Radiolabeling and evaluation of ^{188}Re-RGD as an agent for $\alpha_v\beta_3$ receptor expression

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Abstract Integrin $\alpha_v\beta_3$, a cell adhesion molecule, plays a key role in angiogenesis and tumor cell metastasis and is therefore an important target for new therapeutic and diagnostic strategies. Since RGD bind to $\alpha_v\beta_3$ particularly, a lot of RGD containing peptides were radiolabeled and evaluated as imaging or therapeutic agents. In this study, a new ^{188}Re-RGD peptide analog was synthesized and characterized. Within 30 min, the ^{188}Re-H-E[c(RGDyK)]$_2$ was obtained in radiochemical purity of better than 95%. The His-E[c(RGDyK)]$_2$ had a good integrin-binding affinity (50% inhibitiory concentracer (IC_{50}=46.5×10^{-9} nm) and exhibited active accumulation (7.20±1.06) in S180 tumor. Because of relatively fast washout in blood and normal tissues, the target/non-target ratio was high. Further experiments to improve the tumor targeting efficacy and *in vivo* kinetic profiles are in progress towards developing it into a promising radio agent.

Key words *fac*-[^{188}Re$(H_2O)_3(CO)_3]^+$, RGD peptide, Integrin$\alpha_v\beta_3$

1 Introduction

Integrin $\alpha_v\beta_3$ plays a key role in the progress of angiogenesis, which is essential for tumor growth[1,2]. It is significantly expressed on proliferating endothelial cells[3] and invasive tumor cells of certain cancer types (glioblastoma, melanoma, breast, ovarian and prostate cancers and in almost all tumor vasculature), whereas it is absent on quiescent endothelial cells[4,5]. Consequently, tumor-targeting studies using $\alpha_v\beta_3$ has interested many authors in recent years[6]. Integrin $\alpha_v\beta_3$ binds to the arginine-glycine-aspartic acid (RGD)-containing components, which is present in the interstitial matrix such as vitronectin, fibronectin and thrombospondin [7,8]. Based on RGD tripeptide sequence, there are many studies aimed at inhibiting $\alpha_v\beta_3$ activity and angiogenesis[9]. Especially, the cyclic peptide derivatives have high affinity for $\alpha_v\beta_3$. A series of RGD peptides were radiolabelled by Chen *et al*[10,11]. [^{18}F]FB-E[c(RGDyK)]$_2$ ([^{18}F]FRGD$_2$) showed predominant renal excretion, and in the same animal model the tumor uptake of [^{18}F]FRGD$_2$ was almost twice more than the monomeric tracer [^{18}F]FB-c(RGDyK)[11,12].

Generator-produced ^{188}Re is an attractive radionuclide for targeted radiotherapy due to favorable physical characteristics of high-energy beta particles (E_{max}=2.12 MeV) and 155 keV gamma photons in 15% abundance[13]. In this paper, we radiolablled a dimeric cyclic RGD peptide E[c(RGDyK)]$_2$-His using ^{188}Re *via* *fac*-[^{188}Re$(H_2O)_3(CO)_3]^+$ as a precursor. ^{188}Re-H-E[c(RGDyK)]$_2$ (^{188}Re-H-RGD$_2$) peptide is investigated for further developing it into a radiopharmaceutical agent. The *in vitro* stability is studied and U87MG cells are adopted for the *in vitro* affinity. Using mice models of subcutaneously transplanted S180 tumor, the *in vivo* distribution and tumor imaging of ^{188}Re-H-RGD$_2$ are investigated.

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2 Experimental

2.1 Materials

All reagents were of analytical grade and used without further purification, but borane ammonia complex (Sigma-Aldrich, USA) was of 90% technical purity. Carbon monoxide was obtained from Kunshan XinAn Industry Gas Co. Ltd (China). ^{188}Re-perrhenate was eluted from ^{188}W/^{188}Re generator (our laboratory) using 0.9% saline. The RGD-containing peptides were provided by Dr.Xiaoyuan Chen of National Institute of Biomedical Imaging and Bioengineering National of Health. Plus QMA Sep-Pak cartridges were produced by Waters Corporation (USA). A Dionex P680 pump equipped a PDA-100 ultraviolet detector and a radiometric detector system with a Macherey-Nagel C-18 reversed phase column (5 μm, *Φ*4.6 mm×150 mm) were used to perform High Performance Liquid Chromatography (HPLC) (Bionex USA). TLC analysis was performed using silica gel 60 GF_{254} plates on a Bioscan system AR-2000 with Winscan software of Version 3.09 (Bioscan USA). Radioactive samples from in vivo experiments were measured in a gamma counter (SN-697, Shanghai Rihuan Photoelectronic Instrument Co. Ltd., China). Whole body autoradiography of the animals was performed on a Cyclon system with a storage phosphor screen (Multisensitive Medium, Perkin Elmer, USA).

2.2 Preparation of *fac*-[^{188}Re(CO)$_3$(H$_2$O)$_3$]$^+$

Carbonyl complex *fac*-[^{188}Re(CO)$_3$(H$_2$O)$_3$]$^+$ was prepared by the method in Ref.[14], with simple modification. $BH_3 \cdot NH_3$ (about 5 mg) was put into a 10 mL glass vial, which was sealed and flushed with carbon monoxide for 20 min, before adding the mixture of 6 μL phosphoric acid (85%) and 1 mL ^{188}Re-perrhenate. The vial was heated in water bath at 70ºC for 15 min. A 10-mL syringe was used to keep the H_2 balance in the process. The reaction was terminated immediately by putting the vial into ice bath. Plus QMA Sep-Pak cartridge was used to purify the products. The chelating efficiency was determined by HPLC, at flow rate of 1 mL/min eluting with CH_3OH (solvent A) and TEAP (7 mL triethylamine and 4 mL H_3PO_4 in 1l H_2O, pH=2.25) (solvent B). The gradient of mobile phase was as follows:

0–3 min, 100% B;
3–6 min, 75% B and 25% A;
6–9 min, 66% B and 34% A,
9–22 min, 100% A, and
22–30 min, 75% B and 25% A.

2.3 Radiolabeling of H-E[c(RGDyK)]$_2$ peptide

H-E[c(RGDyK)]$_2$ solution of 100 μL was mixed with 900 μLof freshly prepared ^{188}Re tricarbonyl complex and incubated at 75ºC for 30 min. The radiolabeling efficiency was determined by HPLC, at a flow rate of 1 mL/min eluting with 0.1% trifluoroacetic acid in acetonitrile (TFA/CH_3CN) (solvent A) and 0.1% TFA/H_2O (solvent B). The gradient of mobile phase was as follows:

0−30 min, 95% A and 5% B,
31−35 min, 40% A and 60% B, and
35−40 min, 95% A and 5% B.

2.4 In vitro stability of ^{188}Re-H-RGD$_2$

Stability of the ^{188}Re-H-RGD$_2$ *in vitro* was determined by incubation in PBS or new-born calf serum. The mixture was kept at 37ºC or room temperature (RT) for 0, 1 and 4 h. The radiochemical purity of ^{188}Re-H-RGD$_2$ was evaluated by TLC at various time points. TLC system consisting of acetone/H_2O (*v*/*v* =1/1) and acetonitrile as mobile phase and glass-backed silica gel plates as stationary phase.

2.5 In vitro cell-binding assay

The human glioblastoma U87MG cells were obtained from the Department of Nuclear Medicine, Ruijin Hospital, Shanghai Jiaotong University School of Medicine. The U87MG cells were grown in Dulbecco's medium (Gibco USA) supplemented with 10% fetal bovine serum (FBS). The cells were grown at 37ºC in a humidified atmosphere with 5% CO_2.

The in *vitro* integrin-binding affinity and specificity of ^{188}Re-H-RGD was assessed via displacement cell-binding assays using ^{125}I-echistatin as the integrin-specific radioligand[10]. The cells were harvested, washed twice with PBS, and resuspended

(2×10^6 cells/mL) in medium. Each well of the 96-well plate was seeded with 2×10^5 U87MG cells, which were allowed to attach for overnight. After washed with PBS, the cells were incubated with ^{125}I-echistatin (30,000 cpm/well) in presence of increasing concentrations of different H-E[c(RGDyK)]$_2$ peptide analog (0–500 nmol/L). The total incubation volume was adjusted to 200 μL. After pre-incubation for 2 h, the cells were washed with PBS three times to remove free radioligand. At the end of incubation, the cells were solubilized by incubation with 1N NaOH for 5 min, and the activity from each well was measured using γ-counter. The best-fit 50% inhibitory concentration (IC_{50}) values for the U87MG cells were calculated by nonlinear regression using GraphPad Prism (GraphPad Software, Inc.). Each experiment was performed with triplicate samples.

2.6 Biodistribution of ^{188}Re-H-RGD$_2$ in tumor-bearing mice

The animal experiments were performed in accordance with the Principles of Laboratory Animal Care (NIH publication No. 86-23, revised 1985). Fresh S180 tumor homogenates were transplanted subcutaneously in the right flank of BALB/c mice weighed 19–22 g. Studies were performed when the tumor was approximately 1 cm^3 in volume. About 100 μCi purified ^{188}Re-H-RGD$_2$ in 100 μL of 0.9% NaCl solution was injected via the tail vein. At 1, 4, 24 and 48 h after injection (four mice each time), the mice were sacrificed and the tissues of interest, including tumors, were excised, weighed and counted for radioactivity by the γ-counter. The biodistribution were calculated as percentage of the injected radioactive dose per gram (ID%/g) of tissue wet weight, and target/non target ratio (T/NT ratio) was calculated, too.

3 Results and discussion

3.1 Synthesis of 188Re-H-RGD2

Since the $\{M(CO)_3\}^+$ (M=^{99m}Tc or ^{188}Re) unit reacts with some bidentate and tridentate ligands to displace the substitutionally labile aqua ligands, it allows radiolabeling of low molecular weight biomolecules with high specific activities with retention of biological activity and specificity. In order to radiolabel this RGD peptide with ^{188}Re, *fac*-[^{188}Re$(H_2O)_3(CO)_3]^+$ was chosen to conjugate with His-residue group at the end of this peptide. The yield of *fac*-[^{188}Re$(CO)_3(H_2O)_3]^+$ was over 97% (Fig.1A, peak at min). The radiochemical yield of ^{188}Re-H-RGD$_2$ was over 95% (Fig. 1B, peak at min). The assumed structure was shown in Fig.2.

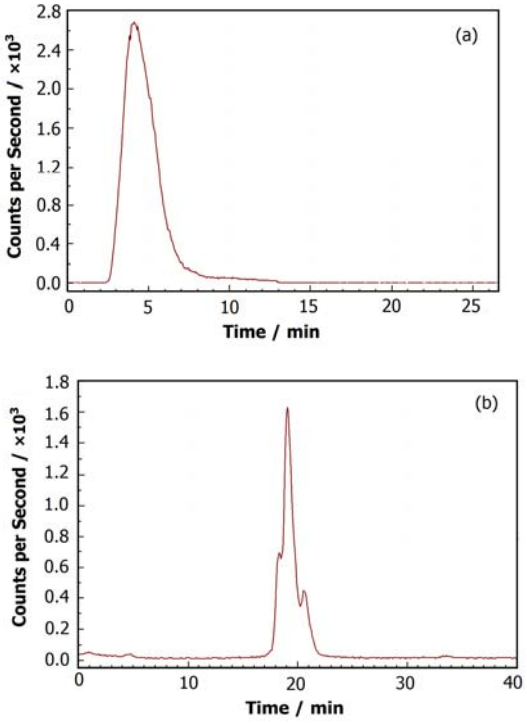

Fig.1 HPLC profiles of fac-[188Re(CO)3(H2O)3]+ intermediate (A) and 188Re-H-RGD2 conjugate (B).

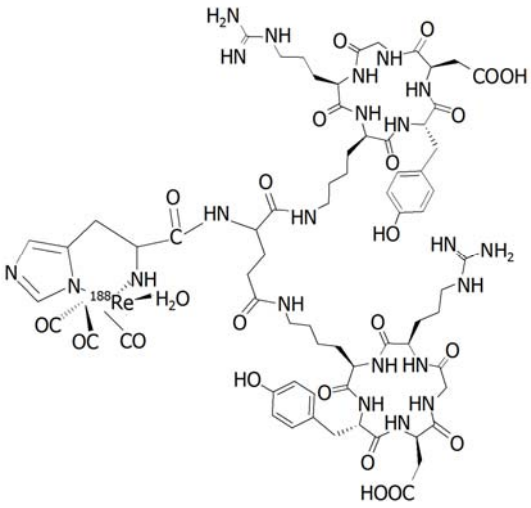

Fig.2 Assumed structure for the radiolabeled 188Re-H-RGD2 conjugate.

3.2 *In vitro* stability of ^{188}Re-RGD$_2$

Stability of ^{188}Re-RGD$_2$ peptide at RT and 37ºC in presence of PBS or new-born calf serum was monitored by Radio-TLC. After a 4-h incubation, the radiochemistry purity was more than 80% in both selected conditions.

3.3 *In vitro* cell-binding assay

Integrin $\alpha_v\beta_3$-positive U87MG cells were used for *in vitro* binding affinity studies, instead of the immobilized integrin. We compared the integrin binding affinity of H-RGD$_2$ by competitive displacement of ^{125}I-echistatin. H-RGD$_2$ inhibited the binding of ^{125}I-echistatin to U87MG cells (Fig. 3). With an IC_{50} value of 86.3 nm (n=3), the H-RGD$_2$ has a binding affinity to integrin $\alpha_v\beta_3$.

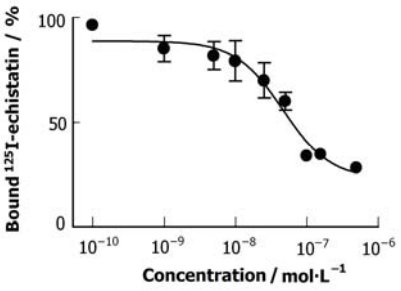

Fig.3 *In vitro* cell-binding assays of H-RGD$_2$ in U87MG cells using ^{125}I-echistatin as radioligand (n=3).

3.4 Biodistribution of ^{188}Re-RGD$_2$ in tumor-bearing mice

These results show that it is worthwhile to evaluate ^{188}Re-RGD$_2$ *in vivo* with tumor-bearing mice. ^{188}Re-RGD$_2$ was injected into mice with induced S180 tumors to perform the biodistribution experiments. The biodistribution data of ^{188}Re-RGD$_2$ are shown in Fig.4. In general, the tumor retention rates of the radiolabeled peptide were 7.20±1.06, 8.82±1.58, 2.06±0.48 and 0.60±0.28 (ID%/g) at 1, 4, 24 and 48 h post injection, respectively. Radioactive levels in the blood were 6.21±1.14 at 1 h, followed by rapid clearance to 0.88±027 at 4 h. High tumor-to-muscle and tumor-to-blood uptake ratios were observed at the 4 h (4.74±1.85 and 1.97± 0.68), which could be due to the clearance of the radioconjugate from non-target tissues. The uptake of radioactivity in normal tissues was observed, and the kidneys exhibited appreciable retention of radioactivity. Kidney accumulation was 6.991.95 at 1 h, and declined to 5.40±061 at 24 h. Similar results were observed for the liver uptake, being 12.02±1.86 and 5.09±1.18 at 1 and 24 h, respectively. These might be due to the rapid clearance ^{188}Re-RGD$_2$ predominantly via the renal and hepatobiliary route.

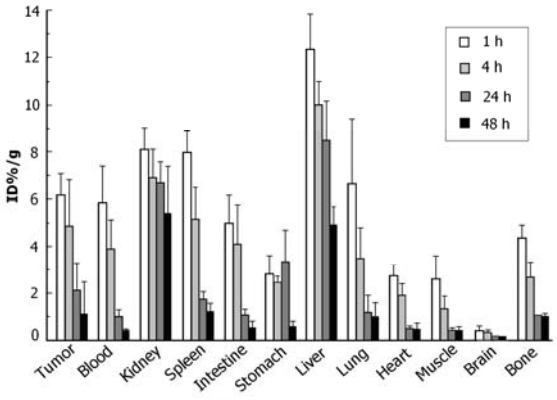

Fig.4 Biodistribution of ^{188}Re-H-RGD$_2$ in mice bearing S180 tumor.

4 Conclusion

H-E[c(RGDyK)]$_2$ peptide was effectively radiolabeled with ^{188}Re. The radiolabeled peptide was stable in vitro. The in vitro studies with U87MG cells showed that the peptide exhibits reasonable binding affinity and specificity. *In vivo* experiments demonstrated that the peptide exhibits specific localization in the tumor and efficient clearance from the circulation primarily through the renal/urinary pathway. These studies suggest that the ^{188}Re-H-RGD$_2$ may form the basis of development for therapy of $\alpha_v\beta_3$ active cancers.

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