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Ga-68 labeled DOTA-rhenium cyclized α -MSH analog for imaging of malignant melanoma

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Abstract

Introduction—Diagnosis of malignant melanoma is critical, since a patient's prognosis is poor. Previous studies have shown that ⁶⁴Cu- and ⁸⁶Y-DOTA-ReCCMSH(Arg¹¹) have the potential for early detection of malignant melanoma by exploiting the sensitivity and high resolution of PET. This encouraged us to investigate DOTA-ReCCMSH(Arg¹¹) labeled with another β^+ -emitting radionuclide, ⁶⁸Ga.

Methods—DOTA-ReCCMSH(Arg¹¹) was successfully labeled with ⁶⁸Ga at pH 3.8–4 at 85 °C. Acute biodistribution and small animal PET imaging studies were performed in B16/F1 melanoma tumor bearing mice.

Results—Biodistribution studies showed moderate receptor-mediated tumor uptake, fast non-target organ clearance, and high tumor to non-target tissue ratios. Pre-administration of D-lysine significantly reduced kidney uptake without affecting the uptake of the agent in the tumor. Small animal PET images showed that the tumor could be clearly visualized at all time points examined (0.5 – 2 h) with the standardized uptake value (SUV) analysis following a similar trend as the biodistribution data.

Conclusions—The preliminary data obtained suggests that ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) is a promising PET imaging agent for early detection of malignant melanoma.

Keywords

PET; Gallium-68; melanoma; MSH receptors

1. Introduction

Malignant melanoma, the most serious form of skin cancer, has become a severe health problem due to an increase in incidence and mortality rate [1,2]. Accurate diagnosis is critical, because unless the primary tumor is excised through surgery, patient prognosis is generally poor [3]. 2-[¹⁸F]fluoro-2-deoxy-D-glucose ([¹⁸F]FDG) [4–6] and melanoma-targeting antibodies or

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antibody fragments [7–9] have been developed for imaging and staging of disease. [^{18}F]FDG positron emission tomography (PET) is limited in detecting melanoma tumors with small foci [5] and some melanoma cells are undetectable with [^{18}F]FDG because they use non-glucose based substrates as an energy source [6], therefore their clinical application has been limited. Radiolabeled antibodies generally lack specificity due to the presence of individual tumor variants and since melanomas tend to become amelanotic as the malignancy progresses, the target antigen is often lost due to the reduction in expression of the specific pigment genes that encode this target [10–12]. The increasing numbers of amelanotic cells in primary tumors or their metastasis increase the possibility that such cells will escape targeting by antibody-based radiopharmaceuticals. Examples of other agents that have been examined clinically for the imaging of melanoma, as alternatives to [^{18}F]FDG, include the PET agents ^{18}F -galacto-RGD [13], 6-[^{18}F]fluoro-L-dopa (^{18}F -DOPA) [6], and 3'- ^{18}F -fluoro-3'-deoxy-L-thymidine (^{18}F -FLT) [14] and the SPECT agents N-isopropyl-p- ^{123}I -iodoamphetamine (^{123}I -IMP) [15], ^{123}I -N-(2-diethylaminoethyl)-2-iodobenzamide (^{123}I -BZA(2)) [16], and ^{123}I -N-[3-(4-morpholino)propyl]-N-methyl-2-hydroxy-5-iodo-3-methylbenzylamine (ERC9) [17].

Recently, alpha-melanocyte-stimulating hormone (α -MSH) peptide analogs were developed as promising agents for both melanoma imaging and radiotherapy. α -MSH is a tridecapeptide that is excreted by the pars intermedia of the pituitary gland and is primarily responsible for the regulation of skin pigmentation [18,19]. α -MSH targets the melanocortin-1 receptor (MC1R), which belongs to a family of G-protein-coupled receptors (MC1R to MC5R) that have been isolated in both mice and humans [20–26]. These receptors are normally expressed on the surface of melanocytes, and it was reported that >80% of human melanoma tumor samples obtained from patients with metastatic lesions express α -MSH receptors [27,28]. It has been reported the MC1R expression ranges from several hundred to around 10,000 receptors per cell [29]. In 2003, Miao et al., reported that MC1R receptor expression ranged from nine hundred (1.49 fmol/million SKMEL28 cells) to around six thousand (9.46 fmol/million TXM13 cells) receptors per cell on human melanoma cells [30], enabling the use of radiolabeled α -MSH peptide analogs as specific melanoma diagnostic and therapeutic agents.

There have been a number of reports on α -MSH peptide-based agents that have demonstrated the ability to image the MCR1 receptor *in vivo* [31–39]. We previously described an α -MSH-targeting peptide system, DOTA-ReCCMSH(Arg¹¹), that was labeled with β^+ -emitting radiometals, as potential agents for the detection of malignant melanoma via PET imaging [40]. The rhenium cyclized peptide system was chosen for further study given its increased stability compared to its linear analog with a concomitant increase in tumor uptake and less renal retention [38,39]. We chose the divalent metal ion ^{64}Cu ($t_{1/2} = 12.7$ h, 17.4% β^+ , 41% EC, 40% β^-) [41] and trivalent metal ion ^{86}Y ($t_{1/2} = 14.7$ h, 33% β^+), both of which can be prepared on a biomedical cyclotron utilizing the $^{64}\text{Ni}(p,n)^{64}\text{Cu}$ and $^{86}\text{Sr}(p,n)^{86}\text{Y}$ reactions, respectively [42,43]. Our results showed that both ^{64}Cu -DOTA-ReCCMSH(Arg¹¹) and ^{86}Y -DOTA-ReCCMSH(Arg¹¹) have the potential for early detection of malignant melanoma by exploiting the sensitivity and high resolution of PET [40]. This encouraging result motivated us to investigate the same peptide DOTA-ReCCMSH(Arg¹¹) radiolabeled with another β^+ -emitting radiometal ^{68}Ga . ^{68}Ga ($t_{1/2} = 68$ min, 88% β^+) is a short-lived positron emitter, the production of which is not dependent on an in-house cyclotron since it is available from commercially available $^{68}\text{Ge}/^{68}\text{Ga}$ generators which have >1 year shelf-lives. The use of ^{68}Ga -labeled peptides is an emerging area of PET radiopharmaceutical development and a number of agents have been translated to the clinic [44]. This report describes our preliminary study on ^{68}Ga labeled DOTA-ReCCMSH(Arg¹¹), and, to the best of our knowledge, this is the first study on ^{68}Ga labeled DOTA conjugated cyclized α -MSH peptide analog. We report small animal PET and biodistribution studies of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) in the B16/F1 tumor-bearing mouse model.

2. Materials and Methods

2.1 General

All chemicals unless otherwise stated were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Water was distilled and then deionized ($18 \text{ M}\Omega/\text{cm}^2$) by passing through a Milli-Q water filtration system (Millipore Corp., Milford, MA). ^{68}Ga was obtained from a $^{68}\text{Ge}/^{68}\text{Ga}$ radionuclide generator (TCI Medical Inc. Albuquerque, MN, 30 mCi generator at delivery). The parent, ^{68}Ge , is accelerator produced and decays with a half-life of 270.8 d by electron capture. This Ge-68 generator uses adsorption chromatography (TiO_2) and is eluted with high purity 0.1 M HCl (TraceMetal concentrated HCl was obtained from Fisher Scientific and diluted with Milli-Q water). At the time of the experiments the generator was over 9-months old and the average elution was 12.5 mCi in 5 mL of eluent (of which 80–90% of the activity was collected in a 1.5 mL fraction). Radioactivity was counted with a Beckman Gamma 8000 counter containing a NaI crystal (Beckman Instruments, Inc., Irvine, CA). Radio-TLC detection was accomplished using a BIOSCAN AR2000 Imaging Scanner (Washington DC).

2.2. Synthesis of DOTA-ReCCMSH(Arg¹¹)

The rhenium-cyclized peptide DOTA-ReCCMSH(Arg¹¹) was prepared as previously described [38]. Briefly, DOTA-CCMSH(Arg¹¹) was synthesized using standard Fmoc/HBTU chemistry on an amide resin with an Advanced ChemTech Omega 396 multiple peptide synthesizer (Advanced ChemTech, Louisville, KY). It was then acetylated with glacial acetic acid at the N-terminus and then deprotected and cleaved from the resin by trifluoroacetic acid (TFA), thioanisole (TIS), water and ethanedithiol (EDT) at room temperature for 3 h. After purification by RP-HPLC, DOTA-CCMSH(Arg¹¹) was redissolved in 60% methanol solution and $\text{ReOCl}_3(\text{Me}_2\text{S})(\text{OPPh}_3)$ was added to give an overall molar ratio of 1:1.5. After adjusting the pH to 8, the reaction mixture was incubated at 75 °C for 2 h. The cyclized peptide, DOTA-ReCCMSH(Arg¹¹) was purified using RP-HPLC and was characterized by electrospray ionization mass spectrometry (ESI-MS) operating in the positive mode. The overall synthetic yield was 10–20% since we purify the rhenium cyclized peptide with HPLC after the reaction.

2.3 Radiolabeling of DOTA-ReCCMSH(Arg¹¹) with ^{68}Ga

2.0 mCi $^{68}\text{GaCl}_3$ in 200 μL 0.1 M HCl was added to 40 μg of DOTA-ReCCMSH(Arg¹¹) in 200 μL 1.25 M ammonium acetate (pH 4.5). The reaction mixture with a final pH of 4 was incubated at 85 °C for 30 min. Quality control was performed with thin-layer chromatography using instant TLC (ITLC-SG) glass microfiber sheets developed with 50:50 10% NH_4OAc to methanol ($^{68}\text{GaCl}_3$: $R_f = 0$; ^{68}Ga -DOTA-ReCCMSH(Arg¹¹): $R_f = 0.9$). Radiochemical purity was also confirmed with reverse-phase HPLC using a Microsorb C₁₈, $4.6 \times 250 \text{ mm}$ column (Varian Inc. Lake Forest, CA) eluted with a gradient of 1% B to 30% B in 12 min then 30% B to 70% B in 5 min at a flow rate of 1.0 mL/min (Solvent A: 0.1% trifluoroacetic acid in water, Solvent B: 0.1% trifluoroacetic acid in acetonitrile).

2.4. Biodistribution Studies

All animal experiments were conducted in compliance with the Guidelines for the Care and Use of Research Animals established by Washington University's Animal Studies Committee. Biodistribution studies were carried out on 18–23 g female C57BL/6 mice (Charles River Laboratories, Wilmington, MA) that had been implanted with 1×10^6 cultured B16/F1 murine melanoma cells in 100 μL subcutaneously into the nape of the neck. Tumors were allowed to grow for 10 days, at which time the animals received $\sim 12 \mu\text{Ci}$ of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) in 100 μL of saline via lateral tail vein injection. Four groups were examined at 2 time points ($n = 5$ per group at 30 min and 2 h). The first two groups received 0.24 μg (115 pmol, 104 mCi/ μmol) of radiolabeled peptide and were sacrificed at 30 min and 2 h time points. To

examine *in vivo* uptake specificity, a third group of mice (2 h timepoint) were pre-injected via lateral tail vein injection with unlabeled peptide to act as a receptor-blockade (20 μ g, 10 nmol) of DOTA-ReCCMSH(Arg¹¹) immediately prior to administering ⁶⁸Ga-DOTA-ReCCMSH (Arg¹¹) (0.24 μ g). This represents a 80-fold increase over the mass of peptide injected with ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹). In an attempt to reduce kidney uptake and retention, a fourth group of mice (2 h timepoint) were pre-injected with D-lysine (15 mg in 100 μ L saline) immediately prior to administering ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) (0.24 μ g). In all studies following euthanasia, tissues and organs of interest were removed and weighed, and the radioactivity was measured in a γ -counter. The percent doses per gram (%ID/g) were then calculated by comparison to known standards.

2.5. Small Animal PET Studies

Whole body small animal PET imaging was performed on a microPET-Focus scanner (Concorde Microsystems, Knoxville, TN) [45]. Imaging studies were carried out on C57BL/6 mice bearing 10-day B16/F1 murine melanoma tumors. The mice were injected via the tail vein with ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) (100 μ Ci, 2 μ g in 100 μ L). These mice were imaged side by side with mice that had been treated with 20 μ g (in 100 μ L saline) of unlabeled peptide (as a blockade) injected via the lateral tail vein immediately prior to administration of ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹). At 30 min, 1 h and 2 h after injection, the mice were anesthetized with 1% to 2% isoflurane, positioned supine, immobilized and imaged. Ten minute static data sets were collected at each time point. Tumor, kidney and liver standard uptake values {(nCi/ml) $\times$ [weigh (g)/injected dose (nCi)]} of the ⁶⁸Ga-activity were generated by measuring regions of interest that encompassed the entire organ from the small animal PET images.

2.6. Statistical Methods

All of the data are presented as mean $\pm$ SD. For statistical classification, a Student's t test was performed using GraphPad PRISM (San Diego, CA). Differences at the 95% confidence level ($p < 0.05$) were considered significant.

3. Results

3.1. ⁶⁸Ga labeling of DOTA-ReCCMSH(Arg¹¹)

DOTA-ReCCMSH(Arg¹¹) was successfully labeled with ⁶⁸Ga at 85 $^{\circ}$ C. It was found that an equal volume of 1.25 M ammonium acetate (pH 4.5), added to ⁶⁸GaCl₃ in 0.1 M HCl, resulted in a final pH of 3.8 – 4. The pH range was found to be critical for the successful labeling of DOTA-ReCCMSH(Arg¹¹) with ⁶⁸Ga. The radiolabeling yield was much lower (<10%) if the final pH of the reaction mixture was 3.2 – 3.5 or when 0.1 M or 0.5 M ammonium acetate (pH 4.5) were used instead of 1.25 M ammonium acetate. Using the optimized radiolabeling conditions, radio-TLC and HPLC confirmed that the radiochemical yield and purity of the labeled peptide was >95% without the need for any HPLC purification procedures. Using the described HPLC conditions, the retention times of uncomplexed ⁶⁸Ga and the product ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) were 2 min and 12 min respectively. The product was produced with a specific activity of 104 mCi/ μ mol.

3.2. Biodistribution Studies

In the acute biodistribution experiments female C57 BL/6 mice bearing 10-day old B16/F1 murine melanoma tumors were used. The biodistribution data for ⁶⁸Ga-DOTA-ReCCMSH (Arg¹¹) (12 μ Ci, 0.24 μ g) at 30 min and 2 h post-injection are listed in Table 1. To determine the *in vivo* specificity of tumor uptake, a blocking study was performed on an additional group which was pre-injected with unlabeled peptide (20 μ g of DOTA-ReCCMSH(Arg¹¹), 10 nmol) and was sacrificed at 2 h post-injection. The tumor uptake of ⁶⁸Ga-DOTA-ReCCMSH

(Arg¹¹) reached a maximum after 30 min (4.31 ± 1.94 %ID/g) and the tumor concentration remained at the same level after 2 h post-injection (4.25 ± 1.41 %ID/g, $p = \text{NS}$) indicating that the tumor retention is high. Pre-injection of 20 μg of unlabeled DOTA-ReCCMSH(Arg¹¹) dramatically reduced the tumor uptake of the radiolabeled peptide (0.42 ± 0.05 %ID/g, $p < 0.01$) at 2 h after injection, strongly supporting a receptor-mediated tumor uptake for the radiolabeled peptide. No other organs excised showed significant difference between the group that received blockade and the group that did not.

The non-target tissue uptake of ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) was high at 30 min post-injection, however, the radioactivity was quickly cleared from all organs at 2 h after injection (e.g. blood uptake: 4.65 ± 0.84 %ID/g at 30 min p.i. (post-injection) vs. 0.40 ± 0.13 %ID/g at 2 h p.i., $p < 0.001$; liver uptake: 1.34 ± 0.09 %ID/g at 30 min p.i. vs. 0.50 ± 0.11 %ID/g at 2 h p.i., $p < 0.00001$; kidney uptake: 16.50 ± 4.30 %ID/g at 30 min p.i. vs. 6.80 ± 1.72 %ID/g at 2 h p.i., $p < 0.01$), resulting in low radioactivity accumulation in normal tissues at the later time point. The fast clearance from normal organs and high tumor retention resulted in favorable tumor to non-target organ ratios at 2 h post-injection. Fig. 1 shows the tumor to blood, tumor to muscle and tumor to skin ratios of ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) (240 ng) and the comparison of the tumor to organ ratios with ⁶⁴Cu-DOTA-ReCCMSH(Arg¹¹) (16 ng) [40] and ⁶⁴Cu-CBTE2A-ReCCMSH(Arg¹¹) (16 ng) [46].

Kidney uptake of ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) was reduced by D-lysine administration. Table 1 showed that the kidney uptake at 2 h post-injection was reduced 53% (from 6.80 ± 1.72 to 3.17 ± 1.41 %ID/g, $p < 0.01$) when 15 mg D-lysine was pre-injected with the radiolabeled peptide, while radioactivity in tumor and other organs was not significantly affected.

3.3. Small Animal PET Studies

C57BL/6 mice bearing 10-day B16/F1 murine melanoma tumors were injected with ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) and microPET images were obtained at 0.5, 1 and 2 h post-injection. Fig. 2 shows the transaxial view from the microPET images at 2 h post-injection. The tumors were visible at as early as 0.5 h after injection (data not shown). However, the background activity was high at 0.5 h post injection, consisting with the high uptake values in non-target organs such as blood, lung, kidney and heart in tissue biodistribution studies. At 2 h post injection, the background activity decreased, only tumor, kidney and bladder were clearly visualized, indicating the fast clearance from non-targeting organs. MicroPET images also show that after administrating 20 μg unlabeled peptide, very little activity can be seen in the tumor at all time points. The dramatic reduction of the tumor concentration by a blockade dose indicates that the tumor uptake is receptor-mediated. Standard uptake values (SUVs) for tumor, kidney and liver were determined at all time points for both blocked and non-blocked mice (Fig. 3). SUVs showed that the tumor concentration of the blocked mice is significantly lower compared to the non-blocked mice ($p < 0.01$) at all time points, and the kidney and liver uptakes between the blocked and non-blocked mice are not statistically different ($p = \text{NS}$).

4. Discussion

Several radiolabeled α -MSH peptides have been investigated for melanoma-specific targeting. Substitution of Met⁴ with Nle⁴ and Phe⁷ with D-Phe⁷ yielded the NDP analogs which showed subnanomolar receptor affinity and resistance to enzymatic degradation [29,47,48]. α -MSH peptides cyclized via a disulfide bond [Cys(4,10), D-Phe⁷] α -MSH [49] and lactam bond formation [Asp⁵, D-Phe⁷, Lys¹¹ α -MSH] [50,51] display increased receptor binding affinity and resistance to proteolysis. A new family of α -MSH analogs was then developed that incorporated the transition metal rhenium and technetium directly into the peptide's structure to generate the stable cyclic α -MSH analog TcO or ReO [Cys^{3,4,10},D-Phe⁷]- α -MSH₃₋₁₃

[ReCCMSH] [38,52,53]. Substitution of Arg¹¹ for Lys¹¹ in ReCCMSH peptide resulted in the analog, ReCCMSH(Arg¹¹), which showed greater tumor uptake and lower kidney accumulation compared to the ReCCMSH [39,54]. We also have shown that radiolabeled DOTA-ReCCMSH(Arg¹¹) is an agonist. It has been reported that both ⁹⁰Y- and ¹⁷⁷Lu-labeled DOTA-ReCCSMH(Arg¹¹) exhibited fast cellular internalization and extended cellular retention in B16/F1 cells.[55]

In our previous study, we investigated DOTA-ReCCMSH(Arg¹¹) radiolabeled with β^+ -emitting radiometals ⁶⁴Cu and ⁸⁶Y, and our results showed that ⁶⁴Cu- and ⁸⁶Y-DOTA-ReCCMSH(Arg¹¹) are ideal potential PET imaging agents for early detection of malignant melanoma [40]. Our promising results encouraged us to investigate the ⁶⁸Ga labeled DOTA-ReCCMSH(Arg¹¹) peptide analog. ⁶⁸Ga is a favorable positron emitter because of its short half life and its production from a commercial generator that can be eluted daily. ⁶⁸Ga and the development of small chelator-coupled peptides may open a new generation of kit-formulated PET radiopharmaceuticals. With manufacturing practice (GMP) produced kits and an onsite generator, it would be possible to produce radiopharmaceuticals as a very cost-effective alternative to cyclotron-based tracers. Therefore, Ga-labeled α -MSH peptide analogs would provide a versatile tool for the diagnosis and therapy of melanoma, including SPECT and PET imaging [34].

In spite of the different positron energies associated with ⁶⁴Cu, ⁸⁶Y and ⁸⁶Ga, very little difference would be expected in the quality of the images produced in a clinical setting due to the inherent resolution of human PET scanners. Any additional noise associated with the positron energy of a particular nuclide could be cleaned up with the application of new reconstruction techniques and algorithms. ⁶⁴Cu, ⁸⁶Y and ⁸⁶Ga have all been used for the PET imaging of cancer with peptide-based agents and have produced images of high quality [44, 56–58]. Even though ⁶⁸Ga has some other advantages over other PET nuclides in terms of availability and applicability, it is important to compare DOTA-ReCCMSH(Arg¹¹) labeled with other metal nuclides, as the choice of metal can have a dramatic effect on the biodistribution of the agent. In our earlier work, animal studies indicated that both ⁶⁴Cu- and ⁸⁶Y- labeled DOTA-ReCCMSH(Arg¹¹) were promising candidates for the detection of melanoma [40]. However, ⁶⁴Cu-DOTA-ReCCMSH(Arg¹¹) demonstrated higher radioactive accumulation in liver and other non-target organs.

Although DOTA is an excellent chelator for many 2+ and 3+ charged metals, ⁶⁴Cu has been shown to dissociate *in vivo* from DOTA and DOTA-conjugates and undergo *in vivo* metabolism [59,60]. The choice of metal nuclide can also dictate the overall charge on the agent and, therefore, the ultimate excretion properties of the agents. For example, ⁶⁴Cu-CBTE2A-ReCCMSH(Arg¹¹) and ⁶⁴Cu-DOTA-ReCCMSH(Arg¹¹) are charged +1 and -1, respectively, and it has been reported that the renal retention of ⁶⁴Cu- and ¹¹¹In-labeled compounds is higher for positively charged peptides and lower for neutral and negatively charged ones [23,61,62]. It is therefore paramount to study a peptide conjugate with a variety of metal nuclides given the significant differences in their *in vivo* behavior. For example, even though, Ga, In and Y are all 3+ metals, ⁶⁷Ga-DOTA⁰-Tyr³-octreotide showed not only 5 times higher binding affinity to the somatostatin-receptor subtype 2 (SSTR2) but also about 2.5 times higher tumor uptake in a mouse model and lower kidney uptake than the ¹¹¹In/⁹⁰Y-DOTATOC analogs [63]. ⁶⁷Ga-DOTA⁰-Tyr³-octreotide was therefore chosen for further clinical evaluation [57].

DOTA-ReCCMSH(Arg¹¹) was successfully labeled with ⁶⁸Ga at 85°C in ammonium acetate buffer. The labeling reaction was pH dependent, with an optimal pH of 3.8 - 4.0, while the labeling yield was significantly reduced (<10%) at higher or lower pH levels. Similar pH dependent labeling conditions were found for ⁶⁸Ga labeling of other DOTA-conjugated

peptides [64,65]. The optimal labeling yield was achieved in the pH range of 3.5 – 4.0 for ^{68}Ga labeling of DOTA-derivatized somatostatin and bombesin derivatives.[64] At higher pH, Ga^{3+} tends to form hydroxyl-aquo complexes, while the complex formation yield decreases at lower pH values [65]. The specific activity of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) using in the biological studies was 104 mCi/ μmol . This labeling efficiency is comparable to that found for a linear α -MSH peptide analog, ^{67}Ga -DOTA-NAPamide, where a specific activity of 200 mCi/ μmol was obtained [34]. However, it is significantly lower compared to that achieved for the ^{86}Y and ^{64}Cu analogs, where a 6-fold (^{64}Cu , 624 mCi/ μmol) and 60-fold higher (^{86}Y , 6240 mCi/ μmol) labeled specific activity was obtained [40]. Moreover, Froidevaux et al., reported a specific activity of 1354 mCi/ μmol for ^{68}Ga -DOTA-NAPamide [34]. The low specific activity achieved in this current study is due to the age of the $^{68}\text{Ge}/^{68}\text{Ga}$ generator used for these studies (see Material and Methods, General 2.1). It would be expected that an increase in specific activity, similar to levels reported by Froidevaux et al. [34], would be achievable with a newer generator.

High specific activity of the radiolabeled peptide is critical for receptor targeting since unlabeled peptide will also compete with the radiolabeled peptide for the receptor binding. One way to increase the specific activity of the radiolabeled peptide is to use the minimum amount of the peptide to achieve the high labeling yield. Another way is to purify the radiolabeled peptide with RP-HPLC. Despite the relatively low specific activity of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) (compared to the ^{86}Y and ^{64}Cu analogs [40]), the acute biodistribution study demonstrated relatively high tumor uptake. Similar tumor concentration at 30 min and 2 h p.i. indicates that the tumor retention is high. Pre-administration of a blocking dose of DOTA-ReCCMSH(Arg¹¹) significantly reduced the tumor uptake by saturating the α -MSH receptor binding site, confirming that the tumor uptake of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) is a receptor-mediated process. Another favorable characteristic of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) is the fast clearance from non-target organs such as blood, liver, lung, heart and muscle from 30 min to 2 h post injection, resulting in low background radioactivity at the 2 h time point. Fig. 1 shows a comparison of the 2 h biodistribution of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) (240 ng), ^{64}Cu -DOTA-ReCCMSH(Arg¹¹) (16 ng) [40] and ^{64}Cu -CBTE2A-ReCCMSH(Arg¹¹) (16 ng) [46]. The tumor to blood ratio of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) (10.9 ± 2.76) is comparable to that of ^{64}Cu -DOTA-ReCCMSH(Arg¹¹) (12.5 ± 2.70 , $p = \text{NS}$) but is lower compared to ^{64}Cu -CBTE2A-ReCCMSH(Arg¹¹) (37.3 ± 10.7 , $p < 0.01$). The tumor to muscle ratio of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) (43.2 ± 15.3) is similar to that of ^{64}Cu -CBTE2A-ReCCMSH(Arg¹¹) (44.6 ± 9.76 , $p = \text{NS}$) and is significantly higher compared to ^{64}Cu -DOTA-ReCCMSH(Arg¹¹) (18.5 ± 3.17 , $p < 0.01$). The ^{86}Y -DOTA-ReCCMSH(Arg¹¹) data [40] is superior to that seen for both ^{64}Cu DOTA- and ^{64}Cu -CBTE2A-ReCCMSH(Arg¹¹) analogs [40,46] as well as that seen for $^{67/68}\text{Ga}$ -DOTA-NAPamide [34], ^{64}Cu -DOTA-NAPamide [32], ^{18}F -FB-NAPamide [33], ^{18}F -FDG [40] and ^{68}Ga -DOTA-ReCCMSH(Arg¹¹). Overall differences in tumor accumulation can be attributed to the amount of peptide mass administered to the animals, differences in tumor types and volumes, and the status of the tumor cells maintained within each laboratory so care must be taken in the comparison of the current studies with those previously reported [32–34,40].

The high nonspecific kidney uptake often hinders the *in vivo* application of radiolabeled peptides and antibody fragments. It has been reported repeatedly that the renal accumulation of peptides or proteins can be reduced by administration of certain amino acids such as lysine and arginine [66]. For example, lysine co-injection was shown to decrease the kidney uptake without significantly interfering with the high melanoma-targeting properties of $^{99\text{m}}\text{Tc}$ -ReCCMSH [53]. The renal accumulation of ^{67}Ga -DOTA-NAPamide was reduced by co-injection of L-lysine without affecting tumor retention [34]. Similarly, our results demonstrated that pre-administration of D-lysine dramatically reduced the kidney uptake while the radioactivity accumulation in tumor and other organs showed no significant change. Moreover,

the level of reduction (53% at 2 h p.i. with 15 mg D-lysine) is similar compared to that obtained with other radiolabeled α -MSH peptide analogs (64% for ^{67}Ga -DOTA-NAPamide at 4 h p.i. with 15 mg L-lysine [34]; 48, 55, 70% for at 0.5, 1, 4 h p.i. for $^{99\text{m}}\text{Tc}$ -ReCCMSH with 30 mg L-lysine [53]). The slight difference of the level of kidney uptake reduction may be related to different time points, the type of lysine (D- vs. L-lysine), lysine administration methods (co-injection vs. pre-injection) and doses (15 mg vs. 30 mg).

Small animal PET imaging studies showed that significant accumulation of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) was clearly visible in the B16/F1 melanoma tumor indicating that the ^{68}Ga -labeled peptide is a viable agent for selectively targeting melanoma *in vivo*. Radioactivity was also present in the kidney and bladder, which serve as the primary route for peptide excretion. The blocking study in the PET imaging confirmed the receptor-mediated property of the tumor uptake of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹). SUVs for tumor, kidney and liver calculated from the PET data are consistent with the trends and relationships observed from the acute biodistribution data. Low SUV values (e.g. 0.20 ± 0.06 ; $\sim 1\%$ ID/g at 0.5 h p.i.) for tumor uptake were observed with the converted %ID/g value were much lower compared to the biodistribution data ($4.31 \pm 1.94\%$ ID/g at 0.5 h p.i.). This is expected because 2 μg of peptide was injected in the imaging study while only 0.24 μg of peptide was administered in the biodistribution study. This indicates that the tumor uptake of the radiolabeled peptide was clearly affected by the mass of the injected peptide, further confirming the receptor-specific property of the tumor accumulation. Higher SUV values would be expected with higher specific activity levels of the ^{68}Ga -DOTA-ReCCMSH(Arg¹¹).

5. Conclusions

^{68}Ga -DOTA-ReCCMSH(Arg¹¹) was evaluated for the PET imaging of malignant melanoma in the B16/F1 tumor-bearing mouse model. The high tumor to non-target tissue ratios, fast clearance from non-target organs, and moderate tumor uptake suggest that ^{68}Ga labeled DOTA-ReCCMSH(Arg¹¹) is a promising agent for the detection of malignant melanoma. The tumor uptake of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) is not optimal due to its low effective specific activity.

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Fig. 1. Selected tumor/organ ratios for ^{64}Cu -DOTA-ReCMSH(Arg¹¹) [40], ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) and ^{64}Cu -CBTE2A-ReCCMSH(Arg¹¹) [46] at 2 h in C57BL/6 mice implanted with B16/F1 tumors. (P = ReCCMSH(Arg¹¹))

Fig. 2.

Transverse (A), coronal (B) and sagittal images (C) images of C57BL/6 mice implanted with B16/F1 tumors at 2 h after tail vein injection of 100 μ Ci (2 μ g) of ^{68}Ga -DOTA-ReCCMSH (Arg¹¹). A mouse that received blockade (left) was co-imaged with a mouse that did not receive blockade (right). Blockade mouse received 20 μ g of unlabeled peptide. The mice were anesthetized with 1 – 2% isoflurane, positioned supine, immobilized and imaged. Ten minute static data sets were collected at each time point. The images shown are 1 mm slices, with the animal in the supine position, and the slices shown are through the center of the tumor volume. The PET images show that the administration of a blockade dose substantially reduces tumor uptake of the agent by such an extent that the tumor is difficult to delineate. (T = tumor, K = kidney, B = bladder)

Fig. 3. SUV data obtained for PET analysis of ^{68}Ga -DOTA-ReCCMSH(Arg¹¹) in C57BL/6 mice implanted with B16/F1 tumors.

Biodistribution of ⁶⁸Ga-DOTA-ReCCMSH(Arg¹¹) (0.24 µg) (%ID/g ± SD, n = 5) in C57BL/6 mice bearing day-10 B16/F1 melanoma tumors at 30 min and 2 h. The third group was pretreated with blockade (20 µg DOTA-ReCCMSH(Arg¹¹)) and the fourth group was pretreated with D-lysine.

Table 1

Tissues	30 min	2 h	2 h with blockade	2 h with D-lysine
blood	4.65 ± 0.84	0.40 ± 0.13	0.63 ± 0.27	0.51 ± 0.17
lung	3.78 ± 0.38	0.40 ± 0.10	0.62 ± 0.21	0.32 ± 0.10
liver	1.34 ± 0.09	0.50 ± 0.11	0.53 ± 0.21	0.45 ± 0.28
spleen	1.34 ± 0.29	0.21 ± 0.04	0.30 ± 0.14	0.27 ± 0.10
kidney	16.50 ± 4.30	6.80 ± 1.72	8.88 ± 2.53	3.17 ± 1.41
muscle	1.16 ± 0.50	0.10 ± 0.03	0.29 ± 0.19	0.08 ± 0.03
heart	2.00 ± 0.41	0.16 ± 0.04	0.30 ± 0.16	0.22 ± 0.11
skin	2.54 ± 0.93	0.31 ± 0.14	0.64 ± 0.11	0.31 ± 0.17
B16/F1 tumor	4.31 ± 1.94	4.25 ± 1.41	0.42 ± 0.05	3.44 ± 1.32