ORIGINAL RESEARCH ARTICLE

Preparation and preclinical study of [68Ga]Ga-(Pip)-Nle-CycMSH$_{hex}$: Optimized production with an in-house 68Ge/68Ga generator

Sepideh Shafiei[1], Leyla Akbari[2], Arezoo Karimian[3], Samaneh Zolghadri[1], Mostafa Erfani[1], Azadeh Mikaeili[1], Zhila Fallah[1], Hassan Yousefnia[1]

[1]Radiation Application Research School, Nuclear Science and Technology Research Institute, Tehran, Iran

[2]Nuclear Engineering Department, Shiraz University, Shiraz, Iran

[3]Faculty of Engineering, Science and Research Branch, Islamic Azad University, Tehran, Iran

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*Corresponding Author:
Dr. Hassan Yousefnia
Address: Radiation Application Research School, Nuclear Science and Technology Research Institute, Tehran, Iran
Email: hyousefnia@aeoi.org.ir

ABSTRACT

Introduction: The early diagnosis of melanoma is crucial for treatment and management of this aggressive malignancy. The present study describes the preparation and preclinical evaluation of 68Ga-radiolabeled DOTA-4amino-(I-carboxymethyl) piperidine (Pip)-Nle-cysMSH$_{hex}$ peptide ([68Ga]Ga-CCZ01048) as a potential aide for PET imaging and early diagnosis of malignant melanoma.

Methods: Various parameters were assessed to optimize the final preparation processes. The radiochemical purity of the final complex was checked using RTLC and HPLC methods. The stability of the radiolabeled complex was studied at 15, 30, 60 and 120-min post-injection. The partition coefficient was also studied. Cellular studies of the labeled peptide were measured using B16F10 cells at different post-treatment intervals. The biodistribution of the labeled compound was evaluated using normal and tumor-bearing mice.

Results: [68Ga]Ga-(Pip)-Nle-cysMSH$_{hex}$ radiolabeled complex was prepared with a specific activity of 118.4 TBq/mmol and radiochemical purity > 99% at optimized conditions. The results of stability studies show that the radiolabeled compound is stable in PBS buffer and human serum after 120 min. the cellular studies demonstrated that a binding affinity of [68Ga]Ga-(Pip)-Nle-cysMSH$_{hex}$ on B16F10 cells and the internalization of the complex increased from about 31% in 30 min to 62% in 240 min post-treatment. The biodistribution studies showed excretion of major portion of the tracer through the kidneys. The remainder of the tracer mostly accumulated at the tumor site. No significant uptake in non-target organs was observed at any interval following injection.

Conclusion: The [68Ga]Ga-(Pip)-Nle-cysMSH$_{hex}$ radiolabeled complex has the potential as a PET imaging agent for evaluation of metastatic malignant melanoma.

INTRODUCTION

Melanoma results from Genetic mutations in melanocytes. This disease is highly associated with genetic predisposition and environmental stressors [1]. Malignant melanoma has the highest death rates of skin cancer because it is aggressive, has high metastatic potential, and is resistant to cytotoxic agents [2-5]. Although melanoma accounts for about 1% of all skin cancer types, it is responsible for more than 50% of all skin cancer deaths[6]. According to the stage of disease, various therapies such as surgical resection, chemotherapy, radiotherapy, photodynamic therapy, immunotherapy and targeted therapy are employed [7]. The crucial keys to managing melanoma are early diagnosis and accurate staging of the disease [8, 9]. Positron emission tomography (PET) is a powerful technique for such purpose [4, 10, 11].

The melanocortin type 1 receptor (MC1R) belonging to the G protein-coupled receptors family is overexpressed on the surface of melanoma metastases [12, 13]. Therefore, this protein is the most promising target for melanoma imaging[14]. It is found that α-melanocyte stimulating hormone (α-MSH) with the minimal sequence of His-Phe-Arg-Trp selectivity bind the MC1R with limitation of a short half-life in humans [15]. Therefore, with this core sequence, different linear and cyclized α-MSH peptides have been designed to improve its stability and receptor binding affinity [2]. The results indicate that cyclic α-MSH peptides are more suited for the MC1R-binding pocket than linear peptides and improve binding affinities [16, 17]. Significant efforts have been made to modify cyclized α-MSH analogs to enhance tumor uptake and provide high contrast between tumor and normal tissue with PET imaging [18-20].

The common radiopharmaceutical [18F]FDG used for PET/CT has limitation in detecting small distant metastatic lesion in malignant melanoma [21]. Therefore, radiopharmaceutical-binding receptors have been developed for more specific imaging. The α-MSH peptides labeled with various positron emitting radioisotopes such as 64Cu [22], 18F [8], 44Sc [23] and 68Ga [24] are used to assess the diagnostic efficacy of these radiopharmaceuticals in melanoma tumors.

Among the emitters, 68Ga with a short half-life (68 min) and high yield of positron emission (88%), is an ideal radionuclide for PET imaging. Since 68Ga can be obtained by the 68Ge/68Ga generator, it is conveniently available and relatively cheap radioisotope [25]. The development of small peptides labeled easily with 68Ga, allows it to be readily converted into radiopharmaceuticals for PET imaging. 68Ga is also has a potential to be employed in combination with 177Lu as a theranostic radiopharmaceutical [26]. Therefore, there is increasing interest in using 68Ga in nuclear medicine.

Skin cancer ranks as the most common cancer in Iran [27]. It is estimated that skin cancer accounts for 7000 new cases every year in Iran [28]. According to studies, men have a higher risk of skin cancer than women. Despite this, women have a higher mortality rate than men [29]. Non-melanoma skin cancer, especially Basal Cell Carcinoma (BCC), is the most common type of skin cancer in the country. Still a study found that BCC is declining, while Squamous Cell Carcinoma (SCC) and melanoma are on the rise[28, 30]. It is estimated that skin cancer is responsible for approximately 15% of all malignancies in Iran [31]. Since metastatic melanoma is resistant to current common therapies, the best way for a cure is early melanoma tumor diagnosis and prompt surgical removal. In fact, the early diagnosis of the cancer can significantly affect the timely treatment and overall patient survival. Therefore, development of a novel PET radiopharmaceutical for early diagnosis and management of melanoma is a research priority in our country.

The special physical characteristics of 68Ga and its availability in the form of 68Ge/68Ga generator make the investigators enthuse in developing novel 68Ga-based radiopharmaceuticals. The 68Ge/68Ga generator production has been well-established in the country and several new radiolabeled compounds have been developed in recent years [32-34]. The malignant melanoma prevalence is high in our country. The cyclized α-MSH analogues has shown high potential to target MC1R overexpressed in metastatic malignant melanoma such as the recently introduced 68Ga-(Pip)-Nle-CycMSHhex (Figure 1) [24]. This study aimed to develop [68Ga]Ga-CCZ01048 obtained from an in-house 68Ge/68Ga generator as a high-potential agent for PET imaging of malignant melanoma. Accordingly, CCZ01048 was successfully labeled with 68Ga in optimized conditions. The stability of the radiopharmaceutical was checked in PBS buffer and human serum. The partition coefficient and the cellular study of the final complex were measured. Finally, the biodistribution of the complex was studied in normal and tumor-bearing mice at different intervals.
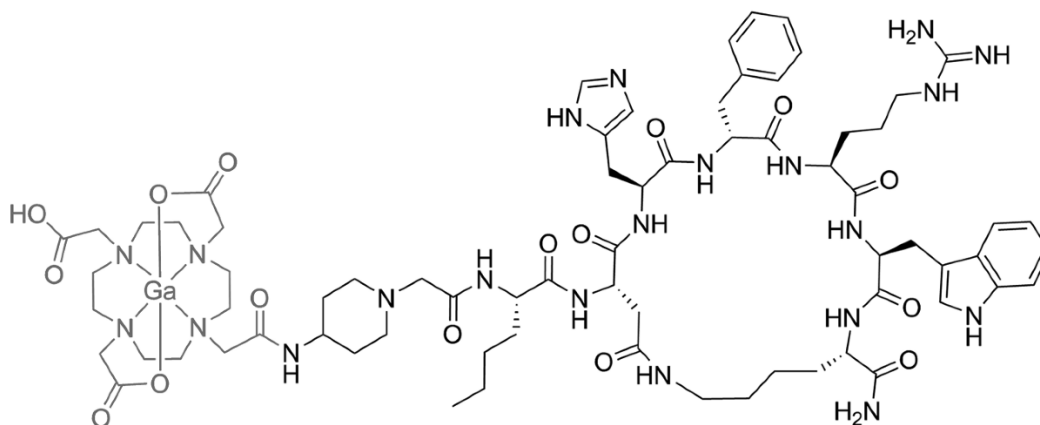
Figure 1. Chemical structure of [^{68}Ga]Ga-CCZ01048

METHODS

A prototype in-house ^{68}Ge/^{68}Ga generator with a nominal activity of 1480 MBq, developed at Pars Isotope Co. (Tehran, Iran), was used as a source of ^{68}Ga. CCZ01048 peptide was prepared from the MedChemExpress LLC (USA). B16F10 murine melanoma cells were obtained from the Pasteur Institute (Iran). All other chemical reagents were purchased from Sigma Aldrich Chemical Co. (UK). The radiochemical purity of the radiolabeled complex was checked using high- performance liquid chromatography (HPLC) method. A C$_{18}$ODS column dimensions of 100 mm × 4.6 mm filled with 5 μm particles was used for the analysis. The activity of the samples was measured by a p-type coaxial high-purity germanium (HPGe) detector (model: EGPC 80–200 R) coupled with a multichannel analyzer system. The 511 keV peak for ^{68}Ga was the basis for the calculations. Animal studies were performed following the United Kingdom Biological Council's Guidelines on the Use of Living Animals in Scientific Investigations, second edition.

Preparation and quality control of radiolabeled CCZ01048

To prepare the radiolabeled compound in optimized conditions, several experiments were done. While the pH of the reaction was changed from 2.8 to 4, other parameters were kept constant. At the best pH, the reaction temperature (50-100 °C) and peptide concentration (10-30 μg) were altered. To find the best reaction time, the radiochemical purity of the radiolabeled compound was checked in different intervals (5-30 min). Finally, in optimized conditions, 3 mL ^{68}Ga was obtained in the form of ^{68}GaCl$_3$ with activity of about 1260 MBq by milking from ^{68}Ge/^{68}Ga generator, with eluting with 0.1 M HCl. The pH of the eluate was adjusted to about 3 by adding 130 mg HEPES to the reaction vial. Then, 15 μg of CCZ01048 (1 mg/mL) was added to the reaction and heated at 95 °C for 15 min. The solution was transferred through a C$_{18}$ Sep-Pak cartridge preconditioned with 5 mL ethanol and 10 mL ultra-pure water, respectively. The cartridge was then washed with 2 mL ethanol and 8 mL saline. The product was filtered through a 0.22 μm Millipore filter. The radiochemical purity was determined by RTLC and HPLC methods. For radiochemical purity evaluation with RTLC, the methanol:saline (5:1) mixture, and Whatman No.2 paper were used as the mobile and stationary phases, respectively. The HPLC was done with the following gradient: A: Ultrapure water + 1% TFA and, B: Acetonitrile, with a flow rate of 2.6 mL.min^{-1} using gradient-elution: 0-3 min, A: 100%, B: 0%; 3-10 min, A: 50%, B: 50%; 10-15 min, A: 0%, B: 100%.

Stability tests

The stability of the final radiolabeled complex was examined at room temperature and in PBS buffer (4 ºC) at the specified time intervals (15, 30, 60, and 120 min). Furthermore, for assessing stability in human serum, a volume of 500 μL of newly obtained serum was introduced to a specimen of the substance. The mixture was then maintained at 37 ºC for approximately 120 min. Over this duration, the radiochemical purity was analyzed using the RTLC method.

Partition coefficient

Partition coefficient (log P) of [^{68}Ga]Ga-CCZ01048 was calculated followed by the determination of P (P = the ratio of specific activities of the organic and aqueous phases). 1 mL of 1-octanol and 1 mL of isotonic acetate-buffered saline (pH = 7) containing approximately 3.7 MBq of the radiolabeled complex were mixed. The mixture

was vortexed for 3 min and left for 5 min. Following centrifugation at >1200 g for 5 min, 50 µL was removed from each phase and counted in an automatic well counter. A 500 µL sample of the octanol phase from the former partitioning was repartitioned two to three times with fresh buffer to ensure that traces of hydrophilic impurities did not influence the calculated P values. The reported log P values are the average of the second and third extractions from three to four independent measurements.

Binding affinity

A test assessed the binding affinity of [68Ga]Ga-CCZ01048, following established procedures with minor modifications [35]. To outline the process briefly, approximately $0.8-1\times10^6$ of B16F10 cells, a mouse melanoma cell line, were seeded per well in a 6-well plate and left to settle overnight in a complete culture medium within an incubator. Subsequently, after 24 hours, the culture medium was removed, and the cells underwent washing, followed by incubation with freshly prepared culture medium for 1 h at 37℃.

The plates were then cooled on ice for 30 min, and various concentrations (ranging from 1 to 100 nM) of [68Ga]Ga-CCZ01048 were introduced to a PBS buffer (pH =7.4). The evaluation of non-specific binding of the radiolabeled peptide took place in the presence of 1 µM of non-radioactive peptide for 30 min at 37 °C. Following this, the cells were re-washed with ice-cold PBS. Subsequently, the cells were collected, and the amount of bound activity was quantified using a gamma counter.

Internalization

Research on internalization involved estimating the rate at which the radiolabeled peptide entered the mouse melanoma cells, was performed according published literature with slight modification [35]. In summary, approximately 1×10^5 cells were placed in each well of the 6-well plates and left to incubate for roughly 24h in a CO_2 incubator. Subsequently, [68Ga]Ga-CCZ01048 (2.5 pmol) was introduced into each well and incubated for 30, 60, 120, 240, and 360 minutes at 37℃.

The culture medium was eliminated after incubation, and the cells underwent washing. They were then subjected to two 5-min incubations with 1 mL of glycine buffer to dissociate any radiolabeled peptide attached to the cell surface, followed by two rinses with ice-cold PBS. Finally, 1mL of a 1N NaOH solution was added to the wells to displace the culture medium and quantify the internally absorbed activity. A gamma counter was utilized to measure both the portion of the radiolabeled peptide bound to the cell surface and the internalized segment.

Tumor implementation

Animal experiments were conducted in compliance with our institution's guidelines and adherence to commonly accepted protocols governing such research. Female C57 mice, aged six weeks obtained from the Pasteur Institute (Iran), were employed in the biodistribution investigation. A 50µL suspension of B16F10 cells (consisting of 1×10^7 cells) was prepared and then subcutaneously injected into the left shoulder of each mouse. Tumors were observed to develop approximately ten days after the injection.

Biodistribution studies

100µL of the final [68Ga]Ga-CCZ01048 solution with about 5MBq radioactivity was administrated intravenously via the tail vein in mice. By counting the 1 mL syringe before and after the injection in a dose calibrator with fixed geometry, the total amount of radioactivity injected in each animal was measured. The biodistribution of the solution among tissues was determined by sacrificing three mice by CO_2 asphyxiation at each particular time interval (15, 30, 60, and 120 min) after injection. Blood samples were taken immediately after the killing. The samples from organs of interest were removed, weighed, and rinsed with normal saline. Their activities were determined with a p-type coaxial HPGe detector coupled with a multichannel analyzer. The biodistribution of [68Ga]Ga-CCZ01048 in various organs was presented as percentage injected dose per gram (%ID/g, mean ± SD) which is calculated by dividing the activity amount of each tissue to the decay-corrected injected activity and the mass of each organ.

Statistical analysis

All experiments were repeated at least three times, and all values were expressed as mean ± standard deviation (Mean ± SD). The data were compared using Student's T-test. $P < 0.05$ was considered statistically significant.

RESULTS

Preparation and quality control of radiolabeled CCZ01048

The radiolabeled compound was prepared with specific activity of 118.4 TBq/mmol at optimized condition. The radiochemical purity of [68Ga]Ga-CCZ01048 was checked by RTLC and HPLC methods, demonstrating a purity >99% (Figures 2 and 3).
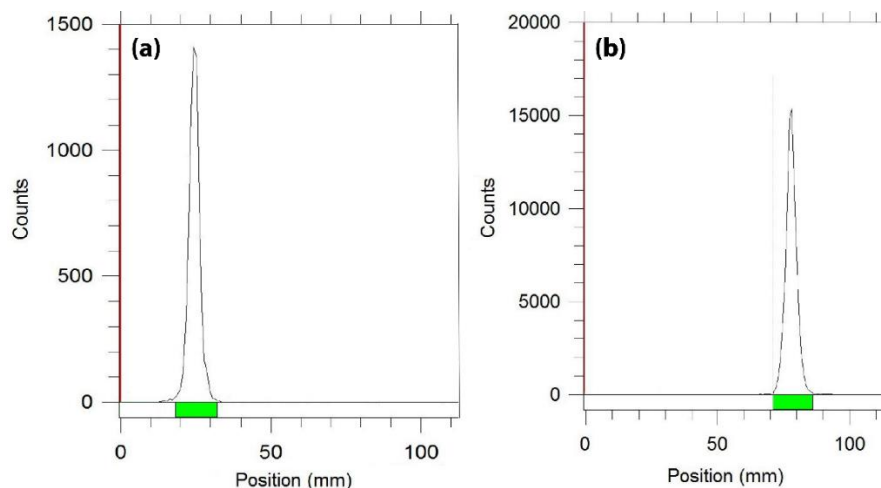
Figure 2. RTLC chromatogram of [^{68}Ga]GaCl$_3$ (a), and [^{68}Ga]Ga-CCZ01048 (b) using methanol:saline (5:1) mixture and Whatman No.2 paper

Stability tests

The stability of the final radiolabeled compound was studied after incubation for 120 min at room temperature, in PBS buffer (4ºC), and human blood serum (37℃), showing the radiochemical purity of 94.5%± 2.1%, 96.1%± 1.2%, and 95.7% ± 2.3%, respectively.
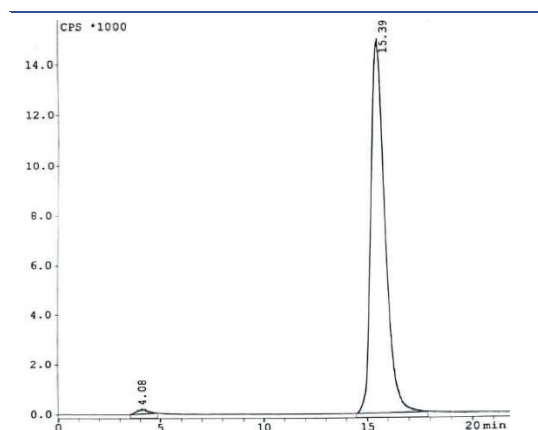

Figure 3. HPLC chromatogram of [^{68}Ga]Ga-CCZ01048

Partition coefficient

The partition coefficient was calculated by dividing the counts in the octanol phase by those in the buffer, and the results were expressed as Log P. The average value for [^{68}Ga]Ga-CCZ01048 was -2.31 ± 0.08.

Cellular studies

The total concentration of MC1Rs expressed on B16F10 cells (B_{max}) and the dissociation constant (K_d) were measured (Figure 4). The saturation binding curve was drowned for [^{68}Ga]Ga-CCZ01048, and the K_d value was determined as 0.74±0.13 nM. The internalization rate of [^{68}Ga]Ga-CCZ01048 was estimated on B16F10 cells at different intervals post-treatment. The results showed that the internalization increased from about 31% (30 min) to 62% (240 min) post-treatment (Figure 5).
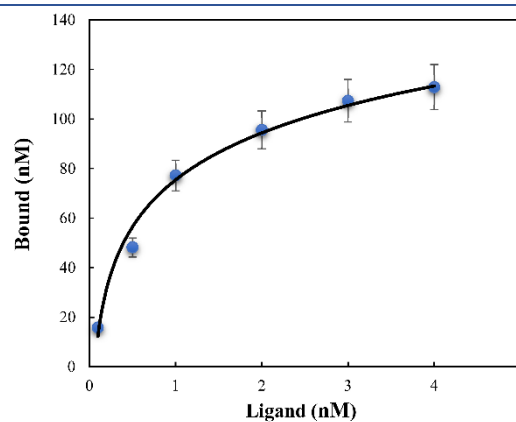

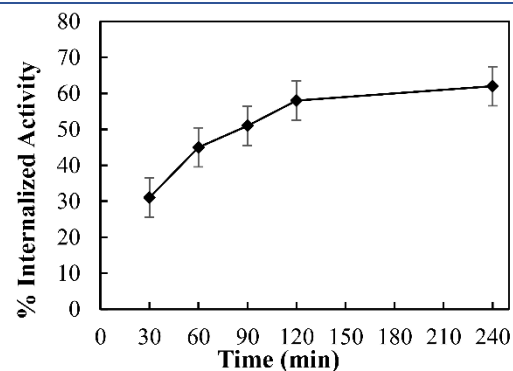**Figure 4.** Saturation binding curve for [^{68}Ga]Ga-CCZ01048

Figure 5. Percentage of internalized activity of [^{68}Ga]Ga-CCZ01048 in B16F10 cells

Biodistribution studies

Biodistribution of [^{68}Ga]GaCl$_3$ and the radiolabeled compound in the normal mice was investigated at 15-, 30-, 60-, and 120-min post-injection. The % ID/g for each organ after injection of [^{68}Ga]GaCl$_3$ and [^{68}Ga]Ga-CCZ01048 is indicated in Figures 6 and 7, respectively.

The biodistribution of [^{68}Ga]Ga-CCZ01048 in tumor-bearing mice was studied at 30-, 60- and 120-min post-injection (Figure 8).
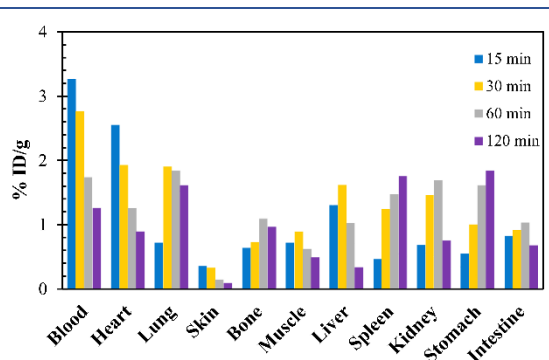

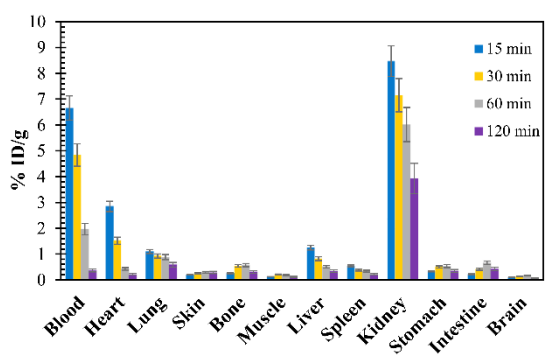**Figure 6.** Biodistribution of [^{68}Ga]GaCl$_3$ in normal mice (n = 3)

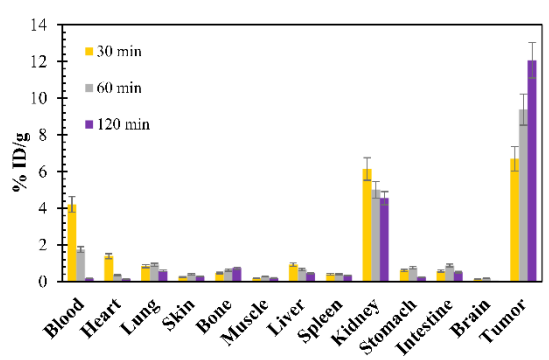**Figure 7.** Biodistribution of [^{68}Ga]Ga-CCZ01048 in normal mice (n = 3)

Figure 8. Biodistribution of [^{68}Ga]Ga-CCZ01048 in tumor-bearing mice (n = 3)

DISCUSSION

In this study, due to the various advantages of PET over single photon emission computed tomography (SPECT) images, [^{68}Ga]Ga-CCZ01048 radiolabeled complex was prepared with radiochemical purity of greater than 99% and specific activity of 118.4 TBq/mmol at optimized condition using an in-house ^{68}Ge/^{68}Ga generator. The study proved that this radiopharmaceutical is highly stable in PBS and human blood serum. The binding affinity study of the complex on the B16F10 cells showed high in vitro binding of the complex to the cells overexpressed MC1Rs with the K$_d$ value of 0.74±0.13 nM. Also, the internalization studies showed that 62% of the bound activity is internalized to the cells after 240 min post-treatment.

The biodistribution pattern of [^{68}Ga]GaCl$_3$ is entirely different from that of [^{68}Ga]Ga-CCZ01048. The accumulation of [^{68}Ga]GaCl$_3$ and [^{68}Ga]Ga-CCZ01048 species in main organs are compared in Figure 9. Although, both [^{68}Ga]GaCl$_3$ and [^{68}Ga]-Ga-CCZ01048 are eliminated fast from the blood circulation, the blood removal of the prepared radiolabeled complex occurs more quickly reaching to nondetectable level after about 2-hour post-injection. The muscle uptake of [^{68}Ga]-GaCl$_3$ was much higher compared to the uptake of [^{68}Ga]Ga-CCZ01048 also the [^{68}Ga]GaCl$_3$ demonstrated higher gastric uptake. However, the renal uptake of the radiolabeled complex was much higher than those of [^{68}Ga]GaCl$_3$ at all-time intervals. The ^{68}Ga was mainly excreted from the gastrointestinal tract (GIT) while the radiolabeled peptide was principally excreted from the urinary tract due to the strong hydrophilic nature of the complex (Log P = -2.31±0.08). The water solubility of the radiocomplex leads to less unwanted non-target tissue uptake and faster renal washout.

The biodistribution of [^{68}Ga]Ga-CCZ01048 in tumor-bearing mice showed increasing tumor uptake over time reaching to maximum level at 120-min post-injection. The target-to-non-target uptake ratios after 30, 60, and 120 min of [^{68}Ga]-Ga-CCZ01048 are presented in Table 1.

Table 1. Tumor to non-target uptake ratios at 30-, 60-, and 120-min post-injection

	30 min	60 min	120 min
Tumor/Blood	1.6±0.2	5.3±0.7	72.2±8.7
Tumor/Muscle	36.0±5.1	32.6±4.1	58.6±6.6
Tumor/kidney	1.1±0.1	1.9±0.3	2.7±0.3

Tumor uptake of [68Ga]Ga-CCZ01048 increased, while the %ID/g of kidney, muscle, and blood decreased during the study. Therefore, the tumor to kidney, muscle, and blood ratios increased over time. The low blood concentration of [68Ga]Ga-CCZ01048 after 120 min indicates the rapid clearance of the compound from the circulation. The considerable value of tumor to non-target uptake ratios after injection of [68Ga]Ga-

CCZ01048 introduces this radiolabeled compound as a promising agent for PET imaging of malignant melanoma. However, this study could be strengthen using a variety of the MC1R overexpressing cells for cellular works, increasing the number of tumor-bearing mice for the animal studies as well as increasing the number of animal PET imaging procedures, not easily accessible in this survey hence a limitation of the study.
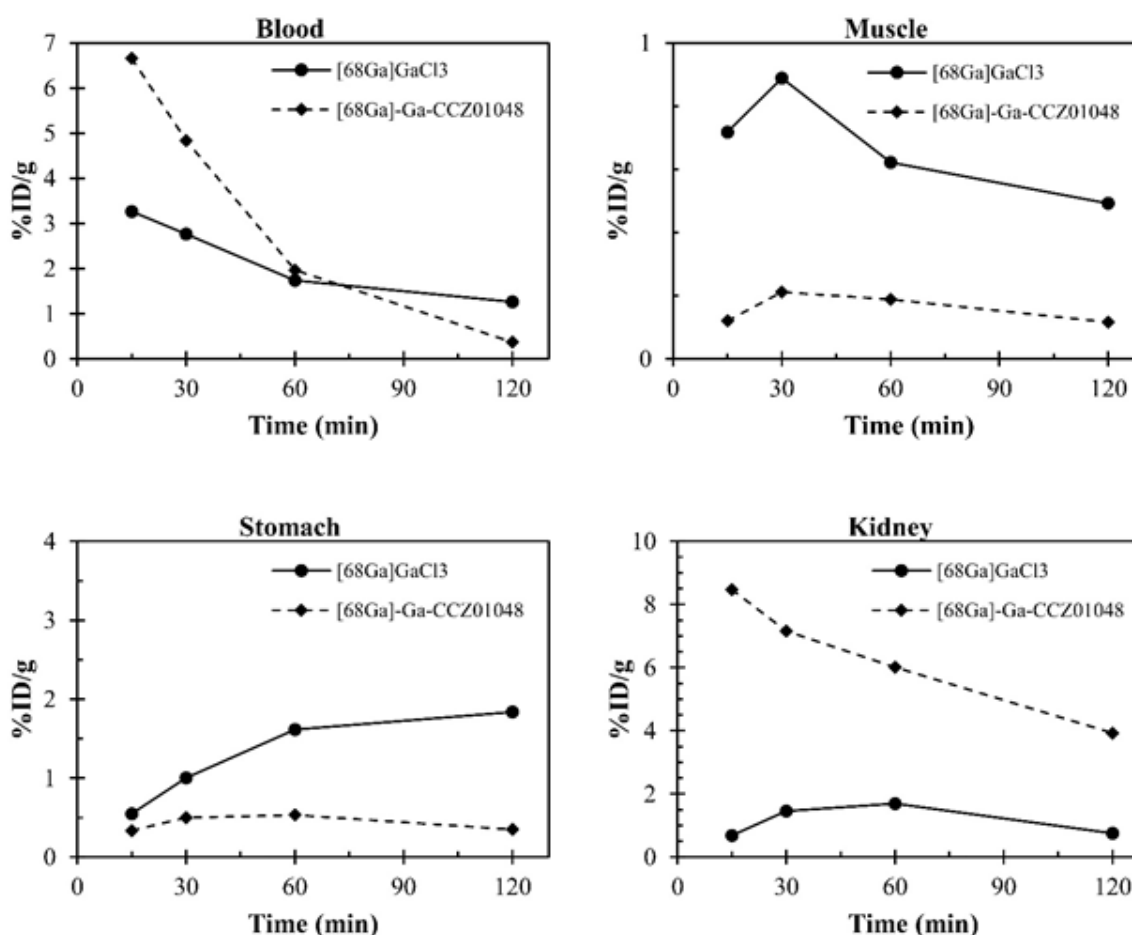

Figure 9. Comparative organ uptake of [68Ga]GaCl₃ and [68Ga]Ga-CCZ01048 in normal mice

CONCLUSION

The prevalence of melanoma in the country emphasizes the importance of early diagnosis of this disease. PET scanning is extremely sensitive for identifying metastatic malignant melanoma. Therefore, studies on radiopharmaceuticals targeting MC1Rs for melanoma imaging are necessary. Leading us to devote this research to the preparation and preclinical evaluation of [68Ga]Ga-CCZ01048 radiopharmaceutical using an in-house ^{68}Ge/^{68}Ga generator hoping to pave the way for future clinical studies. [68Ga]Ga-CCZ01048 was prepared with radiochemical purity more than 99% and specific activity of 118.4 TBq/mmol at optimized conditions. The preclinical results

support the potential of [68Ga]Ga-CCZ01048 as a favorable agent for PET imaging of the metastatic malignant melanoma.

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