

Targeting assay of a fusion protein applied in enzyme prodrug therapy

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Abstract. Tumor growth and metastasis are dependent on angiogenesis. The overexpression of integrin $\alpha_v\beta_3$ on angiogenic vessels and on numerous malignant human tumor cells suggests that these labeled ligands of integrin are potentially suitable for molecular imaging and in targeted therapy of tumors. In previous studies, we added a β -lactamase variant with reduced immunogenicity to the cyclic peptide RGD4C, resulting in the fusion protein RGD4C β L, which is suitable for use in targeted enzyme prodrug therapy (TEPT), a promising treatment for tumors. The targeting of the aforementioned fusion protein serves an important role in TEPT. In the present study, RGD4C β L was labeled with ^{125}I and the targeting effect on integrin-positive tumors was evaluated. The results demonstrated that the ^{125}I -RGD4C β L protein exhibited high levels of accumulation at the tumor site and rapid renal clearance, which revealed the potency and efficiency of RGD4C β L in TEPT.

Introduction

Targeted enzyme prodrug therapy (TEPT) is a novel type of treatment for tumors (1-3), which aims to release cytotoxic agents precisely at the tumor site using pairs of enzymes and corresponding prodrugs, to avoid systemic toxicity (4). The enzymes are directed to the tumor sites by targeted molecules, including antibodies (5), mesenchymal stem cells (6), polymers (7) and genes (8,9). Nontoxic prodrugs, which are substrates of the enzymes, are hydrolyzed by these enzymes in order to release the cytotoxic agents (3,10). Therefore, the conjugates of the targeted molecules and enzymes serve a

key role in TEPT. In previous studies we constructed a fusion protein, RGD4C- β -lactamase (RGD4C β L), which contains 2 motifs: RGD4C and β -lactamase, to develop a targeted enzyme prodrug system for the treatment of tumors (11,12). The targeting motif RGD4C (sequence, ACDCRGDCFCG) delivers β -lactamase, which hydrolyzes lactam-based prodrugs to release the chemotherapeutic agents at the tumor site. RGD4C is a ligand with a high affinity for integrin $\alpha_v\beta_3$, which is overexpressed on various tumor cells and angiogenic vessels. Due to the upregulation of the integrin on tumor cells, $\alpha_v\beta_3$ may be used as a target for treatment (13-18). RGD4C contains 2 disulfide bonds formed by 4 cysteine residues. The affinity of RGD4C for integrin $\alpha_v\beta_3$ is 20-fold greater than similar peptides with a single disulfide bond, and 200-fold greater than linear peptides (13).

RGD4C is suitable for incorporation into proteins and viruses by recombinant technology (19), therefore this cyclic peptide was chosen as the targeting motif. β -lactamase exists in bacteria, exhibits no isozyme in mammals and exhibits a high specificity for hydrolyzing lactam-based prodrugs. This ensures that β -lactamase is useful for targeted enzyme prodrug systems (20,21), and a variant with reduced immunogenicity and high catalytic activity was constructed for the use of β -lactamase in enzyme prodrug therapy (22). This variant was used as the catalytic motif.

Previous studies revealed that the fusion protein RGD4C β L retained a high catalytic activity on the lactam-based prodrug, and exhibited high plasma stability and low immunogenicity (11,12). In the present study, RGD4C β L was labeled with ^{125}I and the properties of the fusion protein were evaluated *in vivo*, to investigate the potency of the protein for use in TEPT.

Materials and methods

Materials. Na ^{125}I was purchased from Perkin Elmer Life & Analytical Sciences (Waltham, MA, USA), and dissolved in pH8-11 350 mCi/ml NaOH solution. Sephadex G-50 was sourced from GE Healthcare Life Sciences (Logan, UT, USA). Medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Hyclone; GE Healthcare Life Sciences. Human sera were obtained from the Tianjin Blood Center (Tianjin, China). All procedures using human sera were approved by the Ethics Committee of the Institute

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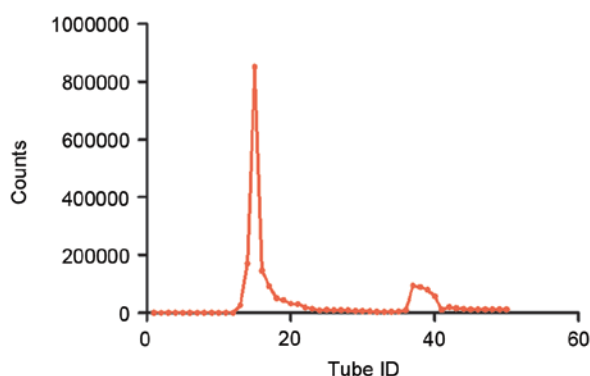


Figure 1. Separation and collection of the ^{125}I -RGD4C β L using column chromatography. The first peak is the ^{125}I -RGD4C β L, and the second is free ^{125}I .

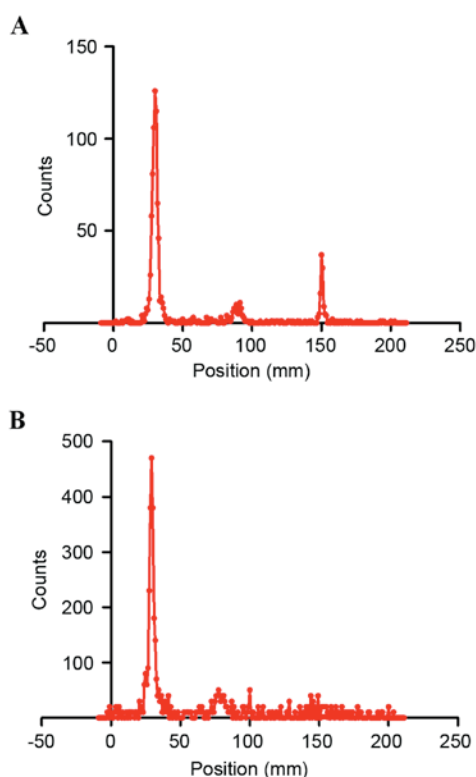


Figure 2. Labeling efficiency and radiochemical purity of the ^{125}I -RGD4C β L. The labeling rate and the radiochemical purity of the ^{125}I -RGD4C β L were determined using radio-thin layer chromatography. (A) The labeling efficiency was calculated to be 86.5%. (B) Following purification, the radiochemical purity of the ^{251}I -RGD4C β L was 100%, which is suitable for the *in vivo* imaging and the biodistribution assay.

of Radiation Medicine of the Chinese Academy of Medical Sciences (Tianjin, China) and all studies using human sera were conducted in accordance with the regulations of the Ethics Committee of the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences. Silica gel plates were purchased from Yantai JIANGYOU Silica Gel Development Co., Ltd., (Yantai, China). All other chemicals were obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The fusion protein RGD4C β L, 4.2 kDa, consisted of β -lactamase combined with ACDCRGDCFCG (RGD4C) by recombinant DNA technology. The fusion gene was cloned into *Escherichia coli* BL21 (DE3), and the protein was purified

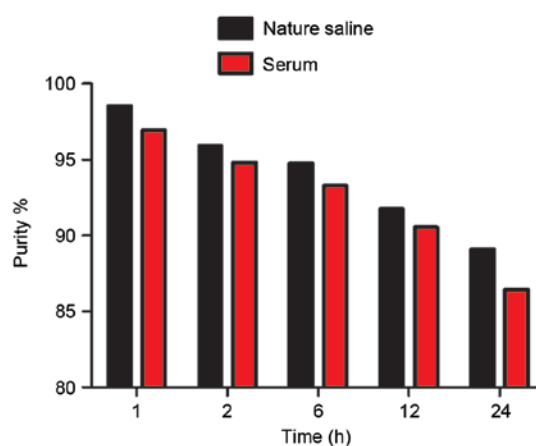


Figure 3. *In vitro* stability. The radiochemical purity of the ^{125}I -RGD4C β L remained at >89 and 86% over 24 h in normal saline at room temperature, 25°C, and in human serum at 37°C, respectively.

with Ni-NTA resin, and was additionally confirmed by western blotting as described previously (11,12).

Cell culture and animals. U87MG and A549 cells were obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China) and cultured in minimum essential medium and RPMI-1640 medium, with 10% fetal bovine serum, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 . Pathogen-free 6-7 week old female BALB/c nude mice were obtained from the Experimental Animal Center of Academy of Military Medical Sciences (Beijing, China). All animal procedures were approved by the Ethics Committee of the Institute of Radiation Medicine of the Chinese Academy of Medical Sciences (Tianjin, China), and all animal studies were conducted in accordance with the regulations of the Ethics Committee of the Chinese Academy of Medical Sciences (Tianjin, China).

Radioiodine labeling. A total of 3 μl Na^{125}I was dissolved in 100 μl phosphate buffer (PB) buffer (pH=7.4) and vibrated. A total of 200 μl protein solution, 0.5 mg RGD4C β L, was then added and the solution was mixed. Subsequently, 50 μl chloramine-t was added and allowed to react at room temperature for 3 min. A total of 50 μl sodium pyrosulfite, 20 mg/ml, was then added to stop the reaction, and the crude product was obtained. The dilution factor of ^{125}I was calculated as: Final volume/initial volume (1:134.3).

Purification and radiochemical purity. The crude product was transferred to a chromatography column filled with Sephadex G-50, which was pre-blocked by bovine serum albumin, and eluted by PB buffer (pH=7.4). The effluent was collected in tubes. The radioactivity in each tube was measured with a γ counter, (2470 WIZARD2, PerkinElmer, Inc., Waltham, MA, USA), and tubes 14-17 were collected. The final product, ^{125}I -RGD4C β L, was obtained and passed through a 0.22 μm Millipore filter to eliminate possible aggregates. Thin-layer chromatography (TLC) was then performed using an AR-2000 radio-TLC Imaging Scanner, Bioscan (Washington, DC, USA) to determine the labeling efficiency and the radiochemical

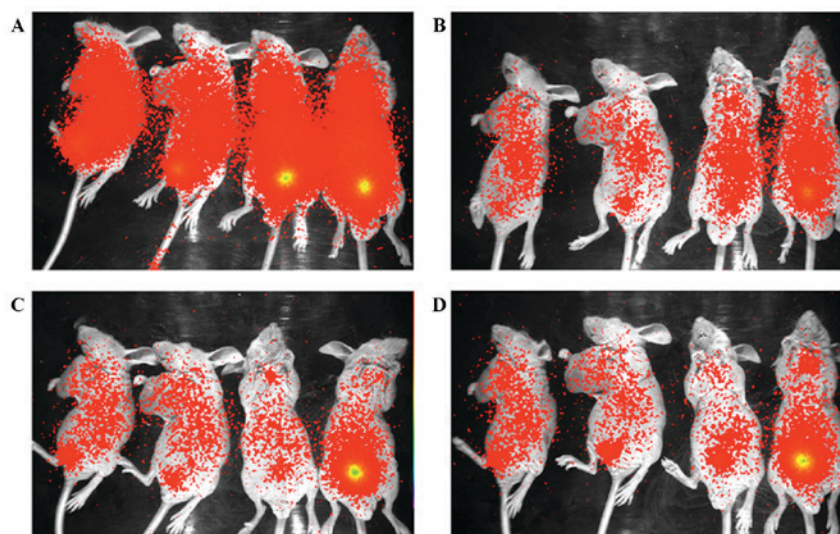


Figure 4. *In vivo* imaging of the ^{125}I -RGD4C β L in nude mice bearing xenografts at (A) 2, (B) 4, (C) 6 and (D) 8 h. The 2 mice on the left of the images were nude mice bearing A549 xenografts, and the 2 mice on the right were nude mice bearing U87MG xenografts. The levels of ^{125}I -RGD4C β L were reduced to background level at 24 h (data not included). Excretion of the ^{125}I -RGD4C β L occurred through the urinary system; as demonstrated in the images, there was intense radioactivity, yellow, in the bladder, which was higher than in the other organs of several mice.

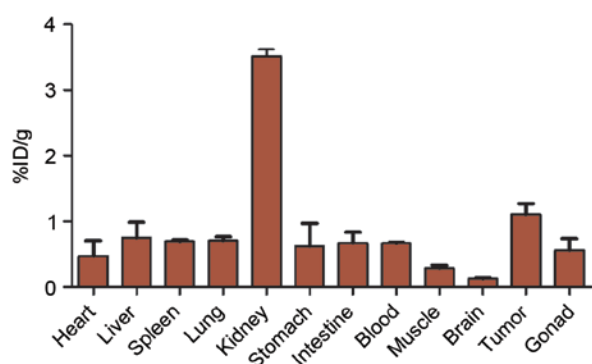


Figure 5. Biodistribution of ^{125}I -RGD4C β L in nude mice bearing A549 xenografts. The data were measured as %ID/g, and were expressed as the mean value \pm standard deviation, $n=4$. The highest dose percentile was in the kidneys, which is consistent with the whole-body imaging results and indicates that the fusion protein was metabolized through the urinary system. The uptake of ^{125}I -RGD4C β L in the tumors was increased compared with in the blood and the muscle tissues, with the ratios of ^{125}I -RGD4C β L levels in tumor vs. blood and in tumor vs. muscle measuring 1.7 and 3.8, respectively, which illustrated the suitability of the ^{125}I -RGD4C β L as an imaging agent for treatment monitoring, and confirmed the viability of RGD4C β L as a targeted enzyme within targeted enzyme prodrug therapy.

purity of the ^{125}I -RGD4C β L, directly subsequent to labeling and purification, using acetone as a developing solvent.

In vitro stability. In total, 2 portions of 100 μl of the ^{125}I -RGD4C β L were added to 500 μl normal saline at room temperature (25°C) and 500 μl human serum at 37°C respectively. The radiochemical purities were assayed by TLC at 1, 2, 6 and 24 h.

Tumor model and in vivo imaging. In the right flank (armpit region) of 6-7 week old BALB/c nude mice, 5×10^6 U87MG or A549 cells were implanted. The tumors were allowed to grow for 3 weeks subsequent to inoculation, then the animals received 7 μCi of the ^{125}I -RGD4C β L, in 200 μl PBS, via the

lateral tail vein under anesthesia. The images of the mice were taken using a small-animal imaging system (Kodak *In-Vivo* Imaging System Fx Pro, Kodak, Rochester, NY, USA) at several time points.

Biodistribution. A total of 4 mice bearing A549 xenografts were injected with 7 μCi of the ^{125}I -RGD4C β L via the tail vein. At 6 h post-injection, mice were anaesthetized, bled and dissected. The blood, tumor, heart, liver, spleen, lung, kidney, stomach, intestine, muscle, brain and gonad, were weighed and measured for radioactivity using a γ counter. The uptake of the ^{125}I -RGD4C β L was expressed as the percentage injected dose per gram body weight (% ID/g).

Statistical analysis. Data were analyzed using Prism software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA). The Student's t-test was used for statistical analysis of all data. Two-sided significance levels were calculated and $P < 0.05$ was considered to indicate a statistically significant difference. Values are presented as the mean \pm standard deviation of triplicate experiments.

Results

Purification and radiochemical purity. Following radio-labeling, the ^{125}I -RGD4C β L was purified using Sephadex G-50 chromatography and the fractions (tubes 14-17) were collected as the final product, as demonstrated in Fig. 1. The radiolabeling efficiency and radiochemical purity were assayed on silica gel plates, using acetone as the developing solvent. The radiolabeling efficiency in the present experiment was 82.6%, as illustrated in Fig. 2 A. The radiochemical purity of final product was almost 100%, as demonstrated in Fig. 2 B.

In vitro stability. Radiochemical purity of the ^{125}I -RGD4C β L remained >89 and 86% periodically, over 24 h in normal saline at room temperature and in human serum at 37°C, as

illustrated in Fig. 3, which demonstrated a positive prognosis for *in vivo* application.

In vivo imaging. Nude mice bearing A549 and U87MG xenografts were injected with 7 μ Ci of the 125 I-RGD4C β L. Fig. 4 demonstrated the results of the *in vivo* imaging at several time points. The colors reflected the distribution of the 125 I-RGD4C β L in mice, where red demonstrated tumor uptake of 125 I-RGD4C β L and yellow demonstrated excretion of 125 I-RGD4C β L by the urinary system.

Biodistribution. The results of the biodistribution of 125 I-RGD4C β L are demonstrated in Fig. 5, and the data is presented as the percentage dose per gram tissue (%ID/g). The distribution of 125 I-RGD4C β L in distinct organs was evaluated by measuring the radioactivity density of each organ at 6 h following intravenous injection. Fig. 5 demonstrates the radioactivities of the primary organs. The highest density of the 125 I-RGD4C β L was observed in the kidney, which suggested renal clearance. The concentration of tumor 125 I-RGD4C β L was increased compared with the blood and muscle.

Discussion

Targeted enzymes are useful in tumor therapy as they convert nontoxic prodrugs into chemotherapeutic agents at the precise tumor sites, avoiding systemic exposure and minimizing side effects, and catalyzing the self-quenching substrate in *in vivo* fluorescence imaging (22,23). The key characteristics of targeted enzymes are their biodistribution and pharmacokinetic properties. These enzymes should exhibit a high uptake level at the tumor site, and should degrade rapidly in the blood and normal tissues, converting the prodrugs in a site-specific manner (2,3,24,25). Therefore, it is important to investigate the *in vivo* distribution and clearance mechanisms of these enzymes.

The present study constructed RGD4C β L as a targeted enzyme for use in TEPT. Previous studies have demonstrated that RGD4C β L possesses a potent catalytic function, low immunogenicity and high stability (11). Flow cytometric studies have also revealed that the targeting effect of RGD4C β L was equal to that of RGD4C, and is blocked by RGD4C *in vitro* (12). Immunofluorescent staining of fluorescein isothiocyanate-RGD4C β L and a binding assay of 99m Tc-RGD4C β L confirmed the affinity and specificity of RGD4C β L on tumor cells, and that the 99m Tc-RGD4C β L exhibited a short half-life in rats. The half-lives of distribution and elimination of 99m Tc-RGD4C β L were 7.8 and 21.9 min, respectively (26), which are favorable for its use in TEPT. In the present study, *in vivo* imaging and biodistribution assays using radioiodine labeling technology were performed, and revealed that RGD4C β L may be efficiently labeled with 125 I, and 125 I-RGD4C β L exhibited good stability in plasma. Biodistribution assays demonstrated that 125 I-RGD4C β L enters tumor sites and remains at an acceptable level within the tumor in comparison with normal tissues. 125 I-RGD4C β L is also removed rapidly from the body by renal excretion; at 24 h post-injection, the radioactivity levels in mice were reduced to background levels (data not included). These results revealed the potency of RGD4C β L within TEPT, and demonstrated

the use of 125 I-RGD4C β L in molecular imaging for treatment monitoring, and the optimization of doses and time schedules in TEPT.

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