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Albumin Affinity Tags Increase Peptide Half-Life In Vivo

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Abstract—Small organic molecules that bind tightly to serum albumin were applied to the amino terminus of an anticoagulant peptide in an effort to increase its protein binding in vivo. The tagged peptides were evaluated for their ability to be retained on liquid chromatographic columns with serum albumins incorporated into the stationary phase. Those which demonstrated significant affinity were administered intravenously to rabbits and found to have significantly increased plasma half-lives. Novel affinity tags were identified by appending a focused library of compounds to a model tetrapeptide and evaluating the resulting compounds' ability to bind to the serum albumin columns. The most promising were synthesized as the full length peptides and again evaluated in vivo. They were found to have still longer half-lives than the first generation compounds.

Phage display technology provides a rapid method by which high affinity peptide ligands can be developed for a variety of protein targets.[1] A number of peptides developed using this approach have been demonstrated to have potent biological activity[2,3] and could potentially serve as therapeutics. Significant obstacles remain, principally those of administration, as the traditional oral route is problematic,[4] and the half-life of peptides is short once in circulation. Gonadotropin releasing hormone (GnRH) analogues and insulin, two well-studied peptide therapies, give some indication of the varying fates of peptides once in circulation.

The GnRH analogues nafarelin, leuprolide, and goserelin are a series of decapeptides[5] approved for treating sex hormone dependent illnesses. Leuprolide has a half-life of 2.9 h in man,[6] typical for the GnRH analogues, and appears to be principally removed from circulation through proteolysis and glomerular filtration. In order to maintain serum levels in patients, leuprolide and goserelin are typically administered continuously through use of a depot injection or an implanted osmotic pump. Insulin has a half-life in man of 5 to 6 min once in circulation due to its degradation following receptor binding, and is therefore administered subcutaneously in an insoluble suspension to prolong its effects through slow dissolution.[7] Recently, an effort has been made to increase the half-life of an insulin analogue through conjugation to fatty acids, which confers weak affinity for serum albumin and therefore slows its absorption from the subcutaneous injection site as well as extending its circulating half-life.[8–10] Insulin detemir, a myrisitoylated insulin analogue of this type, is being evaluated in human clinical trials.[11]

Having previously developed a series of peptides with potent affinity for coagulation factor VIIa (fVIIa) and which act as specific inhibitors of the tissue factor-dependent coagulation pathway,[3] we began to explore the in vivo stability of these compounds. **1a** is a higher affinity analogue of the previously described E-76 peptide and was found to be stable for 24 h at 37 °C in citrated rabbit serum. An initial experiment indicated a half-life in rabbits of less than 10 min following intravenous administration. We therefore decided to examine the extent to which increasing the serum protein binding of these peptides could prolong their half-life.

Our initial efforts to synthesize amino terminally myrisitoylated analogues of **1a** similar to insulin detemir were hampered by the insolubility of the final product,

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and so **1b** and **1c** were targeted based on the tags' similarity to other known albumin binders.[12] Their synthesis was accomplished using Rink amide resin[13] and standard Fmoc chemistry, culminating in the coupling of the appropriate acid to the amino terminus of the peptide. Following cleavage from the resin, each peptide was oxidized with I_2 in acetic acid to form the intramolecular disulfide bond and purified using reverse-phase, high-performance liquid chromatography (RP-HPLC) and its mass verified through electrospray mass spectrometry (Fig. 1).

Peptides **1a**–**c** were examined in a fVIIa binding assay, in which they competed with an amino terminally biotinylated E-76 peptide to bind immobilized fVIIa. We thereby demonstrated that the modified peptides retained their ability to bind their target (Table 1). In addition, peptides **1c** and **1m** showed comparable affinities for fVIIa in the presence of 1% human serum albumin, indicating that they continue to bind their target despite the high concentration of albumin (data not shown).

Standard rabbit serum protein binding measurements showed **1b** and **1c** to be >99% bound, so their ability to bind serum albumins was evaluated using affinity chromatography in which rabbit or human serum albumin was immobilized on a sepharose gel column, the compounds were injected and their retention times determined.[14] The mobile phase consisted of 20 mM phosphate buffer at pH 7.2, 150 mM NaCl, 2 mM β-cyclodextrin[15] and 0.0015% ProClin 300 as a preservative. 20 μL of a 0.1 mM solution of each compound was injected, and the UV absorbance at 295 nm was monitored to determine the retention time. The significantly increased retention of compounds **1b** and **1c** (Table 1) relative to **1a** prompted the further investigation of these compounds in vivo.

Peptides **1a**–**c** were dissolved in 0.9% buffered saline at 2.5 mM concentration, as determined by their UV absorbance at 276, 280, and 288 nm.[16,17] These concentrations were confirmed by amino acid analysis and the peptides were administered via an indwelling catheter in an ear vein at 5 mg/kg to male New Zealand White rabbits. 1.5 mL citrated blood samples were drawn prior to dosing, and then at 1, 2, 5, 10, 15, 20, 30, 40, 60, 120, 360, and 420 min after dosing. Plasma concentrations of each peptide were quantitated through fVIIa binding. Prior to assaying, proteins were precipitated through addition of two volumes of methanol followed by centrifugation. The resulting supernatant

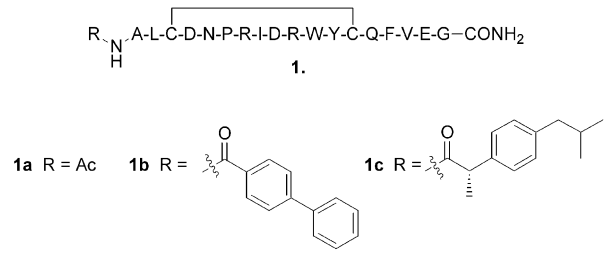


Figure 1. N-terminally acylated peptides **1a**–**c**.

Table 1. Full length peptide assay and albumin column binding data

Compd	IC_{50} (nM)	Rabbit SA $k'/(k'+1)$	Human SA $k'/(k'+1)$
1a	1.1	0.39	0.21
1b	4.0	0.89	0.62
1c	14.6	0.87	0.76
1d	1.0	0.91	0.79
1e	20.4	0.95	0.92
1f	1.6	0.91	0.69
1g	13.4	0.92	0.93
1h	2.9	0.82	0.64
1i	1.8	0.83	0.59
1j	11.8	0.73	0.64
1k	10.0	0.95	0.82
1l	13.4	0.84	0.65
1m	1.0	0.98	0.97

Peptides were evaluated in a fVIIa binding assay and through binding to serum albumins immobilized on columns. $k' = t_r - t_0/t_0$, where t_r is the retention time of the compound and t_0 the retention time of DMSO.

was evaporated on a Speed Vac and reconstituted in assay buffer. The concentrations obtained were then used to compute pharmacokinetic parameters using non-compartmental methods (Table 2). As anticipated, **1b** and **1c**, which were highly protein bound, had clearance rates reduced 7.6- and 22-fold relative to **1a**. The reduction in clearance contributed to an increase in the half lives of these compounds as well, with **1b** and **1c** exhibiting 2.0- and 3.2-fold increases over that of **1a**.

Based upon these encouraging initial results, further albumin affinity tag candidates were sought employing a focused library approach. As it would have been costly and time consuming to prepare full length peptides for each, we decided to model the full length peptide based on its four N-terminal amino acids (ALCD). To eliminate the possibility of forming unwanted disulfide bonds during evaluation of the library, the cysteine needed to be replaced in the model peptide. Serine was considered as a sterically similar residue, and tyrosine and tryptophan were attractive alternatives as their UV absorbance made them easy to detect as they eluted from our albumin affinity columns. In order to assess the degree to which each sequence could mimic the full length peptide **1**, they were coupled to the same acyl groups that had been evaluated in our initial work (Table 3).

Following their synthesis and purification, the tagged peptides were injected onto a column functionalized with human serum albumin column as described above. Only the ALWD peptides were retained with $k'/(k'+1)$ values comparable to the full length peptides (Table 3). This sequence was therefore used in the construction of a library of 94 tagged tetrapeptides for evaluation using the albumin columns.

The four amino acid sequence ALWD was synthesized on Rink amide resin using standard Fmoc chemistry. This resin was then distributed evenly among the wells of a 48-well Robbins FlexChem block and further functionalized using one of five separate chemistries: acylation with acids activated by HATU, reductive amination using aldehydes, carbamate formation using chloroformates, sulfonamides from sulfonyl chlorides,

and ureas derived from the corresponding isocyanate. Each functionalized peptide was cleaved from the resin using TFA and purified by RP-HPLC, followed by characterization using electrospray mass spectrometry.

The tagged tetrapeptides were examined using both human and rabbit serum albumin columns (data not shown), and a subset were chosen for further examination based on having a $k'/(k'+1) > 0.75$ on one or both of the rabbit and human serum albumin columns (Fig. 2). In addition, only the highest affinity tag in a series of structurally related compounds was synthesized initially. The tags were appended to the full length peptide, giving compounds **1d–l**, and their retention on human and rabbit serum albumin columns was then evaluated (Table 1).

Although a number of compounds appeared to bind more tightly than **1c**, sulfonamides **1e** and **1k** were both well retained on the albumin columns and represented a novel linkage between the peptide and the lipophilic region presumed to be interacting with albumin. As albumin is known to bind tightly to anionic lipids, and mindful of the importance of having a free carboxy terminus near the modified region of the insulin derivatives,[8] it was proposed that the improved affinity of the sulfonamides might be due to their ability to present a partial negative charge adjacent to a lipophilic region. To test this hypothesis, the novel tag **5** (Scheme 1), was designed with an acyl sulfonamide adacent to the napthyl group found in **1e**. Acyl sulfonamides are significantly more acidic than the corresponding sulfonamide which ensures essentially complete deprotonation at physiological pH.

The acyl sulfonamide tag **5** was constructed by opening succinic anhydride with 2-napthalenesulfonamide in the presence of DIPEA. The resulting acid was coupled to peptide **1** on resin using HATU, and then cleaved with TFA. The resulting peptide was cyclized and purified as before to give **1m**. As with the previously prepared compounds, this compound was evaluated for its ability to bind to Factor VIIa and for its retention on human and rabbit serum albumin columns (Table 1). As anticipated, **1m** retained its binding activity and was retained significantly longer than previously tested compounds on both albumin columns.

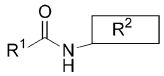
Having demonstrated that **1m** appeared to have significantly improved affinity for rabbit and human albumin, we decided to examine it in vivo, as before. The peptide was dissolved in 50 mM pH 7.4 phosphate buffer, and its concentration determined as before through

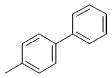
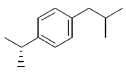
Table 2. PK data from initial experiments

	MRT (min)	Clearance (mL/min/kg)	$t_{1/2}$ (min)	Dose (mg/kg)	n
1a	3.2	33	7.6	5.0	2
1b	17	4.3	15	5.0	2
1c	33	1.5	24	4.9	2

Average pharmacokinetic parameters derived from rabbit studies using non-compartmental methods. MRT is the mean retention time, $t_{1/2}$ is the time during which blood concentration level is halved, and n indicates the number of animals whose data was averaged in the model.

Table 3. $k'/(k'+1)$ values determined for compounds **1a–c** and for model compounds **2–4** on a human serum albumin affinity column



R^1	R^2	1.	2.	3.	4.
CH_3		0.21	0.09	0.11	0.15
		0.62	0.23	0.37	0.82
		0.76	0.22	0.34	0.89

1. ALCDNPRIDRWYCQFVEG-$CONH_2$.
2. ALSD-$CONH_2$.
3. ALYD-$CONH_2$.
4. ALWD-$CONH_2$.

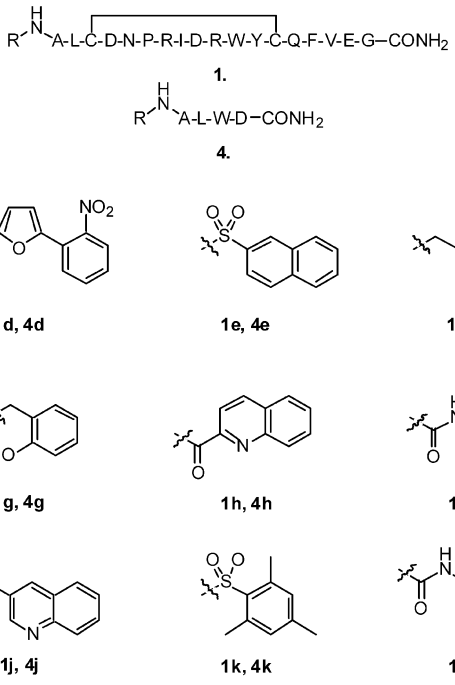
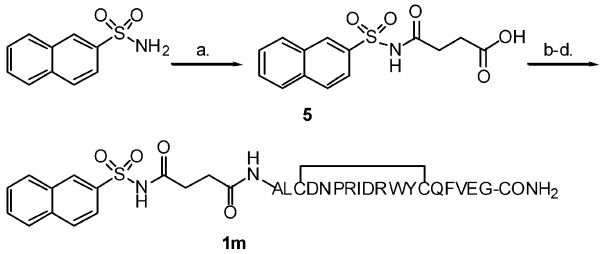


Figure 2. Initial hits among albumin affinity tags in library.



Scheme 1. Synthesis of napthalene acyl sulfonamide peptide **1m**. (a) Succinic anhydride, DIPEA, CH_2Cl_2, 72%; (b) peptide **1** on resin, DIPEA, HATU, DMA; (c) 2.5% TIPSH, 2.5% H_2O in TFA; (d) I_2/HOAc.

Table 4.

	MRT (min)	Clearance (mL/min/kg)	$t_{1/2}$ (min)	Dose (mg/kg)	*n*
1c	19	1.8	17	2.0	2
1m	35	1.6	30	2.0	2

Average pharmacokinetic parameters derived from administration of **1c** and **1m** IV to rabbits. Data analysis was performed as before.

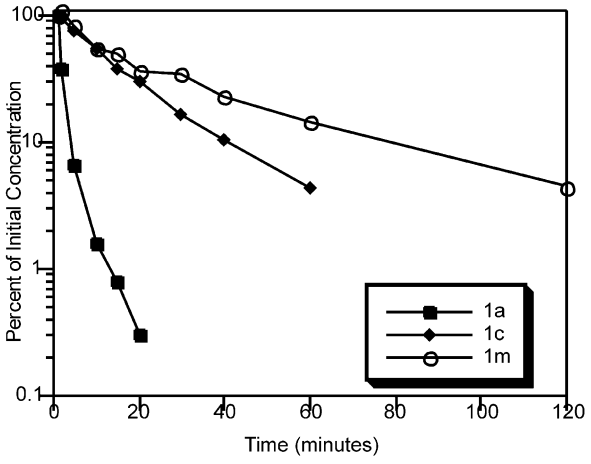


Figure 3. Plot of mean peptide concentration in plasma samples obtained from rabbits as a percentage of initial concentration.

its UV absorbance. Using the previously developed protocol, both **1c** and **1m** were administered to NZW rabbits at 2 mg/kg. Blood samples were drawn and their peptide concentrations determined as before.

A non-compartmental analysis revealed that the tagged peptides performed as designed, with the clearance of **1m** dropping from 1.8 to 1.6 mL/min/kg. A somewhat larger increase in half-life was observed although variation in the administered dose affected these results significantly (Table 4). Whereas peptide **1c** had previously had a 24 min half-life when administered at 5 mg/kg, it was shortened to 17 min when administered at 2 mg/kg. The 30 min half-life of **1m** dosed at 2 mg/kg represents a 3.9-fold improvement in the half-life of the peptide relative to **1a** dosed at 5 mg/kg, without considering the possibility of dose dependence (Fig. 3).

From these results, it is clear that the clearance of a peptide in circulation can be significantly reduced and its half-life thereby increased through attachment of a small molecule tag optimized to bind to serum albumin. The tags that have been successful to this point have an aromatic lipophilic region in common, and the most potent binder places an anionic center adjacent to the lipophilic region, similar to many of the endogenous ligands for serum albumin. In addition, the development of a four amino acid peptide model system for the factor VIIa binding peptide in conjunction with the albumin affinity columns has enabled the rapid synthesis and screening of albumin binding tags to speed the discovery of novel classes of these molecules. It is hoped that further increases in the peptides' affinity for serum albumin will lead to half lives of several h, which would make these compounds more attractive as therapeutics.

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