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Synthesis and In Vitro Analysis of Atrial Natriuretic Peptide–Albumin Conjugates

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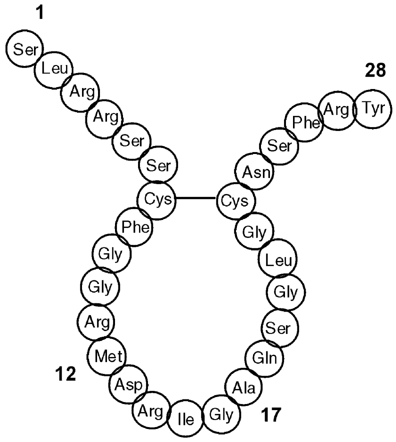
Abstract—Atrial natriuretic peptide (ANP) is a clinically useful anti-hypertensive hormone. Maleimide derivatives of ANP have been synthesized and conjugated to cysteine-34 of human serum albumin. The conjugates were analyzed to assess their stability, receptor binding affinity and ability to stimulate guanylyl-cyclase activity in rat lung fibroblasts.

Natriuretic peptides are natural cardiac hormones responsible for reduction of sodium (natriuresis) and water (diuresis) levels in the blood and inhibition of vasoconstriction.[1] They are used clinically in the treatment of congestive heart failure and are believed to counteract the renin-angiotensin-aldosterone system.[2] Human atrial natriuretic peptide [ANP(1-28), **1**, Fig. 1] is a 28-amino acid peptide that contains a 17-residue ring formed by a disulfide bridge between two cysteine residues. ANP binds to Natriuretic Peptide Receptors NPR-A and NPR-B found on the surface of mammalian cells and causes a cGMP-dependent signaling cascade leading to beneficial natriuretic and diuretic effects. **1** also binds to the Natriuretic Peptide Receptor C (NPR-C) also known as the clearance receptor that is devoid of both kinase and guanylyl-cyclase activity.

Despite the pronounced pharmacological effect of **1**, it suffers from a short plasma half-life due primarily to metabolism by neutral endopeptidase (NEP)[1] and NPR-C[1–3] as well as elimination through the kidney. We believe that we can alleviate the rapid degradation of a natriuretic peptide by conjugating it to a large protein carrier such as human serum albumin (HSA). To demonstrate this hypothesis, conjugation can be performed by the covalent attachment of a maleimide derivative of ANP to cysteine-34 of HSA via a linker (Fig. 2).[4] HSA can help increase the half-life of the native peptide by (1) reducing excretion through the kidney, (2) protecting from degradation by plasma enzymes and (3) bypass the NPR-C elimination mechanism.[4a,b] Consequently, the resulting large molecular weight ANP–HSA conjugate can intuitively lead to reduced biological activity. The present communication reports our in vitro findings from a focused study to



1, Human ANP(1-28)

Figure 1. Structure of human ANP(1-28) **1** and residue positions 1, 12, 17 and 28.

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synthesize derivatives of **1** as albumin conjugates and optimize the NPR-A binding and activation.

Although a large amount of SAR work has already been done since the discovery of **1**, to the best of our knowledge, a limited amount of work has been reported demonstrating retention or loss of activity in vitro when **1** is fused[5] or conjugated[6] to a large protein. Four sites on **1** were chosen to probe the structural features comprising the pharmacophore of the albumin construct as shown in Figure 3. The first site of modification was at the N-terminus leading to compound **2**. The second involved the addition of a lysine residue at the C-terminus (position 29) to accommodate the linker and maleimide on its ε-amine functional group as shown on compound **3**. The third target molecule involved substituting methionine-12 to a lysine residue as illustrated on compound **4**. Position 12 of **1** was chosen due to the fact it is the only non-conserved amino acid when compared to rat ANP (leucine at position 12). The fourth molecule has alanine at position 17 substituted to a lysine residue composed of the linker and reactive group as shown in compound **5**. Using molecular modeling as well as information from an ANP mutant and the CNP (C-type Natriuretic Peptide) structures,[7] the NPR-A X-ray crystal structure[8] and the minimal structural requirement for activity,[9] the residue situated at position 17 was chosen among the loop residues to be the one most distal to the disulfide bond.

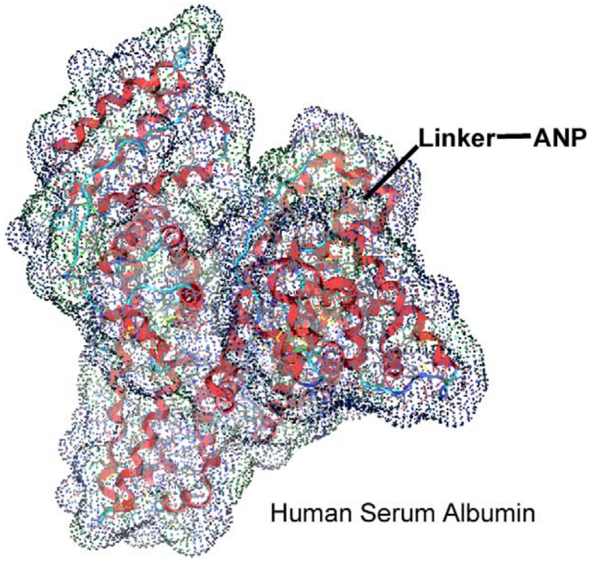


Figure 2. Attachment of ANP to cysteine-34 of HSA.

The linker connecting the maleimide to ANP is a synthetic chain termed AEEA for [2-(2-aminoethoxy)-ethoxy]acetamide.[10]

The general synthetic protocol was the same for all four compounds. Scheme 1 displays the synthesis of **5** accomplished by a combination of liquid- and solid-phase peptide synthesis (SPPS).[11] The SPPS was performed using Fmoc chemistry with *N*-epsilon Alloc lysine protection for selective removal of the orthogonal protecting group with palladium(0)[12] to give intermediate **B**. Commercial Fmoc-AEEA could then be coupled using the standard set of reagents followed by the attachment of maleimidopropionic acid (MPA) to give intermediate **C**. Attempts to perform the cyclization on resin gave poor results. Cleavage of the peptide off the resin gave the bis-acetamidomethylthio intermediate **D**. At this point, the intermediate was purified by semi-preparative HPLC because all cyclization attempts using crude products gave lower yields and further purification difficulties. The cyclization of **D** was done in solution phase using diphenyl sulfoxide, methyltrichlorosilane and anisole in trifluoroacetic acid as solvent,[13] yielding the cyclic disulfide **5**. All compounds produced were C-terminus amides.

The four maleimide derivatives of **1** were obtained in modest yield due to the double purification (3–8% yields overall for 64 synthetic steps). The synthetic results are displayed in Table 1.

Table 2 displays the results from the conjugation of the four maleimide derivatives of **1** with human serum albumin.[14] No species corresponding to the double addition of a maleimide derivative could be detected by LC/MS analysis.

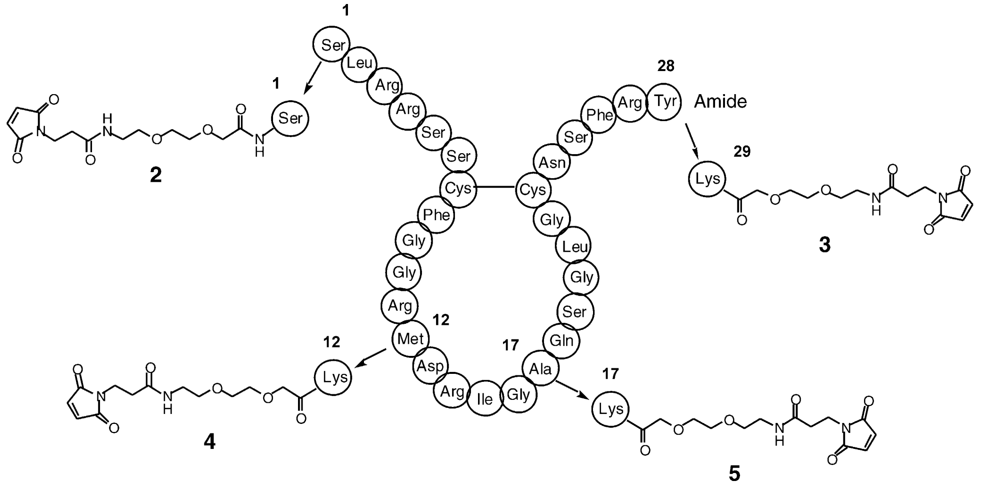
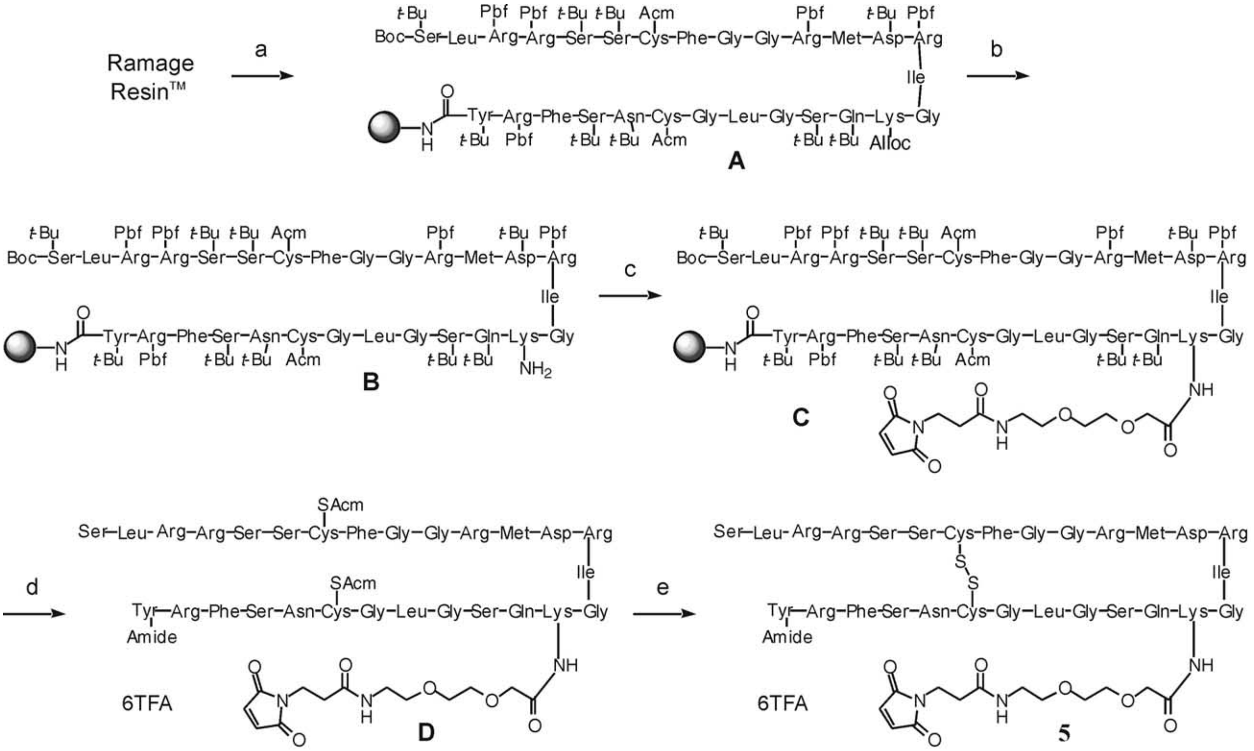


Figure 3. The four maleimide derivatives of **1**.



Scheme 1. Representative synthetic protocol for the preparation of ANP derivatives: synthesis of compound **5**: (a) solid-phase synthesis using Fmoc chemistry and HBTU/Hünig's base as activating agent; (b) Pd(PPh_3)$_4$, *N*-methylmorpholine, acetic acid, benzene and chloroform, ambient temperature; (c) (1) Fmoc-AEEA, Hünig's base, HBTU, DMF; (2) 20% piperidine, DMF; (3) 3-maleimidopropionic acid, Hünig's base, HBTU, DMF; (d) (1) 85% TFA, 5% triisopropylsilane, 5% thioanisole, 5% phenol; (2) purification; (e) (1) Ph_2SO, $MeCl_3Si$, anisole, TFA; (2) purification.

Table 1. Results peptide synthesis

Compd	Predicted M_r	Actual M_r	Purity (HPLC) (%)
2	3373.6	3377.1	93
3	3501.7	3504.0	91
4	3370.6	3372.3	91
5	3430.6	3432.5	99

Table 2. HSA conjugation results

Conjugate	Predicted M_r[a]	Actual M_r	Amount unbound (%)
2-HSA	69,838	69,833	<0.1
3-HSA	69,966	69,962	<0.1
4-HSA	69,817	69,811	<0.1
5-HSA	69,877	69,871	<0.1

[a] Using the measured molecular weight of an albumin standard analyzed by LC/MS prior to conjugate sample analysis.

The albumin conjugates were also analyzed by Western Blot using rabbit anti-hANP IgG (four left rows on Figure 4) and rabbit anti-HSA (right rows in Fig. 4) to demonstrate the presence of an ANP on albumin. The SDS-PAGE separation of the proteins was performed under non-reducing conditions.

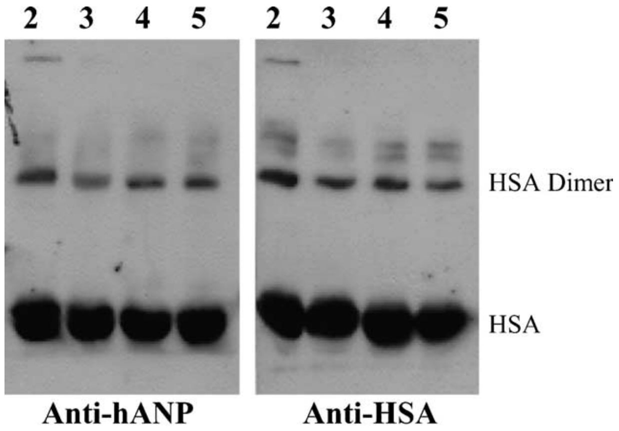


Figure 4. Western Blots of HSA conjugates.

ANP acts upon a membrane bound guanylate cyclase-coupled receptor found in various tissues such as kidney, vascular smooth muscle, adrenal and brain.[15] For the purpose of this study, HSA conjugates of compounds **2**–**5** were tested for their biological responses in a whole cell cGMP production assay on NPR-A expressing rat (Sprague–Dawley) lung fibroblasts[16] as well as in a NPR-A receptor binding assay using guinea pig adrenal glands.[17] The results are displayed in Table 3 and show that the attachment of the ANP molecule at either the N- or C-terminus did not reduce the overall binding to NPR-A by more than 5–6-fold as shown in entries 3 and 4. **2**-HSA conjugate stimulated guanylyl cyclase activity with an EC_{50} of 56.5 nM (entry 3) while that of the native peptide was 22.5 nM (entry 1) in our assay, representing less than a 2.5-fold loss of activity. Conversely, after attachment of the linker to positions 12 and 17 within the cyclic structure of ANP, the binding affinity of the conjugate to the receptor was greatly reduced and consequently, all the guanylyl cyclase

Table 3. In vitro activity results of the HSA conjugates[a,b]

Entry	Compd	cGMP production (rat lung fibroblasts)	NPR-A binding (guinea pig adrenal glands)	
		EC_{50} (nM)	IC_{50} (nM)	KI (nM)
1	**1**	22.5	0.64	0.58
2	HSA	$>10^4$	$>10^4$	$>10^4$
3	**2**-HSA	56.5	2.7	2.4
4	**3**-HSA	297	3.7	3.3
5	**4**-HSA	$>10^4$	5473	4911
6	**5**-HSA	$>10^4$	651	584

[a] Concentration ranges where 10^{-11} to 10^{-5} M.
[b] Analyses were done in duplicate and repeated thrice.

activity was lost (entries 5 and 6). Non-conjugated HSA (entry 2) did not show any intrinsic effect. The results demonstrate that if one chooses the right position of attachment of a peptide to the cysteine-34 on HSA, minimal loss of biological response can be achieved.

The stability of the HSA conjugates for compounds **2** and **3** in human plasma incubated at 37 °C was monitored using LC/MS. Whereby the relative abundance of the mass corresponding to intact conjugate **2**-HSA or **3**-HSA (final concentration 250 μM) was monitored for up to 48 h. Capped albumin was used as internal standard. The graph shown in Figure 5 is a plot of the percentage of conjugate signal (corrected) remaining as a function of time using the signal at time zero as 100%. **1** was completely degraded after 24 h. The major sites of degradation of the peptide have been identified as the bonds on the carboxyl side of arginine-4 and arginine-27 in this particular assay. Alternatively, **2** and **3**-HSA conjugates were protected from degradation where 72 and 78%, respectively, remained after 48 h. No degradation product arising from cleavage next to an arginine residue or other sites was detected in these experiments.

NEP is a membrane bound metallo-protease found primarily in the kidney and is one of the most important enzymes responsible for the in vivo metabolism of ANP.[18] Results from the degradation of **1** with added HSA and **2**-HSA conjugate incubated at 37 °C in the presence of neutral endopeptidase (pig)[18g] at pH 7.2 are shown in Figure 6. The cleavage site of **1** has been identified to be between cysteine-7 and phenylalanine-8[1b,18b] but the product of degradation of **2**-HSA conjugate by NEP could not be identified. Since the mass of a degradant corresponding to a single hydrolysis within the loop was outside the limit of precision of LC/MS, analysis of **2**-HSA was done by radioimmunoassay (RIA) using the anti-ANP IgG described earlier. The results revealed that **1** had a half-life of less than 4 h while the **2**-HSA conjugate half-life was closer to 15 h, thus demonstrating the protection of the ANP portion from degradation by NEP. The capped thiol albumin MS signal remained constant throughout the assay indicating that the mechanism of degradation only affected the conjugate.

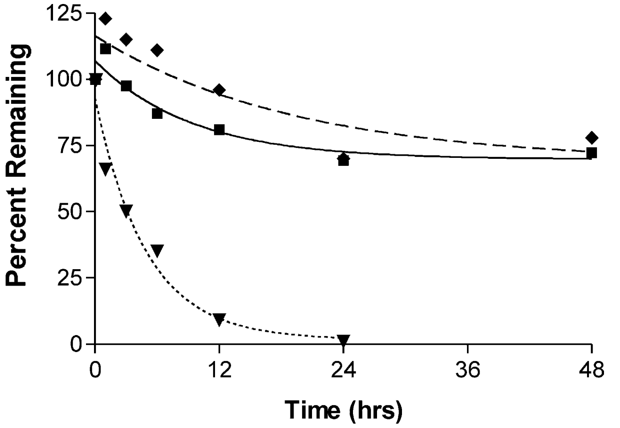


Figure 5. **2**- and **3**-HSA conjugates in the presence of human plasma: ▼: **1**, ■: **2**-HSA conjugate, ◆: **3**-HSA conjugate.

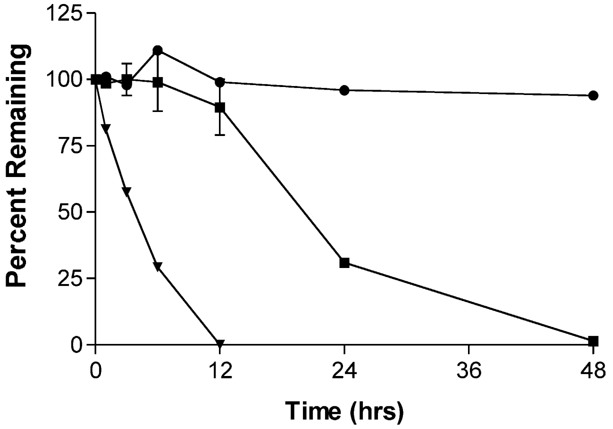


Figure 6. Stability of **2**-HSA conjugate in the presence of NEP: ▼: **1** + HSA (LC/MS analysis); ■: **2**-HSA conjugate (RIA analysis); ●: Capped thiol HSA (LC/MS analysis).

The results from this study demonstrate that the new larger protein structure obtained from the conjugation of ANP to human serum albumin was able to protect the native peptide from degradation by plasma enzymes and neutral endopeptidase. The method described in this communication also demonstrates that attachment at the optimum site of the ANP peptide to albumin led to the discovery of two compounds with respectable biological activity. This implies that the pharmacophore is still available to trigger the desired biological response even though the peptide is irreversibly attached to a large molecule. Studies involving the separation of an ANP–HSA conjugate from the rest of the albumin components and demonstrating in vivo efficacy are presently being explored.

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14. The conjugations were done by adding the test compounds **2**–**5** to commercial albumin (obtained from Cortex BioChem, San Leandro, CA, USA, mixture of mercaptalbumin and capped thiol albumin 60:40 by LC/MS) at pH 7.0 and incubating at 37 °C for 4 h. The conjugates were then purified by size exclusion chromatography using Superdex 200 10/30 (Amersham Pharmacia Biotech) using a 0–2 M sodium chloride gradient over 2 h, subsequently desalted by centrifuge through molecular cut-off filters (30 kDa) and lyophilized to a dry powder. The final protein product is a mixture of capped thiol albumin (M_r 66,650, albumin + cysteine), mercaptalbumin (M_r 66,450) and conjugate (M_r 66,450 + mw of ANP derivative) in a relative proportion of 40:40:20 based on LC/MS (Electrospray) deconvolution of each species and analyses of signal area. Analysis was done on a ZorbaxSBC3 column (Agilent) with 34% acetonitrile (0.1% TFA) from 0 to 5 min followed by gradients of 34–50% acetonitrile (0.1% TFA) over 5 min and 50–95% acetonitrile (0.1% TFA) over 5 min in water (0.1% TFA). The albumin peak (with conjugate) has a retention time of 12 min using this method.
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