Nuclear Medicine and Biology 42 (2015) 109–114

Contents lists available at ScienceDirect

Nuclear Medicine and Biology

journal homepage: www.elsevier.com/locate/nucmedbio

Preparation and preclinical evaluation of ^{66}Ga-DOTA-E(c(RGDfK))$_2$ as a potential theranostic radiopharmaceutical

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ARTICLE INFO

Article history:
Received 28 August 2014
Received in revised form 26 September 2014
Accepted 30 September 2014

Keywords:
Gallium-66
RGD peptides
Integrin $\alpha_v\beta_3$
Angiogenesis
Theranostics
PET imaging

ABSTRACT

Introduction: Integrin $\alpha_v\beta_3$ plays an important role in angiogenesis and is over-expressed in tumoral endothelial cells and some other tumor cells. RGD (Arg-Gly-Asn) peptides labeled with ^{68}Ga ($t_{1/2}$ = 68 min) have showed good characteristics for imaging of $\alpha_v\beta_3$ expression using positron emission tomography (PET). Gallium-66 has been proposed as a PET imaging alternative to ^{68}Ga and given the unique high energy of its emitted positrons (E_{max} 4.15 MeV) it may also be useful for therapy. The aim of this research is to prepare [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ and evaluate in mice its potential as a new theranostic radiopharmaceutical.
Methods: High specific activity ^{66}Ga was produced via the ^{66}Zn(p,n) reaction, and the labelling method of DOTA-E-[c(RGDfK)]$_2$ with ^{66}Ga was optimized. Radiochemical purity was determined by TLC, and *in vitro* stability and protein binding were determined. Serial microPET imaging and biodistribution studies were carried out in nude mice bearing C6 xenografts. Radiation absorbed dose estimates were based on the biodistribution studies, where tumor and organs of interest were collected at 0.5, 1, 3, 5 and 24 h post-injection of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$.
Results: Our results have shown that [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ can be prepared with high radiochemical purity (>97%), specific activity (36–67 GBq/μmol), *in vitro* stability, and moderate protein binding. MicroPET imaging up to 24 post-injection showed contrasting tumors reflecting $\alpha_v\beta_3$-targeted tracer accumulation. Biodistribution studies and dosimetry estimations showed a stable tumor uptake, rapid blood clearance, and favorable tumor-to-tissue ratios.
Conclusions: The peptide conjugated DOTA-E-[c(RGDfK)]$_2$ labeled with ^{66}Ga may be attractive as a theranostic agent for tumors over-expressing $\alpha_v\beta_3$ integrins.

1. Introduction

During the past three decades, several radiopharmaceuticals have been developed for the early diagnosis of cancer through the use of novel molecular targets and predictive biomarkers, especially those aberrantly overexpressed in biological malignancy, invasiveness, metastasis and apoptosis [1–5].

It has been shown that peptides based on the Arg-Gly-Asp (RGD) amino acid sequence have a high affinity and selectivity for $\alpha_v\beta_3$ integrin receptors; specifically, it was found that cyclic analogues of RGD containing 5 amino acids (RGD sequence + a hydrophobic amino acid in position 4 + an additional amino acid in position 5) have the highest $\alpha_v\beta_3$ binding affinities [6,7]. Alpha(V)beta(3) integrin receptors are over expressed on endothelial cells during blood vessel formation affecting tumor growth, invasiveness and metastatic potential, and are therefore potential targets for receptor-mediated tumor imaging and therapy.

New approaches have been addressed in order to improve, with various ligands, the affinity of RGD. Due to the natural mode of interaction between $\alpha_v\beta_3$ and peptides containing the amino acid sequence RDG that may involve multivalent binding sites, the use of multivalent cyclic RGD peptides could improve the binding affinity and tumor uptake. Several research groups have compared the cyclic RGDfK monomer, dimer (E-[c(RGDfK)]$_2$) and tetramer (E-[c(RGDfK)]$_4$) as targeting biomolecule for diagnostic and therapeutic applications [8–11]. The short distances between the cyclic RGD peptides in dimmers (~20 bond distance), make it unlikely simultaneous binding to the adjacent $\alpha_v\beta_3$ receptors. It has been proposed that the binding of one RGD motif to the integrin $\alpha_v\beta_3$ will significantly increase "local concentration" of the multivalent RGD motif in the vicinity of the receptor-binding site leading to an enhanced integrin $\alpha_v\beta_3$ binding rate or the reduced dissociation rate of the cyclic RDG peptide from the integrin $\alpha_v\beta_3$[12]. This may explain the higher tumor uptake and longer tumor retention times of radiolabelled cyclic RGD tetramer and dimmer as compared

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http://dx.doi.org/10.1016/j.nucmedbio.2014.09.010
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to their monomeric analogues [11–13]. The tetrameric RGD peptide labeled with ^{64}Cu ([^{64}Cu]DOTA-E{E-[c(RGDfK)]$_2$}$_2$), showed significantly higher receptor binding affinity than monomeric and dimeric RGD analogues, but demonstrated lower kidney clearance which may be related to the positively charge differences [14].

The positron emitting radionuclide ^{66}Ga ($t_{½}$ = 9.49 h, 56.5% β^+, 43.5% EC) has been proposed as a PET imaging alternative to ^{68}Ga. ^{66}Ga is of special interest because of its relative long half-life which makes it a suitable tracer for the study of long-term physiological processes and labeling of macromolecules with slow pharmacokinetics [15–17]. In addition, the most abundant positrons emitted by ^{66}Ga have a unique high energy (E_{max} 4.15 MeV, mean range 7.6 mm in tissue), which may also be useful for therapy [17].

At the intersection between treatment and diagnosis, interest has grown in combining both paradigms into clinically effective pharmaceuticals. This concept, recently named as theranostics, is highly relevant to agents that target molecular biomarkers of disease and is expected to contribute to personalized medicine [18].

The aim of this research was to prepare, characterize and perform the preclinical evaluation and dosimetry of [^{66}Ga]DOTA-Glu-[cyclo(Arg-Gly-Asp-D-Phe-Lys)]$_2$ ([^{66}Ga]DOTA-E-[c(RGDfK)]$_2$) in order to evaluate its potential as a theranostic radiopharmaceutical for molecular imaging diagnosis and targeted radiotherapy of $\alpha_v\beta_3$ over-expressing tumors.

2. Materials and methods

2.1. Reagents

All reagents used were TraceSelect grade, and water employed to prepare solutions was of Milli-Q grade (18 MΩ-cm) to ensure heavy metal-free aqueous solutions. Ultrapure HCl, HEPES and NH_4OAc (>99.999%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Cation exchange resin (AG50W-X4, 100–200 mesh) was purchased from BioRad (Hercules, CA, USA). Isotopically enriched ^{66}Zn (98.72 %) was obtained from Isoflex (San Francisco, CA, USA). DOTA-E-[c(RGDfK)]$_2$ was purchased from ABX advanced biomedical compounds GmbH (Radeberg, Germany), and centrifugal filters (cellulose, 30,000 MW cut off) and Millex-GV syringe filters (0.22 μm, PVDF, 33 mm) were obtained from Millipore (Bedford, MA, USA).

2.2. Cell lines and animal model

C6 rat glioma cell line was purchased from the American Type Culture Collection (ATCCW CCL-107, Rockville, USA). The cells were grown in RPMI 1640 medium (Invitrogen, USA) supplemented with 10% fetal bovine serum and antibiotics (100 mg/ml streptomycin) and incubated at 37 °C in an atmosphere with 5% CO_2.

Experimental animals were handled observing the technical specifications for the production, care and use of laboratory animals stated in the Official Mexican Norm NOM 0062-ZOO-1999. Studies with mice in this specific work were performed according to protocols approved by the Research and Ethics Committee of the Faculty of Medicine, at the National Autonomous University of Mexico (UNAM).

Female athymic Balb-C nu/nu mice (20–25 g) were supplied by the National Institute of Medical Sciences and Nutrition Salvador Zubiran (INCMNSZ), Mexico City, Mexico. All animals were kept in a pathogen free environment and fed *ad lib*.

C6 xenografts were induced by subcutaneous injection of 1×10^6 cells resuspended in 0.1 ml of phosphate buffered saline, in the dorsal surface of the scapula. The sites of injection were observed at regular intervals for the appearance of tumor formation and progression, and mice were used for *in vivo* experiments when the diameter of tumor reached about 0.5 cm.

2.3. Gallium-66 production

Gallium-66 was produced in a Siemens Eclipse HP cyclotron via the ^{66}Zn (p,n) ^{66}Ga reaction with 11 MeV protons as previously described by Engle et al. [19]. Briefly, ^{66}Zn was electrodeposited on Au backing and then irradiated for 10 to 20 min at a beam current of 10 to 20 μA. Radiochemical separation was performed by ion exchange chromatography using AG 50 W X-4 resin. The reactivity or effective specific activity of ^{66}Ga was determined by titration of [^{66}Ga]$GaCl_3$ with DOTA (1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetraaceic acid).

2.4. Preparation of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$

For radiolabelling of DOTA-E-[c(RGDfK)]$_2$, 25 μl of the conjugated peptide solution (400 μg/ml, 1% EtOH), 25 μl 1.0 M HEPES (pH 7.0), and 25 μl 0.25 M NH_4OAc (pH 5.5), were mixed with 200–370 MBq of ^{66}Ga stock solution (50 μl 0.1 M HCl) and incubated for 20 min at 95 °C in a compact thermomixer (Eppendorf, USA) at 300 rpm [12]. When needed, purification of the final product was performed by SPE using Sep-Pak C18 Light cartridges. The final product was sterilized by passing through a 0.22 μm syringe filter (Millex-GV). The structural formula of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ is shown in Fig. 1 [20].

2.5. Radiochemical purity

The radiochemical purity (RCP) was determined by thin layer chromatography (TLC) using silica gel (SG) strips as stationary phase and 1:1 MeOH:10% NH_4OAc (w/v) as mobile phase [21]. Evaluation of the TLC plates was performed by autoradiography in a Cyclone Plus Storage Phosphor System (Perkin Elmer).

2.6. In vitro stability

To determine the *in vitro* stability of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ in physiological saline and serum, an aliquot (50 μl) of the labelled compound solution was incubated at 37 °C with 1 ml of 0.9% NaCl and fresh human serum. Radiochemical purity stability was evaluated up to 24 h by TLC-SG as described above.

2.7. Protein binding

To determine serum protein binding of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$, and aliquot of the labeled compound (100 μl) was incubated at 37 °C with 1 ml of fresh human serum up to 4 hours. After incubation the solution was analyzed by ultrafiltration (30,000 MW). Protein binding was determined by measuring the activity remaining in the filter, while the unbound [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ passed through the filter.

2.8. MicroPET imaging

Mice bearing glioma C6 tumors were scanned after a tail vein injection of 20 ± 0.5 MBq of [^{66}Ga]DOTA-E[c(RGDfK)]$_2$ under isoflourane anesthesia (1–3%). PET images were acquired in a MicroPET Focus 120 (Concorde Microsystems, Knoxville, TN, USA) at different post injection (p.i.) times (0.5, 1, 3, 5 and 24 h). Scan time was 20 min for images acquired at 0.5, 1, and 3 h p.i., 30 min for images acquired 5 h p.i., and 60 min for images acquired at 24 h. After PET acquisitions animals were sacrificed to perform the biodistribution studies. MicroPET images were reconstructed using a 3-D ordered subset expectation maximization (OSEM 3D) algorithm.

2.9. Biodistribution studies

After PET imaging acquisitions, animals were sacrificed by cervical dislocation at 0.5, 1, 3, 5, and 24 h p.i. (n = 3 per time point), and tissues

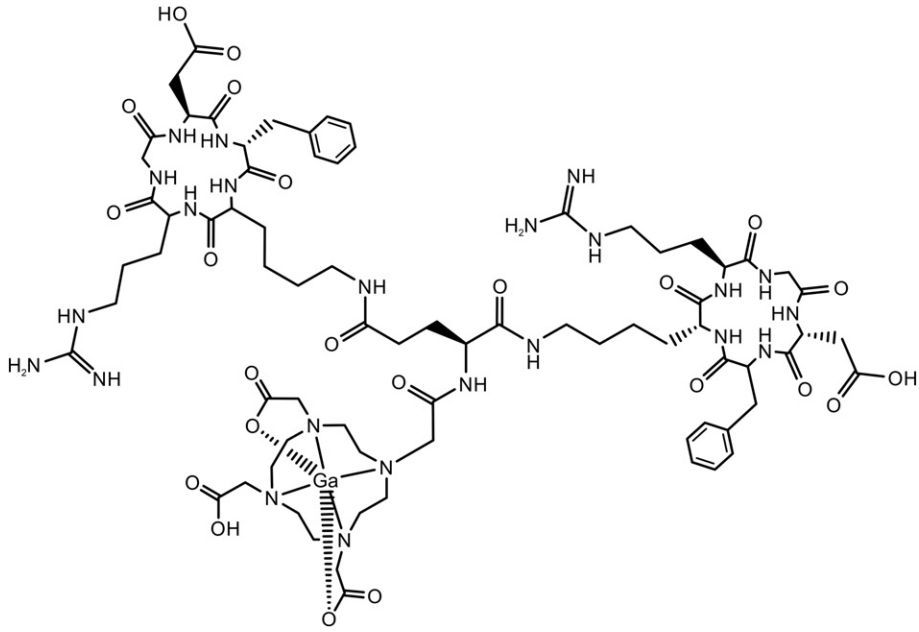


Fig. 1. Structural formula of the DOTA conjugated dimeric-RGD peptide labeled with Ga isotope [20].

of interest (blood, brain, heart, lungs, liver, spleen, blander, kidneys, bowel, muscle, femur and tumor) were removed immediately and weighed. Total blood volume, bone, and muscle mass were estimated as 5.4%, 10%, and 40% of the total body weight, respectively [22,23]. Activity in the different tissues was measured using a NaI(Tl) scintillation detector (ORTEC 905-1, AMETEK, USA). All the data were corrected for physical decay and to calculate uptake in each tissue sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results are expressed as a percentage of injected dose per gram of tissue (%ID/g) and percentage of injected dose per organ (%ID/organ).

2.10. Radiation dosimetry

Mice biodistribution data (%ID/organ) were used for estimation of the radiation absorbed dose for [^{66}Ga]DOTA-E[c(RGDfK)]$_2$. The mean activity in mice organs of interest (liver, spleen, kidneys and tumor) were used to calculate the biokinetic model, residence times and radiation absorbed doses according to the method described by Jimenez et al. [24] and Luna et al. [25]. The absorbed dose to organs was evaluated according to the equation:

$$D(r_k \leftarrow r_h) = \sum_h \widetilde{A}_h \sum_i \Delta_i \Phi_i(r_k \leftarrow r_h)$$

where $\sum_i \Delta_i \Phi_i$ values were estimated in a mouse model by Monte Carlo methodology using the Penmain programme of Penelope 2008 [26] and $\sum_h \widetilde{A}_h$ were estimated using OLINDA/EXM software [27].

2.11. Statistical methods

All of the data are presented as means ± SD.

3. Results

The experimental thin target yield (61 ± 14 mg/cm^2, 11 → 9.7 MeV) for ^{66}Zn targets was 3415 ± 681 MBq/μA with a radionuclide purity >97% at 2 h after the end of bombardment. Typical reactivity or effective specific activity of ^{66}Ga as determined by DOTA titration was in the range of 160–370 GBq/μmol.

The labeling yield of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ was almost quantitative using 5.5 nmol of the conjugated peptide. It was observed that a mass as low as 2 nmol of DOTA-E-[c(RGDfK)]$_2$ was sufficient to reach nearly quantitative labeling. Fig. 2 shows that 7 min of incubation at 95 °C are enough to reach a complexation yield > 95%. Specific activity of the labeled conjugate was in the range of 36–67 GBq/μmol. The RCP as determined by TLC was >97% without the need of purification. With the chromatographic method used, the free [^{66}Ga]$GaCl_3$ stayed at the origin, while the R_f values of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ and [^{66}Ga]DOTA were 0.6–0.7 and 0.7–0.8, respectively.

In vitro studies indicated high stability of the compound in physiological saline and fresh human serum after 1 h of incubation at 37 °C (RCP > 98%) and was degraded to ~95% at 24 h (Fig. 3) On the other hand, protein binding was negligible (<3%) after one hour of incubation in fresh human serum, but increased to almost 20% after 4 h of incubation at 37 °C.

MicroPET Images of C6 glioma tumor-bearing mice at different time points after injection of tracer showed a contrasting tumor reflecting $\alpha_v\beta_3$-targeted tracer accumulation. Typical decay-corrected images at 0.5, 1, 3, 5 and 24 h p.i. are shown in Fig. 4. Note that the C6 tumors were clearly visualized with good tumor-to-background contrast for all time points evaluated. Serial microPET imaging revealed a rapid

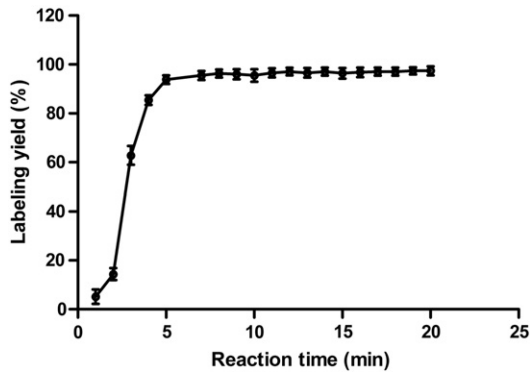


Fig. 2. Effect of incubation time on the complexation yield of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ at 95 °C.

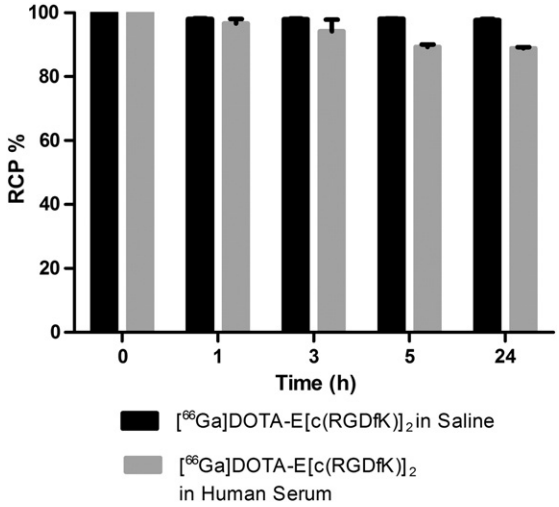


Fig. 3. Stability studies showed high stability of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ up to 24 h in human serum and phisiological saline.

clearance from blood and kidneys and high uptake in the liver and spleen, which was consistent with biodistribution data.

Biodistribution data (%ID/g and %ID/organ) of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ are shown in Fig. 5. High uptake was observed in organs such as liver, kidneys, lung, spleen, heart, bladder, and muscle at early times, but significantly decreased at 5 h p.i. Rapid blood clearance was also observed, reaching a blood uptake of only 2.81 ± 0.32 %ID/g at 1 h p.i. Uptake of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ by the C6 tumor xenograft indicated stable retention of the tracer with minimal wash-out up to 24 h. Tumor-to-muscle ratios (% ID/g) were 1.40 ± 0.97 and 1.78 ± 0.57 at 0.5 h and 24 h p.i., respectively, while tumor-to-blood ratio was 0.20 ± 0.09 at 0.5 h, increasing to 1.97 ± 0.37 at 24 h p.i.

Fig. 6 shows the radiation absorbed doses normalized to unit injected activity (mGy/MBq) and the tumor-to-tissue absorbed dose ratios. As expected, the highest radiation absorbed dose was deposited in the tumor (65.53 ± 8.39 mGy/MBq), followed by the liver (48.06 ± 17.01), kidneys (32.72 ± 4.42), and spleen (27.2 ± 7.29). The biological residence time for the C6 glioma tumors was 5.57 h.

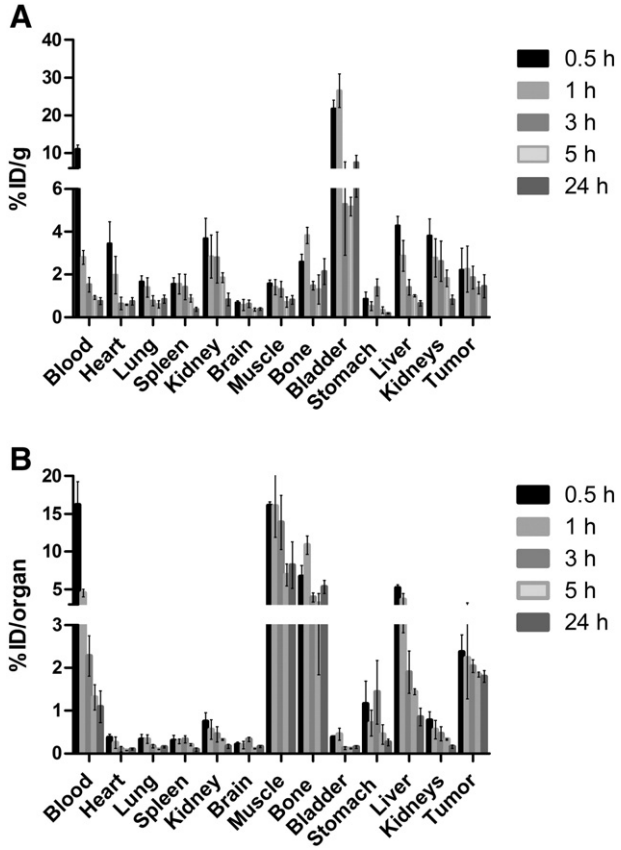


Fig. 5. Biodistribution data for [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ in mice 0.5, 1, 3, 5 and 24 h p.i. Results are shown as A) %ID/g (mean ± SD) and B) %ID/organ (mean ± SD).

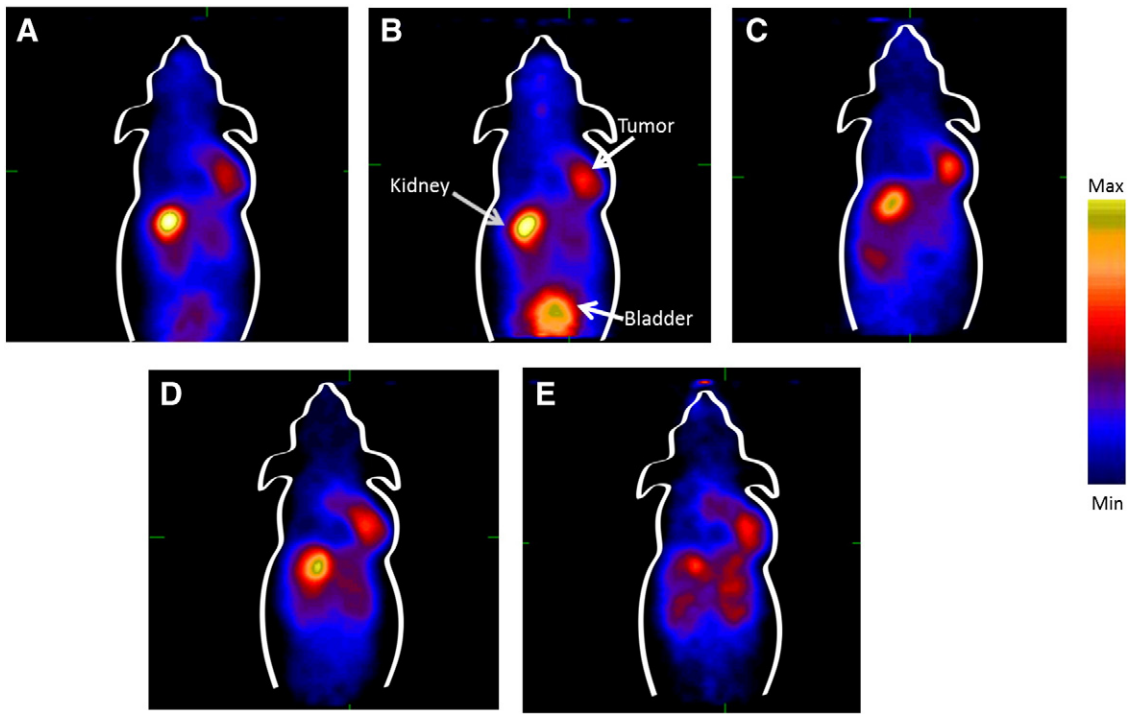
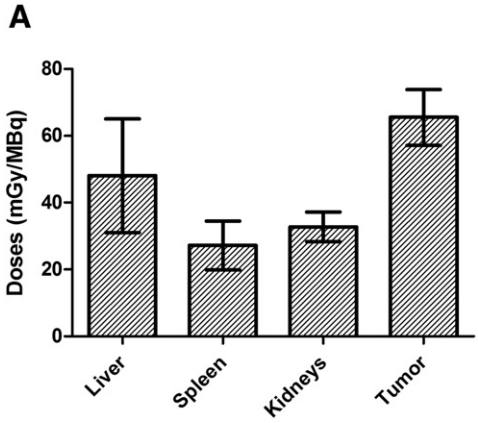


Fig. 4. Coronal microPET images of [^{66}Ga]DOTA -E-[c(RGDfK)]$_2$ in nude mice bearing C6 tumor xenograft at 0.5 h (A), 1 h (B), 3 h (C), 5 h (D) and 24 h (C) after injection of 20 ± 0.5 MBq of tracer under isoflourane anesthesia. Acquisition time was 20 min for A,B, and C); 30 min for D, and 60 min for E.



Tissue	Tumor to tissue absorbed dose ratio
Liver	1.36
Spleen	2.41
Kidneys	2.01
Tumor	1

Fig. 6. A)Estimated radiation absorbed doses normalized to unit injected activity and B) tumor-to-tissue absorbed dose ratios of [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ in nude mice bearing C6 tumor xenograft.

4. Discussion

During the past few years different multivalent cyclic RGD peptide conjugates have been used to target tumors over-expressing $\alpha_v\beta_3$ integrin receptors [25–39]; however, it has been demonstrated that the dimeric conjugate is more suitable for both, imaging and therapeutic applications. Li et al. [34] showed that despite the fact that the tetrameric peptide conjugate labeled with ^{68}Ga had the highest tumor uptake, its poor tumor-to-kidney ratio makes this compound less useful than the dimeric and monomeric counterparts. It was also found that the dimeric and monomeric peptide had similar tumor-to-kidney ratios but the dimer had higher tumor uptake and a prolonged retention time, making it more suitable than the monomer. On the other hand, Luna-Gutierrez et al. [25] investigated the potential of ^{177}Lu-labeled monomeric and dimeric cyclic RGD peptides ([c(RGDfK)]$_n$) for the treatment of tumors over-expressing $\alpha_v\beta_3$ integrins. *In vivo* evaluation in a mouse U87MG xenograft model showed that [^{177}Lu]DOTA-E-[c(RGDfK)]$_2$ had a higher uptake in tumor and a higher tumor-to-kidney ratio than [^{177}Lu]DOTA-E-c(RGDfK), making the dimer more suitable than the monomer for therapeutic applications.

In this light we evaluated the dimeric conjugated DOTA-E-[c(RGDfK)]$_2$ labeled with ^{66}Ga to target tumors over expressing $\alpha_v\beta_3$ integrin receptors. The glioma cell line used in this research was the C6 which is known to have a high tumor density of integrin $\alpha_v\beta_3$ receptors on the surface (1.51×10^{11} number of receptors/mg protein) [28], making this cell line a good candidate to evaluate theranostic applications with peptides containing the amino acid sequence RDG, labeled with the appropriate radionuclide. For imaging purposes, ^{68}Ga had shown to be a suitable radionuclide as it can be easily obtained from a ^{68}Ge/^{68}Ga generator [29–32], while for therapeutic applications the beta emitter ^{177}Lu is promising [34–37]. However, due to its decay scheme and the unique high energy of emitted positrons, ^{66}Ga has the potential to serve a dual role in the development of agents for PET-molecular imaging and radioimmunotherapy drugs for oncology. Additionally, high specific activity ^{66}Ga, in enough quantity and quality for clinical applications, can be efficiently produced in compact biomedical cyclotrons using enriched target material, which is relatively inexpensive (~2–3 USA dll/mg, ^{66}Zn > 99%) [19].

The most important feature of targeted radionuclide therapy is to deliver a tumoricidal dose for tumor ablation, without compromising other vital organs [38]. The [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ evaluated in this research showed good pharmacokinetic characteristics with a relatively stable tumor uptake and a rapid blood clearance. Dosimetry estimations from our biodistribution data showed that critical organs such as liver, kidneys, and spleen can receive a considerable radiation dose per unit injected activity, but in all cases the dose was lower than the one received by the tumor. Of particular interest in this research was to determine the tumor-to-tissue ratios that better define tumor targeting properties.

The 65 mGy/MBq radiation absorbed dose normalized to unit injected activity determined in this research for [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ in C6 tumors was significantly lower than the 230 mGy/MBq reported by Luna-Gutierrez et al. [25] for [^{177}Lu]DOTA-E-[c(RGDfK)]$_2$ in U87MG tumors; however, the tumor-to-tissue ratios reported in both cases are very similar. Note that for comparison purposes the higher density of integrin $\alpha_v\beta_3$ receptors on the surface of U87MG *vs*. C6 cells [28] is compensated in some way by the higher specific activity of ^{177}Lu *vs*. ^{66}Ga [25]. These results suggest that [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ possesses potential for targeted radionuclide therapy of tumors over expressing integrin $\alpha_v\beta_3$ receptors. Additionally, we had shown that, despite the high energy positrons emitted by ^{66}Ga, it is possible to get good quality microPET-images.

Since [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ combines imaging and radiotherapy in one preparation, it could be useful as a theranostic radiopharmaceutical for tumors over-expressing $\alpha_v\beta_3$ integrins. To plan a diagnosis/treatment scheme for an individual patient, the diagnosis and prospective radiation absorbed dose estimates could be made by administering a tracer activity of the radiopharmaceutical and subsequently the larger therapeutic activity. The quantitative patient-specific dosimetry work-up using a diagnostic dose before the therapeutic dose would be useful to identify cancer patients for whom the treatment is most likely to be effective, eliminating those patients for whom it would be unsuccessful ("personalized medicine"). In agreement with other radionuclide therapy protocols, positively charged amino acids could be coinfused to reduce the radiopeptide kidney retention considering that the maximum tolerated dose (MTD) for kidney is 25 Gy, while for spleen and liver the MTD value is almost twice [39].

5. Conclusions

To summarize, ^{66}Ga labeled DOTA-E-[c(RGDfK)]$_2$ was prepared with high yield, specific activity and radiochemical purity. The microPET imaging and biodistribution studies showed high affinity for the $\alpha_v\beta_3$ integrin receptors, with rapid blood clearance; and the radiation absorbed dose estimation suggested sub-toxic doses to critical organs. These results support the idea that [^{66}Ga]DOTA-E-[c(RGDfK)]$_2$ may be attractive as a theranostic agent for tumors over-expressing $\alpha_v\beta_3$ integrins.

Acknowledgements

We are grateful to A. Zarate-Morales and A. Flores-Moreno for cyclotron irradiations and to V.M. Lara-Camacho and M. Avila-Garcia for microPET imaging. This research was supported by CONACYT Grant

179218, UNAM-DGAPA-PAPIIT TA200512 and the International Atomic Energy Agency RC16467.

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