Research Article

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# 3D molecular MR imaging of liver fibrosis and response to rapamycin therapy in a bile duct ligation rat model

Christian T. Farrar[1,†], Danielle K. DePeralta[2,†], Helen Day[1], Tyson A. Rietz[1], Lan Wei[2], Gregory Y. Lauwers[3], Boris Keil[1], Arun Subramaniam[4], Anthony J. Sinskey[5], Kenneth K. Tanabe[2], Bryan C. Fuchs[2,*], Peter Caravan[1,*]

[1]*Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, 149 Thirteenth St., Suite 2301, Charlestown, MA 02129, United States;* [2]*Surgical Oncology, Massachusetts General Hospital Cancer Center and Harvard Medical School, WRN 401, 55 Fruit St., Boston, MA 02114, United States;* [3]*Pathology, Massachusetts General Hospital and Harvard Medical School, WRN 2, 55 Fruit St., Boston, MA 02114, United States;* [4]*Sanofi Genzyme, 49 New York Ave, Framingham, MA 01701, United States;* [5]*Department of Biology, Massachusetts Institute of Technology, 77 Massachusetts Ave., Cambridge, MA 02139, United States*

**Background & Aims**: Liver biopsy, the gold standard for assessing liver fibrosis, suffers from limitations due to sampling error and invasiveness. There is therefore a critical need for methods to non-invasively quantify fibrosis throughout the entire liver. The goal of this study was to use molecular Magnetic Resonance Imaging (MRI) of Type I collagen to non-invasively image liver fibrosis and assess response to rapamycin therapy.

**Methods**: Liver fibrosis was induced in rats by bile duct ligation (BDL). MRI was performed 4, 10, or 18 days following BDL. Some BDL rats were treated daily with rapamycin starting on day 4 and imaged on day 18. A three-dimensional (3D) inversion recovery MRI sequence was used to quantify the change in liver longitudinal relaxation rate ($\Delta R1$) induced by the collagen-targeted probe EP-3533. Liver tissue was subjected to pathologic scoring of fibrosis and analyzed for Sirius Red staining and hydroxyproline content.

**Results**: $\Delta R1$ increased significantly with time following BDL compared to controls in agreement with *ex vivo* measures of increasing fibrosis. Receiver operating characteristic curve analysis demonstrated the ability of $\Delta R1$ to detect liver fibrosis and distinguish intermediate and late stages of fibrosis. EP-3533 MRI correctly characterized the response to rapamycin in 11 out of 12 treated rats compared to the standard of collagen proportional area (CPA). 3D MRI enabled characterization of disease heterogeneity throughout the whole liver.

**Conclusions**: EP-3533 allowed for staging of liver fibrosis, assessment of response to rapamycin therapy, and demonstrated the ability to detect heterogeneity in liver fibrosis.



Keywords: Liver fibrosis; Type I collagen; Molecular imaging; Non-invasive; MRI; EP-3533; Bile duct ligation (BDL).

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* Corresponding authors. Address: Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital and Harvard Medical School, 149 Thirteenth St., Suite 2301, Charlestown, MA 02129, United States. Tel.: +1 617 643 0193; fax: +1 617 726 2422.

*E-mail addresses:* bfuchs@mgh.harvard.edu (B.C. Fuchs), caravan@nmr.mgh.harvard.edu (P. Caravan).

† These authors contributed equally to this work.

*Abbreviations:* MRI, magnetic resonance imaging; MRE, magnetic resonance elastography; R1, longitudinal relaxation rate; T1, longitudinal relaxation time; TE, echo time; TR, repetition time; FLASH, fast low angle shot; IR, inversion recovery; MIP, maximum intensity projection; MPR, multi-planar reconstruction; FOV, field of view; BDL, bile duct ligation; ECM, extracellular matrix; HCC, hepatocellular carcinoma; ROC, receiver operating characteristic; AUROC, area under the receiver operating characteristic curve; 3D, three-dimensional; CNR, contrast-to-noise ratio; HPLC, high performance liquid chromatography; ANOVA, analysis of variance; $CCl_4$, carbon tetrachloride; CPA, collagen proportional area.

## Introduction

Chronic liver disease is a major cause of morbidity and mortality worldwide, and unlike other major causes of mortality, rates of chronic liver disease are increasing rather than declining [1]. Chronic liver disease results from a wide range of etiological factors including viral hepatitis, metabolic dysfunction, alcohol abuse and autoimmune disease. Liver fibrosis is the common result of virtually all chronic liver injuries [2]. During fibrosis, the ongoing cycles of injury and repair lead to accumulation of extracellular matrix (ECM) components rich in fibrillar collagen, and eventually disruption of normal tissue architecture and function [3]. If the underlying cause of disease is suppressed or removed early enough, liver fibrosis has the potential to regress to a lesser stage or even reverse to a normal architecture [4,5]. However, if left unchecked, fibrosis will progress to cirrhosis, an advanced stage of the disease estimated to affect 1–2% of the world's population [6,7]. Accurate assessment of fibrosis stage and early detection of cirrhosis are therefore vital for determining prognosis and guiding management, since doing so identifies those patients at greatest risk of developing complications of cirrhosis for which longitudinal survey is essential.

Biopsy is an imperfect gold standard in assessing liver fibrosis as it suffers from intra/inter-observer variability and is associated with several complications including hospitalization in 1–5% of

cases and mortality in 0.01–0.1% of cases [8,9]. In addition, sampling error is a major issue as the 1–2 pieces of one cm long liver biopsies only assess 1/50,000 of the liver volume [10]. Importantly, repeated biopsies to evaluate disease progression or response to treatment are impractical due to the increased risk of complications and poor patient compliance. For all of these reasons, non-invasive strategies that can repeatedly assess liver fibrosis throughout the entire organ are urgently needed to assess disease stage, monitor treatment response and determine prognosis.

Type I collagen is an attractive target for molecular imaging of fibrosis since collagen deposition is a common outcome regardless of fibrosis cause. Moreover, the collagen concentration increases as fibrosis progresses and its extracellular location makes it readily accessible by the probe. We have previously reported data with a peptide-based Type I collagen specific MR probe, termed EP-3533, for detection and quantification of fibrosis, and demonstrated its utility in cardiac, pulmonary and hepatic models of fibrosis [11–15]. In the liver, molecular imaging of collagen with EP-3533 was much more sensitive to the presence of fibrosis than other MRI measures such as water diffusion and could accurately stage liver fibrosis [14]. EP-3533 was calculated to have a blood half-life of 19 ± 2 min in both fibrotic and control mice consistent with its extracellular distribution and primary renal clearance [14]. By 24 h, the probe was largely eliminated from the body. When tested at 10 μM in a "lead side-effect panel" of 33 different *in vitro* assays, EP-3533 had no measurable effect in terms of inhibiting receptor binding [14]. These pharmacokinetics, biodistribution, and *in vitro* pharmacology studies suggest the potential of this probe for translation to human studies.

Here, we expand upon these studies and demonstrate that EP-3533 can accurately stage biliary fibrosis in BDL rats. While our initial studies were performed on high field small animal scanners, we now show efficacy using a 1.5-tesla clinical MRI scanner in order to further clinical translation. In addition, our previous studies quantified changes in the contrast-to-noise ratio (ΔCNR) between liver and adjacent skeletal muscle after injection of EP-3533 and were typically performed on a just a few liver image slices [11–14]. In the current work we have made fibrosis quantification more extensive by introducing a respiratory-gated three-dimensional (3D) inversion recovery imaging sequence that allows us to measure the change in longitudinal relaxation rate (ΔR1) induced by EP-3533 on a pixel-wise basis throughout the entire liver. Since ΔR1 is linearly proportional to the Gadolinium concentration, it has the potential to provide a more quantitative and robust metric than ΔCNR for the assessment of fibrosis, and to measure disease heterogeneity within the liver. In order to test this, we measured fibrosis in rapamycin treated BDL rats where considerable variability was observed previously in liver fibrosis response to rapamycin [16,17]. The rapamycin experiments therefore provided a rigorous test of the ability of EP-3533 to detect differences in disease progression and response to therapy.

## Materials and methods

### *Animal model*

Liver fibrosis was induced in male CD rats (n = 39) by ligation of the common bile duct (Charles River Labs, Wilmington, MA). Control animals (n = 8) underwent a control procedure. BDL rats were imaged 4 (n = 9), 10 (n = 10), or 18 (n = 8) days following ligation. Rapamycin treated rats (n = 12) were administered 3 mg/kg/day by oral gavage starting on day four and were imaged on day 18. EP-3533 was prepared as reported previously [11]. All experiments and procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

### *Magnetic resonance imaging*

Rats were imaged on a 1.5-tesla clinical MRI scanner (Siemens Healthcare, Malvern, PA) using a home-built, transmit-receive solenoid coil (Supplementary Fig. 1). Animals were anesthetized with 1–2% isoflurane and respiration rate was monitored with a small animal physiological monitoring system (SA Instruments, Inc., Stony Brook, NY). Respiratory-gated, 3D Inversion Recovery (IR) Fast Low Angle Shot (FLASH) images were acquired prior to and one hour following intravenous administration of 10 μmol/kg EP-3533. A non-selective inversion pulse was used and images were acquired with inversion recovery times of 50, 100, 200, 250, 300, 400 and 1000 ms. Image acquisition parameters consisted of an echo time of TE = 2.44 ms, field of view FOV = 120 × 93 mm, matrix = 192 × 150 (0.625 mm in-plane resolution), slice thickness = 0.6 mm, and 36 image slices. A segmented k-space acquisition method consisting of 51 segments was used to reduce the acquisition time. The effective repetition time was dictated by the respiration rate. Anesthesia was adjusted to maintain a respiration rate of 60 ± 5 breaths per minute for an effective repetition time of $TR_{eff}$ = 1000 ± 90 ms. Following imaging, animals were sacrificed and liver tissue was subjected to pathologic scoring of fibrosis and analyzed for hydroxyproline content. Longitudinal relaxation rate (R1) maps were generated from the images using a custom written MATLAB (Mathworks, Natick, MA) program for voxel wise fitting of the inversion recovery signal intensities as a function of the inversion recovery time.

### *Ex vivo tissue analysis*

Formalin-fixed samples were embedded in paraffin, cut into 5 μm thick sections and stained with Sirius Red according to standard procedures. Sirius Red stained sections were analyzed by a pathologist, who was blinded to the study, to score the amount of liver disease according to the method of Ishak [18]. In addition, the collagen proportional area (CPA), as determined by the % area stained with Sirius Red, was quantified from the histology images using ImageJ as per our standard procedures [11–14]. Hydroxyproline in tissue was quantified by high-performance liquid chromatography (HPLC) analysis of tissue acid digests as previously described [19]. Hydroxyproline is expressed as amount per wet weight of tissue.

### *Statistical analysis*

Data are displayed as box plots with the dark band inside the box representing the mean, the bottom and top of the box the first and third quartiles, and the whiskers the minimum and maximum values. Data are reported as the mean ± standard error. Statistical analyses (Analysis of Variance (ANOVA) and Receiver Operating Characteristic (ROC) analysis) were performed using Prism 6 (GraphPad Software, Inc, La Jolla, CA) with $p < 0.05$ considered as significant. Differences among groups were tested with one-way ANOVA followed by the Tukey post-hoc test where appropriate.

## Results

A respiratory-gated, three-dimensional (3D) inversion recovery (IR) Fast Low Angle Shot (FLASH) MRI sequence was used to generate quantitative R1 maps of the entire liver. Representative R1 maps generated from data acquired pre and one hour post-EP3533 are shown in Fig. 1 for an 18 day BDL rat and a control, sham rat. A dramatic increase in R1 is observed following contrast agent injection for the BDL rat, but not the sham rat. As demonstrated by the R1 histogram plot (Fig. 1C), the fibrosis is relatively homogenous in this particular BDL rat with a standard deviation in R1 throughout the liver of only ± 4%