Molecular Magnetic Resonance Imaging of Lung Fibrogenesis with an Oxyamine-Based Probe

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Abstract: Fibrogenesis is the active production of extracellular matrix in response to tissue injury. In many chronic diseases persistent fibrogenesis results in the accumulation of scar tissue, which can lead to organ failure and death. However, no non-invasive technique exists to assess this key biological process. All tissue fibrogenesis results in the formation of allysine, which enables collagen cross-linking and leads to tissue stiffening and scar formation. We report herein a novel allysine-binding gadolinium chelate (GdOA), that can non-invasively detect and quantify the extent of fibrogenesis using magnetic resonance imaging (MRI). We demonstrate that GdOA signal enhancement correlates with the extent of the disease and is sensitive to a therapeutic response.

Almost half of the deaths in the industrialized world can be attributed to diseases with a fibroproliferative component. In the lung, pulmonary fibrosis constitutes a major cause of morbidity and mortality. Idiopathic pulmonary fibrosis (IPF), the most common and most lethal diffuse fibrosing lung disease, is responsible for an estimated 40,000 deaths per year in the US. Pulmonary fibrosis is characterized by the accumulation of myofibroblasts and their production of an excess of extracellular matrix (ECM) proteins. Though this ECM accumulation and remodeling, termed fibrogenesis, is an innate part of the natural wound healing process, under persistent injury excess ECM results in a build up of scar tissue causing the loss of tissue function and potential organ failure and mortality.

High-resolution computed tomography is invaluable for the diagnosis of IPF but cannot distinguish regions of active fibrogenesis from stable scar tissue. Molecular MRI, to quantify the extent of increased ECM components, by direct targeting of the collagen deposition that occurs during scar tissue formation, has previously been used to image fibrosis. While this provides a means to quantify the changes in total fibrotic burden of a patient, collagen imaging does not provide a measure of the dynamic changes occurring at the molecular level during fibrogenesis. Imaging fibrogenesis would identify patients whose disease is progressing and would also enable the monitoring of treatment with drugs that could stop or slow fibrotic progression.

A universal feature of fibrogenesis is the oxidation of ECM lysine residues, chiefly in collagen, to the aldehyde allysine by the lysyl-oxidase (LOX) family of enzymes. Allysine undergoes a series of condensation reactions with other amino acids on neighboring collagen molecules (Supplemental Information, Figure S1) to form irreversible cross-links that stabilize the ECM, and is a fundamental feature across all fibrotic diseases. While lysine oxidation is catalyzed by LOX, the subsequent condensation reactions of allysine are slower. We reasoned that allysine could be a target for imaging fibrogenesis. In active fibrogenesis, an increased pool of allysine would be generated, but in a stable disease state or with therapeutic intervention these allysine moieties would be converted to crosslinks.

To determine if allysine was sufficiently abundant for detection with MRI, we quantified allysine in mouse lung tissue using HPLC. Normal and fibrotic lung tissue was digested at 110°C in 6M HCl for 24 h, and the allysine derivatized with 2-naphthol-7-sulfonate to yield a fluorescent molecule that could be detected and quantified by HPLC. In normal mouse lung, there was 80 ± 6 nmol allysine per gram of lung tissue, approximately 80 μM. In mice injured with bleomycin, the allysine content was increased to 150 ± 16 nmol allysine per gram of lung tissue. Such high micromolar concentrations are readily detectable by Gd-enhanced MRI.

In the design of a probe for MR imaging of fibrogenesis, certain criteria are essential, including: 1) high thermodynamic and kinetic chelate stability, 2) high water solubility, 3) rapid renal excretion, 4) low non-target background uptake, 5) rapid penetration into the tissue interstitial space and 6) target selectivity. To satisfy these criteria, we developed GdOA, an oxyamine-functionalized derivative of GdDOTA for targeting allysine. The GdDOTA core provides a highly stable and inert Gd-chelate (Supporting Information, Figure S2). The amionic and hydrophilic nature of GdOA results in high solubility, reduces nonspecific protein binding, and promotes rapid renal elimination. For target selectivity, an oxyamine was selected, as the oxime formed from the

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Supporting information, including detailed experimental procedures for compound syntheses, relaxivity measurements, binding and stability study measurements, animal model and MR protocols, full methods for analytical assays, and full imaging and assay analysis, and the ORCID identification number(s) for the author(s) of this article can be found under: https://doi.org/10.1002/anie.201704773.
reaction of an aldehyde with an oxamine is known to be more stable to hydrolysis than the analogous hydrazone or imine[9] and is therefore expected to result in a strong MR signal enhancement on binding to allysine.

GdOA (Figure 1a and the Supporting Information, Scheme 1) was prepared by coupling the NHS active-ester of N-Boc-aminooxyacetic acid[10] with an amine-functionalized derivative of DOTAsodium (DOTA-NH$_2$), followed by Boc-deprotection in 1 M HCl, with subsequent Gd chelation at pH 6.8. GdOA purity was assessed by HPLC-ICP-MS analysis, with a single Gd species identified and confirmed as GdOA by HPLC-MS analysis. A six-carbon chain was used as a linker to minimize interaction between the GdDOTA core and oxamine. As a negative control, GdOX was synthesized, which has the same pharmacokinetic properties of GdOA but is incapable of undergoing a condensation reaction with allysine (Figure 1a and the Supporting Information, Scheme 2).

The relaxivity (1.4 T, 37°C) of GdOA was similar when measured in PBS solution (4.25 mm$^{-1}$s$^{-1}$) or in PBS with 3 mg of bovine serum albumin (BSA, 150 μM) indicating very low nonspecific protein binding. However, in the presence of 3 mg of BSA-Alld BSA that had been oxidized with FeCl$_3$/aspartate to generate 16 nmol of aldehydes per milligram of protein[11] relaxivity increased by 90% to 8.10 mm$^{-1}$s$^{-1}$ (Figure 1b); the protein-bound fraction of GdOA had a relaxivity of 16.87 mm$^{-1}$s$^{-1}$. GdOA showed a negligible increase in relaxivity in the presence of BSA or oxidized BSA-Alld Quantification of the GdOA probe bound to BSA-Alld following ultrafiltration and ICP-MS analysis gave a binding constant of 164 μM$^{-1}$ (Figure 1c). To assess inertness, GdOA was challenged with zinc and phosphate and showed no Gd release (Supporting Information, Figure S2).

Next, the binding of GdOA to tissue was assessed. The aorta is rich in allysine as a result of high lysyl-oxidase activity and turnover of elastin and collagen. We measured 7.50 μmol of allysine per gram of porcine aorta using the HPLC assay. We then incubated GdOA or GdOX with segments of aorta (25 mg of aorta tissue, 37°C, 24 h, pH 7). After repeated washing to remove non-specifically bound probe, the aorta-associated Gd was quantified by ICP-MS. GdOA gave a K$_d$ of 360 μM, while GdOX showed no affinity (Figure 1d).

The pharmacokinetics of GdOA and GdOX were assessed in naive mice using MR imaging to measure the blood MR signal wash-out from the left ventricle of the heart. Both probes displayed rapid and almost identical blood clearances with blood half-lives of 5.5 and 6.1 min for GdOA and GdOX respectively, indicating comparable probe pharmacokinetics (Supporting Information, Figure S3). Elimination was exclusively through the kidneys with minimal, transient liver enhancement observed. Biodistribution of GdOA at 1 h after bolus intravenous injection in naive C57Bl/6 mice showed that 95% of the injected dose had already been eliminated from the body (Supporting Information, Table S1).

The ability of GdOA to detect and stage pulmonary fibrosis was then evaluated using a bleomycin lung-injury mouse model. Bleomycin is a chemotherapeutic antibiotic[12] but a major adverse effect of bleomycin is the overproduction of reactive oxygen species in the lung,[13] which can lead to fibrosis.[14] Mice injured with bleomycin rapidly and reliably develop pulmonary fibrosis.[15] We studied four groups of C57Bl/6 mice: 1) mice injured with a single intratracheal administration of bleomycin (Bleo), 2) age-matched healthy mice (Naive), 3) mice injured with bleomycin and then dosed daily with the pan-LOX inhibitor BAPN, (Bleo + BAPN),[16] and 4) mice injured with bleomycin and then dosed daily with PBS as a vehicle control (Bleo + PBS). After 14 days, mice were imaged before and after intravenous injection of 100 μmol·kg$^{-1}$ GdOA (all four cohorts) or GdOX (first two cohorts), the same dose as used for most clinical GdDOTA-enhanced MRI exams.[17]

Bleomycin-injured mice demonstrated increased pulmonary fibrosis as measured by the Ashcroft system of histology scoring (Supporting Information, Figure S4). Bleomycin-injured mice had 3.4-fold higher lung LOX activity (Figure 2a and the Supporting Information, Figure S5), 1.75-fold higher collagen content (Supporting Information, Figure S6), and 2.1-fold higher allysine content (Figure 2b and the Supporting Information, Figure S7) than in naive animals. Treatment with the LOX inhibitor BAPN reduced the lung LOX activity and allysine content to levels observed in naive mice, although...
BAPN had no effect on total lung collagen levels (Figure 2a,b and the Supporting Information, Figure S6).

It is well known that T2* in the lung is very short (ca. 1 ms) because of the magnetic susceptibility gradients caused by the air–tissue interface. Too overcome this signal loss we used an ultrashort echo time (UTE) sequence. T1-weighted UTE MR images were taken before and after injection of GdOA or GdOX (Figure 2c,d,g and the Supporting Information, Figure S8), starting at 12 min when the background blood signal was minimal and lung signal intensity was highest. The lung signal and adjacent skeletal muscle signal were measured before and after probe injection, and the change in lung-to-muscle signal ratio ($\Delta$LMR) (Figure 2e and the Supporting Information, Figure S9) was calculated, where LMR = SI$_{\text{lung}}$/SI$_{\text{muscle}}$ (SI = signal intensity) and $\Delta$LMR = LMR$_{\text{post}}$ - LMR$_{\text{pre}}$.

GdOA injection resulted in a strong lung signal enhancement in bleomycin-injured lungs compared to naive mice. The signal enhancement correlated strongly with the extent of disease (Supporting Information, Figure S10). GdOA injection resulted in a similar, weak lung enhancement in both bleomycin and naive mice, indicating that the oxamine function was required for the higher signal observed in the bleomycin-injured mice. BAPN treatment did not inhibit collagen production (Supporting Information, Figure S6) but did prevent allysine production and crosslinking (Figure 2b). MRI indicated that GdOA is sensitive to the allysine reduction caused by BAPN, further demonstrating the specificity of GdOA (Figure 2h). We also compared the change in liver-to-muscle ratio between naive and bleomycin-treated groups after GdOA and saw no significant difference in signal, suggesting that the increased GdOA lung enhancement observed in bleomycin-mice is disease-dependent (Supporting Information, Figure S11). Ex vivo analysis of lung Gd confirmed the imaging results, with the micromolar Gd lung concentrations measured consistent with the image enhancement observed (Figure 2f and the Supporting Information, Figure S12).

In summary, we showed that allysine is a suitable target for molecular MR detection of fibrogenesis. The novel probe GdOA is stable with respect to Gd release and is able to bind to oxidized collagen present during fibrogenesis. GdOA shows rapid uptake in a disease model of pulmonary fibrosis and demonstrates specific allysine targeting resulting in an enhanced MRI signal in fibrotic tissue. GdOA shows low nonspecific binding and rapid background clearance. GdOA imaging provides a quantitative non-invasive measure of the extent of active fibrogenesis in fibrotic diseases. Targeting allysine as a readout of the rate of fibrogenesis may allow for the determination of fibrotic disease activity across all tissue types.

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Conflict of interest

The authors declare no conflict of interest.

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