

Preparation and Bioevaluation of DOTA-Cyclic RGD Peptide Dimer Labeled with ^{68}Ga

Archana Mukherjee, Aruna Korde, Sudipta Chakraborty, H. D. Sarma, Grace Samuel, and M. R. A. Pillai

Abstract—Radiolabeled cyclic RGD peptides targeting integrin $\alpha_v\beta_3$ are reported as promising agents for the early diagnosis of metastatic tumors. With an aim to improve tumor uptake and retention of the peptide, cyclic RGD peptide dimer E[c (RGDfK)] 2 (E = Glutamic acid, f = phenyl alanine, K = lysine) coupled to the bifunctional chelator DOTA was custom synthesized and radiolabelled with ^{68}Ga . Radiolabelling of cyclic RGD peptide dimer with ^{68}Ga was carried out using HEPES buffer and biological evaluation of the complex was done in nude mice bearing HT29 tumors.

Keywords— ^{68}Ga peptides, Angiogenesis imaging, Cyclic RGD peptides, PET Imaging.

I. INTRODUCTION

RADIOLABELED RGD peptides targeting integrin $\alpha_v\beta_3$ overexpressed during angiogenesis are reported as promising agents for the early diagnosis of aggressively growing tumors [1], [2]. Peptide derivatives labeled with positron-emitting radionuclides are of interest due to the high sensitivity and excellent resolution of the positron emission tomography (PET). Use of ^{68}Ga (β^+ emitter, $t_{1/2} = 67.71$ min) for PET imaging is advantageous mainly due to its availability from $^{68}\text{Ge}/^{68}\text{Ga}$ generator (^{68}Ge , $t_{1/2} = 270.8$ d) which renders its availability independent of an on-site cyclotron [3].

Different radiolabeled analogues of RGD peptides such as mono, di, tetrameric, multimeric RGD derivatives and RGD peptides linked via Gly and PEG are reported [4]. With an aim to improve tumor uptake and retention of the peptide in the target, cyclic RGD peptide dimer E[c (RGDfK)] 2 (E = Glutamic acid, f = phenyl alanine, K = lysine) coupled to the bifunctional chelator DOTA was custom synthesized and radiolabelled with ^{68}Ga .

Radiolabelling of DOTA cyclic RGD peptides is widely reported using ammonium acetate buffer at pH 4.5. However, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)

buffer is reported to be a better alternative for ^{68}Ga labeling consistency and stability [5].

Herein, we report radiolabelling of DOTA cyclic RGD peptide dimer with ^{68}Ga using HEPES buffer at pH 3.9 and biological evaluation of the complex in nude mice bearing HT29 tumors.

II. MATERIALS & METHODS

All radioactivity measurements were made using NaI (TI) scintillation counter. Culture media and supplements were obtained from Sigma chemical company, USA and all other chemicals and solvents were of AR grade, procured from reputed manufacturers from India. Human colon carcinoma HT29 cell line was procured from National Centre for Cell Sciences, Pune, India. Nude mice for tumor induction and biodistribution studies were procured and maintained at animal house facility of BARC. All animal experiments were carried out as per guidelines, regulations and approval from local animal ethics committee.

A. ^{68}Ga Elution

All glasswares were acid washed for ^{68}Ga labeling. $^{68}\text{Ge}/^{68}\text{Ga}$ generator (iThemba labs, South Africa) was eluted with 5ml of 0.1N HCl and metal impurities were removed by passing through commercial ion exchange column. Purified ^{68}Ga was eluted in 0.5ml acetone/HCL (97.6% /0.02N).

B. Radiolabelling

Reagent concentrations and reaction parameters such as temperature and time for radiolabelling of Cyclic RGD peptide dimer were standardized. 25 μg of peptide conjugate in 0.5N HEPES buffer pH 3.9 was radiolabelled with purified ^{68}Ga (~90 MBq) Reaction was carried out at 80°C for 30min.

C. Characterization of the Complex

Complex was characterized by HPLC using gradient elution with solvent A: water + 0.1% TFA and solvent B: Acetonitrile + 0.1% TFA (gradient: 0-20min: 10-90% B, 20-28min 90% B, 28-30min: 90-10% B) and ITLC (Agilent technologies) was carried out in Acetonitrile: Methanol (1:1) solvent.

D. Purification & Stability Studies

^{68}Ga -Cyclic RGD peptide dimer complex was purified by passing through C-18 sep-pak cartridge to remove colloidal species as well as HEPES buffer components. Sep pak column

Archana Mukherjee (archanas@barc.gov.in) Aruna Korde (agkorde@barc.gov.in), Sudipta Chakraborty, Grace Samuel, M. R. A. Pillai are with the Radiopharmaceuticals Division, Bhabha Atomic Research Centre, Mumbai 400085, India. (phone: 91-022-25590614; fax: 091225505151, 551 9613).

H. D. Sarma is with the Radiation Biology and Health Sciences Division Bhabha Atomic Research Centre, Mumbai 400085, India.

P. R. Chaudhari is with the Advanced centre on training, research and education in cancer, Navi Mumbai, India.

(1mL, Millipore) was preconditioned with methanol followed by water. The complex retained on the cartridge was washed with water and purified product was eluted with ethanol. Colloidal species if any gets retained to the cartridge. Elute was reconstituted in saline for animal experiments. Stability of the complex was studied for 3h by HPLC.

E. Development of Animal Model

HT-29 cells were cultured in Ham F12K nutrient medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 50µg/mL streptomycin. All cells were grown to confluence in humidified atmosphere under 5% CO₂ at 37°C. Trypsin-EDTA solution was used for sub-culturing and cell isolation.

HT-29 (Human Colon carcinoma) cells (~10⁷) were injected subcutaneously in the flank region of nude mice. Solid tumor developed after a period of two weeks. Animals bearing tumors were used for studying *in vivo* distribution of ⁶⁸Ga - DOTA-cyclic-RGD dimer.

F. Biodistribution

Biodistribution of ⁶⁸Ga-DOTA-cyclic-RGD dimer was performed in nude mice weighing 15-20 g. 0.1 mL (3-4 MBq) of the complex was injected through tail vein and the animals were sacrificed at 3h p.i. Four animals were used for each time point. The tissues and the organs were excised and activity associated with organs/tissues was measured in a flat type NaI (TI) scintillation counter. Distribution of the activities in different organs was calculated as percentage of injected activity per gram (%ID·g⁻¹) of organ. Radioactivity in blood and muscle was estimated by assuming blood and muscle weight as 8% and 40 % of total body weight, respectively.

G. Imaging Studies

0.1 mL (37 MBq) of the complex was injected through tail vein in tumor bearing animals. PET imaging was performed using small animal micro PET/CT from GE under isoflurane anesthesia. Imaging was done at 2h p.i. of ⁶⁸Ga-DOTA-cyclic-RGD dimer in nude mice bearing HT29 tumors.

III. RESULTS

A. ⁶⁸Ga elution

71±4% of generator eluted activity was obtained after post column purification in 0.5 ml of 98% acetone/0.05M suprapure HCl.

B. Characterization

Structure of DOTA-cyclic RGD dimer is depicted in Fig. 1.

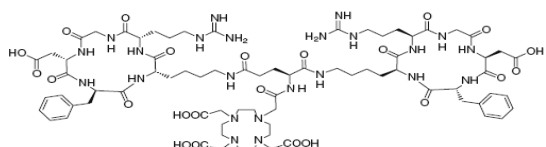


Fig. 1 Structure of DOTA cyclic RGD peptide dimer E [c (RGDfK)]₂

The radiolabelling yield of ⁶⁸Ga labelled RGD peptide dimer was >98%. RC purity was > 99% as per HPLC and complex showed Rt of 15.6 min as shown in Fig. 2.

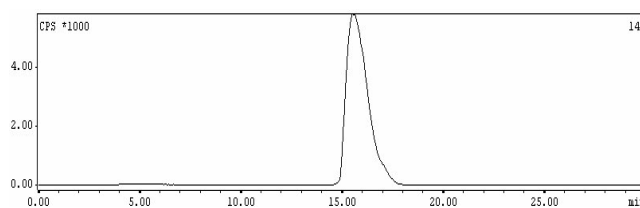


Fig. 2 HPLC radiochromatogram of ⁶⁸Ga- DOTA Cyclic RGD peptide dimer complex

C. Purification and Stability

Sep pak purification could remove impurities from buffer components as confirmed by HPLC with UV detector (Fig. 3).

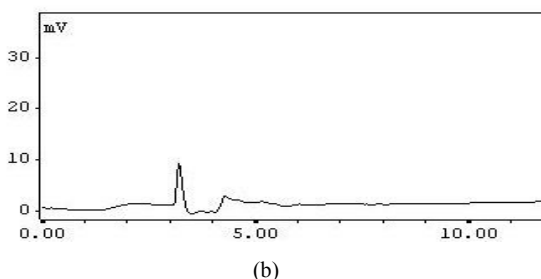
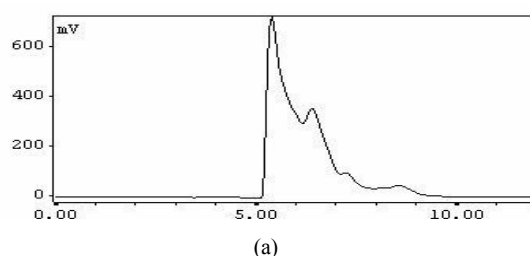


Fig. 3 HPLC chromatogram of ⁶⁸Ga-DOTA-cyclic-RGD dimer using UV detector before (a) and after (b) Sep pak purification

The complex was stable when studied up to 3h by HPLC method (Fig. 4).

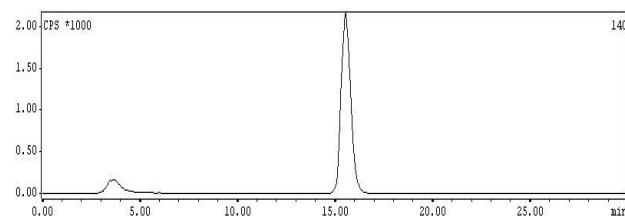


Fig. 4 HPLC radiochromatogram of ⁶⁸Ga- DOTA Cyclic RGD peptide dimer complex 3h after preparation of the complex

D. Biodistribution

Biodistribution data is depicted in Table I. Biodistribution in tumor bearing animals showed excretion of 76.5(8.08) % of the activity via renal route at 3h p.i. Tumor/blood and tumor/muscle ratios were 3.26 and 3.75, respectively.

TABLE I
BIODISTRIBUTION OF ^{68}Ga -DOTA CYCLIC RGD PEPTIDE DIMER COMPLEX IN HT 29 TUMOR BEARING NUDE MOUSE AT 3H P.I

Organ/Tissue	3h % ID/gm
Blood	0.23 (0.11)
Lung	1.62(1.14)
Heart	0.65(0.47)
Stomach	1.87(0.88)
Int + GB	2.34(0.82)
Liver	3.06(0.70)
Spleen	1.93(1.25)
Kidneys	5.40(1.75)
Muscle	0.20(0.18)
Bone	0.05(0.09)
Excreta	76.5(8.08)
Tumor	0.75(0.18)

Figures in the parentheses represents standard deviations,
At each time point 4 animals were used

E. Imaging Studies

Distribution of the complex in PET images was in accordance with the biodistribution data. PET Images of animal injected with the complex are shown in Fig. 5.

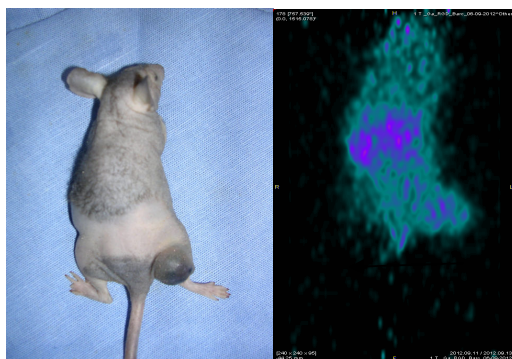


Fig. 5 Uptake of ^{68}Ga -DOTA Cyclic RGD peptide dimer complex in HT 29 tumor bearing nude mouse at 3h p.i

IV. DISCUSSION

Radiolabelling of DOTA-Cyclic RGD peptide dimer using ammonium acetate buffer at pH 4.5 is recently reported by our group. Complex showed ~98.5% radiochemical purity under the optimized conditions as determined by HPLC technique "to be published" [6]. HEPES buffer is reported to effectively maintain pH compared to ammonium acetate buffer that is very crucial for ^{68}Ga labeling of DOTA peptides. Our studies reveal HEPES buffer to be more promising for labeling of DOTA-peptides (unpublished results). Radiochemical purity

of DOTA- Cyclic RGD peptide dimer using HEPES buffer was found to be > 99%. HEPES from the complex was completely removed by Sep pak column. Hence, a semi automated module for radiolabelling of DOTA peptide with HEPES buffer followed by sep-pak purification is a possibility for fast and consistent labeling of DOTA peptides.

Our studies also revealed uptake of radiolabelled complex in HT29 tumors with good tumor/blood and tumor/muscle ratios. However, further studies in animal models bearing integrin over expressing cell lines like M21(melanoma) and U87MG glioblastoma at more time points to follow the distribution of the complex are warranted.

V. CONCLUSION

Preliminary evaluation of ^{68}Ga labelled RGD peptide dimer indicates the potential of the developed agent for possible use in angiogenesis imaging by PET.

ACKNOWLEDGMENT

The authors are thankful to Dr.P.R.Chaudhari for PET imaging studies. The authors are also thankful to IAEA as part of this study was conducted under an IAEA Coordinated Research Project.

DISCLOSURE STATEMENT

The author's research institute is a fully funded by Government of India and the authors have not received funding or grant from external agencies for this research. The authors do not have any conflict of interest.

REFERENCES

- [1]. R. Haubner, "αvβ3-integrin imaging: a new approach to characterize angiogenesis?", *Eur J Nucl Med Mol Imaging* vol. 33, pp. S54–S63, 2006.
- [2]. P.A.Knetsch, M.Petrik, C.M. Griessinger, C. Rangger, M. Fani, C. Kesenheimer, E. V. Guggenberg, & B.J.Pichler, I.Virgolini, C. Decristoforo, R.Haubner, "[^{68}Ga]NODAGA-RGD for imaging αvβ3 integrin expression", *Eur J Nucl Med Mol Imaging*, vol. 38, pp. 1303–1312, 2011.
- [3]. S. Liu, "Radiolabeled Cyclic RGD Peptides as Integrin αvβ3-Targeted Radiotracers: Maximizing Binding Affinity via Bivalency" *Bioconjugate Chem.*, vol. 20 (12), pp. 2199–2213, 2009.
- [4]. I. Dijkgraaf, J. A. W. Kruijtzter, S. Liu, A. C. Soede, W.J. G. Oyen, F.H. M. Corstens, R.M.J. Liskamp, O. C. Boerman, "Improved targeting of the αvβ3 integrin by multimerisation of RGD peptides" *Eur J Nucl Med Mol Imaging*, vol. 34, pp. 267–273, 2007.
- [5]. M. Brom, L. Joosten, W. J.G. Oyen, M. Gotthardt, O.C. Boerman, "Improved labelling of DTPA- and DOTA conjugated peptides and antibodies with ^{111}In in HEPES and MES buffer", *EJNMMI Research* vol.2, pp.4, 2012
- [6]. S. Chakraborty, R.Chakravarty, H.D.Sarma, A. Dash, M.R.A.Pillai, "The Practicality of Nanoceria-PAN-Based (^{68}Ge) (^{68}Ga) Generator Toward Preparation of (^{68}Ga) Ga-Labeled Cyclic RGD Dimer as a Potential PET Radiotracer for Tumor Imaging", *Cancer Biother Radiopharm*, (In press) 2013.