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ORIGINAL RESEARCH ARTICLE

Preparation and preclinical evaluation of a Gd(III)-RGD peptide for MR molecular imaging in non-small cell lung carcinoma (NSCLC)

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ABSTRACT

Introduction: Excessive expression of the αvβ3 integrin receptors is seen in rapidly multiplying endothelial cells, including cancerous growth of various tumors. αvβ3 integrin receptors' specific targeting by peptides containing the RGD motif makes these short sequences a suitable nominee for diagnostic imaging and lung cancer follow-up. A high-affinity RGD-containing peptide is designed. The di-RGD peptide has a greater affinity along with tumor-selective targeting properties. Peptide labeling with gadolinium for magnetic resonance imaging was accomplished, permitting efficient cancer molecular imaging accompanied by high spatial resolution. This peptide will have better sensitivity for the early identification of tumors and is appropriate for follow-up routines.

Methods: DOTA-E(cRGDfK)$_2$ was labeled with Gd(III) effectively. The cytotoxicity to cells was measured. The biodistribution was evaluated in a mouse model for lung cancer. The very early diagnostic capacity of the Gd-RGD peptide was studied using MR molecular imaging.

Results: MR imaging shows high binding specificity of Gd(III)-DOTA-E(cRGDfK)$_2$ to A549 lung tumor in mice. Gd-DOTA-E(cRGDfK)$_2$ did not show cytotoxicity at high concentrations and on different cell lines. Biodistribution studies confirm tumor uptake up to 24h after the injection. The peptide-based contrast agent leaded to an improved tumor contrast enhancement at a dose of 0.1 mmol Gd/kg. The tumor uptake peaks were after 30 min of injection. A clear picture of the tumor was seen in all images.

Conclusion: Gd(III)-DOTA-E(cRGDfK)$_2$ can be used as a peptidic MR imaging contrast agent enabling initial detection of different cancers overexpressing the αvβ3 integrin receptors and can be a prospective candidate in clinical studies of non-small cell lung carcinoma.

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INTRODUCTION

Cancer is the second leading cause of death globally and, it is expected to surpass heart disease to become the No. 1 killer by 2030 [1]. About 7 million people die annually from cancer-related causes per year, and it is estimated that by 2030 there will be more than 16 million new cancer cases every year [2]. Lung carcinoma is amongst the most frequently occurring and fatal cancers affecting either gender, responsible for a great number of deaths because of weak diagnosis of this type of cancer [2]. This carcinoma is categorized into 2 major subtypes: SCLC (Small Cell Lung Carcinoma-15% to 20%) and NSCLC (Non-Small Cell Lung Carcinoma -80% to 85%) [1, 3].

In recent years, targeting tumor cells by RGD tripeptide has been a favorable approach for molecular imaging of lung cancer [4-6]. Integrin αvβ3, which is a receptor expressed in very high levels in many solid tumors but not in normal cells [7]. As αvβ3 levels correlate with tumor metastasis and aggressiveness, it is an important molecular imaging target for early tumor diagnosis. Many labeled linear and cyclic RGD peptides have been evaluated as radiotracers for SPECT and PET imaging [8-17]. As these nuclear imaging strategies are not easily available and require radiotracer administration, magnetic resonance imaging (MRI) can be applied for routine cancer imaging [18]. Besides, to prevent ionizing radiation exposure to patients, alternative methods such as magnetic resonance imaging (MRI) with high-relaxivity contrast agents, including low molecular weight and protein binding agents, dendrimers, liposomes, nanoparticles, and peptides have been developed [19, 20]. The majority of these contrast agents possess the lanthanide ion Gd3+, which creates positive contrast in T1-weighted images [20-24].

In the previous work, we evaluated the ^{68}Ga-DOTA-E(cRGDfK)$_2$ for a soon identification, staging, and post-treatment imaging of NSCLC. Results demonstrated perfect specifications of the radiotracer as an imaging agent in positron emission tomography (PET) [25]. The ability of the imaging agent in tumor targeting was studied in lung cancer mouse model. In addition, the therapeutic capability of the ^{177}Lu- DOTA-E(cRGDfK)$_2$ was assessed. SPECT imaging was performed in lung tumor-bearing mice to certify the potential of this radiopeptide to be applied as both a diagnostic and therapeutic agent [26].

In the current investigation, Gadolinium (Gd) was chosen for DOTA-E(cRGDfK)$_2$ labeling because of its desirable physical and chemical properties in imaging.

METHODS

Non-radioactive DOTA-E(cRGDfK)$_2$ was synthesized by Futurchem (Korea) with a high purity of >99%. Gadolinium (III) chloride hexahydrate was obtained from Alfa Aesar (Germany). All other reagents for chemical synthesis were purchased from Sigma-Aldrich except where indicated otherwise. Non-small-cell lung carcinoma (A549), Swiss mouse embryo fibroblast (NIH-3T3), and human prostate cancer (PC3) cell lines were obtained from Pasture Institute of Iran. All cells are grown in RPMI and DMEM/F12 medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA), and 1% penicillin and streptomycin (Gibco, USA) at 37°C in 5% CO2. Dimethyl Sulfoxide (DMSO) was obtained from DNAbiotech (Iran).

Animals' studies were carried out upon the Research committee of Tehran University of Medical Sciences approval.

Peptide-based MRI contrast agent

For labeling of DOTA-E(cRGDfK)$_2$ with Gadolinium, GdCl3 and DOTA-E(cRGDfK)$_2$ were mixed. To eliminate free Gd, dialysis using a dialysis membrane (MWCO of 0.5-1 KDa, Trial kit Spectra/Por® Biotech CE, USA) against ddH2O was performed for 2h at room temperature. The solution was freeze-dried overnight for further use.

Biodistribution of Gd(III)

The Gd analysis was based quantifying Gd in several samples using an inductively coupled plasma-mass spectrometry (ICP-MS) method. Tissue samples were thawed before analysis. Each sample was weighed, digested, and analyzed for Gd content. Before the elemental analysis, tissue samples of interest were digested in 5 mL nitric acid (32% v/v) for a week.

Cell culture and Cytotoxicity studies of Gd-DOTA-E(cRGDfK)$_2$

Cell viability/cytotoxicity was probed by MTT assay. A549 cells were seeded at 4000 cells density per well in 96-well assay plates. After 24 h of incubation, increasing concentration (0-200 µM) of Gd-DOTA-E(cRGDfK)$_2$ in PBS were added and further incubated for 24 h. the cells' medium was replaced with 25 µL of MTT reagent (0.5 mg/mL in PBS) and incubated for 3 hours at 37°C and 5% CO2. Then, for the dissolution of formazan crystals, 100 µL of DMSO was added into each well, and the absorbance was read at

540 nm using a microplate reader (BioTek® 800™ TS, USA).

To investigate cell viability at different time points, A549, NIH3T3, and PC-3 cells were seeded at a density of 4000 cells/well in 96-well assay plates. Cell viability was assessed at 4h, 12h, and 24h after treatment with 0.1 mmol/L Gd-DOTA-E(cRGDfK)$_2$. The procedure is the same as above.

Animal model

All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Tehran University of Medical Sciences. 6-8 week-old female BALB/c mice with average body weights of 20–25 g were obtained from Royan Insitute of Iran (Amol, Iran). Each mouse was injected subcutaneously in the right dorsal flank with cell suspension (2 million cells) of human lung adenocarcinoma A549 in 100µl phosphate-buffered saline. Mice were kept in standard cages under regulated temperature (25ºC) and humidity (around 50%), twelve-hour light/dark cycles with free access to food and water. Palpable tumors diameters were measured by digital calipers twice a week. When tumors size achieved 50–100 mm3, animals received an injection of 0.1mmol/kg Gd-labeled peptide into the tail vein.

In vivo MR imaging

Mice were placed in a MRI scanner (Siemens MAGNETOM Avanto 1.5T MRI System), and MR images were obtained before injection and 30 min, 1, 2, 3h post-injection using brain coil and MRI protocol (Table 1).

The relaxivity of a MR contrast agent is a crucial parameter, which determines the contrast of images. It shows the proton relaxation rate of the surrounding water as a function of concentration, and it is directly related to the agent's contrast-enhancing capability throughout imaging time. The relaxivity of the peptide-based contrast agents was examined at 37°C in water, pH 7.4 and 1.5T compared to Gd-DOTA (Table 2).

Table 1. MRI protocol

Parameter	T1 **Weighted**
Slice thickness	2 mm
Repetition time (TR)	1070 msec
Echo time (TE)	12 msec
Echo number	1
Field of view	98 * 113 mm
Matrix size	512 * 448
Voxel size	0.22 * 0.22 * 0.22 mm3
Number of excitation	6
Acquisition time	20 min

Table 2. Relaxivities of the peptide-based contrast agents in 1.5 T in water, 37 °C

Agent	**r1 (L/mmol-s)**	**r2 (L/mmol-s)**
Gd-DOTA	2.9	3.2
Gd-DOTA-E(cRGDfK)$_2$	6.8	7.5

Statistical analysis

All data were represented as mean ± SD. The statistical analysis was performed by Student's t-test, and $p < 0.01$ was considered statistically significant.

RESULTS

Biodistribution of Gd(III)

Biodistribution of Gd(III) in the major organs and tissues of the mice at 24 h after intravenous injection of Gd-DOTA-E(cRGDfK)$_2$ at a dose of 0.1 mmol-Gd/kg in tumor-bearing mice is shown in Figure 1.

This figure shows much lower retention of Gd(III) in the main organs, including kidneys, liver, lung, femur, muscle, and spleen 24 h post-injection in comparison to previous reports of Gd-DOTA and significant uptake in the tumor.

Cytotoxicity studies of Gd-DOTA- E(cRGDfK)$_2$

The results of Gd-DOTA-E(cRGDfK)$_2$ cytotoxicity study on cell growth using MTT assay on cell line A549 (non-small lung cancer) showed that viability of cells treated with increasing concentrations of labeled peptide remained unchanged, and the cells maintained their viability up to a concentration of 200 µM.

Therefore, up to concentrations above 200 µM Gd-DOTA-E (cRGDfK)$_2$ does not cause toxicity to cells. The results are shown in Figure 2.

In addition, the MTT assay was performed on three cell lines: A549 (non-small cell lung cancer) NIH-3T3 (healthy fibroblast cell), and PC-3 (prostate cancer). Changes in cell viability at 4, 12, and 24 hours after treatment of different cell lines with a concentration of 0.1 mmol /L of Gd-DOTA-E(cRGDfK)$_2$ are shown in Figure 3. The results showed that Gd-DOTA-E(cRGDfK)$_2$ did not cause toxicity on these cell lines.

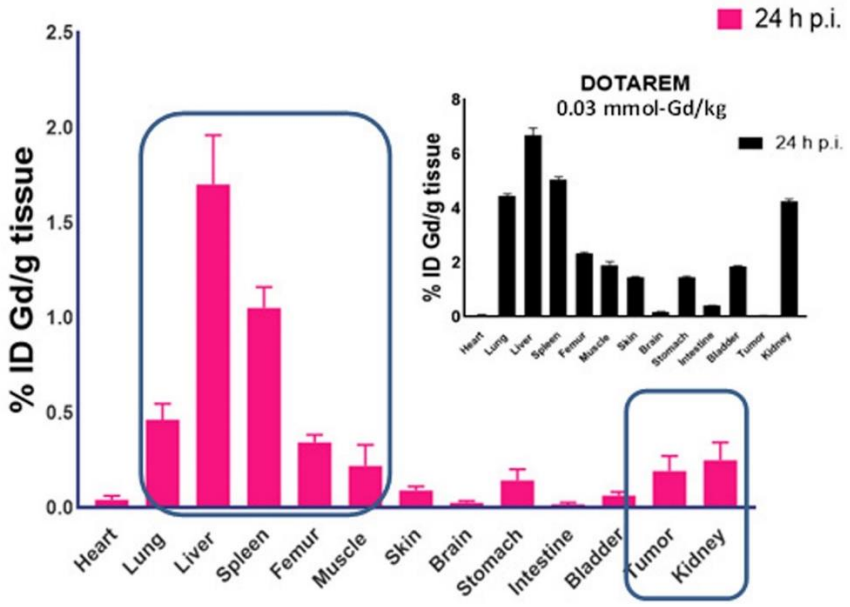

Fig 1. Biodistribution of Gd(III) in the major organs and tissues of the mice at 24 h after intravenous injection of Gd-DOTA-E(cRGDfK)$_2$ at a dose of 0.1 mmol-Gd/kg in tumor-bearing mice

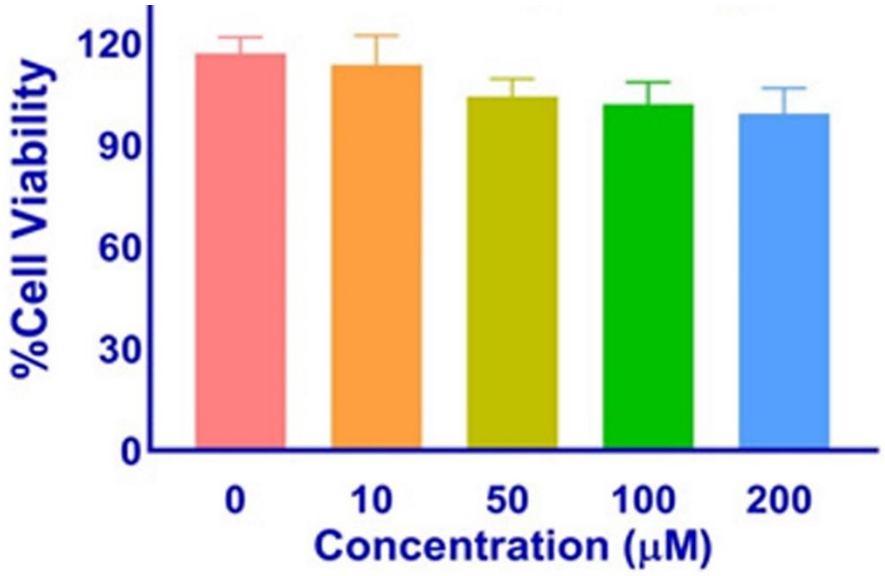

Fig 2. Viability changes of A549 cell line after 24h treatment with different concentrations (10 to 200 µM) of Gd-DOTA-E(cRGDfK)$_2$

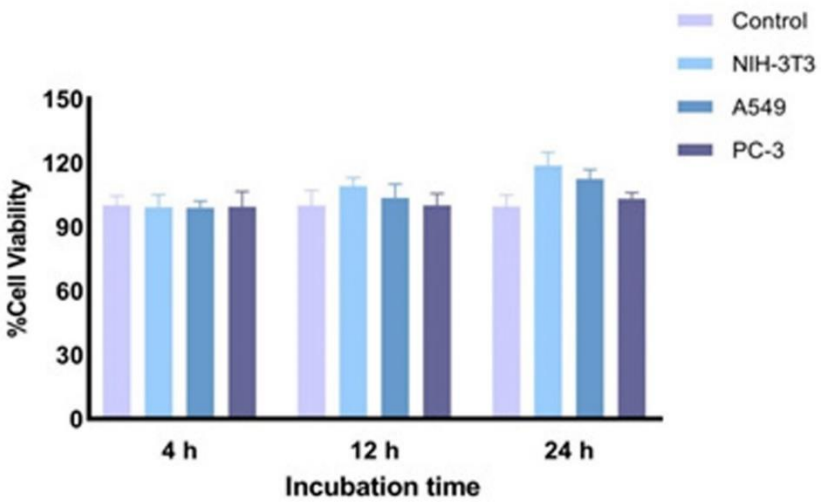

Fig 3. Changes in cell viability at 4, 12, and 24 hours after treatment of different cell lines with a concentration of 0.1 mmol /L of Gd-DOTA-E(cRGDfK)$_2$

In vivo MR imaging

The MRI contrast enhancement of the peptide-based contrast agent was investigated in lung cancer mouse model. The T1-weighted spin-echo sequence before and at 30 min, 1, 2, 3-hour post-injection at a dose of 0.1 mmol/kg was shown in Figure 4.

As shown in the images above, the best image contrast was observed 30 minutes after injection. Gradually the contrast of the images decreased over time. In all post-injection images, the tumor is quite clear, indicating an effective increase in tumor site contrast and tumor-specific targeting of Gd-DOTA-E (cRGDfK)$_2$. Gd-DOTA-E(cRGDfK)$_2$ showed very good tumor targeting and uptake without toxicity. MRI images confirmed effective contrast enhancement at the tumor location. Therefore, this Gd-based contrast agent can be used for NSCLC diagnosis.

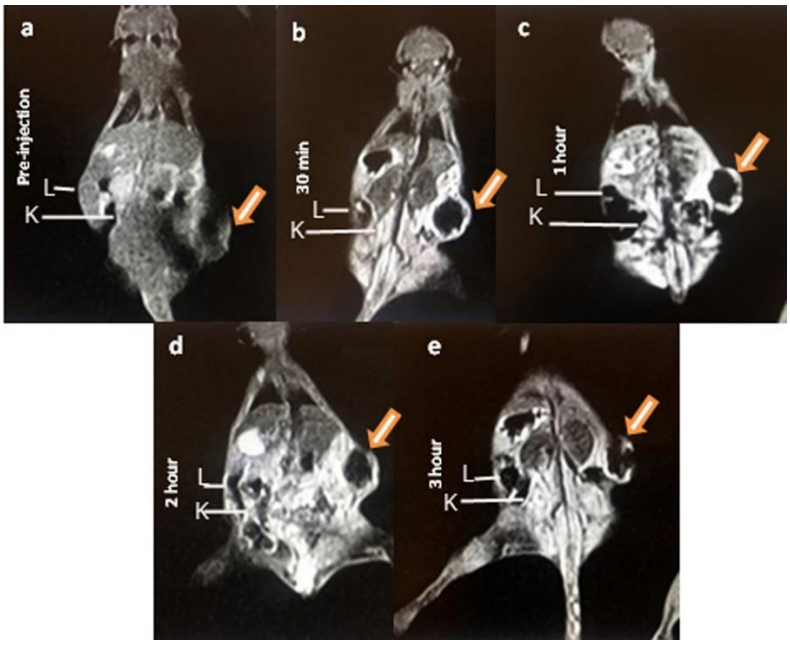

Fig 4. MR imaging of Gd-DOTA-E(cRGDfK)$_2$ in tumor-bearing BALB/c mice at (a) pre-injection (b) 30 min (c) 1h (d) 2h (e) 3 hour post intravenous injection. Tumor is clearly visualized at 30 min, 1h, 2h & 3h post-injection. K- Kidney, L- Liver

Relaxivity

The relaxivities of the peptide-based contrast agents were tested at 37°C in water, pH 7.4 and 1.5T compared to Gd-DOTA was summarized in Table 2.

DISCUSSION

MRI allows high-quality, high-resolution images of tumors and soft tissues. It is routinely used in cancer diagnosis, staging, response to treatment, and image-guided interventions. Nevertheless, the capability of MR imaging for precise identification of cancers such as lung cancer has not been fully exploited due to the non-specificity of available contrast agents. In recent years, targeting tumor cells by RGD tripeptide has been promising strategy for molecular imaging of lung cancer [4-6, 27]. Integrin αvβ3, which is a receptor expressed in very high levels in many solid tumors but not in normal cells [7]. As αvβ3 levels correlate with tumor metastasis and aggressiveness, therefore is an important molecular imaging probe for early tumor diagnosis. Many labeled linear and cyclic RGD peptides have been evaluated as radiotracers for SPECT and PET imaging [8-17]. As these nuclear imaging strategies are not easily available and require radiotracer administration, magnetic resonance imaging (MRI) can be applied for routine cancer imaging [18].

Gadolinium is used as a contrast agent in MRI imaging which has a very strong paramagnetic property that reduces T1 and T2 relaxation times and is seen in T1 images as an increase in signal strength. Clinically, this contrast enhancement by MRI contrast agents is used in the diagnosis and detection of lesions, including tumors, infections, venous arterial malformations, and to a lesser extent infarcts [19-22, 28]. In this study, gadolinium was bound to the E(cRGDfK)$_2$ peptide using a DOTA chelator. A high affinity RGD-containing peptide for αvβ3 integrin receptor is designed. Peptide labeling with gadolinium (Gd) for magnetic resonance imaging was accomplished, which permits efficient MR cancer molecular imaging accompanied by high spatial resolution. The MRI-specific contrast agent has a higher sensitivity for early detection and is also appropriate for follow-up routines.

MR imaging showed high binding specificity of Gd(III)-DOTA-E(cRGDfK)$_2$ to A549 lung tumor in mice. Gd-DOTA-E(cRGDfK)$_2$ did not show cytotoxicity at high concentrations and on different cell lines. Biodistribution studies confirm tumor uptake up to 24 h after the injection. The contrast agent resulted in improved tumor contrast enhancement in mice at a dose of 0.1 mmol Gd/kg. The peptide-targeted MRI contrast agent is promising for high-resolution MR molecular imaging of lung tumors. A clear picture of the tumor was seen in all images. The peptide targeted contrast agent also has a high relaxivity.

In this study, we have demonstrated the potential of MR molecular imaging of non-small cell lung cancer with peptide-based molecular contrast agent in animal tumor models. The strong, extended signal enhancement of the targeted contrast agents in the NSCLC model shows that it is favorable for efficient molecular MR imaging of lung cancer. However, more considerations regarding tissue retention and safety for clinical uses must be made.

Study limitation

The major limitation of our study was the lack of MRI for small animals. One of the biggest challenges of animal MRI concerns its poor resolution, longer durations of measurements, and physical noise caused by animal movements through breathing.

CONCLUSION

Gd(III)-DOTA-E(cRGDfK)$_2$ can be used as a peptidic MR imaging contrast agent enabling initial detection of various cancers overexpressing the αvβ3 cell surface receptors and can be a prospective candidate in clinical studies of non-small cell lung carcinoma. These results suggest that this tumor-targeting peptide can not only be used in nuclear imaging modalities but can also be used as a gadolinium-based contrast agent in MRI imaging.

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