ser-Phe-D-Arg-NH_2 ([Mpr^7,Ala^{20},D-Arg^{27}]rANF$_{7-27}$-NH_2)

Amino acid analysis: Asp 1.58 (2), Glu 0.89 (1), Ser 1.79 (2), Gly 4.09 (4), Ala 2.06 (2), Arg 2.90 (3), Ile 1.97 (2), Leu 0.98 (1), Phe 2.06 (2). FAB mass spectrum: $[M+H]^+$ calc. 2209; found 2209.

2.1.4. H-Ser-Leu-Arg-Arg-Ser-Ser-Cys-Phe-Gly-Gly-Arg-Ile-Asp-Arg-Ile-Gly-Ala-Gln-Ser-Gly-Leu-Gly-Cys-Asn-Ser-Phe-Arg-Tyr-OH (rANF$_{7-28}$)

Amino acid analysis: Asp 1.22 (2), Glu 0.95 (1), Ser 4.73 (5), Gly 5.16 (5), Ala 0.93 (1), Arg 4.88 (5), Tyr 0.99 (1), Ile 2.04 (2), Leu 2.09 (2), Phe 2.10 (2). FAB mass spectrum: $[M+H]^+$ calc. 3060; found 3062 ± 2.

2.2. *Enzymatic degradation*

Rat kidneys were excised, minced and homogenized in ice-cold 50 mM sodium phosphate buffered saline (10 ml/kidney) at pH 7.4. The homogenate was centrifuged for 20 min at $48000 \times g$ at 4°C. The supernatant (100 μl) was added to 10 nmol peptide (freeze dried powder), and incubated at 37°C for various times. Enzymatic activity was destroyed by dipping the sample tubes into boiling water for 2 min. The tubes were centrifuged at 4000 rpm for 2 min. Supernatants (50 μl) were then subjected to reverse-phase HPLC [column: Vydac 218TP54 (25 cm × 4.6 mm i.d.), with a Macrosphere 300 Å C-4, 7 μm, guard column (1 cm × 4.6 mm i.d.)]. Mobile phase: A = H_2O/CH_3CN (85:15) containing 0.01 M ammonium acetate, and B = H_2O/CH_3CN (50:50) containing 0.01 M ammonium acetate. A linear gradient was run from 0% B to 60% B over 30 min at a flow rate of 1.5 ml/min.

3. RESULTS

The compounds were evaluated in several different assays, both in vitro and in vivo, and the results are presented in table 1. In an assay for competition of the binding of ^{125}I-rANF$_{5-28}$ to cultured SHR vascular smooth muscle membranes rANF$_{7-28}$-NH$_2$ had 2-fold less affinity and [Mpr^7, Ala^{20},D-Arg^{27}]rANF$_{7-27}$-NH$_2$ had 7-fold less affinity than rANF$_{1-28}$. In an in vitro vasodilatory assay employing serotonin-contracted rabbit aortic rings, both rANF analogs were 5-fold less potent than rANF$_{1-28}$ in inducing relaxation. In vivo in anesthetized normotensive rats, rANF$_{7-28}$-NH$_2$ was slightly more potent in producing natriuresis/diuresis than rANF$_{1-28}$ while [Mpr^7,Ala^{20},D-Arg^{27}]- rANF$_{7-27}$-NH$_2$ was essentially equipotent with rANF$_{1-28}$. Table 1 lists the bolus dose (nmol/kg, i.v.) required to elicit a 1 ml/10 min diuresis (ED_1) and $U_{Na}V$ of 200 μmol/10 min (ED_{200}). The ED_1 and ED_{200} values reflect half-maximal responses.

The half lives ($t_{1/2}$) of the analogs towards proteolysis by the rat kidney preparation are shown in table 1. The amino and carboxyl modified compound [Mpr^7,Ala^{20},D-Arg^{27}]rANF$_{7-27}$-NH$_2$ has a 27-fold enhanced stability to the proteolytic enzymes present in this tissue preparation.

Table 1

Compound	Binding affinity[a] ($IC_{50} \pm$ SE, pM)	Vasodilatory activity[b] ($ED_{50} \pm$ SE, nM)	Diuresis[c] ($ED_1 \pm$ SE, nmol/kg)	Natriuresis[c] ($ED_{200} \pm$ SE, nmol/kg)	Kidney Homogenate[d] ($t_{1/2}$, min)
rANF$_{1-28}$	2.5 ± 1.0	6.5 ± 2.3	1.80 ± 0.22	1.72 ± 0.19	1.5
rANF$_{7-28}$-NH$_2$	5.0 ± 2.0	31.5 ± 2.4	1.00 ± 0.09	1.18 ± 0.12	3.1
[Mpr^7,Ala^{20},D-Arg^{27}] rANF$_{7-27}$-NH$_2$	18.0 ± 6.0	32.4 ± 4.9	1.53 ± 0.09	1.59 ± 0.11	40.8

[a] Competition for the binding of 0.1 nM ^{125}I-rANF$_{5-28}$ was determined in crude membranes from cultured aortic smooth muscle cells from SHR at 4°C in 50 mM Tris buffer (pH 7.4, 4°C, 90 min) containing 400 μg/ml bovine serum albumin, 40 μg/ml bacitracin, 4 μg/ml leupeptin, 4 μg/ml chymostatin, and 5 μM phenylmethylsulfonyl fluoride, IC_{50}, concentration producing 50% inhibition of maximum specific binding. N, 5–10 determinations

[b] New Zealand white rabbits (3–5 kg) were killed by cervical dislocation. Aortic rings (2 mm) were mounted under 3 g tension in a 3 ml chamber containing Burns Modified Tyrodes solution maintained at 37°C and aerated with 95% O_2–5% CO_2. Each ring was equilibrated for 60 min. Rings were contracted with serotonin (20 μM) and, once the contractile response stabilized, exposed to rANF in half-log increments beginning at 10^{-10} M. Relaxation was determined in six preparations and the linear part of the concentration-response curve was used to calculate the ED_{50} values. $N = 6$

[c] Natriuretic-diuretic assays were performed under Pentobarbital/Inactin® anesthesia in male Sprague-Dawley rats (200–350 g). Catheters were placed in the left carotid artery, the right jugular vein and bladder. The animals were infused with 0.15 ml/min of saline. The protocol was begun when urine flow rate stabilized at 20–150 μl/10 min. Urine was collected for three consecutive 10-min control periods and then rANF was administered intravenously as a 100 μl/100 g body weight bolus. Urine samples were collected for an additional 30 min at 10 min intervals. Only one rANF analog was assayed in each rat. Initial 10 min-periods following rANF injection were compared. Relative activities were determined as the dose that produces 1.0 ml/10 min of urine (ED_1) and 200 μmol/10 min sodium excretion (ED_{200}) using at least 3 data points ($N = 4$) from the linear portion of the dose-response curve. Sodium concentrations were measured by an ion-selective electrode (Orion)

[d] The $t_{1/2}$ (metabolic half-life) values were determined graphically by plotting the log of the peak area $A_{214\ nm}$ vs time assuming first order kinetics

4. DISCUSSION

We set out to design ANF analogs that were stabilized against enzymatic inactivation at the amino- and carboxyl-terminal extensions. The sequence of rANF is shown in fig.1. It consists of a 17 amino acid disulfide linked cyclic structure flanked by amino- and carboxyl-terminal exocyclic extensions. Structure activity studies have shown the following key features: (i) the cyclic core ($rANF_{7-23}$) possesses minimal biological activity [10]; (ii) the entire N-terminal exocyclic tail (residues 1–6) is not required for full activity [11]; (iii) Tyr^{28} may be deleted [11], and (iv) C-terminal carboxamides have good activity [12].

Indeed, the C-terminal carboxamide $rANF_{7-28}$-NH_2 has a biological profile quite similar to the endogenous peptide (table 1). It is, however, rapidly degraded by rat kidney homogenates (table 1).

The initial degradation of $rANF_{7-28}$-NH_2 by soluble proteases of rat kidney homogenates resulted from cleavage of the Arg^{27}-Tyr^{28} amide bond [7]. We reasoned that substitution of Arg^{27} by its D isomer should prevent this hydrolysis. Additionally, in order to protect against aminopeptidase mediated degradation we substituted Cys^7 with 3-mercaptopropionic acid (Mpr). Structure function studies with oxytocin have demonstrated the utility of replacing an N-terminal cysteine with Mpr [13]. This modification is also tolerated in ANF analogs [14–16].

The amino- and carboxyl-terminal modified analog [Mpr^7,Ala^{20},D-Arg^{27}]-$rANF_{7-27}$-NH_2 maintains very high affinity for ^{125}I-$rANF_{5-28}$ binding sites in aortic smooth muscle membranes. These low pM affinity binding sites for ANF have also been reported in rat renal glomerular membranes and in rabbit carotid artery smooth muscle cells [17–19]. They may be receptors that are biologically silent or linked to as yet unknown functions of ANF. [Mpr^7,Ala^{20},D-Arg^{27}]-$rANF_{7-27}$-NH_2 also has vasodilatory and natriuretic/diuretic activity comparable to the endogenous peptide (table 1).

When assayed for stability against kidney homogenate degradation [Mpr^7,Ala^{20},D-Arg^{27}]-$rANF_{7-27}$-NH_2 has greatly enhanced stability. Substitution with D-Arg at position 27 prolongs the in vitro half-life. Disappointingly, [Mpr^7,Ala^{20},D-Arg^{27}]$rANF_{7-27}$-NH_2 does not show an increase in duration of biological activity when assayed in the anesthetized rat. Fig.2 shows that [Mpr^7,Ala^{20},D-Arg^{27}]$rANF_{7-27}$-NH_2 has a time-response relationship that is essentially parallel to $rANF_{1-28}$. The lack of in vivo duration could be due to rapid endopeptidase mediated

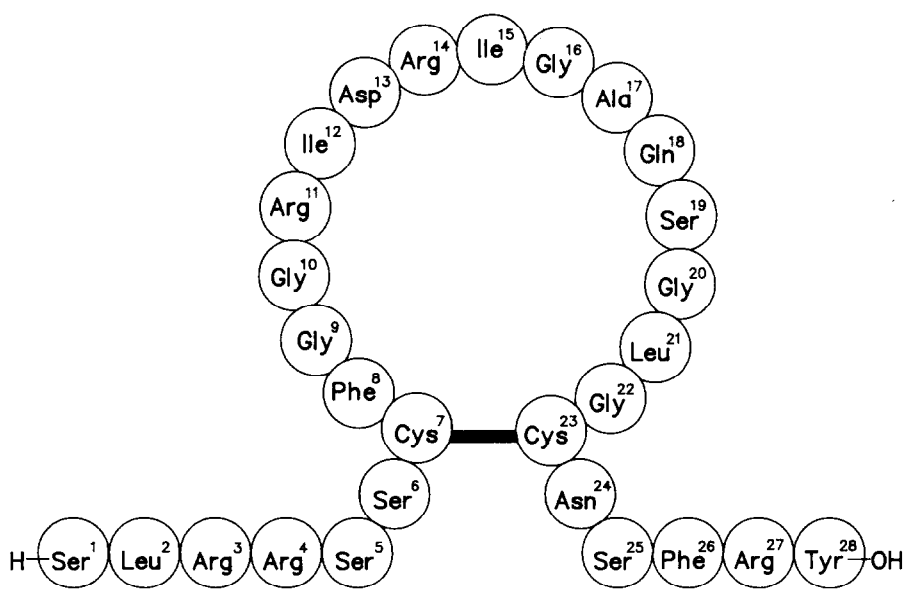

Fig. 1. Structure of $rANF_{1-28}$.

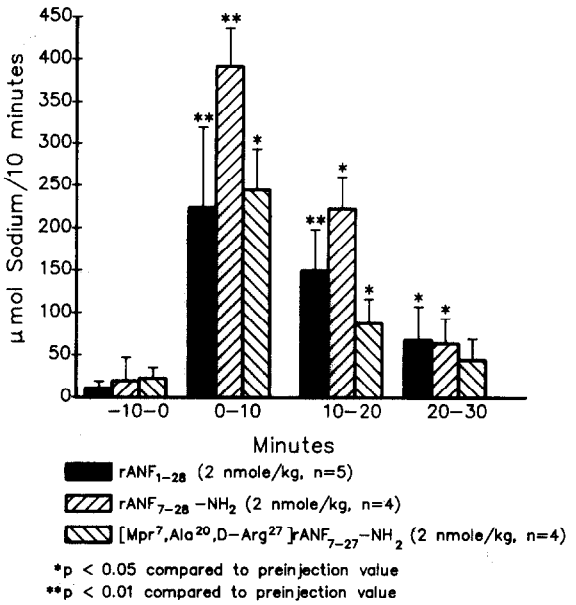

Fig. 2.

cleavages or excretion. The appropriateness of our kidney preparation may be questionable because there is no correlation between in vitro stability and in vivo duration. More likely, the lack of correlation is attributable to the complexity of the in vivo metabolism of ANF.

Recently, several reports describing the metabolism of ANF have appeared. Endopeptidase 24.11 and thermolysin appear to cleave ANF's X-hydrophobic linkages [20,21]. The initial cleavage site appears to be the Cys^7-Phe^8 linkage [21]. Condra et al. [22] have described the in vivo metabolism of ^{125}I-ANF in rats and found that several other amides are susceptable to proteolysis.

In conclusion, we have prepared a novel rANF analog that has enhanced stability towards proteolysis by a rat kidney homogenate preparation. We are currently studying the thermolysin mediated degradation of [Mpr^7,Ala^{20},D-Arg^{27}]-$rANF_{7-27}$-NH_2 in an effort to identify the physiologically relevant cleavage sites.

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