靶向对比剂CLT1-(Gd-DTPA)在小鼠乳腺癌磁共振分子成像效果的研究

Furong Ye[1], Eun-Kee Jeong[2], Denis Parker[2], Zheng-Rong Lu[3*]

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作者单位：
1. Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah, USA;
2. Department of Radiology, University of Utah, Salt Lake City, Utah, USA;
3. Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106, USA

通讯作者：
Zheng-Rong Lu, E-mail: zxl125@case.edu

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[摘要] 本文研究了一个小肽靶向的对比剂CLT1-(Gd-DTPA) 对肿瘤基质中的纤维蛋白——链接纤维蛋白复和物的磁共振分子影像的效能。所用动物模型为带有MDA-MB-231乳腺癌的母系裸鼠，所用仪器为西门子3T磁共振成像仪，对比剂为Gd-(DTPA-BMA)，所用CLT1-(Gd-DPTA)剂量为0.05 mmol/kg。该对比剂特一性地结合到肿瘤并产生明显的对比增强，增强效果至少持续60分钟。相比之下，非特一性对比剂Gd(DTPA-BMA)很快从体内排除，30分钟后几乎无任何增强效果(0.1 mmol/kg)。CLT1-(Gd-DTPA)在血液，肝和肌肉等正常组织中几乎无非特一性结合，其在正常组织中的增强效果和对比剂相当。此研究证明CLT1-(Gd-DTPA)对肿瘤的特一性及其在小鼠乳腺癌模型中的肿瘤增强效果。该靶向性对比剂可以用于乳腺癌的磁共振分子成像。

[关键词] 磁共振成像；分子成像；对比剂；小鼠；乳腺癌

Evaluation of CLT1-(Gd-DTPA) for MR molecular imaging in a mouse breast cancer model

Furong Ye[1], Eun-Kee Jeong[2], Denis Parker[2], Zheng-Rong Lu[3*]
[1]Department of Pharmaceutics and Pharmaceutical Chemistry, University of Utah, Salt Lake City, Utah, USA.
[2]Department of Radiology, University of Utah, Salt Lake City, Utah, USA
[3]Department of Biomedical Engineering, Case Western Reserve University, Cleveland, OH 44106, USA
[*]*Correspondence to: Dr. Zheng-Rong Lu, E-mail: zxl125@case.edu*
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Abstract A peptide targeted contrast agent, CLT1-(Gd-DTPA), was investigated for molecular imaging of fibrin-fribronectin complexes in tumor stroma with magnetic resonance imaging (MRI). The contrast agent was evaluated in female nude mice bearing MDA-MB-231 human breast carcinoma xenografts on a Siemens 3T clinical scanner with a clinical agent Gd (DTPA-BMA) as a non-targeted control. CLT1-(Gd-DTPA) specifically bound to tumor tissue and resulted in significant tumor contrast enhancement at a dose of 0.05 mmol/kg for at least 60 minutes after injection. In contrast, a non-targeted contrast agent, Gd(DTPA-BMA), cleared rapidly from the body with little tumor enhancement after 30 minutes post-injection at a dose of 0.1 mmol/kg. CLT1-(Gd-DTPA) had little non-specific binding in blood and normal tissues, including the liver and muscle, resulting in comparable non-specific enhancement in normal tissues to the control agent. The study has shown that CLT1-(Gd-DTPA) can bind to the tumor tissue, resulting in significant tumor enhancement in a mouse breast cancer model. The targeted contrast agent has a potential for MR molecular imaging of breast cancer.
Key words Magnetic resonance imaging; Molecular imaging; Targeted contrast agent; Mouse; Breast cancer

Introduction

Magnetic resonance imaging (MRI) is a powerful imaging modality for morphological and functional imaging. MRI provides anatomical images of soft tissues with high spatial resolution, but is often limited for molecular imaging because of its low sensitivity[1-3].

Significant efforts have been devoted to the design and development of effective targeted MRI contrast agents for molecular imaging of cancer biomarkers expressed on cancer cell surfaces in the last three decades. Targeting agents, e.g. peptides, antibodies and proteins, have been conjugated to polymers or nanoparticles containing a large number of Gd (III) chelates to increase local concentration of contrast agents and to generate detectable MR signals[4-8]. However, the targeted contrast agents based on these polymers or nanoparticles are too large to be excreted from the body via renal filtration, resulting in prolonged tissue retention. Long-term tissue accumulation of Gd (III) based contrast agents may release Gd (III) ions and cause toxic side effects such as systemic nephrogenic fibrosis[9,10]. An innovative design of safe and effective targeted MRI contrast agents is necessary for satisfying the unmet needs for MR cancer molecular imaging.

We have recently hypothesized that effective MR cancer molecular imaging can be achieved by targeting the molecular biomarkers with high expression in tumor stroma using the agents that can be readily excreted[11-13]. Tumor stroma has a unique extracellular matrix composed of cancer related biomacromolecules needed for cancer cell survival and proliferation. For example, fibrin and fibronectin in tumor stroma are known to associate with increased microvessel permeability and tumor angiogenesis in neoplastic tissues[14,15]. Fibrin and fibronectin are highly expressed and form complexes in the mesh network of malignant tumors. Their complexes could be a suitable biomarker for cancer molecular imaging with MRI. We have recently synthesized and tested a CLT1-(Gd-DTPA) as a targeted MRI contrast agent for cancer molecular imaging[11]. CLT1 is a cyclic decapeptide, CGLIIQKNEC, that specifically binds to the fibrin-fibronectin complexes in various tumor tissues with little non-specific binding to normal tissues[16]. Our initial study has shown that the agent is effective for MR cancer molecular imaging in a mouse colon cancer model. In this study, we further evaluated the efficacy of CLT1-(Gd-DTPA) for cancer molecular imaging in mice bearing MDA-BM-231 breast tumor xenografts.

1 Materials and Methods

1.1 Synthesis of CLT1-(Gd-DTPA)

The CLT1 peptide CGLIIQKNEC was first synthesized using standard solid-phase peptide synthesis from Fmoc-protected amino acids on a 2-chlorotrityl chloride resin. At the end of the peptide synthesis, an excess of DTPA dianhydride in DMSO was reacted with the peptide on the beads at room temperature for 4 hours to conjugate DTPA at the N-terminal of the peptide. The resin was completely washed with water, DMF, dichloromethane and methanol three times each. The CLT1-DTPA was then removed from the resin using a TFA solution (TFA 94%, 1, 2-ethanedithiol 2.5%, triisobutylsilane 2.5%, and water 1%). The product was exposed to air for about 2 hours to allow the formation of disulfide bonds for the cyclic peptide and then purified using preparative HPLC with a C18 column. CLT1-(Gd-DTPA) was finally prepared by complexation of CLT1-DTPA with Gd $(OAc)_3$ at pH 6. Excess Gd $(OAc)_3$ was removed by precipitation at pH 11. The final product was purified by preparative HPLC.

1.2 Animal model

Human breast carcinoma cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC, Manassas, VA). The MDA-MB-231 human breast cancer cells were cultured in Leibovitz's L-15 medium with 2 mM L-glutamine and 10% FBS. Female athymic nu/nu mice (6 weeks old) were purchased from the National Cancer Institute (Frederick, MD). The mice were cared for according to the guidelines of the IACUC, University of Utah. The mice were subcutaneously implanted in both lower flanks with 2×10^6 MDA-MB-231 cells in a mixture of 50 μl culture media and 50 μl Matrigel. Mice were used for MRI study when tumor sizes reached 0.5-0.8 cm.

1.3 MR imaging

MRI study was performed on a Siemens Trio 3T scanner using a human wrist coil[17]. A clinical contrast agent, Gd(DTPA-BMA), was used as a control. A group of 3 mice were used for each contrast agent. The mice were anesthetized by intramuscular administration of a mixture of ketamine (45 mg/kg) and xylazine (6 mg/kg) for MRI. The CLT1-(Gd-DTPA) and Gd(DTPA-BMA) was intravenously injected at a dose of 0.05 and 0.1 mmol/kg, respectively. High resolution 3D images were acquired with a 3D FLASH sequence with 25° flip angle, TR/TE=7.8/2.7 ms, 0.5 mm slice thickness, 120 mm field of view (FOV), $0.5\times0.5\times0.5$ mm^3 voxel size. T1-weighted 2D axial tumor images were

acquired with a 2D spin echo sequence with 90° flip angle, TR/TE = 400/10 ms, 2.0 mm slice thickness, 50 mm FOV, and 0.5×0.5×2 mm^3 voxel size. Contrast enhanced MR images were acquired before and at 1, 5, 10, 15, 20, 30 and 60 minutes after injection. MR images were analyzed with Osirix (http://homepage.mac.com/rossetantoine/osirix/) software. The signal intensity was measured in the tumor periphery and inner core, and the signal to noise ratio (SNR) in the tumor tissues was calculated as SNR= $(SI_{tissue}-S_{Inoise})/SD_{noise}$. MR signal intensity was also measured in the tissue of interest from high-resolution 3D images and SNR was calculated in these tissues. Statistical analysis was performed with Prism software (Version 4.0b, GraphPad software Inc., San Diego, CA) using two-way repeated ANOVA. Bonferroni post-test was used to determine the significant difference in the comparisons among the conjugates. Statistical significance was considered when $P < 0.05$.

1.4 Histological analysis

Immunohistochemistry was performed to evaluate the expression of fibronectin in tumor tissue. Mice bearing MDA-MB-231 tumor xenografts were sacrificed, and tumor tissues were removed and fixed with 3% paraformaldehyde and embedded in paraffin. Tumor tissue was sectioned into 4 μm slices and incubated in 3% hydrogen peroxide, 10% methanol for 10 min at room temperature to block endogenous peroxidase activity. The tumor sections were then boiled in antigen retrieval solution (1 mmol/L Tris-HCl, 0.1 mmol/L EDTA, pH=8.0) for 15 minutes at high power in a microwave and incubated with primary anti-fibronectin antibody (Sigma-Aldrich, cat#F3648) at appropriate dilutions overnight. After washing with PBS buffer, the sections were incubated with biotinylated secondary antibody and a horseradish peroxidase-streptavidin complex for 1 h each. Tissue samples were then colorized with 3, 3' diaminobenzidine (DAB) substrate, counterstained, mounted and visualized with a bright-field microscope.

2 Results and Discussion

The structure of CLT1-(Gd-DTPA) is shown in Figure 1. Gd-DTPA is a clinical MRI contrast agent. The cyclic peptide CLT1 was conjugated to one of the five carboxylic groups of DTPA. The final product had four carboxylates, one amide carbonyl group and three

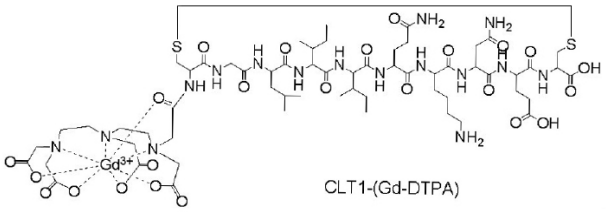

Figure 1 Chemical structure of CLT1-(Gd-DTPA)

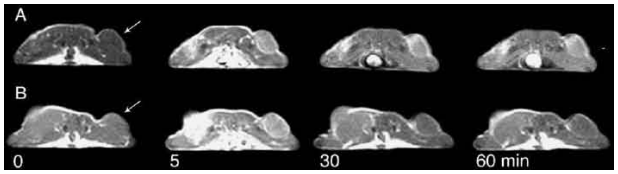

Figure 2 T1-weighted 2D spin-echo images of mice bearing MDA-MB 231 xenografts before and at 5, 30 and 60 minutes after intravenous injection of CLT1-(Gd-DTPA) (A, 0.05 mmol/kg) and Omniscan® (B, 0.1 mmol/kg). Arrows point to the tumor.

amino groups complexed to a Gd(III) ion. It should have a thermodynamic stability higher than the clinical agent, Gd(DTPA-BMA). T1 and T2 relaxivities of CLT1-(Gd-DTPA) were 4.22 and 4.45 $mM^{-1}sec^{-1}$ at 3T, comparable to other Gd(III) based clinical MRI contrast agents.

The effectiveness of CLT1-(Gd-DTPA) for MR molecular imaging of fibrin-fibronectin complexes in tumor stroma was evaluated in female athymic nu/nu mice bearing MDA-MB-231 human breast carcinoma xenografts. Figures 2 shows the axial T_1-weighted 2D spin-echo images of the tumor tissues of the mice bearing MDA-MB-231 tumor xenografts before and after the injection of CLT1-(Gd-DTPA) and Gd(DTPA-BMA). Significant enhancement was observed in tumor tissues for both agents in the first 5 minutes post-injection. Gd(DTPA-BMA) was then cleared from the tumor tissue and tumor enhancement returned to the background level after 30 minutes post-injection. Strong enhancement was still visible in the tumor tissues at 60 minutes after injection for CLT1-(Gd-DTPA). The enhancement of the targeted agent in the tumor periphery was more significant than that in the tumor core. Figure 3 shows the signal-to-noise ratios (SNR) in the tumor periphery before and at various time points after injecting the contrast agents. The SNR in the tumor tissue with Gd(DTPA-BMA) reduced to the background level at 30 minutes post-injection, while approximately 30% increase of SNR was observed at 60 minutes after the injection in the tumor periphery with

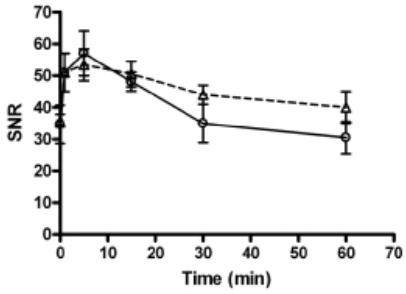

Figure 3 The plots of SNR versus time in tumor periphery before and after injection of CLT1-(Gd-DTPA) (△) and Omniscan® (○).

the targeted contrast agent. The SNR indirectly reflects the concentration of the agents in tumor, with higher SNR indicating higher concentrations of the contrast agents in the tissues. The results indicate the binding and retention of the targeted contrast agent in tumor tissue for significant tumor enhancement.

MR signal intensity with the contrast agents in other regions of interest was also determined in the high-resolution 3D MR images of mice to preliminarily evaluate their biodistribution and pharmacokinetic properties. Figure 4 shows SNR in the blood, liver and muscle of the mice injected with CLT1-(Gd-DTPA) and Gd(DTPA-BMA). CLT1-(Gd-DTPA) had similar blood SNR kinetics as Gd(DTPA-BMA), indicating that the targeted agent had similar pharmacokinetics as the clinical agent with little binding to the soluble fibronectin and fibrinogen in the blood. The blood SNR decreased rapidly for both agents and almost returned to the background level at 60 minutes after the injection. CLT1-(Gd-DTPA) had higher initial SNR in the liver than Gd(DTPA-BMA), possibly due to the lipophilic nature of the peptide. The SNR of the targeted agent in the liver then returned to the similar level as that of Gd(DTPA-BMA) at 60 minutes after the injection. Both agents resulted in minimally increased SNR in the muscle. The results suggest that CLT1-(Gd-DTPA) behaved as a low molecular weight contrast agent and had little non-specific binding to normal tissues.

Immunohistochemistry confirmed the presence of fibronectin in the MDA-MB-231 breast cancer xenografts after in vivo MR imaging. Figure 5 shows the histological images of fibronectin in MDA-MB-231 tumor tissues. The high expression of fibronectin was shown in the tumor stroma with staining of an anti-fibronectin antibody. The abundant presence of fibrin-fibronectin complexes in tumor stroma allowed specific and prolonged binding of a sufficient amount of CLT1-

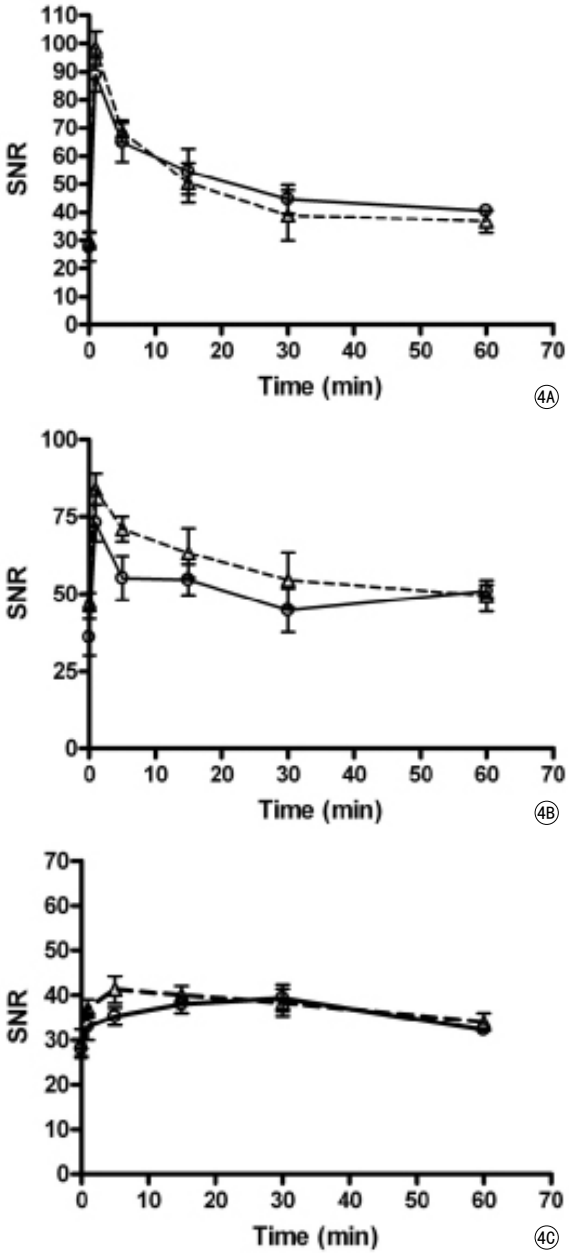

Figure 4 Plots of SNR versus time in the blood (A), liver (B), and muscle (C) of mice bearing MDA-MB 231 xenografts before and after injection of CLT1-(Gd-DTPA) (△, 0.05 mmol/kg) and Omniscan® (○, 0.1 mmol/kg).

(Gd-DTPA) to generate measurable enhancement in the tumor tissue.

It is difficult for contrast enhanced MRI to effectively detect the biomarkers expressed on cancer cells because of its low sensitivity. We have shown that contrast enhanced MRI can be effective for molecular imaging of cancer biomarkers abundantly

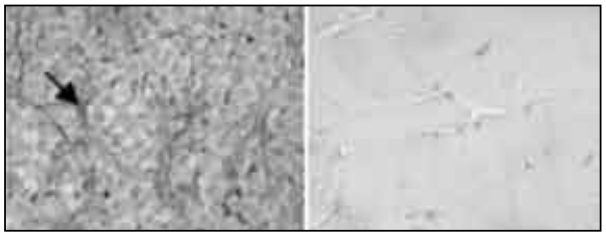

Figure 5 Immunostaining of fibronectin in MDA-MB-231 breast tumor xenografts (right) and muscle tissue (right) with anti-fibronectin primary antibody. The arrow points to the fibronectin in the extracellular space of tumor tissue.

expressed in tumor stroma. The presence of cancer-related biomacromolecules in tumor stroma facilitates cancer cell survival and promotes tumor proliferation and metastasis[14,15]. These biomacromolecules can be used as viable biomarkers for cancer diagnosis and prognosis. Fibrin and fibronectin form clot complexes upon fibrin polymerization in tumor stroma and serve as a provisional matrix for adhesion and migration of cancer cells. Due to the abundant presence of the fibrin-fibronectin complexes in the tumor stroma, a sufficient amount of CLT1-(Gd-DTPA) could specifically bind to the molecular targets. CLT1-(Gd-DTPA) had little non-specific binding to the proteins in the blood and normal tissue. Since the targeted contrast agent was a low molecular weight chelate, the unbound agent could readily be cleared from blood circulation and normal tissues. Consequently, significant tumor enhancement with little background enhancement was observed with the targeted agent since 30 minutes after the injection at a reduced dose. The low molecular weight targeted contrast agent is advantageous as compared to targeted macromolecular contrast agents for further clinical development due to rapid excretion and minimal retention in normal tissues.

CTL1-(Gd-DTPA) resulted in significant enhancement in the tumor periphery of the breast tumor tissue, the regions rich of angiogenic microvessels, similar to the enhancement in the colon cancer model reported in our previous publication[11]. It has been known that the presence of fibrin and fibronectin in tumor extracellular matrix might promote tumor angiogenesis[14,15]. Strong enhancement with the targeted contrast agent in tumor periphery suggested high expression of the fibrin-fibronectin complexes in the highly angiogenic regions of the tumor tissue. This result correlated well to the possible biological functions of fibrin-fibronectin complexes in cancer biology. Accurate characterization of tumor angiogenesis is critical for cancer diagnosis and prognosis and for assessment of tumor response to anticancer therapies. MRI with CLT1-(Gd-DTPA) has a potential to be used for characterizing angiogenesis in breast cancer and for non-invasive evaluation of the efficacy of antiangiogenesis therapy.

3 Conclusion

The targeted contrast agent CLT1-(Gd-DTPA) had minimal non-specific binding in blood and in normal tissues. A sufficient amount of CLT1-(Gd-DTPA) specifically bound in the breast tumor and generated strong and prolonged enhancement in the tumor tissue for effective molecular imaging of breast cancer with MRI. CLT1-(Gd-DTPA) is a promising low molecular weight targeted contrast agent for MR molecular imaging of the fibrin-fibronectin complexes in breast cancer. It has a great potential for the accurate detection and diagnosis of breast cancer.

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Brief introduction of corresponding author:

Dr. Zheng-Rong Lu is M. Frank and Margaret Domiter Rudy Professor of Biomedical Engineering at the Department of Biomedical Engineering, Case School of Engineering, Case Western Reserve University. Dr. Lu received his B.S. and M.S. in Chemistry from Lanzhou University, and Ph.D. in Chemistry from Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences at Lanzhou, China. In 1992, Dr. Lu was hired as an Associate Professor of Chemistry and promoted to Professor of Chemistry shortly after at Wuhan University in China. In 2002, Dr. Lu was appointed to Assistant Professor in the Department of Pharmaceutics and Pharmaceutical Chemistry at the University of Utah. Subsequently, Dr. Lu was promoted to a tenured Associate Professor in 2006. Dr. Lu's research efforts involve molecular imaging, image-guided photodynamic therapy, in vivo imaging of dose forms, polymeric drug delivery systems and multifunctional delivery systems for nucleic acid. He has over 100 peer-reviewed scientific publications, five book chapters and 70 abstracts and four US patents. He is a Principal Investigator of several major grants from the NIH. Dr. Lu serves on the scientific advisory board of Pharmaceutical Research, Molecular Pharmaceutics. Dr. Lu has made a significant contribution in the design and development of novel MRI contrast agents, including biodegradable macromolecular MRI contrast agents and targeted MRI contrast agents, to address the limitations of currently available MRI contrast agents.