Contrast Agents

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Collagen-Targeted MRI Contrast Agent for Molecular Imaging of Fibrosis**

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Fibrosis is the formation or development of excess fibrous connective tissue (largely type I collagen) in a tissue as a result of a reparative or reactive process. Fibrosis, or scarring, is a common outcome in many chronic diseases of the heart, kidney, liver, lungs, or vasculature. Hepatic fibrosis is a result of chronic injury in response to insults such as viral hepatitis, alcohol or drug abuse, and increasingly from non-alcoholic steato hepatitis (NASH).[1] Fibrosis is a hallmark of end-stage renal disease.[2] Pulmonary fibrosis occurs in conditions such as chronic obstructive pulmonary disease.[3] Atherosclerosis involves vascular lesions with fibrous caps, and the thickness of this cap has been implicated in lesion (plaque) rupture resulting in thrombosis.[4] In the heart, hypertrophy from high blood pressure results in increased collagen levels.[5] Following heart attack, necrotic myocytes are replaced by extracellular matrix components, mainly collagen, to form a fibrotic myocardial scar.[6]

For all of these pathologies it would be useful to noninvasively detect, assess, and monitor fibrosis. Treatment decisions hinge on both the identity and severity of the disease. For instance NASH, which afflicts 1–2% of the U.S. population, progresses to cirrhosis of the liver in about 20% of cases. Early detection and accurate characterization of liver fibrosis can improve patient outcomes.[7] Currently liver fibrosis is detected by liver biopsy, but biopsy is not well-suited to screening/monitoring disease because of its cost, associated morbidity, and known lack of accuracy because of sampling variation.[8] In NASH and other pathologies, as new antifibrotic therapies become available, there is a need for a means of noninvasively monitoring fibrosis and the patient's response to therapy.[9,10]

In the case of myocardial infarction (heart attack), it is critical to know the extent of infarction and how much viable myocardium remains. Such information impacts prognosis and helps guide treatment decisions such as whether or not to perform surgery.[11] An improved, noninvasive assessment of myocardial viability would be valuable. After infarction, the heart undergoes remodeling wherein the necrotic myocytes in the infarct zone are replaced by extracellular matrix, a principal component of which is type I collagen. It was reasoned that a collagen-specific magnetic resonance imaging (MRI) contrast agent could act generally as a fibrosis-imaging agent, and specifically as a probe of myocardial infarct size. Collagen is present at relatively high concentrations (1–20 nmol g^{-1}, roughly μM) in many organs[13] and is entirely extracellular, facilitating access by the contrast agent. During fibrosis this concentration can increase tenfold or more.[6] These concentrations are well within the range for detection by MRI with simple gadolinium-based multimers.

MRI could be an ideal modality for detection and monitoring of fibrosis. MRI has much better resolution than γ-imaging techniques, provides good soft-tissue contrast, and, unlike optical imaging, can image deep tissues. Currently, MRI can detect fibrosis at very advanced stages owing to the gross morphological changes involved. However, early detection of fibrosis will likely require a specific contrast agent. A major challenge for molecular targeting with MRI in general is sensitivity.[12] Unless very large particles containing hundreds of gadolinium ions are used, gadolinium-based contrast agents typically require micromolar concentrations to be visualized. The high levels of collagen make it an attractive MRI target for detection. The ability of MRI to localize to a given region or organ overcomes the ubiquity of the target (compare nuclear imaging).

The contrast agent should be small enough such that it can rapidly extravasate from the blood vessels to the interstitial space. The compound must also be eliminated from the body after the diagnostic test; thus it is useful to have a compound that can readily be filtered by the kidneys. For these reasons, it was decided not to employ an antibody-targeting approach[14,15] or to use nanoparticles or polymers with large gadolinium payloads,[16] but rather to focus on relatively low (less than 10 kDa) molecular weight approaches.

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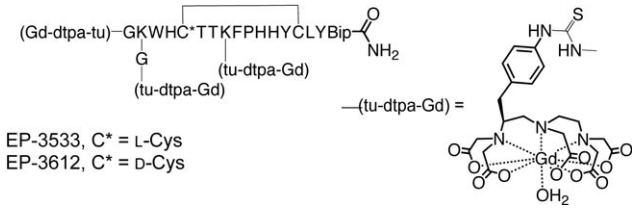
Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

A type I collagen-specific peptide was identified using phage display and subsequently modified to improve affinity for collagen. Conjugate EP-3533 (Scheme 1) consists of a peptide of 16 amino acids, with three amino acids flanking a cyclic peptide of 10 amino acids that is formed through a disulfide bond. Biphenylalanine (Bip) at the amidated C terminus was found to improve collagen binding. Three {Gd(dtpa)} moieties were conjugated to the peptide through thiourea linkages to improve the sensitivity of the contrast agent. Systematic introduction of Lys residues into the peptide demonstrated that the metal complexes were well-tolerated at the N terminus and at position 7 (from the N terminus). Two chelates were conjugated through a lysine residue branched with two glycine linkers at the N terminus. A third chelate was introduced at the lysine side chain within the cyclic peptide. The peptides were synthesized using conventional solid-phase techniques, and the gadolinium complexes (containing a reactive isothiocyanate moiety) were conjugated directly to the peptides in water/acetonitrile.



Scheme 1. Collagen-targeted contrast agents; L-amino acids are designated by one letter code, except where noted; Gd chelates are appended through the N terminus, through branched Lys-Gly residues at the N terminus, and through a Lys side chain within the cyclic portion of the peptide.

Conjugate EP-3533 exhibits nonsaturable binding to type I collagen (Figure 1). To estimate affinity, the data was fit to the simplest model possible, that of N equivalent binding sites, each with a dissociation constant K_d, given by Equation (1).

$$\frac{[\text{bound}]}{[\text{collagen}]} = \frac{N\,[\text{free}]}{K_d + [\text{free}]} \quad (1)$$

This model fit the data well and gave $N = 7.6 \pm 0.7$ equivalent binding sites with a dissociation constant $K_d = 1.8 \pm 1.0$ μM. To demonstrate the specificity of binding, the isomer EP-3612 (Scheme 1) was prepared. Isomer EP-3612 is identical to EP-3533 with the exception that the chirality of one cysteine residue is inverted (D-Cys). Figure 1 clearly demonstrates that the affinity of EP-3612 for collagen ($K_d =$ 400 μM, assuming the same N sites as EP-3533) is two orders of magnitude lower than that of EP-3533.

The relaxivity of EP-3533 and EP-3612 was measured at two field strengths in pH 7.4 phosphate-buffered saline (PBS) or in human plasma. The relaxivities are listed in Table 1 per Gd atom and per molecule. As expected, relaxivities at a given field/medium are similar for both isomers. There is likely some plasma protein binding of these compounds as the relaxivity at 0.47 T is significantly higher in plasma than in PBS. Near the common imaging field of 1.5 T (the 1.41-T data) the relaxivity of these agents is five times higher than [Gd(dtpa)] (magnevist) per Gd atom (15 times higher per molecule).[17] The high relaxivity is likely a result of the larger size of these contrast agents, which results in longer correlation times.[12]

Table 1: Relaxivity of EP-3533 and EP-3612 in PBS or human plasma at 37 °C. Uncertainty estimated at ±10%.

Solution	r_1 [mM^{-1}s^{-1}] at 0.47 T		r_1 [mM^{-1}s^{-1}] at 1.41 T		r_2 [mM^{-1}s^{-1}] at 1.41 T	
	Per Gd atom	Per molecule	Per Gd atom	Per molecule	Per Gd atom	Per molecule
EP-3533/PBS	18.7	56.1	16.1	48.3	25.9	77.7
EP-3533/plasma	27.9	83.7	15.6	46.8	32.5	97.5
EP-3612/PBS	16.7	50.1	15.5	46.5	21.4	64.2
EP-3612/plasma	26.4	79.2	19.3	57.9	33.9	101.7

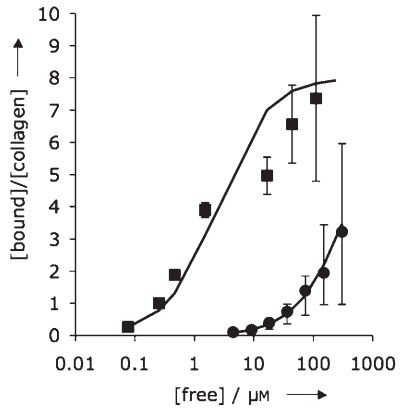


Figure 1. Binding of EP-3533 (squares) and EP-3612 (circles) to type I mouse collagen at 37 °C, demonstrating the specificity of the EP-3533 isomeric form for collagen.

The collagen-targeted contrast agent was evaluated in a mouse model. First, the biodistribution of both EP-3533 and EP-3612 was assessed in control animals at 15 min after injection, and the results are given in Table 2. Blood concentrations were similar as may be expected for the isomers. However, there were significantly higher levels of EP-3533 in the liver, kidney, spleen, heart, and lung. The percentage increase in these organs for EP-3533 relative to EP-3612 correlates reasonably well with the overall collagen content in these organs.[13,18]

After demonstrating positive uptake in the heart relative to its nonbinding control, EP-3533 was evaluated in a mouse model of aged myocardial infarction (heart attack). It is well-established that after infarction, the myocardium becomes remodeled, with collagens I and III and fibronectin replacing the necrotic myocytes. Collagen levels increase several-fold in the infarct zone.[6] In this model[19,20] infarction is induced by

Table 2: Biodistribution of EP-3533 and EP-3612 in mice ($N = 4$) 15 min after injection of 10 μmol kg^{-1}. Data expressed as nmol Gd per gram wet tissue (standard deviation).

Cmpd	Blood	Heart	Kidney	Liver	Spleen	Lung	Femur
EP-3533	14.3 (4.3)	25.5 (3.5)	223 (22)	50.4 (3.1)	77.3 (24)	29.1 (3.6)	1.5 (0.1)
EP-3612	13.3 (0.8)	14.8 (2.1)	93.0 (6.2)	16.8 (1.9)	19.5 (1.7)	20.5 (2.3)	1.5 (0.4)

temporary occlusion of the left anterior descending artery, and then the mouse is allowed to recover for 40 days, during which time the scar formation is complete. Figure 2 shows serial T_1-weighted images acquired prior to, immediately following, and 40 min after tail vein injection of 25 μmol kg^{-1} EP-3533 (A–D) and EP-3612 (E–H). The EP-3612 images were acquired in the same mouse two days after acquisition of the EP-3533 images. Figure 2 demonstrates enhancement of blood and myocardium early after injection using both agents (C, G), and late enhancement of the collagen-rich scar (arrow) by using the collagen-targeted agent (D) but not from using EP-3612 (H). The collagen-rich liver (bottom left) also remains enhanced at 40 min with EP-3533 but not with EP-3612, consistent with the data in Table 2.

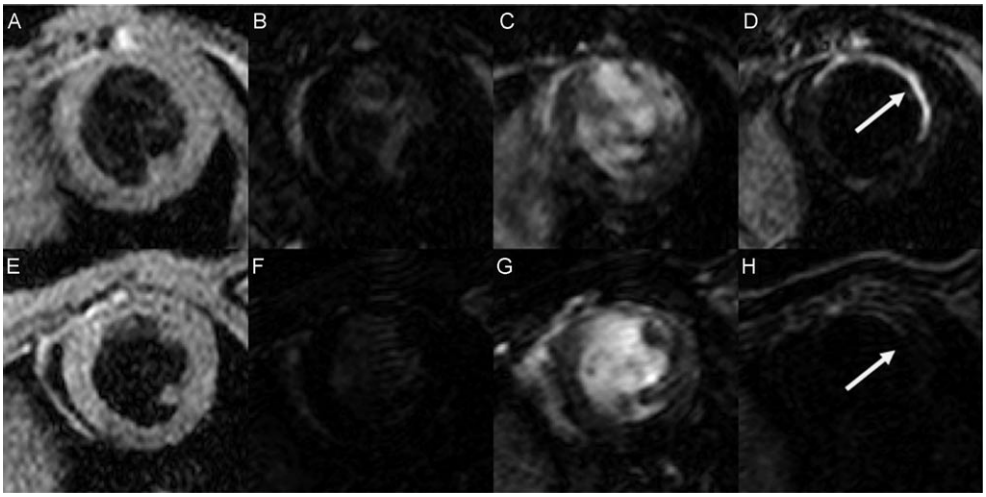


Figure 2. MR imaging of myocardial scar injected with EP-3533 (A–D) and EP-3612 (E–H, image acquired two days later in same mouse). First image (A, E) is a black blood T_2-weighted image acquired before injection of the contrast agent to delineate the anatomy. Images B–D (and F–H) are inversion recovery images taken prior to, 1 min after, and 40 min after injection of the contrast agent.

In summary, EP-3533 represents a new collagen-specific MRI contrast agent. Molecular imaging of collagen with EP-3533 provides specific, high contrast for fibrotic scar versus viable myocardium and blood in a mouse model of chronic infarction. Beyond myocardial scar, this agent may find application in detecting and evaluating a broad array of diseases involving fibrosis and extracellular matrix remodeling.

Experimental Section

Details of compound synthesis, collagen-binding assay, relaxivity, biodistribution, and imaging are given in the Supporting Information.

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