Supplementary Information

A robust collagen-targeting MRI peptide contrast agent for in vivo imaging of hepatic fibrosis

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Materials and methods

Materials. Fmoc-Gly-OH, Fmoc-Leu-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Glu(OtBu)-OH, Fmoc-His(Trt)-OH, Fmoc-Asn(Trt)-OH, Fmoc-6-aminocaproic acid, Rink amide AM resin (200-400 mesh, loading = 0.345 mmol/g), hydroxy benzotriazole (HOBt) and O-(Benzotriazol-1-yl)-N, N, N', N'- tetramethyluronium hexafluorophosphate (HBTU) were purchased from GL Biochemical Company (Shanghai, China). 5(6)-carboxyfluorescein and $GdCl_3 \cdot 6H_2O$ were provided by Aladdin Industries (Shanghai, China). N, N-Diisopropylethylamine (DIEA), Trifluoroacetic acid (TFA), Triisopropylsilane (TIS), tert-Butyl bromoacetate and benzyl bromoacetate were purchased from J&K Chemical Ltd. (Shanghai, China). H&E Stain Kit and goat serum were obtained from Solarbio Science & Technology Co. Ltd (Beijing, China). Cyclen·4HCl, tert-butyl bromoacetate and benzyl 2-bromoacetate were purchased from Beijing InnoChem Science & Technology Co., Ltd (Beijing, China). Pentobarbital sodium was purchased from Kehao Biological Co., Ltd (Wuhan, China). Gadobutrol was obtained from Bayer Co. Ltd (Germany). Collagen Type I Rabbit Polyclonal antibody was purchased from Proteintech Group, Inc (Wuhan, China). All the commercial reagents were of analytical grade and were used without further purification.

Synthesis of the DOTA(tBu)$_3$. Cyclen·4HCl (200 mg, 1.16 mmol) (A) was first dissolved in MeCN (200 mL). Sodium hydroxide (371 mg, 9.28 mmol, 8.0 eq.) was

added, and the resulting white suspension was stirred for 15 min under a nitrogen atmosphere. A solution of tert-butyl bromoacetate (660 mg, 3.40 mmol, 3.0 eq.) was slowly added to the above MeCN (50 mL) for more than 15 min, and the suspension was stirred for an additional 30 min at 25 °C before being refluxed for 16 hrs at 80 °C. The solvent was removed under vacuum and the residue was separated by silica gel column chromatography (MeOH/DCM = 40/1 to 20/1 as eluent) to afford Tri-tert-butyl 2,2',2''-(1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate (C).

C was dissolved in DMF, benzyl 2-bromoacetate and Na_2CO_3 were added to the solution and then stirred at 80 °C. Upon completion of the reaction, a white precipitate was observed and subsequently separated by filtration. The filtrate was then recrystallized from the minimum amount of boiling toluene, and the resulting product was washed with cold diethyl ether to obtain tri-tert-butyl 2,2',2''-(10-(2-(benzyloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-triyl) triacetate (D).

A mixture of D (53.1 mg) and Pd/C (20.1 mg, 0.5 μmol) in MeOH (1 mL) was stirred in a test tube equipped with an H_2 balloon at 25 °C. The reaction was monitored continuously using thin-layer chromatography. After 12 hours, the mixture was filtered and washed with diethyl ether to remove the catalyst. The filtrates were combined and concentrated under vacuum. The resulting product was purified by silica gel column chromatography using a MeOH/DCM eluent gradient ranging from 20/1 to 10/1. The final product, $DOTA(tBu)_3$, was characterized by mass spectrometry and ^1H NMR. m/z calculated 573.7 $[M+H]^+$ for $DOTA(tBu)_3$, found 573.5 $[M+H]^+$. m/z calculated 595.7 $[M+Na]^+$ for $DOTA(tBu)_3$, found 596.5 $[M+Na]^+$ (Fig. S2). ^1H NMR (400 MHz, $CDCl_3$) δ 3.66-1.95 (m, 24H), δ 1.47-1.45 (m, 27H) (Fig. S3).

Synthesis of fluorescent peptide probe. Fluorescent peptide probe FAM-Ahx-LRELHLNNNG (F-ICTP) was synthesized in-house by standard Fmoc solid phase peptide synthesis method using Rink amide AM resin (0.345 mmol/g loading). Stepwise couplings of amino acids were performed using Fmoc-amino acids (4 eq.), HOBt (4 eq.), HBTU (4 eq.), and DIEA (6 eq.). The resin was thoroughly

washed with DMF (3×10 mL) and DCM (2×10 mL) after each coupling step, and the Fmoc protecting group was removed with 20% (v/v) piperidine in DMF. The Chloranil test was used to check the coupling reaction status and Fmoc deprotection status. Once the synthesis of the peptide sequence was complete, the mixture of fluorophore FAM (10 eq.), HOBt (10 eq.), HBTU (10 eq.), and DIEA (16 eq.) in DMF was added to the resin and incubated for 24 hrs at 37 °C to conjugate FAM with the N-terminal of the peptide. TFA/H_2O (95:5) was applied to treat the resin for 2.5 hrs to deprotect the side-chain protecting group and remove the peptide from the resin. The peptide was harvested by precipitation with cold Et_2O. Crude products were collected by re-suspension of the peptide in cold Et_2O, sonication, and centrifugation, and were then purified by dialysis. The purified peptide was lyophilized and stored at -20 °C for future use.

Synthesis of MRI peptide contrast agent. The peptide DOTA-Ahx-LRELHLNNNG (DOTA-Ahx-ICTP) was synthesized using the Fmoc solid phase peptide synthesis method with Rink amide AM resin and stepwise couplings of amino acids using Fmoc-amino acids (4 eq.), HOBt (4 eq.), HBTU (4 eq.), and DIEA (6 eq.). After each coupling step, the resin was washed with DMF and DCM, and the Fmoc protecting group was removed using 20% (v/v) piperidine in DMF. The Chloranil test was used to confirm successful coupling and Fmoc deprotection. After completing the synthesis of the peptide sequence, the mixture of DOTA $(tBu)_3$ (10 eq.), HOBt (10 eq.), HBTU (10 eq.), and DIEA (16 eq.) in DMF was added to the resin and incubated for 24 hrs at 37 °C to conjugate DOTA $(tBu)_3$ with the N-terminal of the peptide. TFA/H_2O (95:5) was applied to treat the resin for 2.5 hrs to deprotect the side-chain protecting group and remove the peptide from the resin. The peptide was then harvested by precipitation with cold Et_2O and purified by dialysis. The peptide DOTA-Ahx-ICTP was dissolved in DI water and complexed with $GdCl_3 \cdot 6H_2O$ at 25 °C overnight to obtain the MRI peptide contrast agent Gd-DOTA-Ahx-ICTP (Gd-ICTP). The identity of the peptides was confirmed by mass spectrometry. m/z calculated 1678.4 $[M+H]^+$ for DOTA-Ahx-ICTP, found 1678.0 $[M+H]^+$ (Fig. S4). m/z calculated 1832.6 $[M+H]^+$ for Gd-ICTP, found 1831.7 $[M+H]^+$ (Fig S5).

HPLC analysis: The separation was carried out using a C18 Superb 5μm 4.6×150 mm(W) column (GL Sciences, Japan) and an UltiMate 3000 system (Thermo Scientific, USA). The mobile phase consisted of water (A) and methanol (B), and a linear gradient was used for Gd-ICTP identification experiment. The separation was initiated by running the system isocratically for 1 min with 90% of mobile phase A. After 5 min, a gradient elution to 85% A phase was performed, followed by another gradient elution to 20% A phase at 5 min. The column was then held isocratically for 2 min. Finally, the column was eluted with 100% B phase for 5 min. The flow rate used for the analysis was 0.5 mL/min, and the injection volume was 20 μL. The column temperature was maintained at 25 ^{o}C, and UV absorbance detection was performed at 230 nm.

Protein binding assay. The binding assay of type I collagen, BSA, pepsin, trypsin and silk protein hydrolysates followed a standard protocol. Solutions of each protein with a concentration of 100 μg/mL were prepared in 10 mM PB (pH 7.4) and added into the wells of a 96-well plate, which was then air-dried. Next, 50 μL of a 20 μM F-ICTP solution in 10 mM PB (pH 7.4) was added to each well, and the plate was incubated at 4°C for 5 hrs to allow for binding. The wells were washed three times with 400 μL 10 mM PB (pH 7.4) for 10 min. Fluorescence (ex: 485 nm, em: 533 nm) was measured on an Infinite M200 (TECAN Corporation, Switzerland). Each binding experiment was repeated three times.

Tissue staining and imaging. The animal experiments were carried out with the approval of the ethics committee of Lanzhou University No.1 Hospital, in compliance with appropriate ethical guidelines and regulations. Hepatic fibrosis was induced in KM mice (n = 60) by treatment with 0.1mL/100g CCl_4 mixture (CCl_4: olive oil=1:1), administered by means of intraperitoneal injection twice per week for 6 weeks. Control animals were treated with olive oil (n = 20). The liver tissues were obtained from these mice and cryopreserved in Tissue-Tek O.C.T. medium, sectioned to 4 μm thickness on glass slides, and air-dried at 25 °C for further analysis.

Tissue sections were added with 10% goat serum (10 mM PBS, pH 7.4) and incubated for 5 min at 25 °C to block nonspecific binding. F-ICTP solution was prepared at a concentration of 15 μM in 10 mM PB (pH 7.4), while the type I collagen antibody solution was diluted 500 times with 10 mM PB (pH 7.4). The tissue slides were coated with 100 μL of the peptide solution and incubated at 4 °C for 4 hrs, covered with parafilm. After removing the parafilm, the solutions on the slides were absorbed using bibulous paper. The tissue slides were then washed with 10 mM PB for 5 min, repeated three times. The anti-quenching agent was added to the tissue slides, and the slides were covered with a cap. The stained tissue sections were then visualized using a Leica DM4000B metallurgical upright microscope (Leica Microsystems Inc., Wetzlar, Germany).

MRI scan: All mice were imaged with a 3.0 T Siemens MRI scanner at the Lanzhou University No.1 Hospital. Animals were anesthetized with pentobarbital and the temperature was maintained with a small animal physiological monitoring system. T1 maps were scanned at pre, 1, 2 and 24 hrs after intravenous administration of 7.5 μmol/kg of Gd-ICTP in both healthy and fibrosis mice. Additionally, T1 maps of fibrosis mice injected with the same dose of gadobutrol were acquired at pre, 1, 2 and 24 hrs.

Histopathological outcomes and biodistribution. The mice used in the MRI study were sacrificed by cervical dislocation after 2 hrs post injection. Formalin-fixed samples were embedded in paraffin, cut into 4 μm thick slices, and stained with hematoxylin and eosin according to standard procedures. The organs, including the lung, kidney, heart, and liver, were immediately removed and rinsed in saline, and then blotted dry. Tissue samples were digested overnight in concentrated nitric acid. All Gd concentrations were determined by Inductively coupled plasma optical emission spectroscopy (ICP-OES).

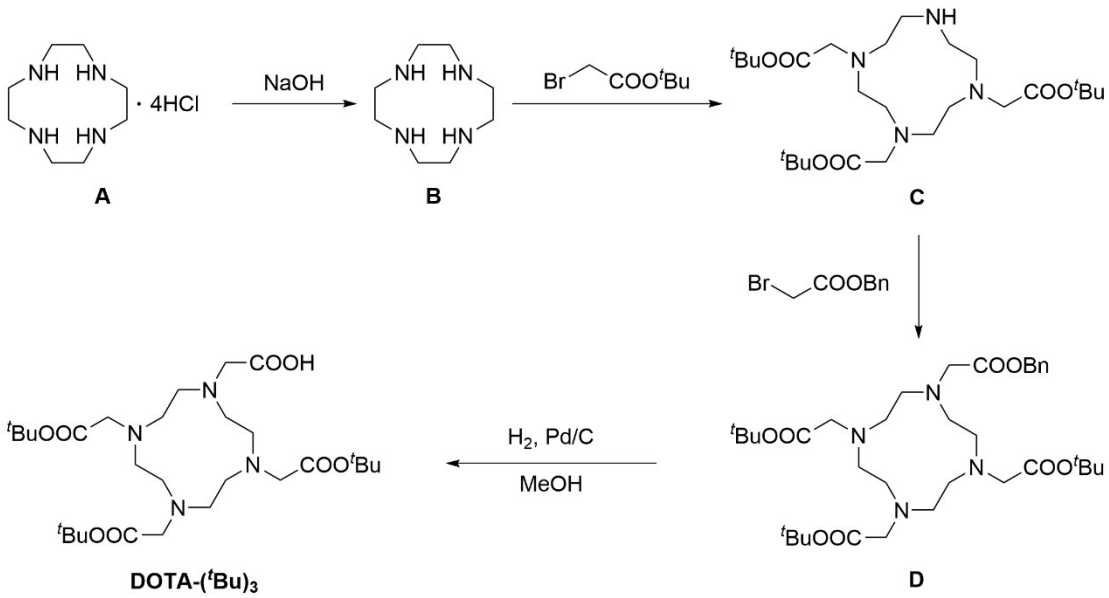

Fig. S1. Schematic diagram of the synthesis of DOTA(tBu)$_3$.

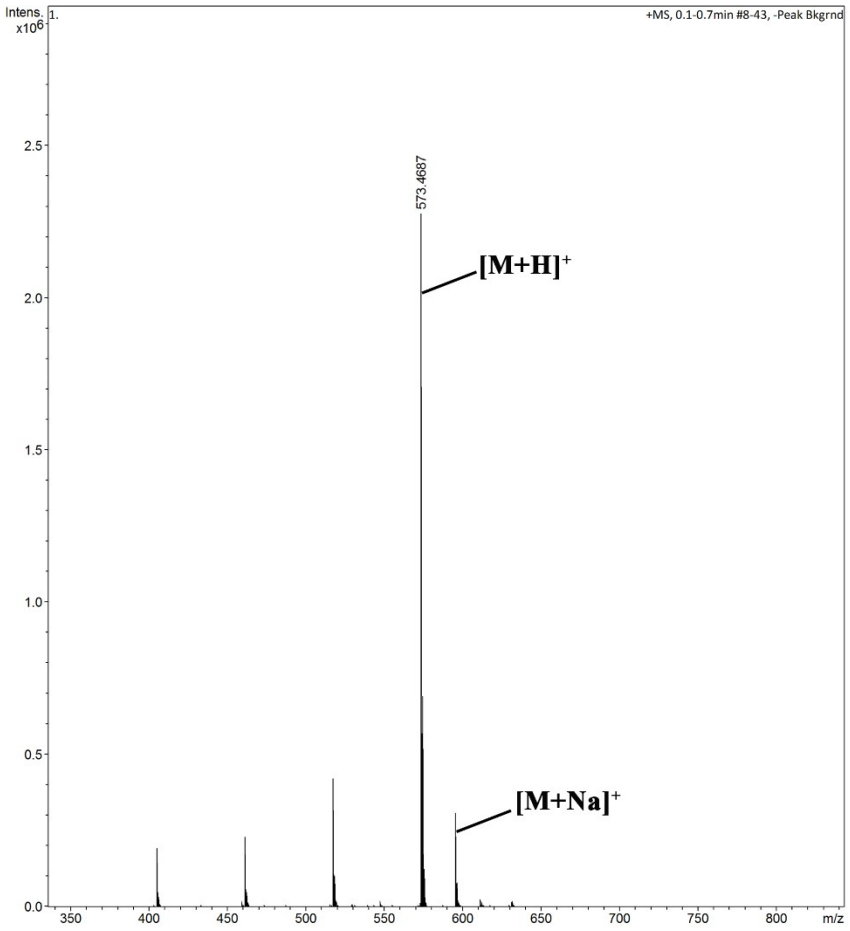

Fig. S2. Mass spectrum of DOTA(tBu)$_3$. m/z calculated 573.7 $[M+H]^+$ for DOTA(tBu)$_3$, found 573.5 $[M+H]^+$. m/z calculated 595.7 $[M+Na]^+$ for DOTA(tBu)$_3$, found 596.5 $[M+Na]^+$.

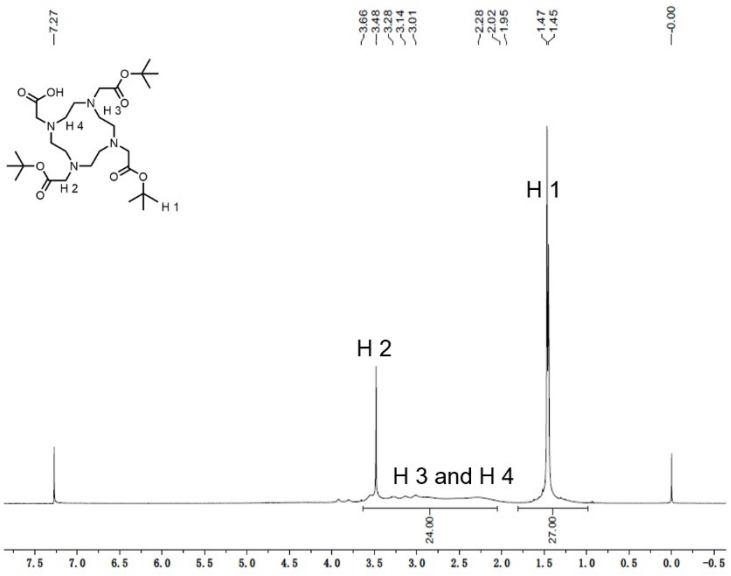

Fig. S3. ^{1}H NMR spectrum of DOTA(tBu)$_3$. 1H NMR (400 MHz, $CDCl_3$) δ 3.66-1.95 (m, 24H), δ 1.47-1.45 (m, 27H).

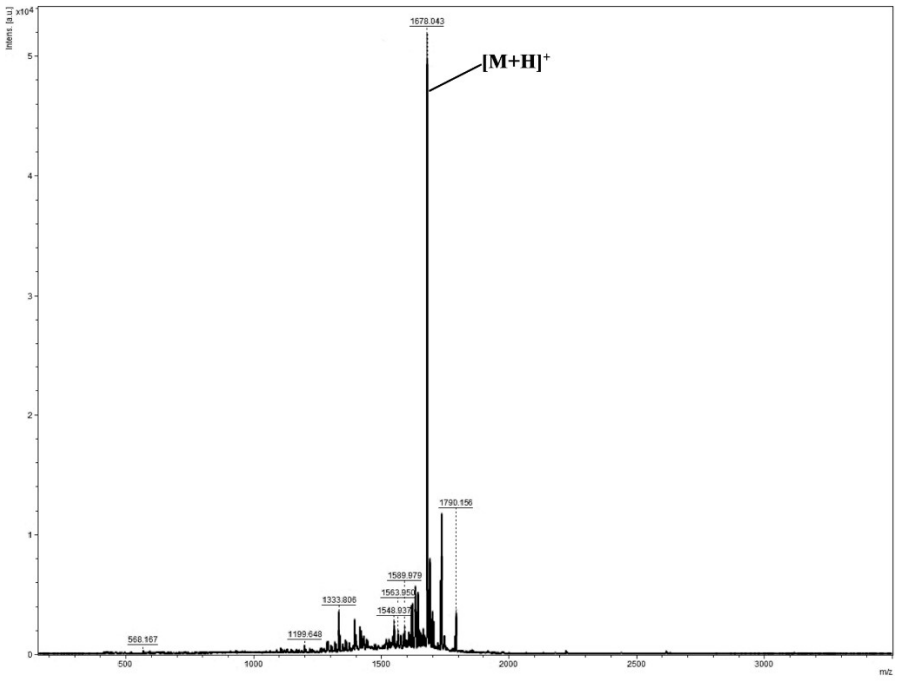

Fig. S4. Mass spectrum of DOTA-Ahx-ICTP. m/z calculated 1678.4 $[M+H]^+$ for DOTA-Ahx-ICTP, found 1678.0 $[M+H]^+$.

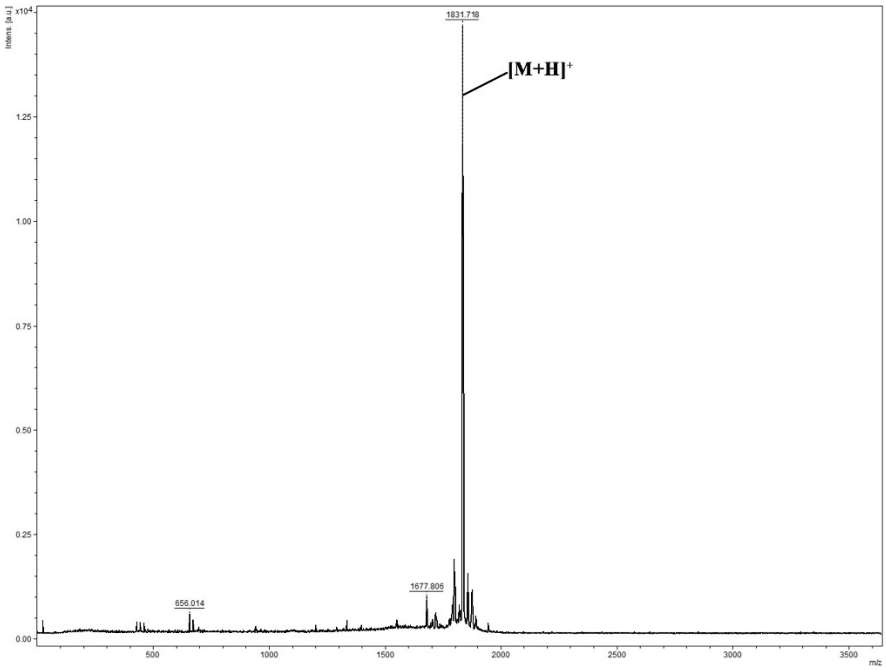

Fig. S5. Mass spectrum of Gd-ICTP. m/z calculated 1832.6 $[M+H]^+$ for Gd-ICTP, found 1831.7 $[M+H]^+$.

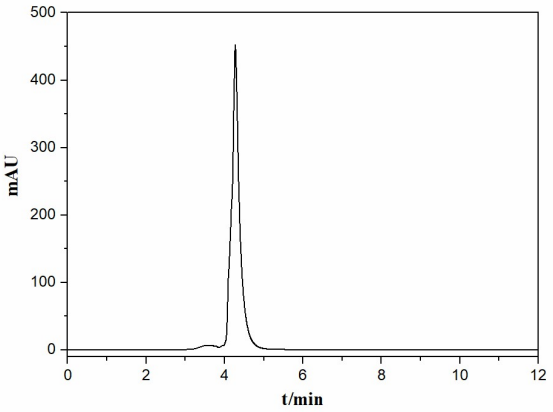

Fig. S6. HPLC of Gd-ICTP.

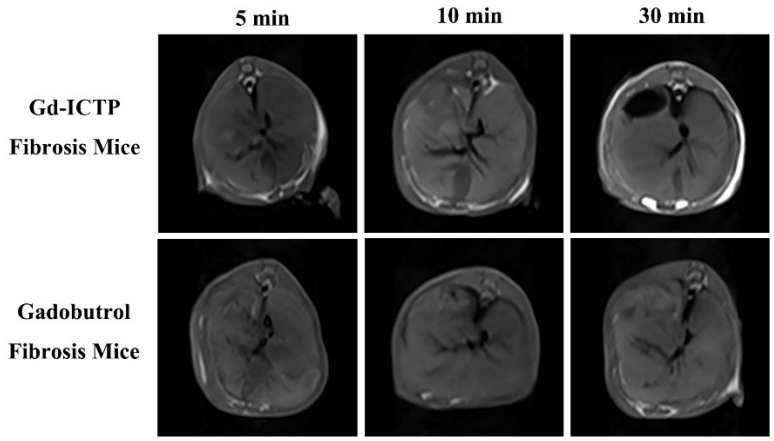

Fig. S7. T1-weighted axial MRI images of hepatic fibrosis mice at 5, 10 and 30 min after administering intravenous injections of the MRI peptide contrast agent Gd-ICTP and Gadobutrol.