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Monovalent and Bivalent Fibrin-specific MRI Contrast Agents for Detection of Thrombus

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Thromboembolic diseases such as stroke, heart attack, and pulmonary embolism contribute significantly to the cost of healthcare in the developed world. Many imaging techniques (ultrasound, magnetic resonance imaging (MRI), gamma scintigraphy, computed tomography) are used to identify thrombus, and the preferred technique often depends on the anatomical region. However these methods typically cannot distinguish thrombus from other pathologies (e.g. atherosclerotic plaque, tumor). It would be useful to have a single, three-dimensional imaging modality that can provide a specific and sensitive diagnosis of thromboembolic disease over the whole body.

Fibrin is a useful MR target because it is the major protein constituent of arterial and venous clots and is present at 10–100 μM in the thrombus, a concentration range compatible with detection by gadolinium-based contrast agents.[1,2] Fibrin-specific antibodies linked to gadolinium-containing nanoparticles have been successfully used to detect thrombi using MRI in animal models.[3,4] EP-2104R, a fibrin-specific peptide derivatized with four Gd chelates, has shown MR imaging efficacy in a variety of animal thrombus models.[5–9] Herein we report a new peptide sequence with specificity for fibrin and a comparison of the fibrin-binding and relaxivity properties of monovalent and bivalent fibrin-specific multimeric MRI contrast agents. In vivo data are presented that demonstrate localization of the bivalent agent in a model of venous thrombosis.

Previous work utilized a cyclic six amino acid peptide[8] for fibrin targeting. The peptide lead that ultimately resulted in EP-2104R was identified by phage display. The phage-display study also identified an 11 amino acid peptide with a seven amino acid cyclic core (CDYYGTC) that had affinity for fibrin. To convert this peptide lead (CDYYGTC) into a fibrin-specific MR contrast agent, we initially derivatized the peptide with a GdDTPA moiety (DTPA = diethylenetriaminepentaacetic acid) at the N-terminus by an amide bond linkage to give EP-782. A control was prepared, denoted EP-821, in which the amino acid sequence (Table 1) was scrambled to eliminate fibrin binding. EP-782 binds with low micromolar affinity to two sites on fibrin, while the scrambled peptide isomer showed no detectable fibrin binding (Table 1). The relaxivity ($r_1 = \Delta(1/T_1)/[\mathrm{Gd}]$) of each compound was determined in Tris (50 mM) buffered saline (TBS), in TBS plus 30 μM human fibrinogen (fgn), or in TBS plus 30 μM fgn that had been converted into fibrin (Table 1). There was no significant difference in EP-782 relaxivity when measured in TBS or TBS + fgn suggesting weak/no interaction with fgn. But the relaxivity in fibrin was > 40% higher consistent with fibrin binding and the receptor induced magnetization enhancement (RIME) effect.[1,10,11] EP-821 showed the same relaxivity, within error, in all three media. The lack of relaxivity enhancement of EP-821 in fibrin demonstrates that the enhancement observed with EP-782 is due to fibrin binding and not a viscosity effect.

Table 1: Compounds with peptide sequence, their relaxivities (r_1) in Tris buffered saline, 30 μM fibrinogen, or 30 μM fibrin, and their stepwise association constants (K_{a1}, K_{a2}).[a]

Compound and peptide sequence	r_1 per Gd [$mM^{-1}s^{-1}$][b]			Affinity [$\times 10^{-5}$ M^{-1}]	
	TBS	fgn	Fibrin	K_{a1}	K_{a2}
EP-782: GdDTPA-G·L·P·C·D·Y·Y·G·T·C·L·D	10.1	10.5	14.9	2.6 ± 0.1	0.46 ± 0.06
EP-821: GdDTPA-G·Y·L·C·G·D·Y·T·L·C·P·D	14.6	13.3	13.0	< 0.01	< 0.01
EP-1084: $(GdDTPA)_4$-(L·P·C·D·Y·Y·G·T·C·Bip·d)$_2$	20.6	ND	27.7	10.3 ± 2.1	2.1 ± 0.3
EP-1086: $(GdDTPA)_4$-L·P·C·D·Y·Y·G·T·C·Bip·d	16.9	ND	26.0	1.7 ± 0.3	0.40 ± 0.07

[a] Amino acids given by one-letter abbreviation. Bip = L-biphenylalanine; d = D-aspartate. Error in r_1 estimated at ±10%. ND = not detectable. [b] 37 °C, 20 MHz; ND = not determined.

Prior work[5–9] indicated that multiple Gd moieties were required for robust MRI signal enhancement of thrombus in vivo. We developed a bifunctional tetrameric GdDTPA-based scaffold, **1**, based on triethylenetetraamine (trien). The trien scaffold is easily differentiated using the Dde protecting group to selectively protect the primary amines (see Supporting Information). Two DTPA moieties were attached to each secondary nitrogen by a short diaminopropionate linker to minimize rotational flexibility and enhance relaxivity, while the primary amines were used to introduce oxime groups for further peptide conjugation. By controlling the stoichiometry

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of peptide conjugation, this scaffold afforded the possibility to test the effect of bivalent versus monovalent fibrin targeting. The EP-782 result indicated that the peptide could be safely modified at the N-terminus without affinity loss. Concurrently, we performed structure–activity studies on the peptide to improve fibrin affinity. The full structure–activity results will be reported separately, but one positive finding was a Leu to biphenylalanine (Bip) substitution to improve fibrin affinity. The C-terminus L-Asp (D) could also be converted into D-Asp (d) without loss of affinity. Nonnatural amino acids can often impart metabolic stability, and by substituting the peptide at the C-terminus with D-Asp and the N-terminus with gadolinium chelates it was anticipated that the resultant complex would be stable in vivo. Chemoselective ligation was used to introduce one or two equivalents of peptide to the DTPA tetramer giving EP-1086 and EP-1084 (Scheme 1). A Ser residue was incorporated at the N-terminus of the peptide and mild periodate oxidation gave an N-terminal α-keto aldehyde, **2**, that reacts selectively with the oxime-containing DTPA-tetramer scaffold at pH 4.6 in water. Complexation with $GdCl_3$ gave the desired product.

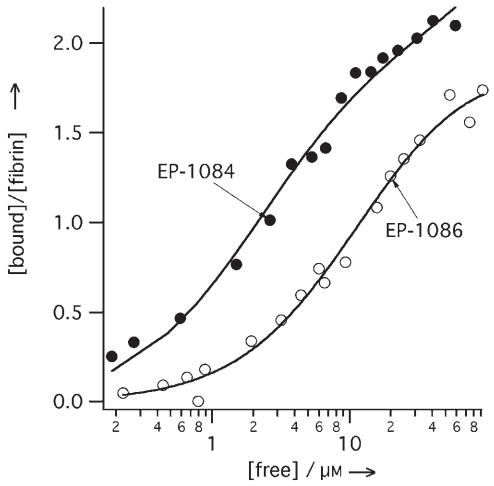
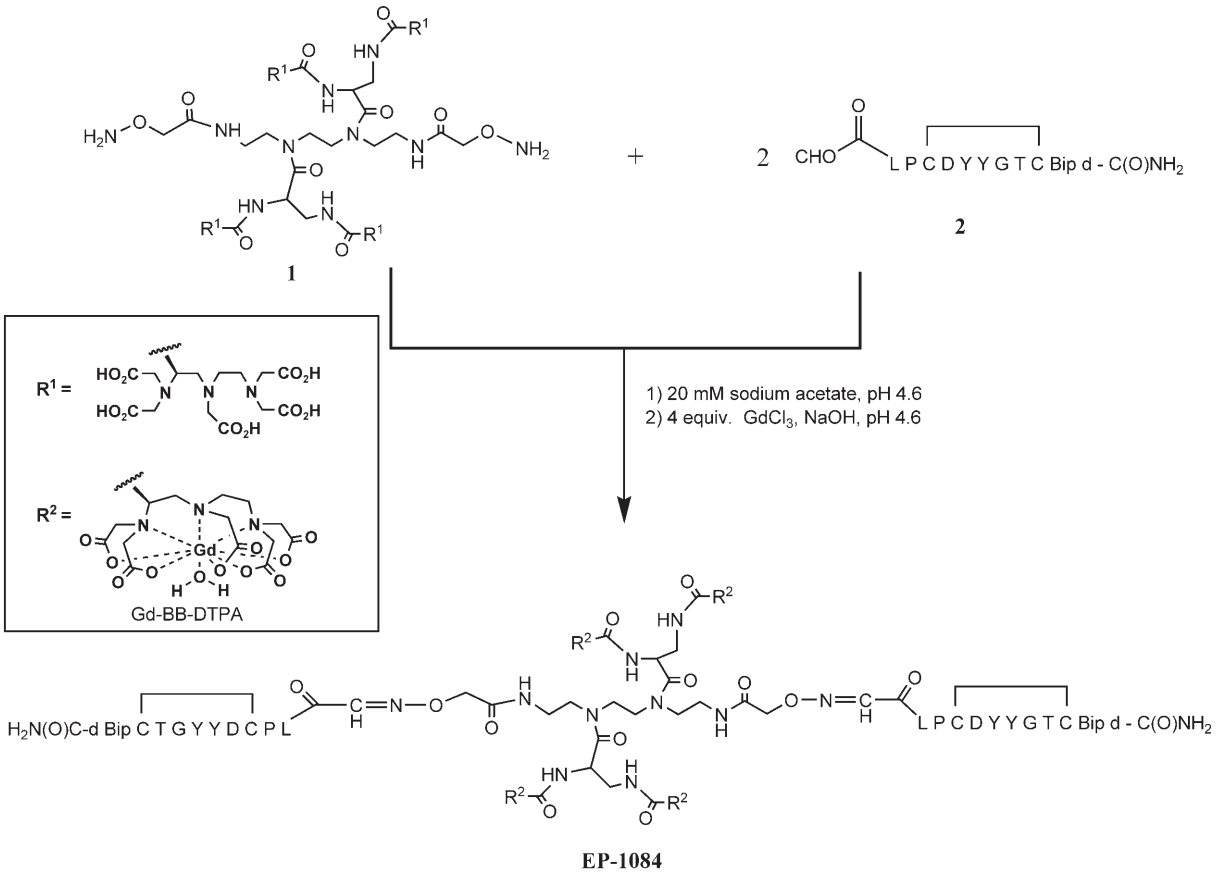


Figure 1. Binding isotherms for EP-1084 (●) and EP-1086 (○) at pH 7.4, 37 °C, TBS.

Fibrin binding data of EP-1084 and EP-1086 shown in Figure 1 demonstrates that both compounds bind to two sites on fibrin and that fibrin affinity is higher for bivalent EP-1084. A stoichiometric binding model [Eq. (1)] with stepwise association constants K_{a1}, K_{a2} (reported in Table 1) were fitted to the data.

$$\frac{[\text{bound}]}{[\text{fibrin}]} = \frac{K_{a1}[\text{free}] + 2\,K_{a1}\,K_{a2}\,[\text{free}]^2 + \ldots + n\,K_{a1}\,K_{a2}\ldots K_{an}\,[\text{free}]^n}{1 + K_{a1}\,[\text{free}] + K_{a1}\,K_{a2}\,[\text{free}]^2 + \ldots + K_{a1}\,K_{a2}\ldots K_{an}\,[\text{free}]^n} \quad (1)$$

The binding curve for EP-1086 was well described by two association constants. For EP-1084, the binding curve did not saturate at [bound]/[fibrin] = 2 and a third, much weaker



EP-1084

Scheme 1.

association constant was required to fit the data, $K_{a3} = (6 \pm 1) \times 10^3\,\text{M}^{-1}$. This likely represents non-specific binding.

The stepwise thermodynamic binding constants K_{a1} and K_{a2} are related to site-specific binding constants, K'_{a1} and K'_{a2}, as $K_{a1} = 2K'_{a1}$ and $K_{a2} = K'_{a2}/2$, and association constants are related to dissociation constants as $K_d = 1/K_a$.[12] For EP-1086, $K'_{d1} = 11.2 \pm 1.6 \approx K'_{d2} = 12.6 \pm 2.4\,\mu\text{M}$ and for EP-1084, $K'_{d1} = 1.9 \pm 0.4 \approx K'_{d2} = 2.4 \pm 0.4\,\mu\text{M}$. The fact that K'_{d1} and K'_{d2} are approximately constant for a given compound strongly suggests that both binding sites are equivalent. This result is consistent with the dimeric subunit structure of this protein. The addition of the second peptide on EP-1084 improves the fibrin affinity five-fold relative to the monopeptide EP-1086.

Nuclear magnetic relaxation dispersion (NMRD) profiles of these compounds in the presence and absence of fibrin are shown in Figure 2. In buffer, r_1 is relatively high for both EP-1084 and EP-1086 because of the increased size of these

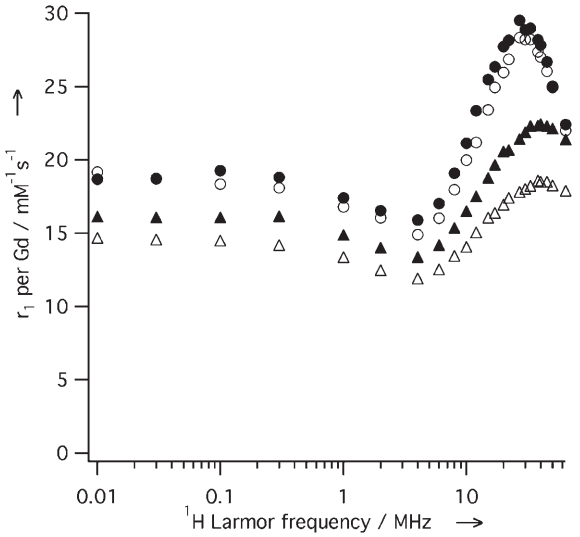


Figure 2. Relaxivity per Gd ion for 60 μM EP-1084 (solid symbols) and 33 μM EP-1086 (open symbols) in TBS (triangles) or in the presence of 30 μM fibrin (circles) at pH 7.4, 35 °C.

contrast agents. In the presence of fibrin, both compounds show increased relaxivity owing to the RIME effect, and the relaxivity maximum shifts to lower frequency, approximately 30 MHz, consistent with slower rotational dynamics. However, the NMRD curves in fibrin are very similar for both compounds suggesting similar rotational dynamics at the GdDTPA moiety. Previously we found that a multilocus binding approach could increase relaxivity in the protein-bound state by rigidifying the molecule upon protein binding.[13] The absence of such an effect here may suggest that only one peptide unit from EP-1084 is binding to fibrin.

The per Gd relaxivity of fibrin-bound EP-1084 is similar to the commercial albumin-binding agent gadofosveset (MS-325)[14,15] and about six-fold higher than extracellular contrast agents such as GdDTPA (24-fold higher per molecule)[16] at the usual MRI field of 1.5 T.

EP-1084 was investigated in a guinea pig venous thrombus model. Prior to thrombus formation ^{125}I-fibrinogen was administered to the animal. A thrombus was formed by isolating a section of the inferior vena cava and injecting thrombin. The clot was aged for 30 min, and then ^{111}In-labeled EP-1084 (2 μmol kg^{-1}) was given as a bolus injection in the jugular vein. Immediately after the EP-1084 was added, ^{99m}Tc-DTPA mixed with 2 μmol kg^{-1} GdDTPA was injected. After another 30 min, blood was drawn and the clot removed. The blood and clot samples were weighed and the amount of each radiolabeled species was determined. The three isotopes used emit at sufficiently different energies that it is possible to quantify each in the presence of the others.

The clot:blood ratio was 6.6 ± 0.4 for ^{125}I-fibrinogen. Guinea pig plasma fgn concentration is 9.7 μM and its hematocrit is 0.44.[17] Assuming conversion of fibrinogen into fibrin in the clot, the mean fibrin concentration in this model is $36 \pm 2\,\mu\text{M}$. EP-1084 showed a 4.4 ± 1.2 clot:blood ratio demonstrating positive uptake in the clot. To ensure that this positive result was not due to a trapping phenomenon, the extracellular tracer GdDTPA was co-administered and gave a clot:blood ratio of 0.64 ± 0.17; the less than unity ratio is consistent with distribution in the clot but no specific uptake. Figure 3 shows the concentration of EP-1084 and GdDTPA in the clot and in blood, highlighting the fact that while the blood levels for both compounds were very similar, there was seven-times more EP-1084 in the clot than GdDTPA.

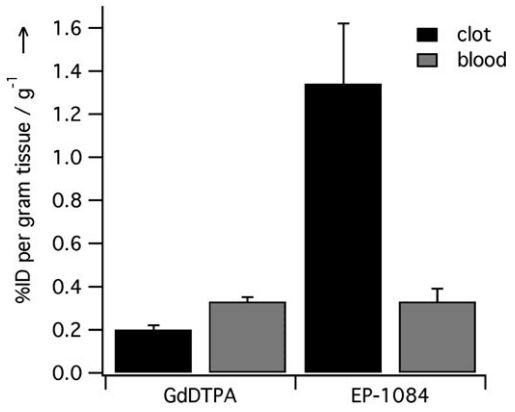


Figure 3. Distribution of GdDTPA and EP-1084 in a venous clot and in the blood pool, 30 min after injection of 2 μmol kg^{-1} of each probe.

The in vivo thrombus uptake and high relaxivity of EP-1084 are compatible with this compound being employed as a thrombus-specific imaging agent. A low dose of EP-1084 (2 μmol kg^{-1}, cf. 100–200 μmol kg^{-1} for most MRI agents) delivered $64 \pm 28\,\mu\text{M}$ Gd to the thrombus. At 1.5 T, $\Delta(1/T_1) = 1.5\ \text{s}^{-1}$ is calculated at the thrombus. The inherent thrombus T_1 depends on the clot age, but in many instances the thrombus $1/T_1$ is very close to that of blood,[18] approximately $0.75\ \text{s}^{-1}$. Under these conditions, a 200% increase is expected in relaxation rate at the thrombus and ready detection of clot.

In conclusion, we have described new fibrin-specific, high relaxivity contrast agents for thrombus MR imaging, and demonstrated thrombus localization in an in vivo model.

Thrombus uptake is consistent with bright spot thrombus MRI and future work will describe in vivo MRI with this class of compounds.

Experimental Section

Details of compound syntheses, fibrin binding and relaxivity assays, and biodistribution are given in the Supporting Information.

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