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Design, synthesis and iodination of an Arg-Arg-Leu peptide for potential use as an imaging agent for human prostate carcinoma

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The aim of this work was to design and synthesize a radioiodinated peptide containing arginine–arginine–leucine (RRL) functionality as a potential imaging agent of tumor angiogenesis. The RRL peptide was synthesized using the Fmoc solid phase method and identified by MS analysis after purification by HPLC. The RRL peptide was labeled with ^{131}I by the Chloramine-T method and purified through Sephadex G-25 column. Furthermore, the ^{131}I labeled peptide was used to evaluate its biodistribution. SPECT imaging was carried out at 24 h after the ^{131}I labeled RRL peptide was injected into BALB/c nude mice bearing human prostate carcinoma. For the first time we have shown that a radiolabelled RRL peptide appears to be useful as a tumor angiogenic endothelium imaging agent for the diagnosis of the human prostate carcinoma.

Keywords: arginine–arginine–leucine (RRL); peptide; radioiodination; human prostate carcinoma

Introduction

Tumor cells, in common with normal tissues, need to receive oxygen and metabolites to survive, so angiogenesis is an essential process for the growth of solid tumors. Moreover, the new vessels are one of the ways through which the tumor can lead to the formation of metastases. The newly formed tumor vessels show characteristics that are different from normal ones. In fact, the vessels are structurally disorganized, tortuous and dilated and they express on their membrane surface specific markers, which can be used for the selective targeting of tumor blood vessels.[1,2] One of the advantages of targeting the endothelial cells that support tumor growth, rather than the tumor cells themselves, is that the endothelial cells are genetically stable and therefore less prone to accumulate mutations.[1] Peptides could find important applications in diagnosis and are good candidates because of their characteristic properties.[3] Numerous peptides have been identified that bind to the tumor angiogenic endothelium, including the arginine–arginine–leucine (RRL) peptide. The tripeptide sequence RRL is a tumor endothelial cells specific binding peptide previously identified using an *in vitro* bacterial peptide display library (FLiTrx) panned against tumor-derived endothelial cell (TDEC) derived from SCC-VII murine squamous cell carcinomas.[4]

Results and discussion

Peptide design and synthesis

A probe specific for tumor vessel imaging was prepared. The probe is an RRL-containing cyclic peptide comprising the RRL sequence bracketed by glycines and terminated with cysteine and tyrosine residues (Tyr-Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys-NH_2), designated WT 1040 (Figure 1A). Reversed-phase HPLC of the crude product yielded a main peak containing > 99% of the absorbance at 220 nm (Figure 1B). ESI-MS of the RRL peptide showed that the molecular weight was 1040.2 before oxidation and was 1038.2 after oxidation. In the control peptide glycines were substituted for the RRL sequence (Tyr-Cys-Gly-Gly-Gly-Gly-Gly-Gly-Cys-NH_2), designated WT 786. Disulfide bonds

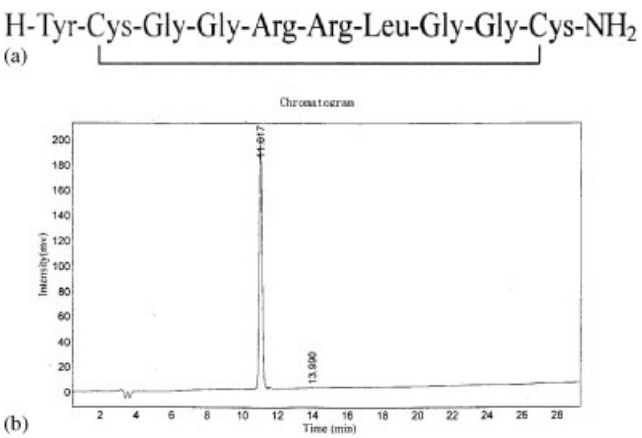

Figure 1. Schematic structure of RRL peptide (a) and reversed-phase HPLC of RRL peptide (b).

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between cysteines on each peptide maintain the cyclic structure.

The peptide sequence motif (Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys) was described by Brown *et al.*[4] for the first time. This peptide contains two cysteines so that a disulfide loop may be formed, thus constraining and mimicking the binding condition of the initial selection process. To determine the endothelial specificity of peptide Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys, *in vitro* binding experiments with TDEC were carried out. In these experiments, fluorescein FITC labeled RRL peptide show the brightest staining and specificity for TDEC. The control peptide, Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys, did not appear to make any change to the binding of the peptide to TDEC. Gregory *et al.*[5] conjugated microbubbles (MB) to the cyclic peptides Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys (MB_{RRL}) and Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys ($MB_{Control}$) via avidin/biotinbridging chemistry. MBs targeted via RRL selectively bound to cultured tumor derived but not normal endothelial cells under physiologically relevant conditions. In comparison, MBs conjugated to a control glycine peptide showed minimal adherence to both cell types, suggesting specific binding interactions between the RRL sequence and its ligand on the endothelial surface. $MB_{Control}$ binding was nonetheless greater than zero, which may indicate a degree of nonspecific adhesion by the cyclic peptide. Gregory *et al.*[5] also injected NH_2 terminal fluoresceinated peptides containing either RRL or the control sequence GGG (Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys and Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys) into athymic mice bearing PC3 or Clone C tumors. The results showed RRL peptide preferentially adheres to tumor vasculature of both Clone C and PC3 tumors *in vivo* whereas the control peptide did not adhere to tumor vasculature. Gregory *et al.*[5] ultrasonically imaged tumor bearing (Clone C and PC3 tumors) mice after MB_{RRL} injection. Results show MBs targeted via RRL generated enhanced ultrasound opacification of tumors. Henry *et al.*[6] conjugated two near-infrared dyes (Alexa 680, 800) to RRL peptide and injected RRL–peptide–Alexa chimera to human PC3 prostate tumor bearing mice or transgenic prostate cancer mice. Whole animal imaging revealed the tumor but did not show an appreciable image signal at any other site or organ.

All studies showed that the RRL peptide targets tumor vasculature and is useful for the specific detection of experimental tumors *in vivo*. Based on their study, we conjugated a tyrosine to the N terminus of the RRL peptide (Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys) and the control peptide (Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys) to allow iodination for the first time. The two peptides were amidated at the C terminus to maintain their stability.

The result of ESI-MS showed that the molecular weight of RRL peptide before oxidation was twice more than that after oxidation. It was caused by the loss of 2 H atoms while forming a disulfide bond between two cysteines. This confirmed indirectly that the RRL peptide contains disulfide bonds.

^{131}I labeled peptides

The RRL peptide was radiolabeled with ^{131}I under optimal conditions. Samples (50 μg) of RRL peptide labeled well at 20°C. Free ^{131}I was present at 40%. The control peptide was labeled similarly. The best RRL peptide/chloramine-T weight ratio was 1:1.80. When the ratio differed from this value the radiolabelling yield decreased. The highest yield was obtained at 20°C, pH 7.4 and 1 min reaction time.

The radiolabelling yield of ^{131}I labeled RRL peptide is about 60%, but the radiochemical purity is >95%. This is enough to detect tumors in the clinic as a molecular probe. The low radiolabelling yield of ^{131}I labeled RRL peptide may be caused by the spatial and stereo structure of RRL peptide.

^{99m}Tc is the optimal radionuclide for SPECT imaging, but the labeling procedure requires $SnCl_2$. The disulfide bond in the RRL peptide may be destroyed in the presence of $SnCl_2$ and this disulfide bond is essential to maintain the stability of the RRL peptide. This is one reason why we chose ^{131}I to label RRL peptide. Another reason is the ^{131}I labeled RRL peptide may be used to cure tumors.[7]

Purification results, radiochemical purity and *ex-vivo* stability of radioiodine labeled RRL peptide

Figure 2 shows the elution curve following chromatography on a Sephadex G-25 column (1 × 20 cm). Figure 3 shows the UV (280 nm) absorbance of eluted fractions. Results showed the first radioactive peak in Figure 2 corresponds to the UV absorbance peak in Figure 3, which was the pure ^{131}I labeled RRL peptide. Thin-layer chromatography of ^{131}I labeled RRL peptide on ITLC-SG (Gelman Sciences, Inc.) showed an Rf of 0–0.1, the Rf of free ^{131}I was 0.9–1.0. The radiochemical purity of purified ^{131}I labeled RRL peptide was >95%. After storage at 37°C for 24 h, the radiochemical purity of purified ^{131}I labeled RRL peptide was >90% (Figure 4).

The radiolabelled RRL peptide can be successfully separated from unbound reactants by chromatography on a Sephadex G-25 column. Results shows there were two radioactive peaks, the first peak was at fraction 7 and the second at fraction 14. Spectrophotometric analysis shows that the first radioactive peak (Figure 2) corresponds to the UV absorbing peak (Figure 3). This confirmed that the first radioactive peak (Figure 2) was ^{131}I labeled RRL peptide. The radiochemical purity of purified ^{131}I

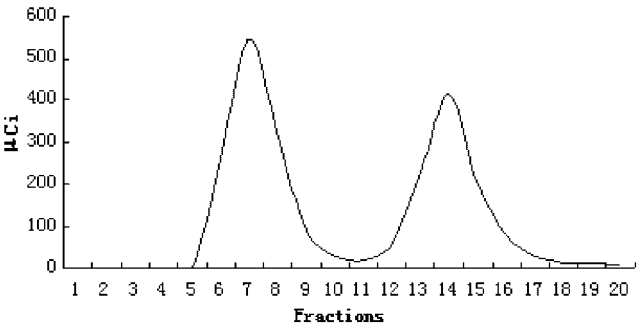

Figure 2. Sephadex G-25 column (1 × 20 cm) chromatography of radioiodine product. Elute rate:0.5 mL/min, 2 min/fraction.

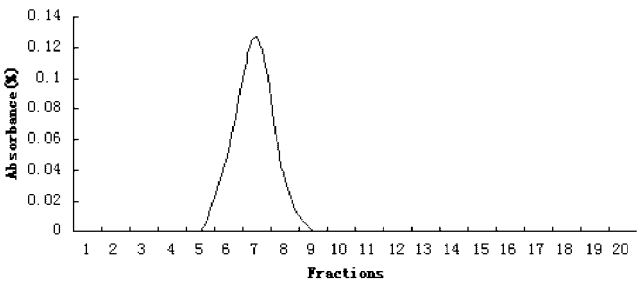

Figure 3. 280 nm UV absorbance of eluted fractions.

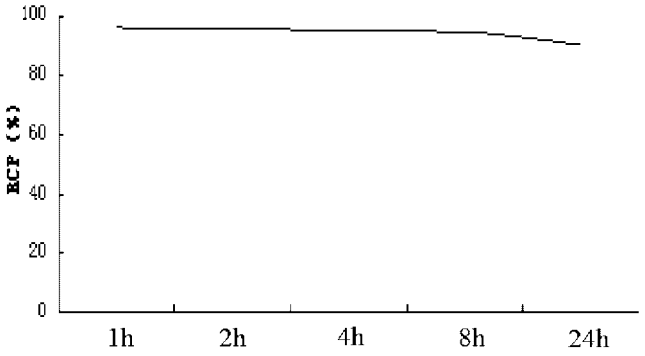

Figure 4. The radiochemical purity of purified ^{131}I labeled RRL peptide was 96.1% at 1 h and 90.3% at 24 h in human blood serum, 37 °C.

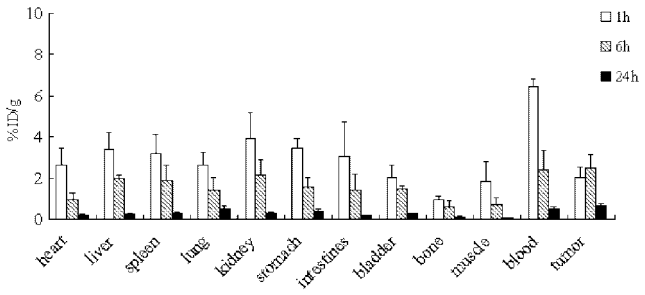

Figure 5. Biodistribution (%ID/g) for ^{131}I labeled RRL peptide in human prostate carcinomabearing BALB/c nude mice.

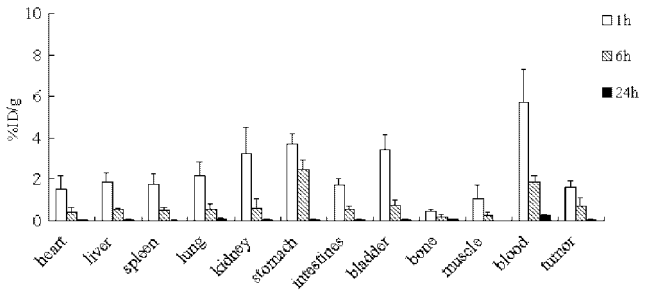

Figure 6. Biodistribution (%ID/g) for ^{131}I labeled control peptide in human prostate carcinomabearing BALB/c nude mice.

labeled RRL peptide was 90.3% in human blood serum at 24 h, 37°C. This suggested the ^{131}I labeled RRL peptide is stable in the human body.

Biodistribution studies

Figures. 5 and 6 summarize the biodistribution of each probe in tumor bearing nude mice at 1, 6 and 24 h after injection. Over time, a decrease in radioactivity was observed in all tissues, with the exception of the tumor. Tumor uptake of ^{131}I labeled RRL peptide was higher than that for ^{131}I labeled control peptide at 6, 24 h. At 24 h after tracer injection, tumor uptake of ^{131}I labeled RRL peptide was 0.65 ± 0.12 %ID/g, whereas it was only 0.06 ± 0.04 %ID/g for ^{131}I labeled control peptide.

In the biodistribution studies, the RRL peptide accumulated in the tumor to a level of approximately 0.65 %ID/g—a higher level than in the other organs. This suggests that ^{131}I labeled RRL peptide is suitable for SPECT imaging.

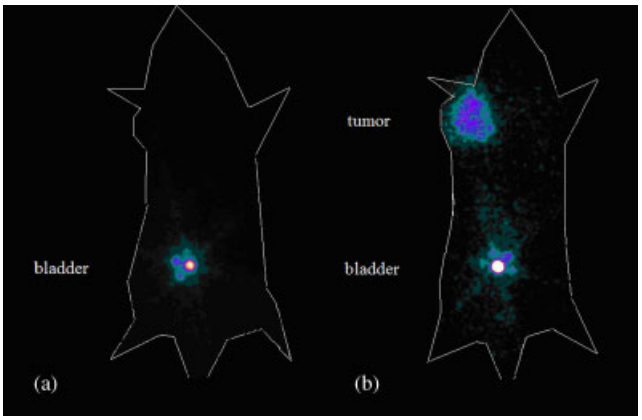

Figure 7. SPECT image at 24 h after intravenous injection of 7.4 MBq ^{131}I labeled control peptide (A) and RRL peptide (B) of human prostate carcinoma-bearing BALB/c nude mice. Contrasting tumor is clearly shown on the right armpit and only additional higher activity concentration was found in bladder (B).

SPECT imaging

The Single photon emission computed tomography (SPECT) imaging at 24 h post-injection of ^{131}I labeled RRL peptide (Figure 7B) clearly shows a contrasting tumor on the right armpit of the mouse with high concentrations of radioactivity. A high concentration of radioactivity was also found in the bladder. The SPECT imaging at 24 h post-injection of ^{131}I labeled control peptide (Figure 7A) only show high concentrations of radioactivity in the bladder.

The result of SPECT imaging shows that the ^{131}I labeled RRL peptide can specifically adhere to the tumor endothelium, compared to the ^{131}I labelled control peptide. The 10-mer RRL peptide has some superiorities as imaging agent: (1) The synthetic process is relatively simple and the synthesis cost is relatively low. (2) The labeling technology of ^{131}I is mature. (3) The molecular weight of the RRL peptide is only about 1040 and it has no or weak immunogenicity in animals or human.

^{131}I has a half-life of 8.02 days and emits not only γ rays but also β rays. This may be valuable to cure tumors. In this study, high concentrations of radioactivity were not found in the unblocked thyroid at 24 h after injection. It may be caused by the following reasons: (1) The labeled compound can target tumor vasculature with high affinity and specificity. (2) The labeled compound is stable and deiodination does not occur easily. (3) The limited resolution of SPECT.

Targeting of tumor-derived vascular endothelial cells has been shown to be feasible. For example, radiolabeled VEGF has been used to visualize gastrointestinal tumors and metastases expressing VEGF receptors.[8] Haubner and Wester have reviewed other targets used for imaging of tumor angiogenesis.[9] Potentially, such imaging could be used to visualize tumor vasculature too, but unfortunately vascular endothelial cells are present in much smaller number than tumor cells. Molecular agents for imaging angiogenesis must therefore bind the targets with high affinity and specificity and be detected at low concentration.[10]

Experimental

Peptide design and synthesis

The sequence of the RRL peptide and the control peptide was based on the reports of Brown *et al.*[4] :(Tyr)-Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys-NH_2 and (Tyr)-Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys-NH_2 (an amino-terminal Tyrosine not present in the native sequence was added to facilitate radioniodination). The 10-mer RRL peptide was assembled by 9-fluorenylmethoxy carbonyl (Fmoc) coupling on NovaSyn TGR resin (0.2–0.3 mmol/g) (Novabiochem) by use of an Apex 396-Automated Muliple Peptide Synthesizer (AAPPTEC). These peptides contain two cysteines so that a disulfide loop was formed, thus constraining and mimicking the binding condition of the initial selection process. The two cysteine side chains in the peptide were cyclized by dissolving the peptide in $NaHCO_3$ (0.01 M, pH 8.5) at 5.0 g/L, and stirring for 24 h at room temperature in the presence of atmospheric oxygen to allow formation of disulfide linkages. The cysteine at the C-terminal of the RRL peptide and control peptide was amidated to protect the peptides from the biocatalyst. Reversed-phase HPLC was carried out using a C18 column (4.6×250 mm) with a flow rate of 1.0 mL/min. For the reversed-phase HPLC procedures the system solvent used was solvent A (0.1% trifluoroacetic in 100% acetonitrile) and solvent B (0.1% trifluoroacetic in 100% water), with a linear gradient from 10 to 100% solvent A over 25 min. The RRL peptide and the control peptide were also investigated by electrospray mass spectrometry (ESI-MS) before and after oxidation. The lyophilized peptide was stored at -20°C.

Radiolabelling

Radioiodination of the RRL peptide Tyr-Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys-NH_2 and the control peptide Tyr-Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys-NH_2 was performed using the Chloramine-T method.[11] The peptides(50 μg) were dissolved in phosphate buffer (PB)(81 μL , 0.5 M, pH 7.4). ^{131}I Na (10 μL, 74 MBq) was added to the solutions. Then CH-T(9 μL, 10 μg/μL) was added to the mixture. The reaction mixture was gently agitated for 1 min at 20°C then quenched by the addition of sodium metabisulfite 45 μL (4.0 mg/mL in 0.1 mol/L PB, pH 7.4). The radiolabelling yield was measured by ITLC-SG thin-layer chromatography developed by acetone.

Purification and radiochemical purity test of radioiodinated RRL peptide

The labeled RRL peptide Tyr-Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys-NH_2 and control peptide Tyr-Cys-Gly-Gly-Gly-Gly-Gly-Gly-Gly-Cys-NH_2 were separated from unbound reactants by chromatography on a Sephadex G-25 column (1×20 cm) at 20°C, first eluted with 1% bovine serum albumin, then eluted with phosphate-buffered saline (PBS, 0.1 M, pH 7.4). Column fractions were collected for 40 min with elution rate: 0.5 mL/min, 2 min/fraction. The radiochemical purity was determined by ITLC-SG thin-layer chromatography, developed by acetone. The intensity of radioactivity of all the fractions were detected by radioactivity meters (National Institute of Metrology) and the peptide content of all fractions was measured at 280 nm using a ND-1000 spectrophotometer (Nanodrop Technologies, Inc.).

Ex-vivo stability test of radioiodine

The purified radioiodine labeled RRL peptide Tyr-Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys-NH_2 was put in human blood serum at 37°C for 24 h, and radiochemical purities were checked on 1, 2, 4, 8 and 24 h.

In vivo animal experiments

Mouse tumor model: Experiments were approved by the Department of Laboratory Animal Science at Peking University and the management of animals corresponded to Animal Ethical Standard. To generate solid tumors, 5×10^6 PC3 cells were injected s.c. into the armpit of athymic nude mice and allowed to grow for 6 weeks until the tumors were $\sim$1.0 cm^3. PC3 cells are derived from metastatic human prostate carcinoma (Chinese Academy of Medical Sciences).

Biodistribution studies: BALB/c nude mice bearing human prostate carcinoma were injected intravenously with 0.74MBq ^{131}I labeled RRL peptide or ^{131}I labeled control peptide. The animals were enthanized and dissected at 1, 6, 24 h after injection of the ^{131}I labeled peptides. Heart, liver, spleen, lung, kidney, stomach, intestines, bladder, bone, muscle, blood and tumor tissues were removed and weighed. The radioactivity in the tissues was measured using a γ counter. Results are expressed as the percentage injected dose per gram of tissue (%ID/g). Each value represents the mean and SD of three animals.

SPECT imaging. 6 BALB/c nude mice bearing human prostate carcinoma (three with RRL peptide and three with control peptide) were each injected with 7.4 MBq of ^{131}I labelled peptide through the tail vein. After 24 h, mice were imaged by SPECT with a single head rotating scintillation camera (MPR from General Electric Company). The camera is equipped with high-energy general purpose collimator (HEGP). The acquisition count is 100 000.

Conclusions

In summary, the RRL peptide Tyr-Cys-Gly-Gly-Arg-Arg-Leu-Gly-Gly-Cys-NH_2 can be synthesized using a solid phase method with high purity (>99%). The peptide can be successfully labelled with ^{131}I using the chloramine-T method. Though the radiolabelling yield is only about 60%, a radiochemical purity of 96.5% can be achieved after purification on a Sephadex G-25 column. The labeled compound is stable in human blood serum at 37°C and the radiochemical purity is 90.3% at 24h. The ^{131}I labeled RRL peptide compared with the ^{131}I labeled control peptide can successfully accumulate in the tumor and image the tumor in human prostate carcinoma bearing nude mice. The ^{131}I labeled RRL peptide is valuable as a molecular probe for detecting tumors.

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