[68]Ga-labeled NODAGA-RGD-BBN heterodimer peptide as a novel radiotracer for dual integrin and GRPR-targeted tumor imaging

N. Amraee[1], B. Alirezapour[2*], M. Hosntalab[1], A. Hadadi[1], H. Yousefnia[2]

[1]*Medical Radiation Engineering Department, Science and Research Branch, Islamic Azad University (IAU), Tehran, Iran*

[2]*Radiation Application Research School, Nuclear Science and Technology Research Institute (NSTRI), Tehran, Iran*

► *Original article*

ABSTRACT

Background: Due to the excellent physical and biochemical characteristics of ^{68}Ga, ^{68}Ga-1,4,7-triazacy–clononane–glutaric acid– 4,7 acetic acid- arginyl-glycyl-aspartic acid- bombesin (^{68}Ga-NODAGA-RGD-BBN) was prepared as a new positron emission tomography (PET) imaging agent, and afterward, the preclinical evaluation of this labeled peptide was studied. **Materials and Methods:** [68]Ga radioisotope was extracted from a [68]Ge/^{68}Ga generator with high radionuclide, chemical and radiochemical purities. Then; the ^{68}Ga-NODAGA-RGD-BBN radiolabeled complex was prepared at optimized conditions. The stability of the complex was evaluated in phosphate-buffered saline (PBS) for at least 2 h. Cell studies of the radiolabeled peptide were also assessed on the gastrin releasing peptide receptor (GRPR)-expressing cell line. Finally; the biodistribution and whole-body scan imaging study of ^{68}Ga-NODAGA-RGD-BBN was studied in normal and tumor-bearing mice. **Results:** The biodistribution and whole-body scan imaging of the radiolabeled compound on GRPR-expressing tumor-bearing mice demonstrated the high uptake in the tumor site at all post-injection intervals. The biodistribution results also demonstrated the major excretion route of the complex is the urinary tract. **Conclusions:** ^{68}Ga-NODAGA-RGD-BBN shows high potential for PET imaging of patients with GRPR-expressing tumors; however, more biological studies are still needed.

INTRODUCTION

Nowadays, cancer is the second leading cause of death in the world [1] and prostate cancer is considered one of the most common cancers in the men [2]. The early diagnosis and timely treatment of cancer are the most important issues in managing the disease.

RGD and BBN are two peptides in the body that consist of 3 and 14 amino acids, respectively [3, 4]. Integrins and gastrin are the receptors of RGD and BBN peptides, correspondingly. Integrins proteins are one the most important factors for regulating angiogenesis, as the key process of tumor proliferation [5, 6]. Since; integrins Alpha-V Beta-3 ($\alpha_v\beta_3$) in the angiogenesis process are overexpressed on the cell membrane of different tumors such as breast cancer [7, 8], ovarian cancer [9] and melanoma [10]; therefore, the integrins can be considered an excellent target for new radiolabeled compounds. The studies confirm that the three amino acid peptides of RGD with the known sequence of arginine-glycine-aspartic can specifically bind to the $\alpha_v\beta_3$ integrins [11].

Integrins and gastrin are overexpressed in a series of human cancers such as breast, prostate, glioma, pancreas, etc. [12, 13]. Therefore; these peptides have been used as excellent carriers to label with diagnostic and therapeutic radionuclides such as ^{64}Cu, ^{68}Ga, ^{18}F, ^{123}I, ^{111}In, and [90]Y [14-17]. Among the PET radionuclides, ^{68}Ga has special nuclear (half-life of 68 min; β^+=89%) and chemical characteristics. Also, ^{68}Ga availability in the form of a [68]Ge/^{68}Ga generator, makes it an excellent candidate for the development of a new PET radiolabeled compound [15, 16].

The recent investigations indicated that the peptide heterodimers are more effective than their monomeric components alone, in the diagnosis of the cancers [17]. The suitable chelator or linker is the other important issue in developing new radiotracers especially in connection of radiometals to peptides [18-24]. NODAGA is one of the newly developed chelators that can firmly bind to the radiometals such as ^{68}Ga radionuclide.

Due to the fascinating physical and chemical characteristics of ^{68}Ga, as one of the most promising PET radioisotopes, and the high specificity of RGD and BBN peptides for tumor detection, this study aimed to label RGD-BBN heterodimer peptide with ^{68}Ga using a new developed chelator of NODAGA and

to study the biological properties of ^{68}Ga-NODAGA-RGD-BBN, as a new PET imaging agent.

MATERIALS AND METHODS

Our investigation is a comprehensive descriptive study based on the preparation, quality control, and biodistribution assay of the ^{68}Ga-NODAGA-RGD-BBN compound.

Materials

All chemicals were purchased from Sigma Aldrich Co. (Germany) and were used without further purification. NODAGA-RGD-BBN heterodimer peptide (purity of more than 99%) was prepared by International Atomic Energy Agency (IAEA) (Vienna, Austria) through F22067 Coordinated Research Project (CRP). The ^{68}Ge/^{68}Ga generator with a nominal activity of 1480 MBq was obtained from Pars Isotope Co. (Tehran, Iran). PC3 and LNCaP cell lines were purchased by Pasteur Institute (Tehran, Iran). The normal and tumor-bearing nude mice were also prepared from Royan Institute (Tehran, Iran).

Peptide radiolabeling

In this study, the ^{68}Ga radionuclide was eluted from a ^{68}Ge/^{68}Ga generator with a nominal activity of 1480 MBq by 0.1 M HCl in the form of ^{68}GaCl$_3$. Radionuclide purity and activities were measured using a NIGC 4020 high pure germanium (HPGe) detector (DSG Co., Germany) equipped with a multichannel analyzer (MCA). Radiochemical purity of ^{68}GaCl$_3$ was checked by radio thin layer chromatography (RTLC) and using ammonium acetate 1%: methanol (1:1) as a mobile phase and silica gel as a stationary phase and 10 mM DTPA as a mobile phase, and Whatman No.2 as a stationary phase. The Chemical purity of the generator eluent was assessed by inductively coupled plasma-mass (ICP-MS) spectrometry.

A stock solution of peptide (1 mg/mL) was prepared by solving 5 mg of the peptide in 5 mL ultrapure water and used for radiolabeling purpose. Different experiments were performed to optimize the radiolabeling conditions. For this purpose, the pH was considered 2 to 5 adjusted by 2-[4-(2-hdroxyethyl) piperazin-1-yl] ethane sulfonic acid (HEPES), while other parameters were constant. At the optimum labeling pH, the reaction temperature was changed from 25 to 95 ºC, while other parameters were constant. At the optimum labeling pH and temperature, the peptide quantity was varied from 10 to 30 µg.

Quality control and Stability studies of radiolabeled compound

Radiochemical purity was checked by high-performance liquid chromatography (HPLC) (Knauer Co., Germany) equipped with Octadecyl-silica (ODS) column, UV, and gamma detectors. Chemical purity was also studied by inductively coupled plasma mass spectrometry (ICP-MS) technique. The radiochemical purity of the final prepared radiolabeled compound was assessed by RTLC and HPLC methods. The 1 M ammonium acetate: Methanol (1:1; V:V) and silica-gel paper were considered mobile and stationary phases, respectively. For HPLC analysis, the C$_{18}$ column (250×4.6 mm, Perfectsil Target ODS-3 5µm) was washed by two solvent systems of A [0.1% TFA:acetonitrile (1:1)] and B [0.1% TFA:water (1:1)] with a linear gradient at a flow rate of 1 mL/min.

The stability of the radiolabeled compound was assessed in PBS buffer (4 ºC), room temperature, and human serum (37 ºC), separately. For this purpose, the radiochemical purity of ^{68}Ga-NODAGA-RGD-BBN was determined using RTLC and HPLC methods for at least 2 h.

Partition coefficient

The activity ratio of organic and aqueous phases was considered as P, to calculate the partition coefficient (log P) of ^{68}Ga-NODAGA-RGD-BBN. A mixture containing 500 µL octanol, 500 µL water, and 37 MBq of the radiolabeled compound was vortexed for 5 min at 37 ºC. The mixture was centrifuged until the aqueous, and organic phases were completely separated, and then each phase was counted separately. The experiment was repeated five times, and the log P was reported as the average of independent measurements.

Cell binding and Cell internalization studies

Binding assay experiment was performed on PC3 and LNCaP as the positive GRPR-expressing cell lines and CHO as a negative GRPR-expressing cell line. 1 × 10^6 cells were seeded in each well of 6-well plates and allowed to adhere overnight at 37 °C. The media was removed and washed with PBS and replaced with 3 mL of complete fresh media; the plates were incubated for 1h at 37 ºC and then put on the ice for 30 min. Finally, different concentration of radiolabeled compound (50-1000 nM for LNCaP and 1-100 nM for PC3) was added to the wells, and the plates were incubated at 4 °C, gently rocking. The cells were washed three times with PBS, trypsinized, and transferred to microcentrifuge tubes. Radioactivity associated with cells was counted in a gamma counter. The percentage of bound radioactivity was calculated as the ratio of bound to the total radioactivity added per well multiplied by 100. Non-specific binding was performed using 1 µM of cold RGD-BBN peptide. The cell internalization follows the below instructions:

PC3, LNCaP, and CHO cell lines (1-2 × 10^4 cells) were seeded into the wells and allowed to adhere overnight. The media was removed, and the ^{68}Ga-NODAGA-RGD-BBN with the concentration of 18 kBq/mL was added to the wells. The plates were incubated for various intervals (30, 60, and 120 min).

The activity of the media was measured separately. 1 mL glycine (0.05 M) was added to each well at any interval and counted to measure the non-specific cell binds, and finally wells were gently washed with PBS, and 1 M NaOH was added to the wells. The final solution was also collected and counted.

Biodistribution in normal and PC3 tumor-bearing mice

In this study, the biodistribution of ^{68}Ga-NODAGA-RGD-BBN was also investigated in normal and PC3 tumor-bearing nude mice. The animals were inoculated subcutaneously with 8×10^5 PC3 cells in 150 μL of PBS for tumor induction. After 20-25 days, the tumors were grown, and 100 μl (3.7 MBq) of the final prepared radiolabeled compound was intravenously injected into the normal and GRPR-expressing tumor-bearing nude mice. For the blocking study, radiolabeled compound with unlabeled peptide [13.5 nmol/kg (2mg/kg) body weight] was co-injected into a group of three PC3 tumor-bearing nude mice. These mice were sacrificed (using CO2 gas) at 30 and 60 min post-injection, whereas the normal BALB/c mice were sacrificed at 15, 30, 60, and 120 min intervals. In the following, the main organs were removed and washed with water. The activity of each organ was measured using the HPGe detector, and the %ID/g of each organ was calculated as the activity of each organ divided by injected activity and the weight of the organ. The entire animal experiments were conducted according to the "*General Principles and Guidelines for Care and Use of Experimental Animals",* Tarbiat Modares University [25].

Imaging studies

The images were acquired with a dual-head single-photon emission computed tomography (SPECT) system (DST-XL, France) equipped with a high-resolution, parallel-hole, high-energy collimator. Furthermore, all animals were kept on a routine day/night standard diet. The normal and PC3 tumor-bearing mice were imaged after intravenous injection of 100 μl (3.7 MBq) of ^{68}Ga-NODAGA-RGD-BBN. The animals were anesthetized by a combination of ketamine:xylazine:PBS (2:1:1), and their whole body scintigrams were obtained at 30 and 60 min post-injection by a SPECT system.

Statistical analysis

For considering statistical analysis, three mice were sacrificed in each time interval (n=3). Quantitative data are expressed as mean value ± SD (standard deviation). Mean values were compared using a one-way analysis of variance (ANOVA) and a Student's *T*-test. P values <0.05 were considered statistically significant.

RESULTS

Quality control of ^{68}GaCl$_3$

Figure 1 shows the gamma spectrum of the eluted ^{68}Ga radioisotope. The result shows all gamma peaks emitting from the ^{68}Ga radioisotope. The activity of the eluted ^{68}Ge measured at least 24 h post elution was less than 0.001 % of the total activity, which is in an acceptable range for radiopharmaceutical development.
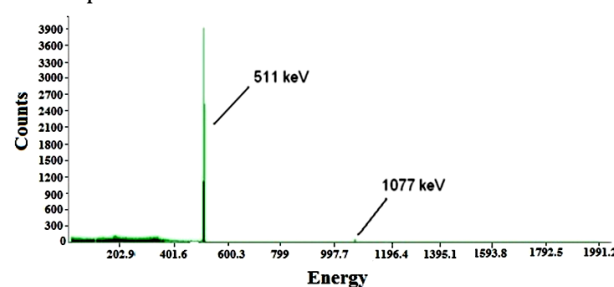

Figure 1. Gamma Spectrum of 68GaCl3 solution. The spectrum shows all gamma peaks emitting from the 68Ga radioisotope (Kilo Electron Volt abbreviated as keV).

Indeed, figure 2 shows the radiochemical quality control result. As the data show, the free ^{68}Ga stays at the origin when ammonium acetate 1%: methanol (1:1) is used as the mobile phase, while it migrates to the higher R$_f$s with 10 mM DTPA. Both mobile phases showed a radiochemical purity of more than 99.9 %.
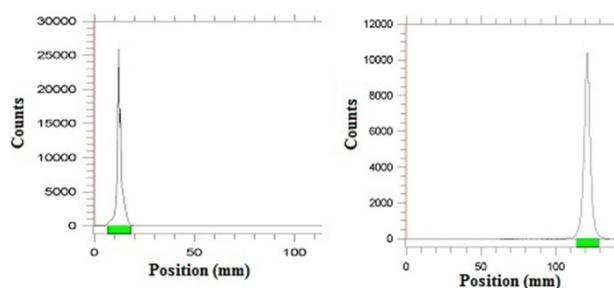

Figure 2. Radio thin layer chromatography (RTLC) of 68GaCl3 using ammonium acetate 1%: methanol (1:1) as a mobile phase and silica gel as a stationary phase (left), and 10 mM DTPA as a mobile phase, and Whatman No.2 as a stationary phase (right).

Radiolabeling of the peptide with ^{68}Ga

Different experiments were performed to optimize the radiolabeling conditions. The optimized condition was as follows: 1295 MBq ^{68}GaCl$_3$ (2 mL) was added to the vial containing 90 mg HEPES and 20 μg NODAGA-RGD-BBN and the reaction was continued at 95 °C for 10 min. The final solution was purified by C$_{18}$ Sep-Pak (Waters) column preconditioned with 5 mL ethanol, 10 mL water, respectively. The column was then washed with 1 mL ethanol and 9 mL of 0.9% NaCl. The complex was obtained with radiochemical purity greater than 98

% (figure 3) and specific activity of 75 GBq/μmol. The free gallium with a more polar nature is removed from the column in less than one minute, while the labeled peptide is removed from the column after 4 minutes.
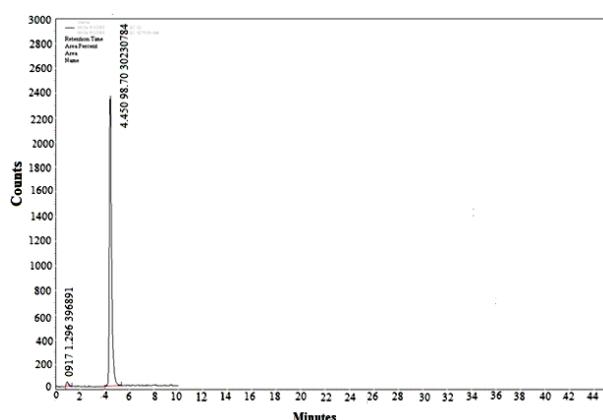

Figure 3. High-performance liquid chromatography (HPLC) of the final radiolabeled compound. The free gallium and the labeled peptide are removed from the column in less than 1 and 4 minutes, respectively.

Stability studies

The stability of the radiolabeled compound was studied in PBS buffer (4 °C), room temperature, and human serum (37 °C). The results showed that at least for 2 h, the radiochemical purity of greater than 96%, 95%, and 90 % in PBS buffer, room temperature, and human serum, respectively.

Partition coefficient of ^{68}Ga-NODAGA-RGD-BBN

The partition coefficient (organic to aqueous phase's activity ratio, P) study was carried out for five independent experiments. The value of -1.14 for the average partition coefficient (log P) for ^{68}Ga-NODAGA-RGD-BBN indicates the hydrophilicity of the radiolabeled compound. According to the chemical formula of the radiolabeled compound, the ^{68}Ga-NODAGA-RGD-BBN has an ionic nature, and therefore, the average partition coefficient is -1.14. The lipophilicity property of the final prepared radioligand can help to the faster kidney washout and unnecessary uptake.

Cell binding and Cell internalization analysis

Binding study of ^{68}Ga-NODAGA-RGD-BBN was performed on PC3 and LNCaP as the positive GRPR-expressing cell lines and CHO as a negative GRPR-expressing cell line. Figure 4 and also figure 5 demonstrate the results of the binding assay for the radiolabeled compound using LNCaP, PC3, and CHO cell lines. Internalization assays were also performed using PC3, LNCaP, and CHO cell lines to demonstrate the internalized activity as a function of time. The result of the internalization assay is shown in figure 6.
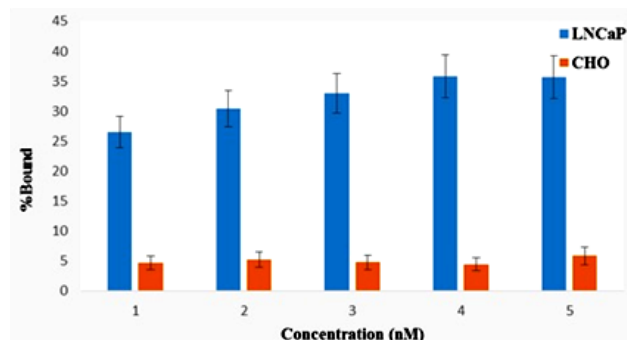

Figure 4. Binding assay of 68Ga-NODAGA-RGD-BBN in different concentrations on LNCaP cell line (as a positive GRPR-expressing cell line) and CHO cell line (as a negative GRPR-expressing cell line).
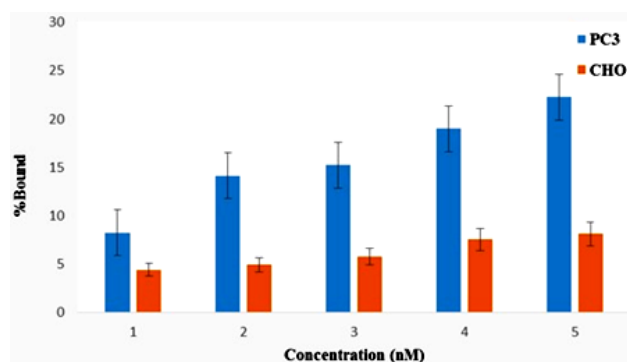

Figure 5. Binding assay of ^{68}Ga-NODAGA-RGD-BBN in different concentrations on PC3 cell line (as a positive GRPR-expressing cell line) and CHO cell line (as a negative GRPR-expressing cell line).
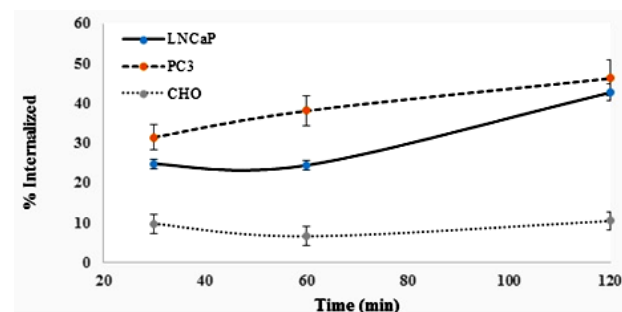

Figure 6. Internalization assay of ^{68}Ga-NODAGA-RGD-BBN on PC3, LNCaP, and CHO cell lines to demonstrates the internalized activity as a function of time.

The binding study demonstrated that the binding would be increased with increasing the PC3 and LNCaP cell concentrations, which is due to the increasing the cell surface receptors. Indeed, the binding on the CHO cell line won't be increased by increasing the cell concentration. As shown in figure 7, more than 40% of the radiolabeled peptide is internalized to PC3 and LNCaP cells only in 2 h post-incubation, while no significant internalization was observed for CHO cells.

Biodistribution in normal and tumor-bearing mice

Biodistribution of ^{68}Ga-NODAGA-RGD-BBN was studied in normal and GRPR-expressing tumor-

bearing nude mice (figures 7 and 8). The results indicated that the radiolabeled complex is removed from the blood quickly and excreted via the urinary tract. Also, the complex demonstrated significant uptake in GRPR-expressing tumor sites. Figure 9 shows the tumor to non-tumor ratios for the radiolabeled peptide at 30 and 60 min after administration of PC3 tumor-bearing nude mice.
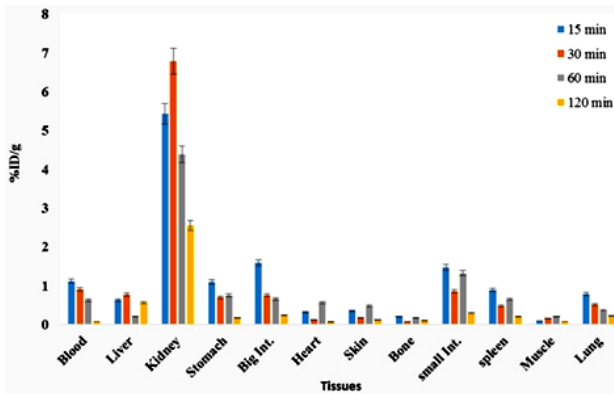

Figure 7. Biodistribution of 68Ga-NODAGA-RGD-BBN in normal BALB/c mice (3.7 MBq, 100 µl, %ID/g ± SD, n=3) up to 120 min post injection, (percentage of injected dose per gram abbreviated as %ID/g).
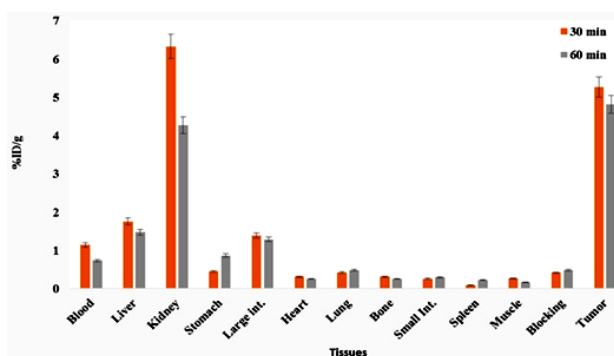

Figure 8. Biodistribution of 68Ga-NODAGA-RGD-BBN in PC3 tumor-bearing nude mice (3.7 MBq, 100 µl, %ID/g ± SD, n=3) up to 60 min post-injection, (percentage of injected dose per gram abbreviated as %ID/g).
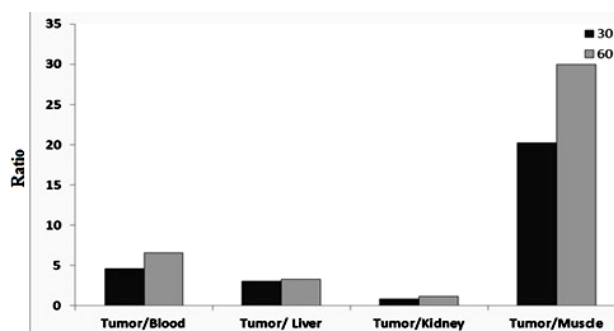

Figure 9. Tumor to non-tumor tissues ratio of 68Ga-NODAGA-RGD-BBN (3.7 MBq, 100 µl, n=3) at 30 and 60 min after administration of the PC3 tumor-bearing nude mice.

Based on data obtained, the kidneys were shown the high uptake at all intervals that confirmed the high water solubility of ⁶⁸Ga-NODAGA-RGD-BBN (logP= -1.14) and the excretion of the radiolabeled compound via a urinary tract. That is in agreement with the biodistribution of ⁶⁸Ga-NOTA-RGD-BBN and

other radiolabeled peptides [21]. The radiolabeled peptide biodistribution assay in GRPR-expressing tumor-bearing mice demonstrated a high uptake in the tumor site at 30 min and 60 min post-injection, which is comparable with the uptake of ⁶⁸Ga-NOTA-RGD-BBN in GRPR-expressing tumors. Indeed, there has been no indication of accumulation in the site of the tumor in the blocking study [21]. Over time increased target to non-target uptake ratio indicates that longer post-injection is more suitable for GRPR-expressing tumor detection after injection of ⁶⁸Ga-NOTA-RGD-BBN via PET imaging.

Imaging studies

The tumor targeting efficacy of ⁶⁸Ga-NODAGA-RGD-BBN was evaluated by whole-body imaging. This assay was carried out using the tumor-bearing mice at 30 and 60 min post-injection. Blocking and unblocking images were captured via a dual-head SPECT system. Figure 10 illustrates the whole-body scan images of the PC3 tumor-bearing mice (a: 30 min and b: 60 min) and blocking (c: 30 min and d: 60 min) of the radiolabeled complex.

The obtained images confirm the biodistribution results. The unblocking image results show the significant accumulation of the radiolabeled compound in the tumor at each time point.
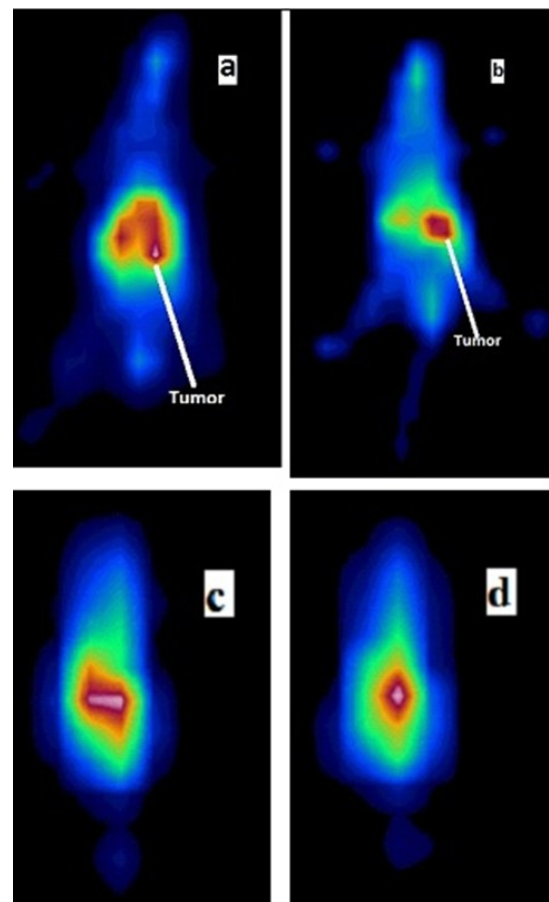

Figure 10. Whole-body scan images of the PC3 tumor-bearing mice (a: 30 min and b: 60 min) and blocking (c: 30 min and d: 60 min) post-injection of 68Ga-NODAGA-RGD-BBN (3.7 MBq, 100 µl).

DISCUSSION

The new radiolabeled compound of ^{68}Ga-NODAGA-RGD-BBN can be prepared with high radiochemical purity, specific activity and stability. The cell binding studies of ^{68}Ga-NODAGA-RGD-BBN on GRPR-expressing PC3 and LNCaP cell lines and CHO cell line as negative control showed that the binding would be increased with increasing the PC3 and LNCaP cell concentrations that is due to the increasing cell surface receptors. Also, the percentage of the binding for the radiolabeled compound to the LNCaP cell line was much higher compared to PC3 cell line at the same concentration which can be due to the more expression of GRPRs on LNCaP cell line. The cell internalization studies of ^{68}Ga-NODAGA-RGD-BBN on PC3, LNCaP and CHO cell lines showed more than 40% of the radiolabeled peptide is internalized into PC3 and LNCaP cells only in 2 h post incubation which is in accordance with the agonist nature of RGD and BBN peptides, while no significant internalization was observed for CHO cells.

However, higher tumor uptake of ^{68}Ga-NOTA-RGD-BBN on T47D, and MDA-MB-435 tumor cells has been reported compared to ^{68}Ga-NOTA-RGD and ^{68}Ga-NOTA-BBN radiolabeled compounds [26], ^{68}Ga-NODAGA-RGD-BBN shows the higher uptake on PC3 cells compared to ^{68}Ga-NOTA-RGD-BBN, which can be due to the differences in the tumor cells and the expression of the receptors. Also, that study showed increased uptake of ^{68}Ga-NOTA-RGD-BBN on T47D, and MDA-MB-435 tumor cells over time for both cell lines [26]. Other study has been compared the cell uptake of ^{68}Ga-NOTA-RGD-BBN, ^{64}Cu-NOTA-RGD-BBN and ^{18}F-FB-PEG3-RGD-BBN on T47D and MDA-MB-435 tumor cells [21] which demonstrated significantly higher uptake of ^{64}Cu-NOTA-RGD-BBN compared to two other radiolabeled compounds. Also, the last study confirmed that the increased uptake with time on both tumor cell lines for all the radiotracers.

It should be noted that due to the water solubility of the radiolabeled compound (average partition coefficient of -1.14), the major excretion route of ^{68}Ga-NODAGA-RGD-BBN is the urinary tract, like other radiolabeled peptides. [68]GaNOTA-RGD-BBN was also shown prominent uptake in the kidneys even at early intervals suggesting the urinary tract as a main route of excretion [26]. The similar observations were reported for excretion of ^{64}Cu-NOTA-RGD-BBN and ^{18}F-FB-PEG3-RGD-BBN [21].

The biodistribution and whole-body scan imaging of the ^{68}Ga-NODAGA-RGD-BBN radiolabeled compound on GRPR-expressing tumor-bearing mice demonstrated the high uptake in the tumor site at all intervals post-injection. It is noteworthy that the whole-body scan images of the blocking and PC3 tumor-bearing mice confirmed the biodistribution results. The biodistribution of the radiolabeled compound also shows the fast removal of the radiolabeled complex from the blood. Also, those results demonstrated higher GRPR expression might result in a higher uptake of the radiotracer, which is in agreement with this study. ^{68}Ga-NOTA-RGD-BBN has been demonstrated the tumor uptake of 5.26±0.32 %ID/g at 60 min post-injection [26] which is comparable with the tumor uptake of 5.05±0.19 % ID/g (60 min post-injection) for ^{68}Ga-NODAGA-RGD-BBN. Other study on ^{18}F-FB-PEG3-RGD-BBN showed the tumor uptake of 1.81± 0.34 vs 1.59±0.65 %ID/g at 60 min post-injection in T47D and MDA-MB-435 tumor models, respectively [21].

According to the obtained results, the radiolabeled compound has favorable characteristics, including good radiochemical purity, high stability and considerable affinity to the GRPR receptor, significant internalization to the target cells, fast blood clearance, and significant tumor uptake at each time point of the study.

CONCLUSION

The ^{68}Ga-NODAGA-RGD-BBN complex can be prepared radiochemical purity of >98% and specific activity of 75 GBq/µmol at optimized conditions. This new agent has a high affinity to the GRPR-expressing tumor cells and can be considered as a great potential agent for PET imaging of GRPR-expressing tumors; however more biological studies are still needed.

Conflict of interest: The authors declare that they have no conflict of interests, scientific or otherwise in the publication of this article.

Ethical consideration: The entire animal experiments were conducted according to the "*General Principles and Guidelines for Care and Use of Experimental Animals",* Tarbiat Modares University.

Author contribution: The authors confirm contribution to the manuscript as follows: study conception and design: B. Alirezapour, H. Yousefnia; data collection: N. Amraee; analysis and interpretation of results: B. Alirezapour, M. Hosntalab; draft manuscript preparation: B. Alirezapour, H. Yousefnia. All authors reviewed the results and approved the final version of the manuscript.

REFERENCES

1. www.euro.who.int-"world health organization, regional office for Europe".

2. Ferlay J, Shin HR, Bray F, Forman D, *et al.* (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer,* **127**(12): 2893-917.

3. Fischer R, Hill RM, Roland F, Diana W (1969) Effects of psychodysleptic drug psilocybin on visual perception: Changes in brightness perference. *Experientia*, **25**: 166-169.

4. Nagalla SR, Brenda JB, Arnold MF, Bradford W, Gibson JE, Taylor JZ, *et al.* (1996) There are three distinct forms of Bombesin: IDENTIFICATION OF [Leu13] BOMBESIN,[Phe13] BOMBESIN, AND [Ser3, Arg10, Phe13] BOMBESIN IN THE FROG BOMBINA ORIENTALIS *Journal of Biological Chemistry,* **271**(13): 7731-7737.

5. Tonini T, Francesca R, Pier PC (2003) Molecular basis of angiogenesis and cancer. *Oncogene,* **22**(42):6549-6556.

6. Hanahan D, Robert AW (2011) Hallmarks of cancer: the next generation. *Cell,* **144**(5): 646-674.

7. Axelsson R, Tore BG, Juan CC, Brian JM, Study G (2010) An open-label, multicenter, phase 2a study to assess the feasibility of imaging metastases in late-stage cancer patients with the αv β 3-selective angiogenesis imaging agent 99mTc-NC100692. *Acta Radiologica,* **51**(1): 40-46.

8. Liu L, Yan S, Shi G, Tiefeng Ji, *et al.* (2014) 99mTc-3PRGD2 scintimammography in palpable and nonpalpable breast lesions. *Molecular imaging,* **13**(5): 7290-2014.

9. Shinderman ME, Cohen K, Weingarten C, *et al.* (2016) The thyroid hormone-αvβ3 integrin axis in ovarian cancer: regulation of gene transcription and MAPK-dependent proliferation. *Oncogene,* **35** (15): 1977-1987.

10. Huang R and Einar KR (2018) Integrins as therapeutic targets in the organ-specific metastasis of human malignant melanoma. *Journal of Experimental & Clinical Cancer Research,* **37**(1): 1-14.

11. Li, Q, Caili Z, Wenqiang T, *et al.* (2017) Novel amino-pyridine functionalized chitosan quaternary ammonium derivatives: Design, synthesis, and antioxidant activity, *Molecules,* **22**(1): 156.

12. Moody TW, Lingaku L, Irene RA, Tatiana I, *et al.* (2021) Bombesin receptor family activation and CNS/neural tumors: Review of evidence supporting possible role for novel targeted therapy. *Frontiers in Endocrinology, 12.*

13. Moreno P, Irene RÁ, Terry WM, Robert TJ (2016) Bombesin related peptides/receptors and their promising therapeutic roles in cancer imaging, targeting and treatment. *Expert Opinion on Therapeutic Targets,* **20**(9): 1055-1073.

14. Fournier P, Véronique DP, Samia AM, *et al.* (2012) Comparative study of 64Cu/NOTA-[D-Tyr6, βAla11, Thi13, Nle14] BBN (6-14) monomer and dimers for prostate cancer PET imaging. *EJNMMI research,* **2**(1): 1-15.

15. Smith CJ, Volkert WA, Hoffman TJ (2003) Gastrin releasing peptide (GRP) receptor targeted radiopharmaceuticals: a concise update. *Nuclear Medicine and Biology,* **30**(8): 861-868.

16. Hajiramezanali M, Atyabi F, Mosayebnia M, *et al.* (2019) 68Ga-radiolabeled bombesin-conjugated to trimethyl chitosan-coated superparamagnetic nanoparticles for molecular imaging: preparation, characterization and biological evaluation. *Int Journal Nanomedicine,* **14**: 2591–2605.

17. Habibi F, Faghihi R, Zollghadri S, Yousefnia H , *et al.* (2022) Estimation of human absorbed dose of 68-Ga-Citrate based on biodistribution data in rats: Comparison with 67-Ga-Citrate. *Int J Radiat Res,* **20**(1): 151-156.

18. Shanehsazzadeh S, Lahooti A, Yousefnia H, *et al.* (2015) Comparison of estimated human dose of 68Ga-MAA with 99mTc-MAA based on rat data. *Annals of Nuclear Medicine,* **29**(8): 745-753.

19. Shanehsazzadeh S, Yousefnia H, Jalilian AR, *et al.* (2015) Estimated human absorbed dose for ⁶⁸Ga-ECC based on mice data: comparison with ⁶⁷Ga-ECC. *Annals of Nuclear Medicine,* **29**(6): 475-481.

20. Li, Q, Caili Z, Wenqiang T, *et al.* (2017) Novel amino-pyridine functionalized chitosan quaternary ammonium derivatives: Design, synthesis, and antioxidant activity. *Molecules,* **22**(1): 156.

21. Li ZB, Zhanhong W, Kai C, Eun KR, Xiaoyuan C (2008) ¹⁸F-labeled BBN-RGD heterodimer for prostate cancer imaging. *Journal of Nuclear Medicine,* **49**(3): 453-461.

22. Jackson AB, Prasant KN, Tammy LR, *et al.* (2012) 64Cu-NO2A-RGD-Glu-6-Ahx-BBN (7-14) NH2: a heterodimeric targeting vector for positron emission tomography imaging of prostate cancer. *Nuclear medicine and biology,* **39**(3): 377-387.

23. Fournier P, Veronique DP, Samia A-, *et al.* (2011) Comparative study of 64Cu/NOTA-[D-Tyr6, βAla11, Thi13, Nle14] BBN (6-14) monomer and dimer for breast cancer PET Imaging, 1575-1575.

24. Varasteh Z, Ola A, Irina V, Gunnar L, *et al.* (2013) *In-vitro* and *in-vivo* evaluation of a 18F-labeled high affinity NOTA conjugated bombesin antagonist as a PET ligand for GRPR-targeted tumor imaging. *PloS one, 8*(12).

25. Medical Ethics Committee, Scholl of Medical Sciences of the Tarbiat Modares University on 28th Farvardin, 1385 / 18th Rabi al-Awwal, 1427 (2006). General Principles and Guidelines for Care and Use of Experimental Animals.

26. Liu Z, Gang N, Fan W, Xiaoyuan C (2009) ⁶⁸Ga-labeled NOTA-RGD-BBN peptide for dual integrin and GRPR-targeted tumor imaging. *European journal of nuclear medicine and molecular imaging,* **36**(9): 1483-1494.