

Video Article

MR Molecular Imaging of Prostate Cancer with a Small Molecular CLT1 Peptide Targeted Contrast Agent

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Abstract

Tumor extracellular matrix has abundance of cancer related proteins that can be used as biomarkers for cancer molecular imaging. In this work, we demonstrated effective MR cancer molecular imaging with a small molecular peptide targeted Gd-DOTA monoamide complex as a targeted MRI contrast agent specific to clotted plasma proteins in tumor stroma. We performed the experiment of evaluating the effectiveness of the agent for non-invasive detection of prostate tumor with MRI in a mouse orthotopic PC-3 prostate cancer model. The targeted contrast agent was effective to produce significant tumor contrast enhancement at a low dose of 0.03 mmol Gd/kg. The peptide targeted MRI contrast agent is promising for MR molecular imaging of prostate tumor.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50565/>

Introduction

Effective imaging of cancer related molecular targets is of great significance to improve the accuracy of earlier cancer detection and diagnosis. Magnetic resonance imaging (MRI) is a powerful clinical imaging modality with high spatial resolution and no ionization radiation¹. However, no targeted contrast agent is available for clinical MR cancer molecular imaging. Innovative design and development of targeted MRI contrast agents would greatly advance the application of MR cancer molecular imaging. Significant efforts have been made to develop targeted contrast agents for MR imaging of the biomarkers expressed on the surface of cancer cells. Due to relatively low sensitivity of MRI and low concentration of these biomarkers, it is a challenge to generate sufficient contrast enhancement for effective MR molecular imaging using small molecular targeted contrast agents^{2,3}. In order to obtain sufficient enhancement, various delivery systems such as liposomes, nanoparticles and polymer conjugates with a high payload of paramagnetic Gd(III) chelates have been prepared to increase local concentration of contrast agents at the target sites^{4,5}. Although these delivery systems were able to generate significant tumor enhancement in animal models, their large sizes resulted in slow and incomplete elimination from the body, resulting in prolonged accumulation of toxic Gd(III) ions, which may cause serious safety concerns⁶. Recently, some studies have shown that the limitations of MRI for molecular imaging can be overcome by selecting proper molecular biomarkers with high local expression in the lesions and using small molecular agents that can be readily excreted^{7,8}. The key feature of these agents is that they target molecular markers abundantly present in the diseased tissues with little presence in normal tissues. A high concentration of contrast agents can bind to these targets, resulting in sufficient contrast enhancement for effective MR molecular imaging. Since their size is smaller than the renal filtration threshold, unbound contrast agents can readily be excreted from the body with reduced background noise. We have selected a universal cancer-related biomarker, clotted plasma proteins, which abundantly exist in tumor stroma, and are rarely present in normal tissues⁹. We synthesized a targeted contrast agent containing a small targeting peptide CGLIIQKNEC (CLT1), which showed strong specific binding to the PC3 prostate tumor model¹⁰, and four Gd-DOTA monoamide chelates. Here, we provide a methodology for MR cancer molecular imaging to detect tumors in mice.

Protocol

Protocol adapted from a prior study¹¹.

1. Conjugation of Gd-DOTA to CLT1 Peptide

1. Using standard solid-phase peptide synthesis, synthesize CLT1 peptide (CGLIIQKNEC) from Fmoc-protected amino acids on a 2-chlorotrityl chloride resin (1.0 mmol).
2. After adding final amino acid, cyclize the linear peptide on-resin with thallium(III) trifluoroacetate (1.09 g, 2.0 mmol, 2 equivalents) in DMF (20 ml) at 0 °C for 2 hr.
3. Next, conjugate PEG and lysine sequentially to the N-terminus of CLT1 peptide (1.0 mmol) on resin by reacting sequentially with Fmoc-NH-PEG-COOH (3.0 mmol), Fmoc-Lys(Fmoc)-OH (3.0 mmol), and a second batch of Fmoc-Lys(Fmoc)-OH (6 mmol).
4. Remove Fmoc with piperidine. Add DOTA-tris(t-Bu) (4.58 g, 8.0 mmol, 2 equivalents to each amino group) to react with the four free amines from the lysine dendrimer on the resin for 2 hr.
5. Finally, cleave and deprotect the product from the resin by treating with a cocktail of trifluoroacetic acid, water, and triisobutylsilane (TIS) (20 ml, 95/2.5/2.5) for 8 hr at RT. Remove the resin by filtration followed by washing with trifluoroacetic acid (TFA). Add the combined filtrates dropwise to cold ethyl ether (200 ml), centrifuge, and wash with ethyl ether 4x. Dry under vacuum to give a colorless solid (1.99 g, 61% yield).
6. Purify the crude product with preparative HPLC using a gradient of 0–40% solvent B (0.1% TFA in acetonitrile) in solvent A (0.1% TFA aqueous solution) for 20 min and 40–90% solvent B in solvent A for 10 min.
7. Dissolve CLT1-dL-(DOTA)₄ (250 mg, 0.077 mmol) in DI water (15 ml) and adjust the pH to 6 using 1 M NaOH. Add Gd(OAc)₃·4H₂O (472 mg, 0.462 mmol, 1.5 equivalents to DOTA monoamide) in portions to the solution, while maintaining the pH at 6 using 1 M NaOH. Stir the reaction solution at RT for 48 hr.
8. Complex the residual Gd(III) with ethylenediaminetetraacetic acid (EDTA) (90 mg, 0.31 mmol), and purify the crude product with a size exclusion column to give the final product CLT1-dL-(Gd-DOTA)₄ (169 mg, 57%).

2. Characterization of Contrast Agents

1. Measure Gd(III) content with inductively coupled plasma-optical emission spectroscopy.
2. Acquire matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra in linear mode with 2,5-dihydroxybenzoic acid (2,5-DHB) as a matrix.
3. Measure relaxation times of the aqueous solution of the contrast agents of CLT1-dL-(Gd-DOTA)₄ and a nontargeted control agent sCLT1-dL-(Gd-DOTA)₄ with different concentrations at 60 MHz (1.5 T) with a relaxometer at 37 °C. Use an inversion-recovery pulse sequence to measure T₁ and a Carr-Purcell-Meiboom-Gill sequence with 500 echoes to measure T₂. Calculate T₁ and T₂ relaxivities of the agents from the slopes of the plots of 1/T₁ and 1/T₂ versus the Gd concentrations.

3. Cell Culture and Development of Animal Model with Orthotopic PC3 Prostate Tumor

1. Culture PC3 prostate cancer cells with constitutive expression of green fluorescence protein (GFP) in RPMI medium supplemented with 5% fetal bovine serum and penicillin/streptomycin/fungizone, harvest by trypsinization and resuspend at density of 2.5 × 10⁴ cells/μl PBS.
2. Maintain male NIH athymic nude mice (4–5 weeks old) at the Athymic Animal Core Facility, Case Western Reserve University (CWRU) according to an animal protocol approved by the CWRU Institutional Animal Care and Use Committee.
3. The surgical surfaces were sprayed with 2% chlorhexidine solution and then covered with absorbent drapes. Sterile surgical gloves were worn during the procedure. Before animal surgery, i.p. inject Avertin (250 mg/kg) to anesthetize the mice. Alternatively, ketamine/xylazine / acepromazine can be used at a concentration of 50/5/1 mg/kg. Ophthalmic ointment was applied to the mouse to maintain adequate moisture during the anesthesia.
4. Start the surgery by cutting a small incision through the skin and peritoneum along the lower midline at a length of about 1 cm using a sterile scalpel. A sterile surgical drape was covered around the incision site during survival surgeries.
5. Gently exteriorize and stabilize the prostate dorsal lobes to expose the prostate. Inject the suspension of PC3-GFP cells in PBS (20 μl) into the prostate with a 30 gauge needle. Finally close the incision with wound autoclip¹².
6. For post-procedure monitoring, monitor animals twice per day for 1 week. Signs of illness include lack of eating, dark urine collecting around urethra, and unsteady gait which could indicate infection or occlusion of the bladder in the staples.
7. After 10 days, remove the staples using a staple remover. Monitor the animals daily to assess tumor size and any evidence of pain such as weight loss, behavioral deviations, and motor defects. Euthanize the animals which showed a tumor size exceeding the ethically allowed limit or evidence of pain during tumor growth.

4. Confirmation of Tumor Binding Specificity of the Peptides with Fluorescence Imaging and Histology

1. After tumor cell inoculation, allow approximately 4 weeks of tumor growth before beginning with the imaging. Prior to injection of the peptide probes, acquire GFP fluorescence images with live mice on a fluorescence imager to verify the presence of tumor.
2. I.v. inject Texas Red labeled peptides to the tumor bearing mice at a dose of 10 nmol/mouse.
3. Sacrifice the mice 2 hr later by cervical dislocation, collect the tumor and major organs, and immediately image on a fluorescence imager. Use green light filters for GFP (excitation: 444–490 nm; emission: 515 nm long-pass filter; acquisition settings: 500–720 in 10 nm steps) and red light filters for Texas Red (excitation: 576–621 nm; emission: 635 nm long-pass filter; acquisition settings: 630–800 in 10 nm steps). 10 msec exposure time for GFP and 150 msec for Texas Red.
4. Immediately after measurement of whole tissue fluorescence, collect tumor tissues, fix with formalin, and cryosection into 5-μm slices.
5. After rinsing with PBS, mount with 1 drop of mounting medium containing 4',6-diamidino-2-phenylindole (DAPI), image immediately on a confocal laser scanning microscope.

5. Magnetic Resonance Imaging (MRI)

1. Approximately 4 weeks after tumor cell inoculation, allow the tumors to grow up to 0.3-0.6 cm in diameter. Use a 7 T MRI scanner with a volume radio frequency (RF) coil for MRI study.
2. Anesthetize the mouse with a 2% isoflurane-oxygen mixture in an isoflurane induction chamber. Ophthalmic ointment was applied to the mouse to maintain adequate moisture during the anesthesia.
3. Insert a 30 gauge needle into a 1.0 m long tubing filled with heparinized saline. Place the self-made catheter in the mouse tail vein.
4. Place the mouse into the magnet and keep under inhalation anesthesia with 1.5% isoflurane-oxygen via a nose cone.
5. Place a respiratory sensor connected to a monitoring system on the abdomen to monitor rate and depth of respiration. Maintain the body temperature at 37 °C by blowing hot air into the magnet through a feedback control system.
6. Begin with sagittal section images using a localizing sequence to identify the tumor location (TR/TE = 200/3.7 msec, FOV = 3.0 cm, slice thickness = 2.2 mm, slice number = 16, average = 2, flip angle = 45°, matrix = 128 x 128).
7. Use a 2D T1-weighted gradient fat suppression sequence to acquire 2D axial images for CE-MRI (TR/TE = 151.2/1.9 msec, FOV = 3.0 cm x 3.0 cm, slice thickness = 1.2 mm, slice number = 12, average = 1, flip angle = 80°, matrix = 128 x 128).
8. After pre-injection baseline MR image acquisition, begin to inject the targeted agent or control agent at a dose of 0.03 mmol Gd/kg by flushing with 200 µl of saline.
9. Continue to acquire CE-MRI images at different time points for up to 30 min.

6. Image Processing and Analysis

1. Use imaging software for image analysis.
2. Draw the regions of interest (ROIs) over the whole tumor and the kidneys in the two-dimensional imaging plane and measure average signal intensity.
3. To quantify the CE-MRI data, measure tumor or kidney contrast enhancement (ΔSNR) by calculation of the increase in post-contrast SNR over pre-contrast SNR using the following equation: $\Delta\text{SNR} = (S_t/\sigma_t) - (S_0/\sigma_0)$, where S_0 and S_t denote the signal in the tumor or the kidneys before and after contrast, and σ_0 and σ_t are the standard deviation of noise measured from the background air before and after contrast.
4. Calculate the p values using the student's two-tailed t-test, assuming statistical significance at $p < 0.05$.

Representative Results

Figure 1 depicts synthesis of the targeted contrast agent CLT1-dL-(Gd-DOTA)₄ and the overall scheme of the experiment. CLT1-dL-(Gd-DOTA)₄ shows much higher relaxivity than clinical Gd-DOTA (Table 1). At 1.5 T, the T_1 relaxivity per gadolinium of CLT1-dL-(Gd-DOTA)₄ in PBS (pH 7.4) is approximately 3 times higher than that of Gd-DOTA¹⁰. Maestro imaging confirms the strong specific binding of Texas Red labeled CLT1 (CLT1-TR) to tumor with little binding to normal organs and tissues, while the non-specific scrambled peptide (sCLT1-TR) shows very little tumor binding (**Figure 2**). **Figure 3** shows typical pre and post-injection contrast enhanced (CE) T_1 -weighted images. The targeted agent results in greater and longer enhancement in tumor tissue compared to non-targeted scrambled agents. Contrast enhancement in the urinary bladder gradually increases over time, indicating that the contrast agents were excreted via renal filtration. Quantitative signal analysis reveals that the targeted agent produced more significant signal enhancement in the tumor tissue than the control agent ($p < 0.05$) up to 30 min. The biodistribution study shows the agent has minimal Gd retention in the main organs and tissues 2 days after injection. Our preliminary data shows that CLT1 targeted MRI contrast agents can be specifically delivered to the tumor at relatively low dose (one third of clinical dose).

	r_2 (mM ⁻¹ .s ⁻¹)		r_1 (mM ⁻¹ .s ⁻¹)		Gd content (mmol-Gd/g)
	per Gd	per molecule	per Gd	per molecule	
CLT1-(Gd-DOTA) ₄	10.1 ± 1.0	40.4 ± 3.0	13.0 ± 3.1	52.0 ± 9.6	1.05 ± 0.10
sCLT1-(Gd-DOTA) ₄	11.5 ± 1.4	46.0 ± 5.0	12.4 ± 0.3	49.6 ± 1.3	0.97 ± 0.08
Gd-DOTA ₄	2.9*	2.9*	3.2*	3.2*	

Table 1. Physicochemical properties of the targeted and scrambled MRI contrast agents at 1.5 T, 37 °C (Relaxivities for Gd-DOTA in water at 37 °C are from reference ¹⁰).

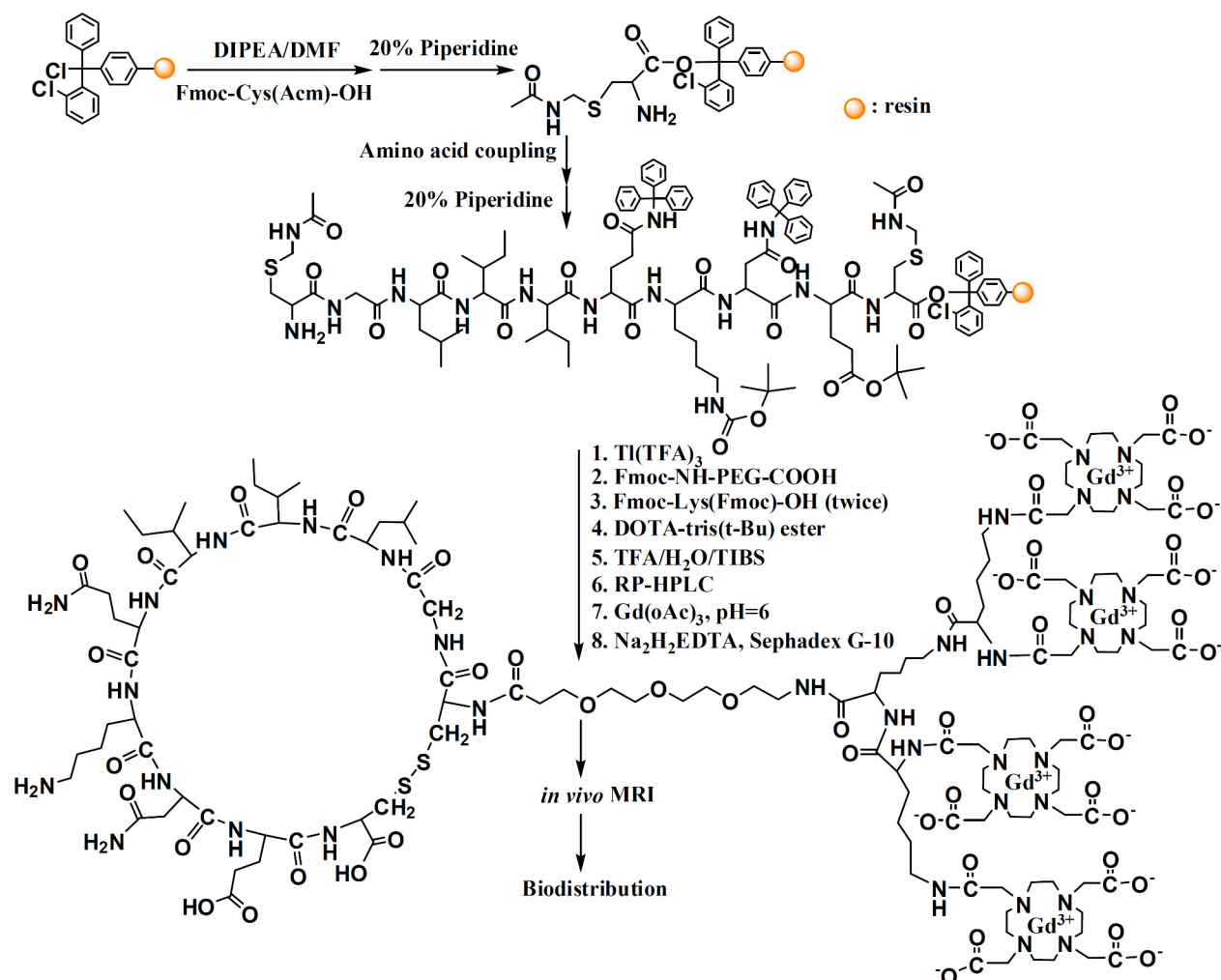


Figure 1. Graphical depiction of the synthesis procedure and overall experiment. [Click here to view larger figure.](#)

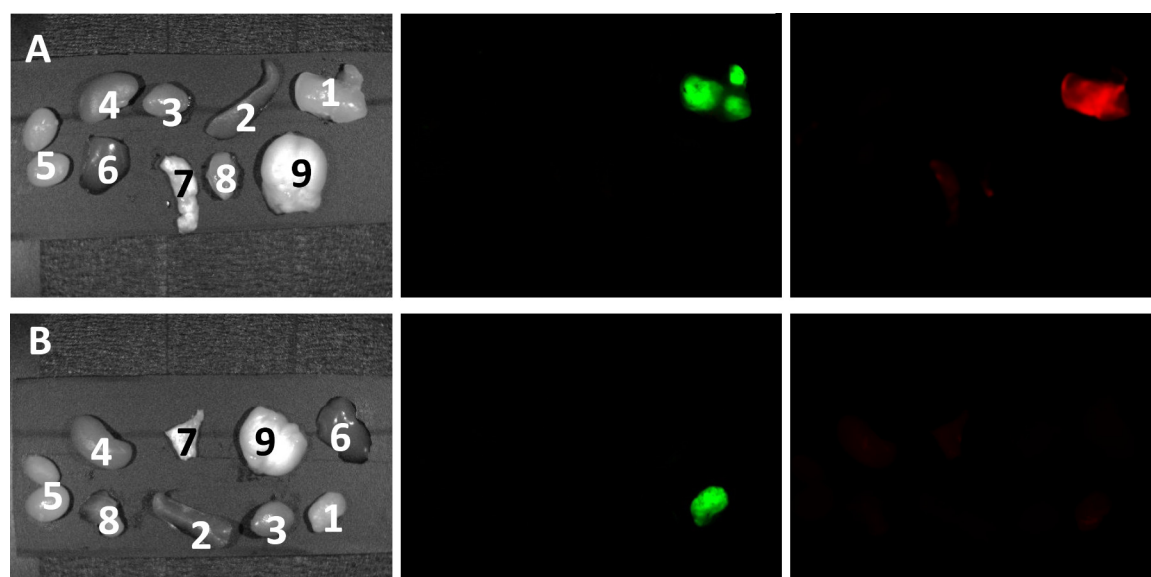


Figure 2. Tumor binding of CLT1-TR. Targeted CLT1-TR (A) and non-targeted sCLT1-TR (B) were injected intravenously to athymic nude mice bearing orthotopic PC3-GFP prostate at a dose of 10 nmol/mouse. After 2 hr, tumors and various organs were collected and imaged. Green fluorescence images are from PC3-GFP tumor cells. Red fluorescence images are from CLT1-TR (A) and sCLT1-TR (B) probes. 1. tumor; 2. spleen; 3. heart; 4. kidney; 5. testicle; 6. liver; 7. lung; 8. muscle; 9. brain. [Click here to view larger figure.](#)

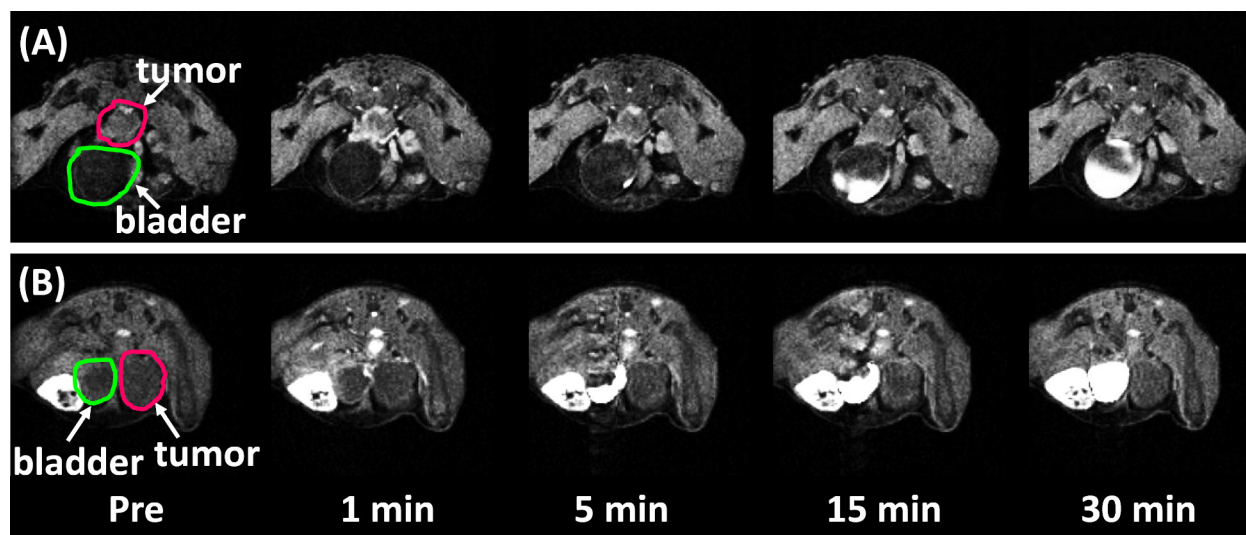


Figure 3. Representative T1-weighted axial 2D gradient images of orthotopic PC-3 human prostate tumor before and after intravenous injection of CLT1-dL-(Gd-DOTA)₄ (A) and sCLT1-dL-(Gd-DOTA)₄ (B) at 0.03 mmol Gd/kg in nu/nu mice. Tumor labeled with red circle and bladder labeled with green circle. [Click here to view larger figure.](#)

Discussion

Critical Steps

Selection of Proper Biomarker and Targeting Small Peptide

To successfully develop a targeted contrast agent with small size, two key points need to be considered. First, it is important to select proper molecular biomarkers which are abundantly present in diseased tissues with little presence in normal tissues. Our selected cancer-related biomarker, clotted plasma proteins, meets this requirement. Second, the selected targeted small molecular agents should have high binding affinity for the biomarkers so that sufficient contrast agents can bind to these targets, resulting in enough contrast enhancement. In this study, we chose the clot-binding peptide CLT1 that shows strong specific binding to the clotted plasma proteins in the tumor¹¹.

Contrast Agent Synthesis

The peptide synthesis involves oxidizing two thiol groups in the linear peptide molecule to form final cyclic CLT1 peptide. Whilst peptide cyclisation is usually conducted in solution at low concentration to minimize potential aggregation, dimerization and oligomerization, on-resin cyclization takes advantage of the pseudo-dilution phenomenon as well as the removal of reagents by simple washing and filtration¹³. In our synthesis procedure, we find that on-resin cyclization using thallium(III) trifluoroacetate works well with high yield and little dimerization, although thallium(III) trifluoroacetate is very toxic and care must be taken while handling it. A second approach uses 20% DMSO in solution at low peptide concentration. However more steps are needed to remove DMSO, and lyophilizing the large volume solution takes longer. By using either method, it is crucial to make sure of the complete formation of intramolecular disulfide bond. When complexing the peptide ligand with Gd, the free thiols can induce substantial aggregations because trace metals in the buffer catalyze air oxidation, resulting in the overoxidation of cysteine to form polymerization¹⁴. This usually dramatically decreases the yield and purity of final product. To make sure complete disulfide bond formation, first we need to use mild oxidation conditions to avoid formation of intermolecular disulfide bond resulting in dimer or higher oligomer formation. Secondly, long enough reaction time should be maintained to make sure that all thiol groups are oxidized before the Gd conjugation step.

Animal Model and Cannulation of Mouse Tail Vein

Preparation of the animal tumor model and tail vein cannulation are also very important aspects of this procedure. During the surgery, the tools/bench must be kept clean and sterile to avoid potential infection which could injure or kill the mice. In order to avoid moving the mouse before and after injection, it is necessary to cannulate mouse tail vein. To prepare the catheter for cannulation, break a 30 gauge needle from the hub and insert the blunt end into a 1.0 m long tubing, and connect another end of the tubing to a syringe loaded with 1% heparinized saline. Push the syringe to fill up the tubing. Dilate the tail with warm water (~105 °F). Insert the needle into the vein. Blood backflow into the tubing indicates successful insertion of the catheter. To verify that the needle is in the vein, gently push the syringe and the saline can be smoothly injected, the vein is cleared with the injection of a small amount of saline. Fix the catheter in place with tape before moving the mouse to the scanner.

Limitations and Possible Modifications

There are several limitations in our current study. First, from the preliminary results we notice that CLT1 washes out very quickly from the targeting site although it shows excellent binding to the tumor. *In vivo* proteolytic degradation likely causes the instability of CLT1. Modification of CLT1 to protect against proteolytic degradation should improve its stability and prolong its wash-out time *in vivo*. Using D- type amino acids to replace natural L- type amino acids is a potential strategy to achieve this goal. Second, the current study doesn't clarify which exact components in the clotted plasma proteins the CLT1 peptide binds to. Finally, we synthesize the contrast agents mainly using solid phase synthesis with relatively low yield and high cost. Developing a liquid phase synthesis method should increase the yield and lower the cost.

Applications

We are developing techniques for accurate detection of small malignant tumors with contrast enhanced MRI. MRI with the targeted agent provides a more effective and safer alternative for cancer molecular imaging.

Disclosures

No conflicts of interest declared.

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