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Phosphate Ester Serum Albumin Affinity Tags Greatly Improve Peptide Half-Life In Vivo

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Abstract—A series of phosphate ester based small molecules designed to 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 exhibited high affinity for both rabbit and human serum albumin when passed through liquid chromatographic columns with serum albumins incorporated into the stationary phase. The peptides were then administered intravenously to rabbits and found to have a greater than 50-fold increase in plasma half life. The highest affinity peptides showed a reduction in bioactivity consistent with their sequestration away from their protein target in the presence of 0.1% rabbit serum albumin.

Phage display offers a rapid and convenient technology for identifying and optimizing peptides which bind to a target protein.[1] A number of these peptides have been shown to have potent biological activity,[2] and could potentially serve as therapeutics. There are still significant obstacles to oral delivery of peptides, including their rapid proteolysis and poor permeability in the gut.[3] These issues have been addressed through novel delivery technologies, and thus close analogues of peptide hormones such as somatostatin and gonadotropin releasing hormone are used clinically by delivering them through an osmotic pump or other slow release formulation.[4] Even if the problems of peptide delivery can be solved, their rapid clearance once in circulation remains an unaddressed obstacle to their general therapeutic use.

Recently, we reported an increase to peptide half-life in vivo by attaching serum albumin binding tags to the amino terminus of an anticoagulant peptide **1a**.[5] Appending a napthalene acylsulfonamide tag to give peptide **1b** increased its half-life nearly four-fold relative to peptide **1a** (Fig. 1). From this initial success, it was clear that this approach had merit, but it was unclear whether the maximum effect had been achieved. We therefore set out to design tags with higher affinity for serum albumin to determine whether we could further reduce their clearance and increase their circulating half life.

In the course of our initial work, we noted that serum albumin binding has also been investigated in the development of magnetic resonance imaging (MRI) contrast agents. Contrast agents are employed during MRI procedures as a tool to enhance the magnetic susceptibility of water molecules in the body, and their effectiveness is increased through association with a macromolecule.[6] A rationally designed contrast agent, MS-325 (Fig. 1), has been developed which binds to human serum albumin (HSA).[7] This molecule is bifunctional, with the diethylenetriaminepentaacetate (DTPA) portion of the molecule serving to bind the gadolinium ion while the diphenylcyclohexanol phosphate ester moiety binds to HSA. We envisioned incorporating this and similar phosphate esters into albumin affinity tags to determine if they could further extend the in vivo half-life of peptide **1c**.

As we had done previously, we designed our tags to be coupled to the amino terminus of peptide **1** through an

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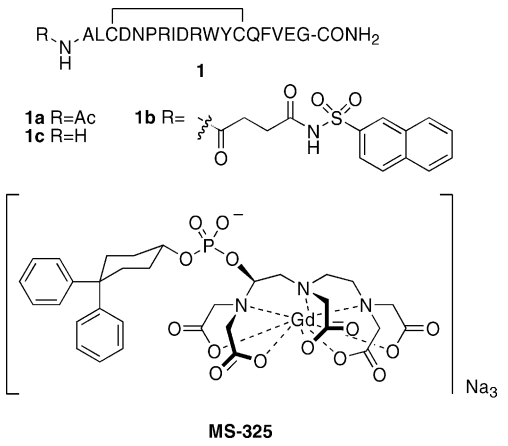
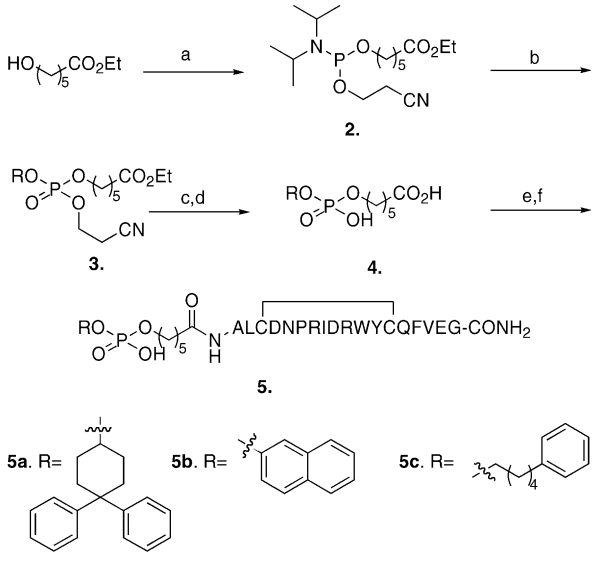


Figure 1. Previously described anticoagulant peptides **1a–c** and serum albumin binding MRI contrast reagent MS-325.

amide linkage. In this case, a six carbon linker was used to couple the peptide and the phosphate ester portions of the molecule (Scheme 1). Ethyl 6-hydroxyhexanoate was coupled with 2-cyanoethyl *N*,*N*-diisopropyl-chlorophosphoramidite,[8] giving phosphoramidite **2**. Three different alcohols, selected for their demonstrated affinity for albumin as well as similarity to the previously prepared **1b** were then coupled and oxidized to give phosphodiesters **3a–c**. Subsequently, the cyanoethyl protecting group was removed using ammonia in methanol and the esters cleaved to the free acids **4a–c**. Each acid was then coupled to the amino terminus of peptide **1** on resin with PyBOP and DIPEA.[9] The peptides were cleaved from the resin using standard TFA conditions, then oxidized with I_2 in acetic acid to form the intramolecular disulfide bond. Purification using reverse phase high performance liquid chromatography (HPLC) gave compounds **5a–c** which each eluted as a single peak on an analytical HPLC column and exhibited the desired M^+ ion by electrospray mass spectrometry.



Scheme 1. (a) 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite, DIPEA, CH_2Cl_2; (b) ROH, 1-H tetrazole, *t*-butylhydroperoxide, CH_3CN; (c) 2M NH_3 in MeOH; (d) LiOH, THF, H_2O; (e) peptide **1c** unoxidized and with protected sidechains on resin, DIPEA, PyBOP, DMA, CH_2Cl_2; (f) 2.5% TIPSH, 2.5% H_2O in TFA; (g) I_2/HOAc.

Following their synthesis, peptides **5a–c** were examined in a coagulation factor VIIa (fVIIa) ELISA binding assay[5] to determine how the albumin affinity tags affected their ability to bind their protein target (Table 1). All the peptides examined bound with similar affinity to fVIIa in the presence of 1% ovalbumin or 0.1% HSA as a bulking agent. However, when the assay was done in the presence of 0.1% rabbit serum albumin (RSA), activity was reduced for peptides **1b**, **5a** and **5c**, indicating that RSA binding might be reducing the ability of the tagged peptides to bind fVIIa by sequestering the bound peptide from fVIIa. This effect is commonly observed in small molecules which bind to serum proteins, and indicates that compound **5a** may bind more tightly to RSA than our previously synthesized compounds.

As had been done previously for compounds **1a** and **1b**, compounds **5a–c** were evaluated for their ability to bind to both HSA and RSA using an affinity chromatography assay (Table 1). HSA and RSA were immobilized on sepharose gel columns,[10] and 20 μL of a 0.1 mM solution of the compounds injected while the UV absorbance at 295 nm was monitored to determine the retention time of each. The mobile phase consisted of 20 mM phosphate buffer at pH 7.2, 150 mM NaCl, 2 mM β-cyclodextrin[11] and 0.0015% ProClin 300 as a preservative. Compounds **5a–c** were all strongly retained on the columns, as expressed in their $k'/(k'+1)$ values near the theoretical maximum of one. Conjugate **5a** binds less well to HSA than **5b** and **5c** which were similar to **1b**. Compounds **5a–c** were indistinguishable from **1b** on the RSA column, indicating that the sensitivity limit of this approach may have been reached.

Peptides **5a–c** were subsequently evaluated along with the previously described peptides **1a** and **1b** in vivo. According to the previously described procedure,[5] the peptides were administered at 2 mg/kg intravenously to male New Zealand White rabbits. Citrated blood samples were drawn prior to dosing and then at regular intervals thereafter. Following sample workup, the plasma concentration of each peptide was evaluated by comparing the sample activity in the fVIIa binding assay to that of known standards, which were handled identically. In assaying both the samples and the standard, 1% bovine serum albumin was used as the bulking agent, which did not significantly affect the IC_{50} values of the peptides (data not shown). The concentrations obtained were used to compute pharmacokinetic parameters using a non-compartmental model (Table 2). The newly designed peptides had sharply reduced clearance rates and significantly increased half-lives in vivo (Fig. 2). The longest acting peptide, **5a**, had a half-life of 222 min, 7.4-fold longer than **1b**, the longest acting peptide in our previous work and 53.1-fold longer than the unmodified peptide **1a**. Peptides **5b** and **5c** showed improvements of 8.3-fold and 18.9-fold respectively, over **1a**.

From these results, it is clear that optimizing the albumin binding properties of our affinity tags has resulted in a dramatically reduced clearance rate and a com-

Table 1. Affinities of tagged peptides for fVIIa in the presence of different bulking proteins, and albumin column binding data

Compd	IC_{50}, (nM) 1% ovalbumin	IC_{50}, (nM) 0.1% HSA	HSA $k'/(k'+1)$	IC_{50}, (nM) 0.1% RSA	RSA $k'/(k'+1)$
1a	9.2	11	0.21	9.7	0.39
1b	3.6	2.5	0.97	24	0.98
5a	4.0	3.4	0.78	90	0.97
5b	1.9	2.0	0.92	9.2	0.97
5c	2.0	2.4	0.92	22	0.98

$k' = (t_r - t_0)/t_0$, with t_r being the retention time of the compound and t_0 the retention time of DMSO.

Table 2. PK parameters calculated from the concentrations of peptide over time in rabbit blood samples

Compd	Clearance (mL/min/kg)	$t_{1/2}$ (min)	Dose (mg/kg)	*n*
1a	26.8	4.18	1.11	1
1b	1.64	30	2.0	2
5a	0.185	222	2.0	2
5b	0.707	34.8	2.0	2
5c	0.505	79.1	2.0	2

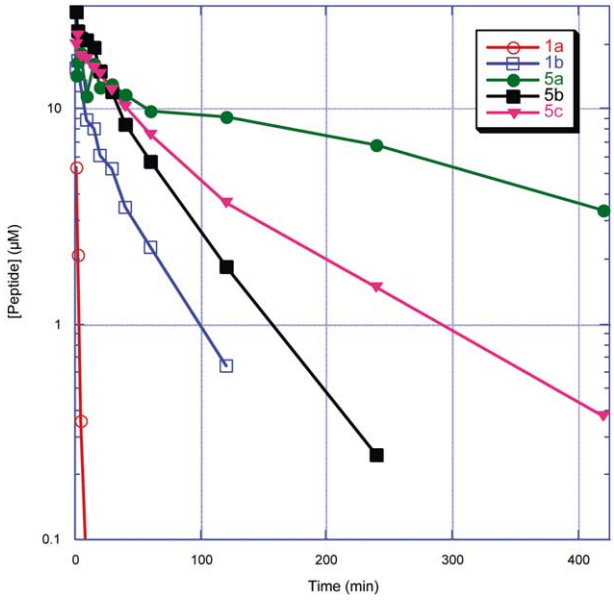


Figure 2. Average concentration of peptides **1a**, **1b** and **5a–c** in rabbit plasma samples.

mensurately longer half life. Conjugate **5a** is especially effective in this regard with a half life in circulation of nearly 4 h. However, as the affinity of the tags for serum albumin has increased, a reduction in their ability to bind fVIIa in the presence of RSA has also been observed. This indicates that the peptide is less active or inactive when bound to RSA, despite the inclusion of a flexible six carbon linker between the albumin binding phosphate ester and the bioactive peptide.

Despite this potential drawback, the general approach of reducing the clearance rate of peptides in vivo by appending tags to them which confer affinity for serum albumin has been further validated through these experiments. Conjugate **5a** exhibits a greater than 50-fold increase in half life over the unmodified peptide **1a**, and its 222 min half life allows for animal studies with a managable dosing schedule as well as a reduction in both the amount of peptide required and the variation in serum peptide levels between doses. Additional experimentation with both tether composition and length is underway and may allow us to access these improvements in reducing clearance while maintaining the potent bioactivity of the peptides.

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